Multiple Autophosphorylation Sites of the Epidermal Growth Factor Receptor Are Essential for Receptor Kinase Activity and Internalization

CONTRASTING SIGNIFICANCE OF TYROSINE 992 IN THE NATIVE AND TRUNCATED RECEPTORS*

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The role of epidermal growth factor (EGF) receptor autophosphorylation sites in the regulation of receptor functions has been studied using cells transfected with mutant EGF receptors. Simultaneous point mutation of 4 tyrosines (Y1068, Y1086, Y1148, Y1173) to phenylalanine, as well as removal of these sites by truncation of the carboxyl-terminal 123 amino acid residues, resulted in reduced receptor phosphorylation of an in vivo specific substrate phospholipase C-γ1 to less than 50% compared to the wild-type receptor. The internalization rate constant, K, was also significantly lower in these mutants (0.15/min) compared to cells transfected with wild-type receptor (0.27/min). Additional mutation of tyrosine 992 to phenylalanine in the truncated receptor mutant (DC-123F) further decreased the receptor internalization rate to a minimal level (K = 0.07–0.10/min), equivalent to the K measured for cells expressing kinase-negative receptor (A721). Moreover, tyrosine kinase activity of the DC-123F receptor toward phospholipase C-γ1, compared to wild-type receptor, was reduced by 90%. Taken together, these results show that EGF receptor lacking five autophosphorylation sites functions similar to a kinase-negative receptor. Mutation of tyrosine residue Y992 alone in the context of full length EGF receptor, however, did not affect receptor internalization or kinase activity toward phospholipase C-γ1. These data indicate that tyrosine 992 is critical for substrate phosphorylation and internalization only in the context of the truncated receptor, and that minor autophosphorylation sites, such as Y992, may act as compensatory regulatory sites in the absence of the major EGF receptor autophosphorylation sites.

Binding of epidermal growth factor (EGF)† to its receptor

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† The abbreviations used are: EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; SH2 domain, Src homology region 2; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

results in a variety of early and late cellular responses that ultimately produce mitogenesis (1, 2). Specific structural changes in the receptor molecule induced by the ligand binding are likely to occur for these mitogenic signals to be transmitted to points of cellular response, such as the nucleus. The tyrosine kinase activity of the EGF receptor, activated by EGF binding, is essential for inducing the intracellular events necessary for mitogenesis (3, 4). In addition to phosphorylating intracellular substrates, receptor kinase activity is required for autophosphorylation of multiple tyrosine residues, all localized near the carboxyl terminus of the receptor. The presence of these tyrosine residues is essential for maximal biological activity of the receptor (5). Tyrosine-phosphorylated carboxy-terminal fragments of the EGF receptor have been shown to represent an association site for phospholipase C-γ1, mediated by the Src homology 2 (SH2) domains of phospholipase C-γ1 (6, 7). A similar interaction probably occurs with other receptor phosphorylation substrates that contain SH2 domains (7).

In addition to autophosphorylation and substrate phosphorylation, other events also occur rapidly after the binding of EGF to its receptor. One such response is the internalization and down-regulation of EGF receptors. EGF-receptor complexes, that initially are diffusely distributed on the cell surface, accumulate in costed pits and are rapidly internalized within endosomes. After internalization, EGF and its receptor are mainly (unlike nutritive-type receptors) sorted to the degradative pathway (8–10), although some recycling does occur (11, 12). Efficient targeting to the degradative compartment is a general feature of ligand-activated growth factor receptors (13, 14).

Molecular properties of the EGF receptor that mediate internalization and intracellular trafficking are poorly understood. Deletion analysis indicates that a domain necessary for receptor internalization is located at the boundary of the kinase domain and the carboxyl terminus (15). While receptor kinase activity has been shown to be essential for rapid down-regulation of EGF receptors (15–18), there is disagreement as to whether slow internalization (15, 17) or intracellular sorting to the recycling versus degradative pathway (16, 18) is responsible for reduced receptor degradation in the absence of kinase activity.

