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Human epidermal growth factor receptor residue covalently cross-linked to epidermal growth factor

(ligand/affinity labeling/cross-linking)

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ABSTRACT **An epidermal growth factor (EGF) receptor monoclonal antibody (mAb), mAb LA22, was used to analyze the covalent coupling of human EGF receptors to mouse EGF by the amine-reactive cross-linking agent disuccinimidyl suberate. A soluble M_r 105,000 truncated form of the receptor secreted by A-431 epidermoid carcinoma cells and consisting of the ligand-binding extracellular domain was cross-linked to ^{125}I-labeled EGF. Digestion of this complex with an endoproteinase that specifically cleaves at the COOH side of glutamyl residue released a single radiolabeled glycosylated fragment of M_r 18,000 that reacted with mAb LA22. As the epitope for mAb LA22 resided between Ala-351 and Asp-364 of the mature receptor, this result localized the cross-linked receptor residue(s) to the 47-amino acid interval from Phe-321 to Glu-367. The receptor residue(s) involved in the covalent coupling of rat ^{125}I-labeled transforming growth factor α was similarly localized to this region of the receptor. This receptor interval, which included two glycosylated asparaginyl residues at positions 328 and 337, contained but three amino acid residues that were potentially reactive with disuccinimidyl suberate: Lys-332, Lys-333, and Lys-336. Characterization of mAb LA22-reactive ^{125}I-EGF-labeled receptor fragments generated by an endoproteinase specific for the COOH side of lysyl residue placed the NH_2 termini of the two smallest fragments between the glycosylated residues Asn-328 and Asn-337. These results indicated that disuccinimidyl suberate cross-linked the NH_2 group of EGF residue Asn-1 to the human EGF receptor residue Lys-336. Our results further suggest that EGF and transforming growth factor α, two members of the EGF family of peptide growth factors, interact with closely apposed or identical features of the receptor.**

Epidermal growth factor (EGF) receptor, which is responsible for transducing the biological effects of EGF, is a M_r 170,000 glycoprotein (1–4) containing two functional domains linked by a transmembrane region (5). The extracellular domain is heavily glycosylated (6–8) and possesses a single ligand-binding site (9), and the intracellular portion of the receptor includes a protein-tyrosine kinase domain (5). Ligand binding to the extracellular domain stimulates the kinase activity, which is thought to be essential for signal transduction, normal receptor trafficking, ligand-induced mitogenesis, and transformation (10–14). The extracellular domain of the EGF receptor contains 51 cysteine residues, which are clustered in two regions. These cysteine-rich regions divide the extracellular region of the receptor into four subdomains: domain I is the NH_2-terminal region, domains II and IV are the cysteine-rich regions, and domain III is the region between the two cysteine-rich regions (15). The feature of cysteinyl residue clustering in the extracellular domain is also shared by the receptors for insulin, insulin-like growth factor I, nerve growth factor, and low-density lipoprotein (1).

Transforming growth factor α (TGF-α) is a 50-amino acid polypeptide growth factor (M_r 6000) that is 35% homologous with EGF, including 6 conserved cysteine residues (16, 17). TGF-α and EGF have very similar 3-dimensional structures as determined by 2-dimensional NMR studies (18), and TGF-α has been shown to have a similar affinity for the EGF receptor as EGF (19). Although Massague *et al.* (20) reported that TGF-α interacted with a M_r 60,000 protein as well as the EGF receptor, it is generally believed that TGF-α exerts its biological activity via the EGF receptor. EGF and TGF-α have been demonstrated to have similar activities; however, there are several cases where these two ligands exhibit differences in biological activity such as in the release of calcium from murine calveria and rat fetal long bones, angiogenesis, and the proliferation of human epidermal cells (for reviews, see refs. 21 and 22).

