Mutagenesis Reveals a Role for Epidermal Growth Factor Receptor Extracellular Subdomain IV in Ligand Binding*

(Received for publication, May 17, 1999, and in revised form, July 9, 1999)

Marian L. Saxon‡§ and David C. Lee‡¶

From the ‡Department of Biochemistry and Biophysics and the ¶Lineberger Comprehensive Cancer Center, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599-7260

The extracellular domain of the epidermal growth factor (EGF) receptor (EGFR) comprises four subdomains (I–IV) and mediates binding of several different polypeptide ligands, including EGF, transforming growth factor-α, and heparin-binding EGF. Previous studies have predominantly implicated subdomain III in ligand binding. To investigate a possible role for sequences in subdomain IV, we constructed several mutant EGFRs in which clusters of charged or aromatic amino acids were replaced with alanine. Analysis of stably transfected Chinese hamster ovary cells expressing mutant EGFRs confirmed that they were present on the cell surface at levels approaching that of the wild-type receptor. Although tyrosine phosphorylation of most mutants was markedly induced by EGF, a cluster mutation (mt25) containing four alanine substitutions in the span of residues 521–527 failed to respond. EGF-induced tyrosine phosphorylation of an alternative mutant (∆EN) with amino acids 518–589 deleted was also greatly diminished. Larger doses of EGF or heparin-binding EGF induced only weak tyrosine phosphorylation of mt25, whereas the response to transforming growth factor-α was undetectable. These results suggest that mt25 might be defective with respect to either ligand binding or receptor dimerization. Quantitative analyses showed that binding of $^{125}$I-EGF to mt25 and ∆EN was reduced to near background levels, whereas binding of EGF to other cluster mutants was reduced 60–70% compared with wild-type levels. Among the mutants, only mt25 and ∆EN failed to form homodimers or to transphosphorylate HER2/Neu in response to EGF treatment. Collectively, our results are the first to provide direct evidence that discrete subdomain IV residues are required for normal binding of EGF family ligands. Significantly, they were obtained with the full-length receptor in vivo, rather than a soluble truncated receptor, which has been frequently used for structure/function studies of the EGFR extracellular region.

The epidermal growth factor (EGF) receptor (EGFR; ErbB1), a large transmembrane glycoprotein with ligand-inducible tyrosine kinase activity, is a member of a conserved receptor family that includes HER2/Neu/ErbB2, HER3/ErbB3, and HER4/ErbB4 (1–3). Shared characteristics of ErbB receptors include an extracellular (EC) region with two cysteine-rich repeats, a single transmembrane domain, and a cytoplasmic sequence containing a tyrosine kinase and autophosphorylation sites (4). ErbB receptors dimerize upon ligand binding (5, 6), and this is critical for conversion to the high affinity binding state as well as for intermolecular receptor transphosphorylation (7, 8). Homodimers as well as various combinations of heterodimers are formed, depending on the relative levels of the four receptors as well as the activating ligand (9, 10). Because ErbB receptors contain different phosphotyrosine motifs, heterodimerization is believed to expand potential signaling diversity or intensity.

ErbB receptors are bound and activated by members of a ligand superfamily characterized by a conserved, three-disulfide loop structure (the EGF-like motif) that is required for high affinity receptor binding (Ref. 11; reviewed in Ref. 12). This superfamily includes the EGF and neuregulin subfamilies. Besides its namesake, the EGF subfamily includes transforming growth factor-α (TGF-α), amphiregulin, heparin-binding EGF (HB-EGF), betacellulin, and epiregulin. These ligands all bind EGFR, although a subset also directly binds ErbB4 (10, 13, 14). The second ligand family, the neuregulins, comprise a set of isoforms derived from three distinct genes by alternative splicing (15–18). Neuregulins bind and activate ErbB3 and ErbB4, but not EGFR (16, 17, 19). No direct ErbB2-binding EGF or neuregulin ligand has yet been identified. Instead, this receptor might function as a preferred heterodimerization partner, mediating activation of other ErbB proteins via sequential interaction and transphosphorylation (9, 20). These complex ligand/receptor interactions indicate that ErbB, EGF, and neuregulin proteins are most appropriately viewed as components of an intricate, highly regulated signaling network.

The EC region of EGFR and other ErbB receptors is divided into four subdomains. Subdomain I corresponds to the N terminus, whereas subdomain III is flanked by subdomains II and IV, the cysteine-rich repeats. Evidence to date indicates that EGFR subdomains I and especially III are the major determinants of ligand binding (21). Mutant EGFR proteins lacking subdomain I (22) or containing insertion mutations in subdomain III (23) have markedly lower affinity for EGF and TGF-α. Additionally, substitution of subdomain III of chicken EGFR with that of human EGFR restores high affinity binding toward murine EGF, characteristic of the human receptor (24, 25). Finally, cyanogen bromide mapping has identified sequences in

hamster ovary; PBS, phosphate-buffered saline; BSA, bovine serum albumin; mAb, monoclonal antibody; DMEM, Dulbecco’s modified Eagle’s medium.