We have recently shown that simultaneous autophosphorylation of three carboxyl-terminal tyrosine residues (Y1068, Y1148, Y1173) is necessary for rapid internalization and degradation of EGF receptor as well as for maximal kinase activity toward phospholipase C-γ1 (20, 21). In addition to these three main autophosphorylation sites, the EGF receptor

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has been reported to be phosphorylated at two other minor sites, Y1086 and Y992 (22, 23). Autophosphorylation of Y1086 was detected in the intact receptor (22), while autophosphorylation of Y992 was detected in an EGF receptor carboxy-terminal truncation mutant that lacks the other four autophosphorylation sites (5, 23). However, the functional role of these sites has yet to be elucidated. In this manuscript, we have addressed the physiological role of these minor autophosphorylation sites for receptor kinase activity, and receptor internalization and down-regulation in the context of full length and, in the case of Y992, truncated EGF receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—EGF was isolated from the mouse submaxillary gland according to the method of Savage and Cohen (24) and iodinated as described by Carpenter and Cohen (8). The specific activity was 100,000 cpm/ng. A polyclonal antibody to the human EGF receptor was described previously (25). Polyclonal antibody to phosphotyrosine and monoclonal antibody to EGF receptor were purchased from Zymed Inc. Rabbit polyclonal antibodies to phospholipase C-1γ were described previously (25). Polyclonal antibodies to phosphotyrosine were from Gibco-BRL. Rabbit polyclonal antibody to phospholipase C-yl was from Sigma. 125I-Protein A was purchased from ICN. Nitrocellulose and Immobilon paper were from BASF or Millipore Inc., respectively.

**Mutant Construction**—EGF receptor mutants Dc-123 (truncation of the carboxy-terminal 123 amino acids) and kinase-negative (pMI-60, point mutation of lysine 72 with alanine) were previously described (19) as were a four-point mutation mutant, R1086C-Y992C (1086-1148-1173F), phenylalanine substitution of tyrosine 1086 and, in the intact receptor (22), while autophosphorylation of Y992 was detected in an EGF receptor carboxyl-terminal truncation mutant, R1086-1148-1173F (pM1-11). A single-stranded template cDNA lacking the last 123 amino acids (5) was cloned back in the pC0-11 vector. To substitute tyrosine 992 with phenylalanine in the context of EGF binding at 4°C were estimated by Scatchard analysis as more than 3-4% of the total counts. Specific binding is reported.

**Internalization of 125I-EGF**—To evaluate the rate constant (k1) for receptor internalization, cells, cultured in 12-well dishes were incubated with 125I-EGF in "binding medium" (20, 21) at 37°C for 1-6 min. After the indicated times, the medium was aspirated, and the monolayers were washed three times with DMEM to remove unbound ligand and then incubated for 5 min with 0.2 M acetic acid (pH 2.8) containing 0.5 M NaCl at 4°C. The acid wash was combined with an additional short rinse with the same acidic solution to determine the amount of surface-bound 125I-EGF. Finally, the cells were lysed in 1 M NaOH to quantitate internalized radioactivity.

**Non specific binding** was measured for each time point in the presence of 200-fold molar excess of unlabelled EGF and was not more than 15% of the total counts. Specific binding is reported.

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**Phosphorylation of the EGF Receptor, Phospholipase C-yl and Total Cell Proteins in Intact Cells**—Cells grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum and gentamicin. Cells were plated at a density of 25–30 × 10^3/cm^2 and used when confluent (100–150 × 10^3/cm^2).

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**Autophosphorylation of EGF Receptor** has been reported to be phosphorylated at two other minor sites, Y1086 and Y992 (22, 23). Autophosphorylation of Y1086 was detected in the intact receptor (22), while autophosphorylation of Y992 was detected in an EGF receptor carboxy-terminal truncation mutant that lacks the other four autophosphorylation sites (5, 23). However, the functional role of these sites has yet to be elucidated. In this manuscript, we have addressed the physiological role of these minor autophosphorylation sites for receptor kinase activity, and receptor internalization and down-regulation in the context of full length and, in the case of Y992, truncated EGF receptors.

