Analysis of Deletions of the Carboxyl Terminus of the Epidermal Growth Factor Receptor Reveals Self-phosphorylation at Tyrosine 992 and Enhanced in Vivo Tyrosine Phosphorylation of Cell Substrates*

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The human epidermal growth factor receptor (EGFR) contains a large C' terminal distal to the protein tyrosine kinase domain that is conserved among members of its extended family. To investigate the C' terminus, a series of mutant EGFR cDNAs encoding progressive C'-terminal deletions were prepared and expressed in null recipient B82L cells. In vivo self-phosphorylation was retained in receptors truncated to residues 1052 and 1022 which lack the three identified sites of tyrosine self-phosphorylation. Receptors truncated to residue 991 did not undergo in vivo self-phosphorylation. Purified 1022 truncated receptor was self-phosphorylated to the extent of 1 mol of phosphate/mol of receptor protein. The deduced additional site of tyrosine self-phosphorylation at residue 992 was confirmed by tryptic phosphopeptide mapping and protein sequencing. EGFRs deleted to give C'-terminal residues 1052, 1022, 991, and 973 exhibited enhanced EGF-stimulated tyrosine phosphorylation of cell substrates in vivo, whereas deletion at residue 944 abolished all detectable EGF-stimulated protein tyrosine phosphorylation. These results indicate that ligand-induced self-phosphorylation is limited to the C' terminus of the EGFR and suggest that this region of the holoreceptor has an inhibitory function.

On ligand binding the epidermal growth factor receptor (EGFR) undergoes self-phosphorylation (1, 2). Three sites of in vivo self-phosphorylation have been identified as residues 1068, 1148, and 1173 (3) located in the C' terminal distal to the conserved core protein kinase domain (4). Kinetic analysis indicates that self-phosphorylation is competitive with exogenous peptide substrates, with self-phosphorylation reducing the $K_m$ for peptide substrates (5). The C' terminus is proposed to be a regulatory domain with self-phosphorylation removing an alternate substrate/inhibitory constraint. In support of this hypothesis Lin and Clinton (6) found that dephosphorylation of activated EGFRs reduced their protein tyrosine kinase activity. Moreover, an antipeptide antibody, directed against a sequence of the kinase core, bound and inhibited only self-phosphorylated EGFR, suggesting this part of the catalytic domain was exposed on self-phosphorylation. In contrast others have reported that self-phosphorylation does not affect the protein tyrosine kinase activity of EGFR (8, 9). Mutation of identified phosphorylatable tyrosine residues to phenylalanine has yielded conflicting results (10, 11).

To clarify the role of tyrosine self-phosphorylation and of the C' terminus in EGFR action, a progressive series of C'-terminal truncated EGFR cDNAs were constructed and expressed in recipient cells lacking endogenous EGFR mRNA or protein (12). In addition to previously identified self-phosphorylation sites, self-phosphorylation at Tyr-992 is identified. Removal of the C' terminal 195 or 213 amino acids results in a receptor which lacks self-phosphorylation, but which exhibits enhanced in vivo EGF-stimulated phosphorylation of protein substrates on tyrosine residues. These studies indicate that self-phosphorylation of the EGFR is limited to the C'-terminal regulatory domain and self-phosphorylation of the kinase domain is not required for activity. Because removal of the C' terminus enhanced EGF-stimulated tyrosine phosphorylation in vivo, this domain is proposed to serve an inhibitory function.

MATERIALS AND METHODS

Construction and Expression of Mutant EGFRs—All C'-terminal truncated EGFRs were constructed in the context of wild-type T154, K712 human EGFR cDNA (13) as described by Chen et al. (14) using oligonucleotide directed mutagenesis to create both a stop codon and a restriction site (15). Mutated cDNAs were reinserted into the pxER expression vector under control of the SV40 promoter/enhancer. The expression vector contains a mutant dihydrofolate reductase gene as a selectable marker (16). DNA transfections into mouse B82L cells were performed using calcium phosphate precipitation (17), and clonal lines were selected and amplified using methotrexate. Mutant EGFRs expressed in clonal cell lines are identified by their C'-terminal amino acid residue based on cDNA sequence (18): i.e. c'1052, c'1022, c'991, c'973, and c'944.