This paper is available on line at http://www.jbc.org

28356 This paper is available online at http://www.jbc.org
subdomain III that are cross-linked to ligand (26, 27) or recognized by ligand-competitive antibodies (27, 28).

ErbB EC domains may also mediate receptor dimerization. The EGFR EC domain forms ligand-dependent homodimers in solution (7, 29, 30), and mutation or deletion of sequences in the EC juxtamembrane region of HER2/Neu induces constitutive receptor activation via inappropriate disulfide bonding (31, 32). Additionally, EC domain interactions are required for formation of heterodimers of wild-type HER2/Neu and EGFR (3, 33, 34) that are signaling-competent (35). Despite their apparent importance, EC domain sequences mediating receptor dimerization have not been well defined. Moreover, the manner in which binding of various ligands differentially regulates ErbB receptor dimerization or underlies ligand-specific differences in bioactivity is also unclear.

To further define sequence motifs in the EGFR EC domain that are involved in ligand binding and/or receptor dimerization, we performed site-directed mutagenesis. In contrast to most previous studies that manipulated soluble truncated receptor forms corresponding to the EC domain, we utilized full-length EGFR and extended the analyses to examine HER2/Neu transphosphorylation. Our initial focus was the potential role of EGFR subdomain IV sequences in receptor homo- and heterodimerization. However, we were surprised to find that a small cluster of mutations in subdomain IV dramatically impaired the binding of several EGFR ligands. Additionally, mutation of other subdomain IV clusters also reduced ligand binding to 30–40% of control. Our results thus unexpectedly implicated this portion of the extracellular domain in ligand/receptor interactions.

**EXPERIMENTAL PROCEDURES**

**Reagents**—All restriction and modification enzymes were purchased from New England Biolabs Inc. (Beverly, MA) unless otherwise noted. R408 was from Promega (Madison, WI).

**EGFR Construction and Mutagenesis**—Full-length human EGFR cDNA, kindly provided by Dr. Glenn Merlino (National Institutes of Health, Bethesda, MD), was subcloned into pcDNA3 (Invitrogen, San Diego, CA) to generate pc3-EGFR. The latter vector was used for both mutagenesis and expression.

Charged-to-alanine mutagenesis (36, 37) of EGFR subdomain IV was performed as described previously (38) using the Mut-Ena Phagemid In Vitro Mutagenesis Version 2 Protocol (Bio-Rad) and the following primers: pr23, 5'-GACATCCGCGCAAGAGCAGCTGCGGCGTCGCGCCCGCAGGCAGC-3'; pr24, 5'-CTACCATCCAGAACGTTGACGCTGCTGCACACCTGCGCTACATATTCCGGCAAGAGACGCTGCGGCTC-3'; and pr25, 5'-GGACTTCCAGACCTACTTCCAGACCAG-3'. Site-specific mutagenesis was achieved by using Borst restriction site in the vector sequence (38), thus allowing for elimination of WT templates. Second-strand synthesis products were passaged through BMH cells (Promega) to inactivate the uracil templates, and Borst-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α resistant clones were screened for surface expression by using Dosper reagent (Roche Molecular Biochemicals). Geneticin (Life Technologies, Inc.)-resistant clones were screened for surface expression using the Muta-Gene Phagemid (American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α cells (Life Technologies, a division of American Type Culture Collection, Manassas, VA), cultured as described above. BstI-resistant DNAs were amplified in DH5α Cells were transfected with pLXSN-Neu were serum-starved 36 h after LipofectAMINE treatment and 12–16 h later stimulated with 20 ng/ml EGF for 2 min. Receptors were immunoprecipitated with either ERCT antibody or 5 μg/ml anti-HER2/Neu mAb 1 as described above. Immune complexes were boiled in 100 μl of 2× SDS-polyacrylamide gel electrophoresis sample buffer and samples and samples were resolved on duplicate gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked with either 5% milk or 3% BSA in Tris-buffered saline and 0.1% Tween 20 and probed with ERCT antibody or anti-phosphotyrosine antibody RC20 (Transduction Laboratories, Lexington, KY), respectively. 3H- or 125I-labeled anti-EGF antibody 11 or anti-HER2/Neu antibody C-18 (both from Santa Cruz Biotechnology, Santa Cruz, CA), respectively.