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RESULTS

Previously, we have shown that multiple mutation of the three major autophosphorylation sites of EGF receptor (Y1086, Y1148, Y1173) caused impaired internalization and down-regulation of the receptor (20, 21). In contrast, single or double mutations of these sites did not appreciably alter receptor function. To assess the role of the minor autophosphorylation sites, Y1086 and Y992, in EGF receptor endocytosis, site-directed EGF receptor mutants were constructed in which these tyrosine residues were replaced by phenylalanine. The mutations were introduced in the context of the full length receptor in which the three main autophosphorylation sites (Y1068, Y1148, Y1173) had previously been mutated to phenylalanine (F4) or in truncated receptor in which those sites (Y992, Y1068, Y1086, Y1148, and Y1173) had previously been mutated to phenylalanine (F4). Receptor mutants with phenylalanine substitution of tyrosines 992 in the full length receptor; Dc-123, deletion of the carboxyl-terminal 123 amino acids; Dc-123F, deletion of the carboxyl-terminal 123 amino acids and phenylalanine substitution of Y992; Dc-214, deletion of carboxyl-terminal 214 amino acids.

Previously, we have shown that multiple mutation of the three major autophosphorylation sites of EGF receptor (Y1086, Y1148, Y1173) caused impaired internalization and down-regulation of the receptor (20, 21). In contrast, single or double mutations of these sites did not appreciably alter receptor function. To assess the role of the minor autophosphorylation sites, Y1086 and Y992, in EGF receptor endocytosis, site-directed EGF receptor mutants were constructed in which these tyrosine residues were replaced by phenylalanine. The mutations were introduced in the context of the full length receptor in which the three main autophosphorylation sites (Y1068, Y1148, Y1173) had previously been mutated to phenylalanine (F4) or in truncated receptor in which those sites had been deleted (Dc-123F) (Fig. 1). All mutant receptors were transfected into NIH 3T3 cells. In addition to the kinase-negative mutant (27), the Dc-214 receptor which is missing all five autophosphorylation sites and the putative internalization region (15) was used as a negative control in the internalization studies. As determined by $^{125}$I-EGF binding assay at 4 °C, most of the mutant receptors were expressed at levels of 70,000-160,000 receptors per cell. F4 receptor, however, was expressed at a slightly higher level (230,000 receptors per cell). In addition, one clone of cells expressing Dc-123F receptor displayed a significantly higher complement of receptors (500,000-600,000 per cell). Cl-17 cells (30), which express a comparable amount of wild-type receptors (400,000 per cell), were used as control for this mutant.

$^{125}$I-EGF Internalization—To measure the specific rate of $^{125}$I-EGF internalization, cells were briefly exposed to $^{125}$I-EGF (1 ng/ml) at 37 °C. The amount of internalized versus surface-bound ligand was determined by an acid wash (pH 2.8) as described under "Experimental Procedures." In Fig. 2A, the apparent rate of $^{125}$I-EGF internalization was expressed as the rate of accumulation of intracellular $^{125}$I-EGF relative to the amount of the surface-bound $^{125}$I-EGF. The amount of internalized $^{125}$I-EGF in cells expressing wild-type receptors exceeded the amount of surface-bound ligand after 4–5 min of continuous incubation at 37 °C. Kinase-negative cells displayed a 3-fold lower apparent rate of internalization. Internalization of $^{125}$I-EGF was reduced approximately 2-fold in both F4 cells and Dc-123F compared to wild-type receptor expressing cells. The rate of internalization of the Dc-123F receptor was less than that of the Dc-123 receptor and nearly equal to that of kinase-negative receptor.