Immunodetection of in Vivo Tyrosine Phosphorylated Proteins and of EGFRs—Cells expressing mutant EGFRs were treated without or with 100 nM EGF for 2 min at 23 °C. Hot Laemmli sample buffer was added and 4 × 10⁶ cell equivalents of protein were separated on 7.5 or 10% SDS-polyacrylamide gels and transferred to Immobilon. Alternatively, EGFRs in cell extracts were measured by immunodetection under linear assay conditions, and the volumes of cell extract were adjusted to load equivalent amounts of mutant EGFR receptor protein onto each gel lane. Phosphotyrosine containing proteins were detected using 125I-labeled FY-20 monoclonal anti-phosphotyrosine antibody and autoradiography (13). EGFRs were subsequently de-
tected on the same membranes using a 1:400 dilution of polyclonal rabbit anti-human EGFR serum. Alternatively, EGFRs were detected using a 1:10,000 dilution of a rabbit anti-peptide serum generated against EGFR receptor residues 643–688 (generously provided by Dr. Tony Hunter, The Salk Institute). Immunostaining of blots was performed using either biotinylated protein A, horseradish peroxidase coupled avidin (Vector Labs), with a 3,3′-diaminobenzidine as substrate or goat anti-rabbit IgG conjugated with alkaline phosphatase and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate as a substrate (Promega).

For measurement of in vitro tyrosine self-phosphorylation of mutant EGFRs, cells were treated without or with 100 nM EGF for 2 min at 23 °C. Cells were rapidly lysed at 4 °C in buffer containing 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 12 mM 2-mercaptoethanol, 0.1 mM Na3VO4, 10 μM ammonium molybdate, 50 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride and 20 μg/ml leupeptin. EGFRs were immunoprecipitated using 528 monoclonal anti-human EGFR IgG specific for the ligand binding domain (20, 21). Washed immunoprecipitates were dissolved in hot Laemmli sample buffer, run on 7.5% SDS-polyacrylamide gels, transferred to Immobilon, and immunodetection was carried out.

Identification of Sites of Tyrosine Self-phosphorylation—Holo EGFR protein was purified from A431 cells and c’1022 EGFR protein was purified from B82 cells using immunoaffinity chromatography on 528 IgG-Sepharose with competitive elution with EGF (22). The stoichiometry of self-phosphorylation of the c’1022 receptor was determined by incubation at 30 °C in buffer containing 20 mM Hepes, pH 7.4, 5 mM MgCl2, 2 mM MnCl2, 0.1 mM Na3VO4, 0.25 mg/ml bovine serum albumin, and 7 μM [γ-32P]ATP (4 × 10^6 cpm/pmol). The amount of c’1022 EGF receptor used was determined from densitometric measurement of Coomassie Blue staining of varying amounts of receptor protein on SDS-polyacrylamide gels relative to staining of known amounts of holoEGFR receptor protein (22). Aliquots of 4.5 pmol of receptor were removed at various times up to 60 min and spotted onto paper disks (Whatman) which were fixed, washed in trichloroacetic acid, and counted. For sequencing, protein (35 pmol) which was self-phosphorylated using 6 μM [γ-32P]ATP (100,000 cpm/nmol) was dialyzed against 20 mM Tris-HCl, pH 7.5, 2 mM EDTA and exhaustively digested with V8 protease. 32P-Labeled peptides were separated by two runs on a reverse phase HPLC column (Aqua pore BU-300, 200 × 2.1 mm) using a 3–55% acetonitrile gradient containing 0.1% trifluoroacetic acid (3). The peaks containing 32P-labeled phosphopeptides were subjected to amino-terminal sequencing on a gas phase protein sequencer. A portion of each cycle (60%) containing phenylthiohydantoin derivatives was analyzed by HPLC, and the remainder was counted to determine 32P release.

Tryptic maps of sites of tyrosine self-phosphorylation in holo- and truncated EGFRs were analyzed using membranes prepared from clonal expressor cell lines. Membranes containing 150 pmol of each receptor were solubilized in 20 mM Hepes, pH 7.4, 10% glycerol, 1% Triton X-100 and centrifuged for 5 min at 5 °C. Solubilized EGFRs were immunoadsorbed to 528 IgG-Sepharose and self-phosphorylated with 10 μM [γ-32P]ATP (22,500 cpm/pmol) for 60 min at 5 °C. Gels were washed extensively, and EGFRs were eluted with 8 M urea in 0.2 M Tris-HCl, 10 mM dithiothreitol. Immunoprecipitated self-phosphorylated EGFR was reduced, carboxymethylated, digested extensively, and digested with trypsin (3). Tryptic phosphopeptides were resolved on HPLC.

RESULTS

Identification of an Additional Site of Tyrosine Self-phosphorylation Located in the C' Terminus—To determine which C’-terminal truncated EGFRs are self-phosphorylated in vivo, cells expressing mutant EGFRs were treated with EGF, homogenized in buffer containing a mixture of phosphatase inhibitors and EGFRs immunoprecipitated using 528 monoclonal IgG specific for the N’-terminal ligand binding domain. Fig. 1 shows that phosphotyrosine is present in the isolated holo- c’1052, and c’1022 EGFRs but is absent from c’91 and c’973 EGFRs. Only 1 tyrosine residue is present between residues 991 and 1022 (10); by deduction the site of in vivo self-phosphorylation in c’1022 EGFR is located at Tyr-992.