**EGF Binding Assay**—Subconfluent cells in 12-well plates were incubated in triplicate with 15 μCi/ml (20 ng/ml) 125I-EGF (ICN Pharmaceuticals, Irvine, CA) in DMEM, 0.1% BSA, and 20 μl of DMEM/BSA/HEPES for 2 h at 4 °C. Duplicate wells were incubated with a 1000–2000-fold excess of unlabeled EGF to measure nonspecific binding. Wells were washed three times with PBS and solubilized in 0.5 ml of 1 N HCl, and assayed. To correct for cell-surface receptor expression, duplicate plates were also incubated with 5 μg/ml anti-EGFR mAb 1 plus mAb 3 or mAb 11 in DMEM/BSA/HEPES for 2 h at 4 °C. They were then washed, and 0.5 μCi/well 125I-protein A (ICN Pharmaceuticals) was added for 1 h. Some wells were incubated without antibody to correct for nonspecific binding of protein A. Cells were washed and processed for radioactive counting and protein determination as described above. 3H- or 125I-labeled anti-EGF antibody 11 or anti-HER2/Neu antibody C-18 (both from Santa Cruz Biotechnology, Santa Cruz, CA), respectively.

**Dimerization Assay**—Subconfluent cells grown in 100-mm dishes were serum-starved, incubated with or without 200 ng/ml EGF in DMEM/BSA/HEPES for 2 h at 4 °C, washed, and incubated with 5 mM sodium orthovanadate, 50 μM sodium molybdate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotonin. Lysates were centrifuged for 10 min at 14,000 rpm, and supernatant protein concentrations were determined using the Bio-Rad protein assay. Samples (120–200 μg) were immunoprecipitated and immunoblotted with anti-EGFR antibody ERCT (a gift from Dr. H. Shetlon Earp, University of North Carolina, Chapel Hill, NC) as described previously (40). To detect cell-surface EGFR, live cells were incubated with 5 μg/ml EC domain-specific mAb 1 plus mAb 3 or mAb 11 (Lab Vision Corp.) for 2 h in 3% BSA/PBS/Ca2+/Mg2+ at 4 °C. Washed cells were harvested in Triton X-100 lysis buffer and centrifuged. Immunoprecipitates were collected with protein G-agarose beads prior to Western blot analysis.

**Ligand Stimulation**—Subconfluent cells were serum-starved for 16–24 h in nonessential amino acids-supplemented DMEM and stimulated for 2 min with human recombinant EGF (Upstate Biotechnology, Inc., Lake Placid, NY), TGF-α, or HB-EGF (R&D Systems, Minneapolis, MN). Cells were washed with PBS and lysed in Triton X-100 lysis buffer, and EGFR was immunoprecipitated by incubating 350–500 μg of protein sequentially with ERCT antibody and protein G-agarose. Immune complexes were boiled in 100 μl of 2× SDS-polyacrylamide gel electrophoresis sample buffer, and samples were resolved on duplicate gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked with either 5% milk or 3% BSA in Tris-buffered saline and 0.1% Tween 20 and probed with ERCT antibody or anti-phosphotyrosine antibody RC20 (Transduction Laboratories, Lexington, KY), respectively. 3H- or 125I-labeled anti-EGF antibody 11 or anti-HER2/Neu antibody C-18 (both from Santa Cruz Biotechnology, Santa Cruz, CA), respectively.

**Dimerization Assay**—Subconfluent cells grown in 12-well plates were incubated in triplicate with 15 μCi/ml (20 ng/ml) 125I-EGF (ICN Pharmaceuticals, Irvine, CA) in DMEM, 0.1% BSA, and 20 μl of DMEM/BSA/HEPES for 2 h at 4 °C. Duplicate wells were incubated with a 1000–2000-fold excess of unlabeled EGF to measure nonspecific binding. Wells were washed three times with PBS and solubilized in 0.5 ml of 1 N HCl, and equal portions were used to measure bound radioactivity and protein concentration. Protein was trichloroacetic acid-precipitated, resuspended in 12.5 μl of 1 N NaOH, neutralized with 12.5 μl of 1 N HCl, and assayed. To correct for cell-surface receptor expression, duplicate plates were also incubated with 5 μg/ml anti-EGFR mAb 1 plus mAb 3 or mAb 11 in DMEM/BSA/HEPES for 2 h at 4 °C. They were then washed, and 0.5 μCi/well 125I-protein A (ICN Pharmaceuticals) was added for 1 h. Some wells were incubated without antibody to correct for nonspecific binding of protein A. Cells were washed and processed for radioactive counting and protein determination as described above. 3H- or 125I-labeled anti-EGF antibody 11 or anti-HER2/Neu antibody C-18 (both from Santa Cruz Biotechnology, Santa Cruz, CA), respectively.
EGFR Mutagenesis and Ligand Binding