Under the conditions used for the internalization assay, the influence of recycling of $^{125}$I-EGF on the ratio of the intracellular and surface pools of the ligand is minimal (21, 33, 34). Nevertheless, the values of the specific internalization constant, $k_a$, calculated according to a model without terms for recycling are underestimated (34). Therefore, we analyzed data obtained from the experiments performed as in Fig. 2A using a mathematical model that takes into account recycling of $^{125}$I-EGF (21). To evaluate the value of $k_a$ at different surface receptor occupancies, cells were incubated with $^{125}$I-EGF at concentrations ranging from 0.1 to 100 ng/ml. The most dramatic differences in the specific internalization rates for the various receptor mutants were observed when low (0.1–5.0 ng/ml) concentration of $^{125}$I-EGF was used, i.e., at low surface receptor occupancy. The values of mean $k_a$ calculated from experiments in which the mean surface EGF receptor occupancy was less than 25,000 are presented in Fig. 2B. The value of $k_a$ was highest in wild-type receptor cells (0.22–0.30/min) and lowest in kinase-negative receptor expressing cells ($k_a = 0.06–0.11/min). As an additional control, cells expressing the Dc-214 truncation receptor that lacks all five autophosphorylation sites and a proposed internalization domain (15) were used. In agreement with the data obtained with transfected B82 cells (15), the internalization of this mutant ($k_a = 0.07–0.09/min)$ was similar to that for the kinase-negative receptor.

The internalization rate was also reduced for F4 and De-
123 receptors, indicating that the tyrosines in the carboxyl terminus are important for EGF receptor internalization. The values of $k_r$ measured for these mutants (0.14–0.15/min) were similar to the value previously obtained with a receptor mutant (F3) mutated at three tyrosine autophosphorylation sites (20, 21). Importantly, the internalization of $^{125}$I-EGF was particularly reduced in cells bearing the Dc-123F receptor (Fig. 2B). The internalization rate constants measured in the 2 clones expressing different numbers of receptors (160,000 and 600,000 per cell) were comparable to the values for kinase-negative and Dc-214 receptors (0.07–0.10/min). Although mutation of Y992 in the truncated receptor significantly decreased receptor internalization, mutation of Y992 in the context of the full length receptor did not affect internalization of $^{125}$I-EGF.

The value of $k_r$ decreased with the increase of $^{125}$I-EGF concentrations applied, in wild-type, F4, and Dc-123 receptor expressing cells, but not in kinase-negative and Dc-123F cells (data not shown). At high occupancy of surface EGF receptors (above 50,000 per cell), the $k_r$ value of the wild-type and F990 receptor was 0.10–0.20/min. Cells expressing F4, Dc-123, Dc-123F, and kinase-negative EGF receptors internalized $^{125}$I-EGF with a similar rate ($k_r = 0.07–0.11$/min). At one clone given high occupancy, the $k_r$ value of wild-type or F990 receptor was 1.5 to 2 times higher than that of the other mutant receptors.

**Down-regulation of EGF Receptor**—Since differences in the EGF internalization rates have been proposed to correlate with the differences in the rates of the receptor down-regulation (15, 17, 20, 21, 34), we have examined down-regulation of the various autophosphorylation site mutants. To monitor EGF-induced EGF receptor down-regulation, transfected cells were incubated at 37 °C with a saturating concentration of EGF for 30, 60, or 120 min. The surface-bound, unlabeled EGF was then removed by a mild acid wash (pH 4.5), and the number of residual binding sites at the cell surface was measured by incubating the cells with a saturating concentration of $^{125}$I-EGF at 4 °C. As shown in Fig. 3, EGF induced a rapid down-regulation of 80% of the wild-type receptors, albeit at a slightly slower rate in cells expressing a higher number of receptors (C1-17) (Fig. 3B) than in cells expressing 100,000 receptors per cell (Fig. 3A). Since levels of receptor expression are an important variable in determining the rate and extent of receptor down-regulation, we compared cells expressing mutant receptors at 70,000–160,000 receptors/cell with cells expressing 100,000 wild-type receptors per cell (Fig. 3A). For mutants expressed at a higher level (more than 220,000 receptors/cell), comparison was made with a wild-type receptor expressed at similar high levels (C1-17, 400,000/cell) (Fig. 3B).