To better understand the mechanism of receptor activation, the localization of the ligand-binding region of the receptor is very important. Lax *et al.* (23) have cleaved with cyanogen bromide human EGF receptors cross-linked to ^{125}I-labeled EGF and have used anti-peptide antibodies to identify a labeled M_r 50,000 receptor fragment. This peptide included residues 284–543, which was largely located in receptor domains III and IV. They then further localized the ligand-binding region to domain III by functional analysis of chimeric chicken/human receptor molecules (24). Similarly, our research has indicated that domain III participated in ligand binding by identifying within this region the epitopes of three EGF-competitive monoclonal antibodies (mAbs) (25).

In an effort to determine further the functional significance of domain III we analyzed ^{125}I-EGF covalently cross-linked to the EGF receptor-related protein (ERRP) secreted by A-431 human epidermal cells (26). The cross-linker used was the homobifunctional coupling agent disuccinimidyl suberate (DSS), which acylates free primary NH_2 groups (27, 28). After a series of enzymatic digestions and immunoprecipitation with immobilized EGF receptor mAb LA22 (25), we have identified Lys-336 as a cross-linked ERRP residue. This suggests that the NH_2-terminal region of domain III is intimately involved in ligand binding. Cross-linking with ^{125}I-labeled TGF-α gave similar results: immobilized LA22 antibodies precipitated a radiolabeled M_r 18,500 fragment generated by an endoproteinase that specifically cleaved at the COOH side of glutamyl groups (Endo Glu-C) (29). This result supports the idea that TGF-α and EGF recognize identical or overlapping binding sites on the EGF receptor.

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Abbreviations: EGF, epidermal growth factor; TGF, transforming growth factor; ERRP, EGF receptor-related protein; DSS, disuccinimidyl suberate; mAb, monoclonal antibody; ^{125}I-EGF and ^{125}I-TGF-α, ^{125}I-labeled EGF and TGF α, respectively; Endo Glu-C and Endo Lys-C, endoproteinases; Endo-F, endoglycosidase F.

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MATERIALS AND METHODS

Iodination of EGF and TGF-α. Receptor grade EGF (Upstate Biotechnology, Lake Placid, NY) and synthetic rat TGFα (Peninsula Laboratories) were iodinated with Na ^{125}I (Amersham) by the chloramine-T method (30) to specific activities of 4.0×10^5 cpm/ng and 3.5×10^5 cpm/ng, respectively.

The Culture of A-431 Cells and Preparation of ERRP. A-431 cells were cultured in Dulbecco's modified Eagle's/F-12 medium, and ERRP was purified from A-431-conditioned medium with an affinity column of immobilized 528 IgG, as described (25).

Cross-Linking and Enzymatic Digestion. ERRP was incubated with ^{125}I-EGF or ^{125}I-TGF-α at a ratio of 1000:1 (wt/wt) for 2 hr at room temperature. DSS (Pierce) was added to a final concentration of 1 mM. After 20 min, the reaction was stopped by the addition of reduction and alkylation buffer (1 M Tris, pH 8.4/6 M guanidine hydrochloride/5 mM EDTA). The detailed procedures for reduction and alkylation were described by Crabb *et al.* (31). The alkylated cross-linked material was dialyzed in either Endo Glu-C digestion buffer (0.05 M NH_4HCO_3, pH 7.8) or Endo Lys-C digestion buffer (0.1 M NH_4HCO_3, pH 9.0). Cleavage by Endo Glu-C (Boehringer Mannheim) (29) occurred at an enzyme/protein ratio of 1:40 (wt/wt) for 14 hr at room temperature, and cleavage by Endo Lys-C (Boehringer Mannheim) (33) was performed at a ratio of 1:40 (wt/wt) for 10 hr at 37°C.