RESULTS

EGFR Mutagenesis—We replaced selected clusters of charged or aromatic amino acids with alanine residues throughout the EC region, focusing particularly on sequences previously implicated in ligand binding or exhibiting significant homology among ErbB family members. For each of 20 clusters, 3–5 charged or aromatic residues were simultaneously replaced with alanine in a full-length human EGFR cDNA using the method of Kunkel (42). Cytomegalovirus-directed expression vectors containing mutant or WT EGFR proteins or no insert were then transfected into CHO cells, and Geneticin-resistant colonies were selected. Colonies were screened for cell-surface receptor expression via an enzyme-linked immunosorbent assay (ELISA) using a mixture of mAb 1 and mAb 3 monoclonal antibodies to the receptor’s EC domain (mAb 1 and mAb 3 or mAb 11). Cells were then lysed, and protein G-agarose precipitated immune complexes were blotted with ERCT antibody.

Expression of Mutant EGFR Proteins—Fig. 2 shows Western blot analysis of representative CHO cell clones expressing WT EGFR or EGFR subdomain IV mutants. Total EGFR was detected by blotting immunoprecipitated receptor with an antibody (ERCT) directed against its C-terminal sequences. Cell-surface EGFR was identified by incubating live cells with monoclonal antibodies to the receptor’s EC domain (mAb 1 + mAb 3 or mAb 11). Cells were then lysed, and protein G-agarose-precipitated immune complexes were blotted with ERCT antibody.

As expected, WT EGFR was predominantly detected as a diffuse band of ~170 kDa corresponding to fully glycosylated, surface-localized EGFR (Fig. 2, upper panel). Mutant EGFR proteins were instead typically present in two forms: the broad 170-kDa band and a discrete band of 160 kDa that likely corresponds to nascent EGFR still retained in intracellular membranes (44, 45). The ratio of 170/160-kDa forms varied with different mutants and was highest in the case of the single-point mutant mt23. The deletion mutant ΔEN was predictably smaller and detected as both diffuse 160- and discrete 120-kDa bands. For all mutants, similar receptor profiles were detected using multiple cell clones and an alternative anti-C terminus antibody. Incubation of live cells with either mAb 1 + mAb 3 or mAb 11 confirmed cell-surface localization of the diffuse 170- or 160-kDa (ΔEN) receptor bands. The latter analyses confirmed roughly comparable cell-surface expression of wild-type and mutant EGFR proteins.

EGF-induced Tyrosine Phosphorylation—To assess whether subdomain IV mutations affected EGF-induced tyrosine phosphorylation, serum-starved cells were stimulated for 2 min with 10 ng/ml EGF. EGFR proteins were then immunoprecipitated with ERCT antibody and immunoblotted with anti-phosphotyrosine antibody RC20. To compare the levels of the various EGFR proteins, blots were reprobed (or in some cases, parallel blots were probed) with anti-receptor antibodies. As shown in Fig. 3 (upper panel), EGF treatment rapidly induced tyrosine phosphorylation of WT EGFR as well as the 170-kDa surface form of mt23, mt24, mt26, and mt27. Interestingly, basal phosphorylation of mt24 was observed in the absence of EGF, but was not seen with the WT receptor. Most striking,
Comparing the various mutants, only mt23 (the single-point mutant) bound normalized levels of $^{125}$I-EGF that were identical to those of WT EGFR. This is consistent with the fact that mt23 also displayed normal levels of receptor activation. In contrast, normalized binding to mt24, mt26, and mt27 was reduced to 30–40% of WT receptor levels. Most important, however, binding of $^{125}$I-EGF to the unresponsive mt25 and ΔEN receptors was reduced to near background levels. These results indicate that subdomain IV sequences in the region from amino acids 507 to 589 influence ligand binding, with residues altered in mt25 having the greatest effect.

**Dimerization of WT and Mutant EGFRs and Transphosphorylation of HER2/Neu**—Since subdomain IV of ErbB proteins has been implicated in receptor dimerization (32), we compared the ability of WT and selected mutant receptors to dimerize following EGF treatment. Cells were exposed to 200 ng/ml EGF for 2 h, and receptor oligomers were stabilized using bis(sulfosuccinimidyl) suberate, a noncleavable cross-linking reagent (46). Cells were then lysed, and receptor complexes were immunoprecipitated with ERCT antibody. Western blotting of the immunoprecipitates with ERCT antibody revealed dimeric ligand-dependent dimers of WT EGFR and mt27 (Fig. 6), with dimers of mt23 and mt24 also observed (data not shown). Consistent with its reduced cell-surface expression and EGF binding, mt26 showed detectable but diminished ligand-induced dimerization. In contrast, dimerization of mt25 was undetectable, whereas a low basal level of ΔEN dimerization was not enhanced by ligand treatment.