Fig. 3A shows that following the 2-h incubation with EGF, the extent of receptor down-regulation was highest in wild-type (more than 80%) and lowest in kinase-negative receptor expressing cells (35–40%). The amount of surface receptors was maintained at these levels during an additional 2-h incubation with EGF (data not shown). The rate and extent of down-regulation of the Dc-214 mutant was similar to that of the kinase-negative receptor. Down-regulation was also substantially impaired in the F4 and Dc-123 mutants. Mutation of Y992 in the Dc-123 receptor, however, caused an additional decrease in the extent of down-regulation (Fig. 3A), whereas the mutation of Y992 in the full length receptor did not affect this process (Fig. 3B). Measurements of EGF-induced degradation of the $[^{35}$S]methionine metabolically labeled EGF receptor also confirmed the reduced rate of Dc-123F down-regulation compared to wild-type receptor. EGF caused a 7- to 10-fold decrease in the half-life of wild-type receptor (from 8–12 h to 1–1.5 h), but only a slight decrease in the half-life of Dc-123F receptor (from 10 to 8.5 h). The effect of EGF on Dc-123F receptor degradation was, therefore, similar to that measured for kinase-negative receptor, since EGF decreased the half-life of kinase-negative receptor from 8 to 7 h.

These data demonstrate that EGF receptor down-regulation and degradation are affected by the removal of multiple autophosphorylation sites in a manner comparable to the initial internalization process. The inhibitory effect of tyrosine mutations or carboxyl-terminal truncations on the overall endocytic process suggests that the receptor carboxyl terminus is an important determinant for quantitative subcellular distribution of the EGF receptor in the presence of EGF.

**EGF Receptor Mutations and Receptor Autophosphorylation**—To determine whether substitutions or truncations of tyrosine residues in receptor mutants, that alter ligand-induced receptor internalization, influence EGF-induced receptor autophosphorylation, transfected cells were incubated with EGF at 4 °C for 1 h. This protocol obviates the potential differences, in the various mutant receptor cell lines, in the rate of ligand-receptor association and receptor internalization on receptor activation and autophosphorylation. Immunodetection of phosphotyrosine on the EGF receptor revealed very little or no detectable tyrosine phosphorylation in cells not treated with EGF (Fig. 4). After incubation with EGF, autophosphorylation increased to different extents in the different EGF receptor mutants. Mutation of all four tyrosine residues in the F4 receptor resulted in a substantial decrease (82%) in ligand-stimulatable receptor autophosphorylation compared to the wild-type receptor. Removal of these 4 tyrosines by truncation of the carboxyl-terminal 123 amino acids (Dc-123) led to a greater decrease (91%) in autophosphorylation. This difference, although small, was repeatedly observed and suggests that unmapped tyrosine residues located within the truncated carboxyl terminus of the Dc-123 receptor could be phosphorylated in the F4 receptor. Although the EGF-induced receptor autophosphorylation recovered from
the Dc-123 receptor was low, mutation of Y992 in the truncated receptor (Dc-123F) resulted in an additional decrease in the amount of phosphorysosine per receptor molecule to less than 5% of the autophosphorylation activity detected in the wild-type receptor. Mutation of Y992 in the full length EGF receptor (FgQ2), however, did not significantly affect the extent of receptor autophosphorylation. It should be pointed out that the low amount of EGF-induced phosphorylation of the Dc-123F receptor may indicate autophosphorylation of novel tyrosine residues, such as Y1043, Y974, or Y845. With polyclonal (Fig. 4) as well as monoclonal (data not shown) phosphorysosine antibodies, very weak tyrosine phosphorylation of the kinase-negative receptor was also detected in the presence of EGF. Tyrosine phosphate on the kinase-negative receptor could be attributed to transphosphorylation by endogenous wild-type receptors in NIH 3T3 cells or to autophosphorylation and immunoprecipitation of endogenous mouse EGF receptors. Binding studies indicate the presence of no more than 3,000 mouse receptors per cell, less than 5% of the minimal level obtained with cells transfected with human EGF receptor mutants.