Immunoprecipitation of ERRP Fragments. The protease activity in digests was inactivated by boiling for 2 min, and immunoprecipitation was performed by adding LA22 IgG2a (25) conjugated to Affi-Gel 10 (Bio-Rad) (1 mg of antibody/ml of gel). After a 1½-hr incubation at 22°C, the supernatant was removed, and the gel was washed twice with 1% bovine serum albumin in phosphate-buffered saline and twice by phosphate-buffered saline or, for further cleavage, by either Endo Glu-C digestion buffer or Endo Lys-C digestion buffer. The gels washed with digestion buffers were boiled for 2 min to release peptides from the antibodies. Proteases were then added to the gel slurry and incubated for 10 hr at room temperature for Endo Glu-C and at 37°C for Endo Lys-C.

SDS/PAGE. SDS/PAGE was done under reducing conditions according to Laemmli (32). The gels were dried and exposed to XAR-5 film (Kodak). Cross-linked fragments were detected by autoradiography at −70°C.

RESULTS

ERRP secreted by A-431 cells contains the entire extracellular domain of the EGF receptor (5). To identify receptor cross-linking sites, ^{125}I-EGF was covalently cross-linked to ERRP with DSS (Fig. 1), and the ^{125}I-EGF–ERRP complex was cleaved by proteases. The reduced and alkylated ^{125}I-EGF–ERRP complex was first digested with Endo Glu-C, which specifically cleaves at the COOH side of glutamyl residues. The enzyme was then heat-inactivated, and the cleavage products were immunoprecipitated with immobilized LA22, an anti-EGF receptor mAb, the epitope of which is located within a 14-amino acid sequence from Ala-351 to Asp-364 in the mature receptor (25). Bound peptides were released by boiling in SDS/PAGE sample buffer and were analyzed by electrophoresis and autoradiography. A single radiolabeled M_r 18,000 band was detected (Fig. 2, lane 1), indicating that the epitope of mAb LA22 was close to the cross-linked receptor residue(s). To ensure complete cleavage, the M_r 18,000 fragment was treated again with Endo Glu-C. No further shift in mobility or decrease in band intensity was seen (Fig. 2, lane 2).

As reported (25), the smallest Endo Glu-C ERRP fragment containing the mAb LA22 epitope consisted of the 47 amino acids from Phe-321 to Glu-367 (Fig. 3); this M_r 15,000 peptide included a M_r of ≈9000 in N-linked carbohydrates distributed between Asn-328 and Asn-337. The M_r 18,000 fragment

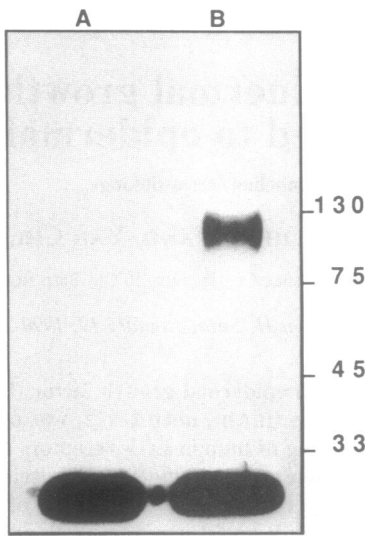

FIG. 1. Specificity of cross-linking of ^{125}I-EGF to ERRP. One microgram of ERRP was incubated with 1 ng of ^{125}I-EGF with (lane A) or without (lane B) 1 μg of cold EGF for 2 hr at 37°C, and 1 mM of DSS was added subsequently for 20 min at 4°C. Reactions were terminated with SDS/PAGE sample buffer, and reaction products were electrophoresed in 7% polyacrylamide gel and detected by autoradiography.