For a more sensitive, functional dimerization assay, we examined the ability of WT and mutant receptors to transphosphorylate HER2/Neu in an EGF-dependent manner. Stable WT and mutant clones were transiently transfected with low levels of pLXSN-Neu (39) and 48 h later treated with 20 ng/ml EGF for 2 min. The respective ErbB receptors were then immunoprecipitated with ERCT antibody or anti-HER2/Neu mAb 1, and the levels of receptor phosphotyrosine and protein were compared by Western blotting. Consistent with previous results, EGF treatment induced marked tyrosine phosphorylation of WT, mt23, mt24, and mt27 EGFR proteins and lower phosphorylation of mt26, but failed to induce phosphorylation of mt25 and ΔEN.

HER2/Neu displayed basal phosphorylation in most cell populations, with the exception of the ΔEN and vector clones, which showed lower HER2 expression. This basal phosphorylation (typical in transient transfectants with high levels of expression) was nevertheless increased by EGF in cells stably expressing WT EGF-R or most mutant receptors. In contrast, EGF-induced phosphorylation of HER2 was not observed with mt25 and ΔEN. These results are consistent with the EGFR activation profile and further indicate that none of the subdomain IV mutations inhibit EGFR homo- or heterodimerization independent of effects on ligand binding.

**DISCUSSION**

Previous evidence predominantly implicated EGFR subdomain III, and especially its C-terminal portion, in ligand binding. CNBr mapping identified a single receptor fragment that was cross-linked to iodinated EGF (28). Encompassing residues 294–543, this fragment spanned subdomain III and the N-terminal portion of subdomain IV. In agreement, a slightly smaller proteolytic fragment bound EGF or TGF-α with dissociation constants similar to those of intact soluble EGFR (47).

More refined mapping of epitopes for EGF-competing antibodies identified a continuous 14-amino acid sequence (residues 351–364) within subdomain III (28), and a 47-amino acid fragment encompassing this epitope was cross-linked to EGF (27). These physical analyses were corroborated by a functional as-
EGFR or the indicated mutant receptors (e.g. panel), CHO cell clones expressing either low or high levels of wild-type receptors were treated with or without 100 ng/ml EGF (vector). Shown is the ratio of bound EGF to cell-surface EGFR levels. Lower (cpm/mg of protein) represent the average of triplicate samples.

Proteins, clones were incubated for 2 h with or without 5 mM mAb to quantitate cell-surface expression of EGFR proteins, clones were incubated for 2 h with or without 5 ng/ml mAb 11 and then for 1 h with lz-125I-protein A and processed for counting. Values (cpm/mg of protein) represent the average of triplicate determinations. Middle panel, to quantitate cell-surface expression of EGFR, clones were incubated for 2 h with or without 5 ng/ml mAb 11 and then for 1 h with lz-125I-protein A and processed for counting. Values (cpm/mg of protein) represent the average of triplicate samples. Lower panel, shown is the ratio of bound EGF to cell-surface EGFR levels. V, vector.

Fig. 5. EGF binding to wild-type and mutant receptors. Upper panel, CHO cell clones expressing either low or high levels of wild-type EGFR or the indicated mutant receptors (e.g. 23 = Mt23) were incubated with lz-125I-EGF for 2 h, and samples were processed for counting as described under "Experimental Procedures." Nonspecific binding was assessed in the presence of a 1000–2000-fold excess of unlabeled EGF. Values (cpm/mg of protein) represent the average of triplicate determinations. Middle panel, to quantitate cell-surface expression of EGFR proteins, clones were incubated for 2 h with or without 5 ng/ml mAb 11 and then for 1 h with lz-125I-protein A and processed for counting. Values (cpm/mg of protein) represent the average of triplicate samples. Lower panel, shown is the ratio of bound EGF to cell-surface EGFR levels. V, vector.

say in which subdomain III of chicken EGFR was replaced with the corresponding region of the human receptor. In contrast to human EGFR, the chicken receptor binds murine EGF with 100-fold reduced affinity. However, a chicken chimera that contained subdomain III of human EGFR bound EGF in a manner indistinguishable from that of the mammalian receptor (24). Collectively, these results argue strongly that subdomain III contributes major ligand-binding determinants.