Examination of the sequence of the EGFR reveals that Tyr-992 is immediately preceded by a glutamic acid residue sus-ceptible to cleavage by V8 protease. To identify the additional site of self-phosphorylation, c’1022 EGFR was purified using immunoaffinity chromatography (22). A single protein band of the expected size compared to holoEGFR was obtained (Fig. 2A). A time course of self-phosphorylation of the purified c’1022 EGFR revealed a stoichiometry of 1 mol of 32P incorporated per mol of receptor protein compatible with a single site (Fig. 2B). Purified self-phosphorylated c’1022 EGFR (Fig. 2A) was digested with V8 protease and 32P-labeled peptides separated on HPLC. A single major phosphopeptide peak was identified and sequenced. Radioactivity was released at cycle 1 with the observed amino acid sequence in cycles 1–5 of Tyr-Leu-X-Pro-Gln in agreement with the predicted site of cleavage of Glu-991 (Fig. 2C).

c’1052 and c’1022 EGFRs both undergo EGF-stimulated self-phosphorylation in vivo and in vitro (Figs. 1 and 3). Because tyrosine is present at residue 1045, it was possible that this residue represented an additional site of self-phosphorylation. To examine this possibility, membranes were isolated from cells expressing holo- or mutant EGFRs, and tryptic phosphopeptide maps of immunoprecipitated self-phosphorylated EGFRs were compared. Four major tryptic phosphopeptides and a minor one were identified in the holo EGFR (Fig. 3A), in good agreement with reported stoichiometry of self-phosphorylation of about 4 mol of phosphate/mol of
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EGFR (22). The sites designated P1, P2, and P3 represent previously identified sites (3). These three sites were, as expected, absent from c'1052 and c'1022 receptors. Tryptic phosphopeptide maps of c'1052 and c'1022 EGFRs were identical, revealing only the two unidentified peaks (P4a and P4b) seen in the holoreceptor (Fig. 3, B and C). Only a trace amount of self-phosphorylation of c'991 EGFR occurred (Fig. 3A, inset), and no distinct tryptic phosphopeptide peaks were identified (Fig. 3D). Thus, it is unlikely that another major autophosphorylation site exists N'-terminal to residue 991. Because only the single newly identified site of self-phosphorylation at residue 992 is present between residues 991 and 1022, the two tryptic phosphopeptide peaks seen in c'1022 presumably represent alternate tryptic cleavage sites rather than 2 phosphorylated tyrosines. There were no additional peaks in the digest of c'1052 EGFR, i.e. the profiles of tryptic phosphopeptides of c'1052 and c'1022 EGFRs were identical, arguing that Tyr-1045 is not a site of self-phosphorylation. Examination of the sequence surrounding Tyr-1045 indicates it lacks most features seen in tyrosine kinase substrates (23).

**Fig. 2.** Identification of the site of tyrosine self-phosphorylation in c'1022 EGFR. A, silver-stained polyacrylamide gel of immunoaffinity purified holoreceptor (lane a) and c'1022 (lane b) EGFRs. Autoradiogram of self-phosphorylated c'1022 EGFR (lane c). B, time course of self-phosphorylation of c'1022 EGFR. C, %P release during sequencing of HPLC-isolated V8 protease phosphopeptide of c'1022 EGFR. The identified amino acid present at each cycle is shown.

**Fig. 3.** Comparison of tryptic phosphopeptide maps of self-phosphorylated mutant EGFRs. Tryptic %P-labeled phosphopeptides of immunoisolated self-phosphorylated EGFRs were analyzed by HPLC. Inset in A is an autoradiogram of the EGFRs used for the analysis. This was overexposed to enhance lane D containing c'991 EGFR. The background of %P seen in B-D corresponds to large peptide peaks observed at A220 (not shown) and presumably represents nonspecific trapping of radioactivity.

Effect of C'-terminal Truncations of EGFR Protein on Tyrosine Phosphorylation of Cell Proteins—EGFR cDNAs were mutated to create the C'-terminal deletions shown in Fig. 4A and activity of mutant EGFRs were measured after treating cells with EGF by using a monoclonal anti-phosphotyrosine antibody to identify proteins phosphorylated on tyrosine residues in vivo. Fig. 4B shows that addition of EGF markedly stimulated phosphorylation of tyrosine residues on proteins. Immunostaining with anti-EGFR serum confirmed expression of mutant receptors of the expected molecular weights (Fig. 4C). Comparison of EGF-dependent substrate phosphorylation with the amount of immunoidentified EGFR protein indicated increased in vivo protein tyrosine kinase activity when the C'-terminus of the receptor was removed. C'-terminal truncation to residue 944 resulted in loss of all detectable kinase activity.