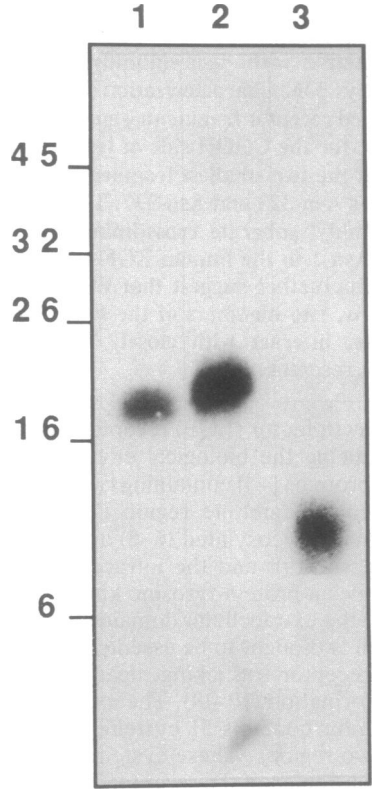

FIG. 2. Immunoprecipitation of the cross-linked fragments produced from ^{125}I-EGF–ERRP complexes by Endo Glu-C. The reduced and carboxymethylated cross-linked complex was digested by Endo Glu-C and immunoprecipitated by immobilized mAb LA22. The immunoprecipitated fragment was either run on 15% SDS/PAGE (lane 1), or subjected to further treatment with Endo Glu-C (lane 2) or Endo-F (lane 3) before electrophoresis. The dried gel was exposed to x-ray film. Numbers at left represent M_r markers ($\times 10^{-3}$).

observed here most likely consisted of this 47-amino acid peptide with a further M_r 3000 contributed by EGF residues Asn-1–Glu-24. To confirm this idea, the M_r 18,000 fragment was treated with a mixture of endoglycosidase F and N-glycosidase F (Endo-F). Endo-F treatment reduced the molecular weight of the radiolabeled fragment by ≈9000 (Fig. 2, lane 3). This result was consistent with our previous observation that the Endo Glu-C fragment was shifted from M_r 15,000 to M_r 6000 by Endo-F treatment (25).

According to the amino acid sequence deduced from cloned EGF receptor cDNA (5), this 47-amino acid fragment includes only three lysine residues—Lys-332, Lys-333, and Lys-336—which are potentially available to react with the amine-specific cross-linker DSS (Fig. 3). To determine which of these residues were cross-linked to EGF, ^{125}I-EGF–ERRP complexes were cleaved with Endo Lys-C, which specifically cleaves peptide bonds on the COOH side of lysyl residues. At least five LA22-reactive radiolabeled fragments were detected (Fig. 4, lane A), which indicated incomplete digestion. The two smallest Endo Lys-C fragments immunoprecipitated by immobilized LA22 antibodies were a peptide of ≈M_r 12,500 and a prominent radiolabeled M_r 18,000 peptide. When deglycosylated with Endo-F, both bands shifted in M_r by ≈1000–2000 (Fig. 4, lane B). As this shift did not equal or exceed 9000, both ERRP fragments evidently contained only a single glycosylated residue, Asn-337, which was the most proximal to the LA22 epitope (Ala-351–Asp-364) of the two glycosylation sites between Phe-321 and Glu-367 (Fig. 3). This experiment further indicated that the oligosaccharides N-linked to Asn-328 and Asn-337 contributed M_r 7000–8000 and M_r 1000–2000, respectively, to the mass of ERRP.

Because the NH_2 termini of the M_r 12,500 and the M_r 18,000 ERRP fragments resided between Asn-328 and Asn-337, the cleavage specificity of Endo Lys-C indicated that His-334 was the NH_2-terminal residue of both fragments (Fig. 3) and that Lys-336 was the sole receptor residue that could be cross-linked by DSS to EGF. As EGF possesses no lysyl residues, it was most likely cross-linked to ERRP via the NH_2 group of Asn-1. Although the COOH-terminal residues of the ERRP fragments could not be identified umambiguously, the molecular masses of the ^{125}I-EGF–peptide complexes were consistent with the M_r 12,500 fragment terminating with Lys-372 or Lys-375 and the M_r 18,000 fragment ending at Lys-407 (Fig. 3). The existence of the radiolabeled M_r 18,000 fragment suggested that the cleavage of ERRP by Endo Lys-C at residues 372 and 375 was very inefficient.