Although the involvement of subdomain II in ligand binding has considerable support, other portions of the extracellular region have also been implicated. Conservation of sequence between subdomains I and III implies that the former might contribute to ligand binding. Indeed, a mutant EGFR devoid of subdomain I exhibited a 10-fold lower affinity for EGF compared with the wild-type receptor (22). Subdomains II and IV may also contribute to ligand/receptor interactions. Harte and Gentry (23) reported that soluble EC domain receptors containing subdomain II insertions of 4–5 hydrophobic or charged amino acids bound EGF normally, but not TGF-α. This finding raised the possibility that the affected sites selectively regulate the binding of different EGF family members. Moreover, two mutants containing subdomain II or IV insertions at sites equidistant from the center of subdomain III showed increased ligand binding. Affinity was unaltered, leading the investigators to suggest that disruption of these sites promoted the binding of more than one ligand molecule.

Additional evidence implicates subdomain IV or the following juxtamembrane sequence. A naturally occurring R497K mutant, first identified in human lymphocytes and several cancer cell lines, displayed only low affinity binding of TGF-α, but retained both high and low affinity binding of EGF (43). (Surprisingly, the less conservative replacement of Arg-497 with alanine in the present study (mt23; Fig. 1) did not appreciably affect the binding of either EGF or TGF-α, nor did it affect receptor activation by these ligands.) On the other hand, a more disruptive insertion of 23 amino acids in the EC juxtamembrane region reduced EGF binding, although receptor dimerization was still observed (48).

Here, we provide the strongest evidence to date that residues within EGFR subdomain IV influence interactions between the receptor and its ligands. The deletion mutant ΔEN, which lacks 72 amino acids (positions 518–589) from the C-terminal half of subdomain IV, but retains the juxtamembrane sequence, bound little to no EGF. Furthermore, several mutant EGFR proteins, each of which contained three to five alanine substitutions in the subdomain region from amino acids 507 to 589, all displayed 60–70% reductions in EGF binding compared with the wild-type receptor. Most significantly, mt25, which harbored only five charged/aromatic-to-alanine substitutions in a 7-amino acid stretch in the middle of subdomain IV (and was encompassed by the ΔEN deletion), bound dramatically reduced levels of EGF, TGF-α, and HB-EGF. The latter was particularly important since the ΔEN deletion is predicted to leave unpaired cysteines at both the beginning and end of the.
deletion (49, 50) and hence could dramatically affect the conformation of the EC region.

How the critical subdomain IV residues contribute to ligand binding is unclear. Possibly, these residues directly contribute to a ligand-binding pocket or instead participate in interactions with subdomain III that indirectly affect the conformation of the binding pocket. The latter possibility is consistent with models of the EGFR EC region that predict that the four subdomains comprise largely independent structures that interact to form a binding site through EC domain folding (21). Interestingly, alignment of subdomains II and IV relative to cysteine residues reveals that the residues altered in mt25 fall within a 14-amino acid sequence not represented (i.e., a gap) in subdomain II (23). Thus, subdomain IV may contribute unique motifs required for proper ligand/receptor interactions. Consistent with this speculation, Summerfield et al. (51) mapped the receptor site proximal to the C terminus of bound EGF to the interface between subdomains III and IV.

An alternative explanation for our results, suggested by the crystal structure of an insulin-like growth factor I receptor EC domain fragment (50), is that the mt25 mutations might introduce a negative influence on ligand binding. Thus, a critical interaction between corresponding portions of that receptor’s EC region appears to involve the insertion of a tryptophan (Trp-492) from the cysteine-rich region (equivalent to EGFR subdomain IV) into a hydrophobic core formed by the preceding subdomain. Since EGFR contains an equivalent conserved tryptophan, this residue may play a critical role in interactions between EGFR subdomains III and IV. EGFR Trp-492 is located <30 amino acids upstream of the mt25 mutations, raising the possibility that even the limited alterations of mt25 constrain the ability of Trp-492 to interact with subdomain III. This “negative influence” scenario may be consistent with findings that subdomain III alone bound EGF and TGF-α with a Kd indistinguishable from that of the soluble EGFR EC domain (6, 47). It may also be consistent with the modest, ligand-dependent phosphorylation of the ΔEN mutant observed in Fig. 3. On the other hand, the soluble EC domain has markedly reduced affinity for ligands compared with the full-length receptor (41), raising the possibility that the contributing role of mt25 sequences would not be apparent with EC domain fragments.