Tyrosine Kinase Activity of the Wild-type and Mutant EGF Receptors—It has been proposed that ligand-induced activation of receptor tyrosine kinase activity leads to substrate phosphorylation that is required for rapid internalization of EGF-receptor complexes (17). However, cellular substrates that have a role in internalization are not known. Therefore, we tested the in vivo kinase activity of the EGF receptor mutants using phospholipase C-γ1 as a specific cellular substrate (35–37). Wild-type EGF receptors produced marked EGF-dependent tyrosine phosphorylation of phospholipase C-γ1 as detected with phosphotyrosine antibody, while kinase-negative receptor did not detectably phosphorylate this substrate (Fig. 5). Compared to wild-type receptors, the kinase activity of the F4 and Dc-123 EGF receptors toward phospholipase C-γ1 was decreased by approximately 50–60%. Mutation of tyrosine 992 in the Dc-123 receptor resulted in an additional substantial decrease of EGF-induced phospholipase C-γ1 phosphorylation. In this mutant phospholipase C-γ1 phosphorylation was reduced by 90% compared to wild-type receptor. However, the same mutation did not affect the ability of the full length receptor (FgQ2) to phospholipase C-γ1. These data imply that phosphorylation of Y992 is important for receptor-induced phospholipase C-γ1 phosphorylation only in the absence of other major autophosphorylation sites.

We also examined EGF-induced tyrosine phosphorylation of total cellular proteins in cells expressing wild-type and Dc-123F cells by immunoblotting total cellular extracts with phosphotyrosine antibody. As shown in Fig. 6, EGF stimulated the tyrosine phosphorylation of a number of proteins in cells expressing wild-type receptors. In contrast, tyrosine phosphorylation of individual proteins was drastically reduced in cells expressing the Dc-123F receptor. Several tyrosine-phosphorylated proteins detected in wild-type receptor-transfected cells were totally undetectable in Dc-123F cells, even after prolonged exposure of the anti-phosphotyrosine blot. These results enhance the conclusion that the kinase activity of Dc-123F receptor mutant toward phospholipase C-γ1 as well as other intracellular substrates is dramatically reduced. In agreement with a very low kinase activity, biological and transforming activity of Dc-123F receptor was also very low. Ability of inducing EGF-dependent foci in NIH 3T3 cells was reduced by 92% compared to wild-type receptor.

Finally, it should be pointed out that the low kinase activity of Dc-123F was not due to inefficient EGF-inducedimerization of this receptor. The extent of dimerization, measured by chemical cross-linking of 125I-EGF-receptor complexes (27), was similar in cells expressing wild-type and Dc-123F receptors (data not shown).

Fig. 4. Autophosphorylation of EGF receptor. Cells were treated with or without EGF (100 ng/ml) for 1 h at 4°C and solubilized, and the EGF receptor immunoprecipitated with a polyclonal EGF receptor antibody. After electrophoresis and transfer to nitrocellulose, the phosphorysosine (pY) content of mutant receptors was detected with polyclonal antibody. Panel A shows an autoradiogram of the immunoblot. To quantify the number of receptors expressed on the surface of each cell line, 125I-EGF binding was performed in parallel cultures. Binding to mutant receptors was normalized to that in wild-type receptor cells (100%). Percent binding for F4 receptor was 95%, for kinase-negative receptor (kin−), 120%; for Dc-123 receptor, 149%; for FgQ2 receptor, 90%; for Dc123F receptor, 97%; and for F4 receptor, 73%. Panel B, the amount of phosphorysosine per occupied EGF receptor expressed as percent of the amount detected in wild-type receptor. Bars in B correspond to the average value from four to six independent experiments performed as in A. Error bars represent standard deviations.