To eliminate the possibility that cleavage by Endo Lys-C was occurring at residues other than lysine, the immunoprecipitated fragments generated with Endo Lys-C were digested with Endo Glu-C. Three radiolabeled fragments were expected if Endo Lys-C were acting in a specific manner: an M_r 18,000 band corresponding to receptor fragments from either Phe-321 or Asp-323 to Glu-367, which would result from any fragment bearing the LA22 epitope terminating NH_2 terminal to Lys-333 and COOH terminal to Lys-372 (Fig. 3); an M_r 9000 receptor fragment corresponding to His-334–Glu-367, which would be produced from fragments with His-334 as the NH_2-terminal amino acid and any lysyl residue

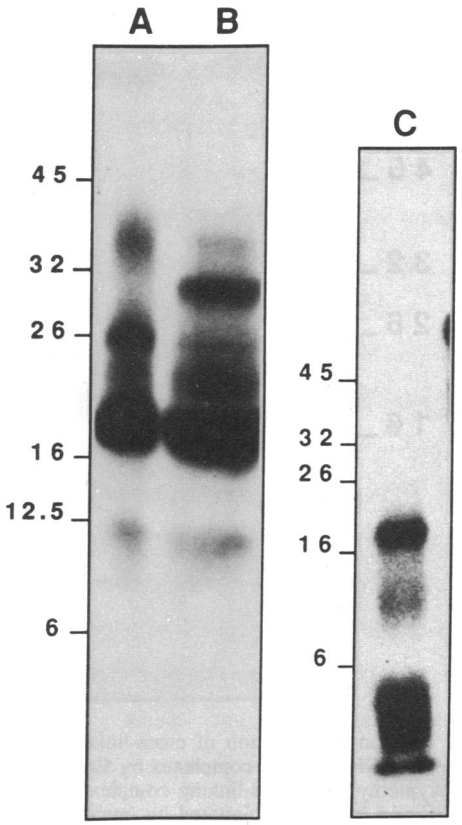

FIG. 4. Cleavage of ^{125}I-EGF–ERRP complexes by Endo Lys-C. Reduced and carboxymethylated cross-linked complexes were subjected to cleavage by Endo Lys-C. The resulting fragments were immunoprecipitated by immobilized mAb LA22, and the immunoprecipitate was either directly run on 15% SDS/PAGE (lane A) or further treated with Endo-F (lane B) or Endo Glu-C (lane C), respectively, before electrophoresis. The dried gel was exposed to x-ray film. Numbers at left of lanes represent M_r markers ($\times 10^{-3}$).

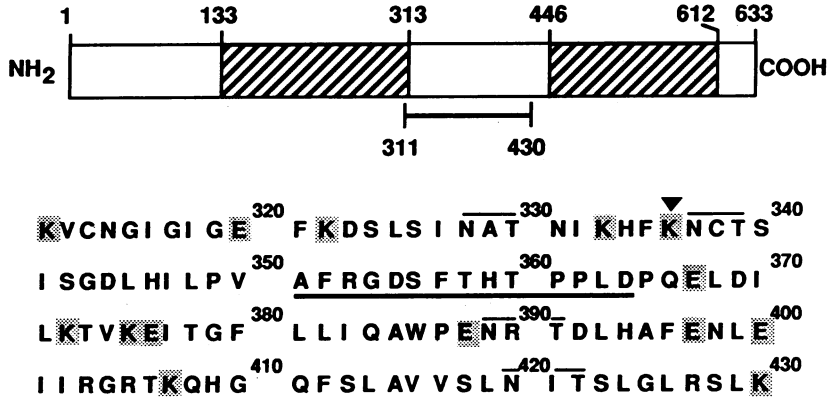

FIG. 3. Schematic representation of ERRP. The cysteine-rich regions from Cys-133 to Cys-313 and from Cys-446 to Cys-612 are hatched. The amino acid sequence (in one-letter code) deduced from ERRP cDNA (5) for residues 311–430, donated by the bar, is presented below. Consensus sequences for potential asparagine-linked glycosylation sites are overlined. The epitope for mAb LA22 is underlined (25). Glutamic acid (E) and lysine (K) residues are boxed. Lys-336 is marked by an arrowhead.