Interestingly, our mutational analysis did not identify residues that affect receptor dimerization without affecting ligand binding. Thus, only mutants with negligible EGFR binding failed to homodimerize or transphosphorylate HER2/Neu in a ligand-dependent manner. Nevertheless, a variety of evidence supports a role for subdomain IV in ErbB interactions. This includes the finding that a naturally occurring mutant EGFR that is expressed in various human epithelial tumors and lacks subdomains I and II heterodimerized with HER2/Neu (52). Additionally, deletion of cysteines and surrounding residues within subdomain IV of HER2/Neu resulted in constitutive oligomerization of this ErbB receptor (32). Since we did not perform saturation mutagenesis of subdomain IV, residues critical for dimerization but not ligand binding may have been missed. Alternatively, dimerization may depend on the contribution of multiple motifs distributed throughout the extracellular and intracellular regions of ErbB receptors. Clearly, further studies of ErbB EC domains and their roles in ligand binding and receptor homo- and heterodimerization are warranted.

Acknowledgments—We thank Ron Swanstrom for advice regarding mutagenesis strategies and Noreen Luetteke for a critical evaluation of this manuscript.

REFERENCES

1. Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Lieberman, T. A., Schlessinger, J., Francke, U., et al. (1985) Science 230, 1129–1139.
2. Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J., and Shoyab, M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5065–5069.
3. Plowman, G. D., Green, J. M., Calos, J. M., Carlson, G. W., Rothwell, V. M., and Buckley, S. (1993) Nature 366, 473–475.
4. Earp, H. S., Dawson, T. L., Li, X., and Yu, H. (1995) Breast Cancer Res. Treat. 35, 115–132.
5. Heldin, C. H. (1995) Cell 80, 213–223.
6. Lemmon, M. A., Tu, Z., Ladbury, J. E., Zhou, M., Finchais, D., Lax, I., Engelsing, D. M., and Schlessinger, J. (1995) EMBO J. 16, 281–294.
7. Hurwitz, D. R., Emanuel, S. L., Nathan, M. H., Sarver, N., Ulrich, A., Felder, S. L., Lax, I., and Schlessinger, J. (1991) J. Biol. Chem. 266, 22035–22043.
8. Sluskin, A., Lemmon, M. A., Ulrich, A., and Schlessinger, J. (1994) J. Biol. Chem. 269, 9752–9759.
9. Graus-Porta, D., Beeri, R. R., Daly, J. M., and Hynes, N. E. (1997) EMBO J. 16, 1647–1655.
10. Riese, D. J., Bermingham, Y., Raaij, T. M. V., Buckley, S., Plowman, G. D., and Schlessinger, J. (1996) Oncogene 12, 345–353.
11. Toyoda, H., Komurasaki, T., Uchida, D., Takayama, Y., Isebe, T., Okuyama, T., and Hanada, K. (1995) J. Biol. Chem. 270, 7485–7500.
12. Lee, D. C., Fenton, S. E., Berkowitz, E. A., and Hissung, M. A. (1995) Pharmacol. Rev. 47, 51–85.
13. Beeri, R. R., and Hynes, N. E. (1996) J. Biol. Chem. 271, 6071–6076.
14. Ellenius, K., Paul, S., Allison, G., Sun, J., and Klagsbrun, M. (1997) EMBO J. 16, 1268–1278.
15. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacs, S. S., Luo, Y., Trail, G., Hu, S., Sibergh, S. M., Levy, R. B., Koski, R. A., Lu, H. S., and Yarden, Y. (1992) Cell 69, 559–572.
16. Wen, D., Suggs, S. V., Karunagaran, D., Liu, N., Cupples, R. L., Luo, T., Janssen, A. M., Ben-Baruch, N., Trelilinger, D. B., Jacobsen, Y. L., Meng, S.-Y., Lu, H. S., Hu, S., Chang, D., Yang, W., Yanighahrda, D., Koski, R. A., and Yarden, Y. (1994) Mol. Cell. Biol. 14, 1909–1919.
17. Chang, H., Riese, D. J., Gilbert, W., Stern, D. F., and McManus, U. J. (1997) Nature 387, 509–512.
18. Zhang, D., Slivkovsky, M. X., Mark, M., Frantz, G., Akita, R., Sun, Y., Hillan, K., Crowley, C., Brush, J., and Godowski, P. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9562–9567.
19. Carraway, K. L., III, Weber, J. L., Unger, M. J., Ledesma, J., Yu, N., Gammann, M., and Lai, C. (1997) Nature 387, 512–516.