Fig. 5. Tyrosine phosphorylation of phospholipase C-γ1. Cells were treated with or without EGF (100 ng/ml) for 1 h at 4°C and solubilized, and phospholipase C-γ1 was immunoprecipitated with polyclonal phospholipase C-γ1 antibody. After electrophoresis and transfer to nitrocellulose, phosphorysosine (pY) and phospholipase C-γ1 were detected with polyclonal antibodies to each. The number of EGF receptors was determined in parallel culture by measuring the binding of 125I-EGF (100 ng/ml) for 1 h at 4°C. Panel A shows an autoradiogram of the immunoblot with phosphorysosine antibody, panel B an autoradiogram of the immunoblot with phospholipase C-γ1 antibody, and panel C indicates the amount of phosphorysosine per phospholipase C-γ1 molecule normalized to the number of occupied EGF binding sites present in the various mutants (expressed as percent of the amount detected in cells expressing wild-type receptor). Bars in panel C correspond to the average value from three to six independent experiments, and error bars represent standard deviations. Left and right panels show different experiments.
phototyrosine antibody. The number of receptors occupied by EGF, determined by measuring 125I-EGF binding on parallel cultures, the level of receptor autophosphorylation compared to the F3 mutation of all three sites was necessary to allow rapid receptor internalization of all cells expressing wild-type receptors (Cl-17, 400,000 receptors/cell) and Dc-123F receptors (600,000 receptors/cell). Cells were treated with or without EGF (100 ng/ml) for 1 h at 4 °C and solubilized. An aliquot of cell lysate was electrophoresed on 7.5% gel and transferred to nitrocellulose followed by blotting with phosphotyrosine antibody. The number of receptors occupied by EGF, determined by measuring 125I-EGF binding on parallel cultures, was 180% in Dc-123F receptor expressing cells compared to wildtype receptor expressing cells.

**DISCUSSION**

In present report, we have extended studies of the roles of EGF receptor tyrosine autophosphorylation sites by analyzing the influence of two minor sites of phosphorylation, Y1086 and Y992, on receptor kinase activity and internalization. We previously demonstrated that simultaneous autophosphorylation of three principal autophosphorylation sites (Y1173, Y1148, Y1068) located near the carboxyl terminus of the EGF receptor positively regulates biological and tyrosine kinase activity of the receptor (5, 20, 21). In addition, phosphorylation of all three sites was necessary to allow rapid receptor internalization and down-regulation (20, 21). Single or double mutations of any three tyrosines did not, however, significantly influence receptor functions.

The triple tyrosine mutant receptor (F3, 1068-1148-1173F), however, did retain residual biological activity (5, 20, 21) indicative of a partial compensatory role for the minor sites of tyrosine phosphorylation. Substitution of one of these minor sites, Y1086, with phenylalanine in the context of the F3 full length EGF receptor or removal of this site together with the other major autophosphorylation sites by truncation of the carboxyl-terminal 123 amino acid residues (F4 and Dc-123 mutants, respectively) resulted in a further decrease in the level of receptor autophosphorylation compared to the F3 mutant (Fig. 2 and Refs. 5 and 21). The tyrosine kinase activity and internalization rate of the F4 and Dc-123 receptors were reduced approximately 50% compared to wild-type receptors (Figs. 2 and 5). Very similar reductions were measured previously in F3 receptor expressing cells (5, 20, 21). These results indicate that neither Y1086 nor other potential minor tyrosine phosphorylation sites located within the carboxyl-terminal 123 amino acid residues accounted for the significant residual tyrosine activity and internalization of F3 receptors. It should be pointed out that we have reproduced the same results with four conservative point mutations (F4) and with a corresponding deletion mutant encompassing these 4 tyrosine residues (Dc-123). This indicates that deletion of 123 amino acid residues did not significantly alter the conformation of the remaining intracellular domain of the receptor and allowed us to analyze the role of tyrosine 992 without the possible interference of other minor sites present in the carboxyl terminus.