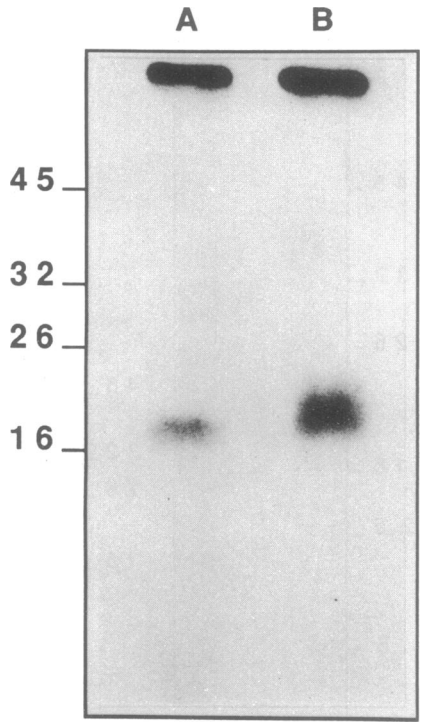

FIG. 5. Immunoprecipitation of cross-linked fragments generated from ^{125}I-TGF-α–ERRP complexes by Endo Glu-C. Reduced and carboxymethylated cross-linking complexes were cleaved by Endo Glu-C and immunoprecipitated by immobilized mAb LA22. The immunoprecipitate was either directly run on 15% SDS/PAGE (lane 2) or subjected to further retreatment by Endo Glu-C before electrophoresis (lane 1). Radiolabeled fragments were detected by autoradiography. M_r markers ($\times 10^{-3}$) are at left.

at the COOH terminus (Fig. 3); and a M_r 3000 peptide representing EGF residues 25–53 that would be released by the protease. The Endo Glu-C digest was resolved by SDS/PAGE into three bands of M_r 18,000, 9500, and 3000 (Fig. 4, lane C). Thus, the original Endo Lys-C cleavages occurred specifically at lysyl residues, even though the reaction did not go to completion.

We applied the same strategy to investigate the EGF receptor binding site for TGF-α. After cross-linking ^{125}I-TGF-α to ERRP with DSS, the complex was subjected to reduction and alkylation and Endo Glu-C cleavage. An M_r 18,500 band was detected by autoradiography after immunoprecipitation with LA22 antibodies (Fig. 5). Complete cleavage was confirmed by the lack of further digestion with additional Endo Glu-C. This antibody-reactive Endo Glu-C-generated ERRP fragment was most likely the same as that cross-linked with EGF. That is, the band was composed of a 47-residue receptor fragment (Phe-321–Gln-367) of M_r 15,000 with a M_r $\approx$3000 TGF-α fragment from Val-1 to Gln-26 or Lys-29 to Ala-50. These data indicated that the receptor site cross-linked with TGF-α was also in close proximity to the LA22 epitope and was included within the same 47-amino acid ERRP fragment.

DISCUSSION

We have previously used the truncated M_r 105,000 form of the EGF receptor secreted by A-431 human epidermoid carcinoma cells to define the epitopes of three EGF-competitive mAbs including mAb LA22 (25). In this report, we used the truncated form of the receptor to identify the receptor cross-linking site for EGF. The truncated receptor (ERRP) is identical in amino acid sequence to the extracellular domain of the full-length human EGF receptor, but it includes 17 nonidentical COOH-terminal amino acids starting from residue 617 (5). Because ERRP was reactive with both EGF and conformation-dependent EGF receptor mAbs raised against intact receptors (unpublished results), ERRP retained the conformation of the extracellular domain of the intact receptor.