20. Takah, E., Waterman, H. C., Levkovitz, G., Karunagaran, D., Lari, S., Ratakin, B. J., and Yarden, Y. (1996) Mol. Cell. Biol. 16, 5276–5287.
21. Ulrich, A., and Schlessinger, J. (1990) Cell 61, 203–212.
22. Lax, I., Bellot, P., Houseger, A. M., Schmidt, A., Ulrich, A., Givol, D., and Schlessinger, J. (1990) Cell Regul. 1, 173–188.
23. Harte, M. T., and Gentry, L. E. (1995) Arch. Biochem. Biophys. 322, 378–389.
24. Lax, I., Bellot, P., Howk, R., Ulrich, A., Givol, D., and Schlessinger, J. (1989) EMBO J. 8, 421–427.
25. Lax, I., Fischer, R., Ng, C., Segre, J., Ullrich, A., Givol, D., and Schlessinger, J. (1991) *Cell Regul.* 2, 337–345
26. Lax, I., Burgess, W. H., Bellot, F., Ullrich, A., Schlessinger, J., and Givol, D. (1988) *Mol. Cell. Biol.* 8, 1831–1834
27. Wu, D., Wang, L., Chi, Y., Sato, G. H., and Sato, J. D. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 3151–3155
28. Wu, D., Wang, L., Sato, G. H., West, K. A., Harris, W. R., Crabb, J. W., and Sato, J. D. (1989) *J. Biol. Chem.* 264, 17469–17475
29. Wu, D., Wang, L., Sato, G. H., West, K. A., Harris, W. R., Crabb, J. W., and Sato, J. D. (1989) *J. Biol. Chem.* 264, 17469–17475
30. Zhou, M., Felder, S., Rubenstein, M., Hurwitz, D. R., Ullrich, A., Lax, I., and Schlessinger, J. (1993) *Biochemistry* 32, 8193–8198
31. Siegel, P. M., Dankort, D. L., Hardy, W. R., and Muller, W. J. (1994) *Mol. Cell. Biol.* 14, 7068–7077
32. Siegel, P. M., and Muller, W. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 8878–8883
33. Spivak-Kroizman, T., Rotin, D., Pinchasi, D., Ullrich, A., Schlessinger, J., and Lax, I. (1992) *J. Biol. Chem.* 267, 8056–8063
34. Qian, X., Dougall, W. C., Hellman, M. E., and Greene, M. I. (1994) *Oncogene* 9, 1507–1514
35. Qian, X., O'Rourke, D. M., Fei, Z., Zhang, H. T., Kao, C. C., and Greene, M. I. (1999) *J. Biol. Chem.* 274, 574–583
36. Bass, S. H., Mulkerrin, M. G., and Wells, J. A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 4496–4502
37. Wells, J. A. (1991) *Methods Enzymol.* 202, 390–411
38. Briley, G. P., Hissong, M. A., Chiu, M. L., and Lee, D. C. (1997) *Mol. Biol. Cell* 8, 1619–1631
39. Biese, D. J., van Raaaij, T. M., Plewman, G. D., Andrews, G. C., and Stern, D. F. (1995) *Mol. Cell. Biol.* 15, 5770–5776
40. Schroeder, J. A., and Lee, D. C. (1996) *Cell Growth Differ.* 9, 451–464
41. Kashles, O., Yarden, Y., Fischer, R., Ullrich, A., and Schlessinger, J. (1991) *Mol. Cell. Biol.* 11, 1454–1463
42. Kunkle, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 488–492
43. Morita, T., Koo, M., Ghe, S., Speek, L., and Koc, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 1027–1022
44. Soderquist, A. M., and Carpenter, G. (1984) *J. Biol. Chem.* 259, 1586–1594
45. Mayes, E. L., and Waterfield, M. D. (1984) *EMBO J.* 3, 531–534
46. Partis, M. D., Griffiths, D. G., Roberts, G. C., and Beechey, R. B. (1983) *J. Protein Chem.* 2, 263–277
47. Kohda, D., Odaka, M., Lax, I., Kawasaki, H., Suzuki, K., Ullrich, A., Schlessinger, J., and Inagaki, F. (1993) *J. Biol. Chem.* 268, 1976–1981
48. Sorokin, A. (1995) *Oncogene* 11, 1531–1540
49. Abe, Y., Odaka, M., Inagaki, F., Lax, I., Schlessinger, J., and Kohda, D. (1998) *J. Biol. Chem.* 273, 11150–11157
50. Garrett, T. P., McKern, N. M., Lou, M., Frenkel, M. J., Bentley, J. D., Lovrecz, G. O., Ellem, T. C., Cosgrove, L. J., and Ward, C. W. (1998) *Nature* 394, 395–399
51. Summerfield, A. E., Hudd, A. K., Lukas, T. J., Guyer, C. A., and Staros, J. V. (1996) *J. Biol. Chem.* 271, 19656–19658
52. O'Rourke, D. M., Nute, E. J., Davis, J. G., Wu, C., Lee, A., Murali, R., Zhang, H. T., Qian, X., Kao, C. C., and Greene, M. I. (1998) *Oncogene* 16, 1197–1207