Although our data show that the distal carboxyl-terminal tyrosine residues (Y1068, Y1148, Y1173) are important for receptor endocytosis, multiple mutations, or truncation of this domain achieved only a 50% reduction in the internalization rate. In the absence of these major autophosphorylation sites, another domain (residues 973–1063, including tyrosine 992) becomes regulatory for receptor internalization. Mutation of Y992 in the Dc-123 receptor (Dc-123F) significantly reduced residual internalization of the receptor to the point that the internalization rate of the Dc-123F mutant was comparable to the minimal internalization rate of Dc-214 and kinase-negative receptors. This observation indicates that Dc-123F is unable to undergo rapid internalization, although it possessed an intact tyrosine kinase domain and the domain that has been proposed to contain an internalization signal (residues 973–1022) (15). These data also indicate that there is a requirement of at least 1 carboxyl terminus tyrosine residue for rapid internalization of the EGF receptor.

Tyrosine 992 was also critical for tyrosine phosphorylation of the cellular substrates by the EGF receptor kinase, when the other principal autophosphorylation sites were removed. In the presence of EGF, the receptor mutant Dc-123F had very little kinase activity toward phospholipase C-γ1 and other substrates in total cell lysates. However, some proteins in the total cell lysate were significantly phosphorylated by Dc123F receptor in response to EGF. These proteins may represent substrates, such as annexin I (38, 39), that do not associate with the EGF receptor through interaction of their SH2 domains with the phosphotyrosine residues in the EGF receptor. By contrast, mutation of tyrosine 992 in the full length EGF receptor did not affect phospholipase C-γ1 phosphorylation or internalization. This is in agreement with previous reports demonstrating that single mutations of other autophosphorylation sites did not significantly influence receptor kinase activity and internalization (5, 20, 21, 40).

Since the inhibition of internalization correlates with the inhibition of kinase activity in these receptor mutants, our data support the notion that receptor kinase activity is involved in the internalization process (17). Several nonmutually exclusive mechanisms can be envisaged. First, it is possible that kinase activity is necessary only to produce autophosphorylation. Phosphate addition to multiple tyrosines could induce conformational changes in the carboxyl-terminal domain and lead to efficient internalization by allowing receptor interaction with a component(s) of the coated pit. Thus, exposure of EGF receptor sequences responsible for the internalization would be achieved by tyrosine phosphorylation of the native receptor. Large carboxyl-terminal truncations of the EGF receptor, as in a receptor mutant lacking 164 amino acid residues (15) can produce a similar effect exposing internalization sequences.

Second, in addition to autophosphorylation, exogenous substrate phosphorylation may be a requirement in the internalization process. It might be that component(s) of the coated pits must be phosphorylated on tyrosine for the receptor to be trapped in these specialized membrane regions. Whether such interactions are mediated by coated pit substrates containing SH2 domain remains to be determined. Finally, there may be more than one step in receptor interaction with coated pits and, therefore, more than one function performed by the carboxyl terminus that requires the receptor kinase activity. Recent studies on polymeric Ig, mannose 6-phosphate, and transferrin receptor endocytosis (41–43) suggest that the presence of additional sequences other than the tyrosine-containing internalization motif is necessary for rapid internalization of these receptors.

Taken together, our results indicate that by simultaneous removal of five autophosphorylation sites it is possible to produce a receptor mutant that, in many ways, functions as a
kinase-negative receptor. For at least some receptor functions, the absence of any one or two tyrosine phosphorylation sites can be compensated for by phosphorylation of other tyrosines in the carboxyl terminus. Further studies would reveal how these phosphotyrosine-containing sequences allow receptor association with specific substrates and how the receptor interacts with internalization apparatus.

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Multiple autophosphorylation sites of the epidermal growth factor receptor are essential for receptor kinase activity and internalization. Contrasting significance of tyrosine 992 in the native and truncated receptors.

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