Because the mutant selection procedure results in differing...
expression of the various EGFRs, analysis of equal numbers of B82 cells reflects equal concentrations of substrates but not equal concentrations of receptors. To compare tyrosine phosphorylation of cell substrates relative to the concentration of receptor, the amount of EGF receptor was equalized using immunoquantitation with an anti-peptide serum directed against a sequence common to all the mutant receptors (residues 645-688) (Fig. 5A). When an amount of cell extract yielding equivalent amounts of EGFR was analyzed, increased tyrosine phosphorylation of cell substrates by C'-terminal truncation mutants was confirmed (Fig. 5B). The results using c'991 and c'973 EGFRs indicate that self-phosphorylation of the protein tyrosine kinase core domain is not required for ligand-dependent protein tyrosine kinase activity.

**DISCUSSION**

The C' terminus of the EGFR is proposed to be a regulatory domain containing three major sites of tyrosine self-phosphorylation (5). An additional site of self-phosphorylation is identified as Tyr-992. This was deduced from finding that C'-terminal truncation at residue 1022 resulted in an in vivo self-phosphorylating receptor. Only a single Tyr is located between residues 991 and 1022. This deduction was confirmed by stoichiometric incorporation of 1 mol of phosphate/mol of purified c'1022 receptor and by direct identification of Tyr-992 as the site of phosphorylation in purified c'1022 EGFR protein.

Margolis et al. (24) recently identified Tyr-1086 as an additional site of self-phosphorylation in the EGFR C' terminus. The study of Margolis et al. (24) and the present one differed in HPLC gradient separation of tryptic phosphopeptides. In the present study the tryptic peptide containing Tyr-1086 would not be well separated from P1 and P2 containing Tyr-1173 and Tyr-1148, respectively, whereas in the study of Margolis et al. (24), the tryptic phosphopeptide containing Tyr-992 would not be separated from P3 containing Tyr-1068. Both Tyr-992 and -1086 are located in the C' terminus distal to the core protein tyrosine kinase domain.
Because both c'991 and c'973 EGFRs exhibit strong in vivo EGF-dependent stimulation of cell protein tyrosine phosphorylation, self-phosphorylation of the core kinase domain does not appear involved in enzyme activation. Increased EGF-dependent tyrosine phosphorylation of cell substrates by C' terminal truncated EGFRs may depend on several factors. Removal of the inhibitory C' terminus may increase catalytic activity. Although deletion of this C'-terminal regulatory domain appears to more strongly increase the kinase activity of EGFR in vivo, self-phosphorylation of holoEGFR is likely the major mechanism for removing inhibitory constraints on enzyme activity. Deletion of the C' terminus may increase protein tyrosine kinase activity in vivo more than self-phosphorylation because the latter can be reversed by tyrosine phosphatases (25) and by reversibility of the kinase reaction (26). C'-terminal truncation may also remove a non-competitive structural component which sterically reduces kinase activity and allows greater substrate access to the catalytic site and permit phosphorylation of substrates which do not normally access the holoenzyme. Prolonged exposure of gels from cells expressing holoreceptors reveals many of the same tyrosine phosphorylated substrates as seen in cells expressing mutant receptors, but the analytic capacity of one-dimensional gels is limited. Both increased EGF-dependent tyrosine kinase activity and recognition of additional substrates may contribute to enhanced in vivo tyrosine phosphorylation of substrates.

Changes in ligand-induced receptor down-regulation (15) are not involved in enhanced substrate phosphorylation. EGF treatment was limited to 2 min at 23°C where significant receptor down regulation does not occur (13, 15). Only C' terminal truncation at residue 973 abrogates ligand-induced receptor down-regulation (15), whereas enhanced EGF-dependent tyrosine phosphorylation is observed with all of the C'-terminal truncated EGFR receptors. Tyr-992 is located at the acidic, predicted helical region required for ligand-induced receptor internalization and may contribute to this function (15).

Limited mutational analysis of the effects of C'-terminal truncations have yielded conflicting results. Khazaie et al. (27) reported that removal of 202 C'-terminal amino acids from erb-B and EGFR enhanced the biological effect of fibroblast transformation, but Velu et al. (7) reported that removal of either of 9 or 63 C'-terminal amino acids decreased EGF-dependent transformation. We suggest that because the C' terminus is a strong inhibitory domain, truncation must be extensive enough to relieve this inhibition. One must analyze a large series of C'-terminal mutations to understand the diversity of functions dictated by this region of the EGFR (15).

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