Cleavage with Endo Glu-C of ERRP covalently linked to ^{125}I-EGF with DSS resulted in a single LA22-reactive fragment of M_r 18,000 (Fig. 2). This fragment was previously identified as a 47-amino acid peptide consisting of residues Phe-321 to Glu-367 (25). The colocalization to this ERRP fragment of the cross-linked receptor residue(s) and the LA22 epitope, encompassed by the 14 amino acids from Ala-351 to Asp-364 (25), indicated that the lysyl residues at positions 322, 333, and 336 were the only potential sites of covalent linkage to EGF. EGF, which possesses no lysine residues (2), had only the NH_2 group of Asn-1 available for cross-linking by DSS. Thus, it was apparent that EGF was covalently linked by DSS through its NH_2 terminus to one or more of three receptor lysyl residues.

Unlike Endo Glu-C, the protease Endo Lys-C generated several immunoreactive ERRP fragments covalently linked to ^{125}I-EGF. This incomplete digestion may have resulted from the modification of some lysyl residues by DSS, by the rapid self-digestion of the enzyme, or by the differential recognition of lysyl residues in the substrate (33). Identification of all of the partial digestion products was not possible due, in part, to a lack of information about the mass of the N-linked glycans at Asn-389 and Asn-420. However, the smallest two LA22-reactive fragments, with apparent M_r values of 12,500 and 18,000, were identified as having NH_2-terminal residues between Asn-328 and Asn-337 based on decreases in M_r of 1000–2000 resulting from Endo-F treatment. As the carbohydrates NH_2-linked to Asn-328 and Asn-337 together contributed M_r 9000 to the mass of ERRP (Fig. 2), the M_r 1000–2000 shift in the LA22-reactive ERRP fragments indicated that both fragments contained only the glycosylated asparaginyl residue nearest the LA22 epitope. Between the ERRP residues Asn-328 and Asn-337 were two Endo Lys-C cleavage sites, Lys-333 and Lys-336, both of which had been established as potential cross-linked residues. However, the recovery of the radiolabeled M_r 12,500 and M_r 18,000 Endo Lys-C-generated ERRP fragments was consistent only with enzymatic cleavage occurring at Lys-333 and cross-linking to ^{125}I-EGF through Lys-336 (Fig. 3).

Winkler et al. (34) proposed two models for binding of TGF-α to the EGF receptor based on the differential effect of EGF receptor antibody 13A9 on binding of EGF and TGF-α to the receptor. Under one model the EGF receptor could have two distinct binding sites for TGF-α and EGF. These two sites could be spatially separated such that competition between the two ligands was caused by conformation changes induced by binding. Under the second model the two ligands bind to sites that are partially or completely overlapping. Our results with protease-digested covalent ^{125}I-TGF-α–ERRP complexes described above and the observation that four EGF receptor mAbs, including mAb LA22, competitively inhibited ^{125}I-TGF-α binding to intact A-431 cells (unpublished results) suggest that EGF and TGF-α recognize overlapping or identical regions of the receptor.

The EGF receptor mAb LA22, which recognizes the 14-amino acid receptor sequence from Ala-351 to Gln-364, mutually competed with EGF for binding to human EGF receptors (25). These results suggested that this 14-amino acid receptor sequence or immediately adjacent sequences participated in the ligand-binding region of the receptor. The identification of Lys-336 as a receptor residue that can be covalently cross-linked to EGF further substantiates this conclusion, as this lysyl residue is only 15 amino acid residues from the LA22 epitope (Fig. 3). In addition, the

cross-linking of TGF-α and EGF to the same 47-residue Endo Glu-C ERRP fragment, Phe-321–Gln-367, implicates this region of the receptor in binding TGF-α. Thus, the NH_2-terminal part of receptor subdomain III plays an important role in interactions with EGF and at least one other member of the EGF family of peptide growth factors.

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