Identification of Residues of the Epidermal Growth Factor Receptor Proximal to Residue 45 of Bound Epidermal Growth Factor*

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A triple mutant of murine epidermal growth factor (mEGF), N1Q/H22Y/R45K-mEGF, was constructed by site-directed mutagenesis, expressed, purified, and characterized for use in an affinity cross-linking study to identify aminoacyl residues of the EGF receptor adjacent to a residue in the carboxyl-terminal domain of bound EGF thought to be important in distinguishing between EGF and transforming growth factor-α in their recognition by the receptor. Cyclization of Gln1 to form pyroglutamate (pE) limited the site of cross-linking in the mutant to Lys45, permitting identification of receptor residues that are proximal to this residue of bound EGF. The resulting N1pE/H22Y/R45K-mEGF was shown to be comparable to wild-type mEGF in receptor binding and stimulation of receptor autophosphorylation. 121I-Labeled N1pE/H22Y/R45K-mEGF was reacted with the heterobifunctional cross-linking reagent sulfo-N-succinimidyl-4-(fluorosulfonyl)benzoate, and the resulting modified EGF was incubated with A431 membrane vesicles bearing EGF receptors. Incubation resulted in specific cross-linking of the labeled N1pE/H22Y/R45K-mEGF to EGF receptors. The resulting cross-linked complex was then partially purified, denatured, reduced, and carboxamidomethylated. Digestion with endoprotease LysC resulted in a unique radiolabeled peptide that could be immunoprecipitated using antibodies to mEGF. This immunoprecipitated fragment was purified by gel electrophoresis and subjected to microsequencing. The resulting sequence was matched to that of a LysC fragment of the receptor, which begins with Thr464 and is near the interface of receptor subdomains III and IV. Loss of signal at cycle 2 suggests that the point of attachment of cross-linked N1pE/H22Y/R45K is Lys465 of the receptor.

Epidermal growth factor (EGF)1 and TGFα mediate their biological responses by binding to the EGF receptor in target cells (reviewed in Refs. 1-3). While the three-dimensional structures of mEGF (4, 5), hEGF (6), and TGFα (7) are known from high field NMR studies, no three-dimensional structure is yet available for the EGF receptor. Much structural information about the receptor has been gleaned, however, by mapping onto the derived primary sequence of the receptor (8) information from chemical, immunochemical, and molecular biological studies of the receptor (reviewed in Refs. 9 and 10). In the present study, we focus on the extracellular, ligand-binding domain of the receptor, identifying residues of the receptor adjacent to the carboxyl-terminal domain of bound EGF, a region shown to be important in the discrimination of EGF and TGFα by the EGF receptor (11). Murine EGF has no lysyl residues (12), so the α-amino terminus is the only primary amino group. We exploited this aspect of the chemistry of the hormone in a study in which the N terminus of wild-type mEGF was modified with SSFSB, a heterobifunctional cross-linking reagent, and affinity cross-linked to the receptor (13). The site of cross-linking was identified by Edman degradation of a purified fragment of the receptor containing the cross-link to EGF, revealing Tyr101 as a receptor residue adjacent to the N terminus of bound EGF (14).

In order to expand the range of information that could be derived from this affinity cross-linking approach to identify residues of the EGF receptor adjacent to the carboxyl-terminal domain of bound EGF, we designed, expressed, and characterized a triple mutant of mEGF, N10/H22Y/R45K-mEGF. The mutation of Arg10 → Lys introduced a lysyl residue into the carboxyl-terminal domain as a target for cross-linking. The Asn1 → Gln mutation allowed chemical cyclization of the resulting Gln1 to pGlu, blocking the amino terminus both to reaction with SSFSB and to Edman degradation. The mutation of His22 → Tyr, the homologous residue in hEGF (15), resulted in the conservative replacement of the only His in mEGF. His residues are known to react rapidly with sulfo-N-succinimidyl esters to form transient N-acyl imidazoles (16), an undesirable side reaction for our affinity cross-linking experiments. Incubation of A431 cell membrane vesicles with SSFSB-modified N1pE/H22Y/R45K-mEGF resulted in a high yield of affinity cross-linking of the mutant mEGF to the receptor. Purification of the resulting complex, followed by denaturation, reduction, carboxamidomethylation, and limit digestion with endoprotease LysC, resulted in a fragment of the receptor cross-linked to the full-length, reduced, and carboxamidomethylated mu-

1 The abbreviations used are: EGF, epidermal growth factor; hEGF, human EGF; mEGF, murine EGF; AcCN, acetonitrile; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; pGlu, pyroglutamate; SSFSB, sulfo-N-succinimidyl-4-(fluorosulfonyl)benzoate; TGFα, transforming growth factor-α; WGL, wheat germ lectin; RIPA, radiolmmune precipitation; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
tant mEGF. This adduct was purified and subjected to Edman degradation, resulting in the identification of Lys465 as the site of cross-linking to the receptor. In addition to identifying this specific site, our strategy of preparing site-directed mutants of mEGF blocked at the amino terminus by a pGlu and containing a single Lys provides a general approach to mapping residues adjacent to bound EGF.

EXPERIMENTAL PROCEDURES

Materials—wild-type mEGF was prepared as described (17). EGF species were radiolabeled by the method of Carpenter and Cohen (18). Shed membrane vesicles were prepared from A431 cells by modifications (19) of the method of Cohen et al. (20). SSFSB was synthesized as described (14). EGF antiserum was a generous gift from Prof. S. Cohen (Vanderbilt University). HPLC-grade acetonitrile and methanol were from Burdick and Jackson. Other reagents were from BioRad, as was Bio-Gel P-2 gel filtration resin (fine; 45–50 μm). Sequencing membranes were from Beckman. SDS was from Serva. Thioglycolic acid (sodium salt) was from Sigma; methylamine (40% solution) was from Aldrich. All other reagents were purchased from Sigma and were reagent grade or better.

Affinity Cross-linking of 125I-N1pE/H22Y/R45K-mEGF to EGF Receptor Residues Near the C Terminus of Bound EGF

EXPERIMENTAL PROCEDURES

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Preparation of N1Q/H22Y/R45K-mEGF—Site-directed mutagenesis (21) was used to mutate the codons for Asp1 to Gln and for Arg45 to Lys in a mEGF synthetic gene previously mutated to contain the His22→ Tyr base changes (22). Mutations were confirmed by dideoxy DNA sequencing (23), and the mutated mEGF DNA was cloned into the pN-III-ompA3 (24) secretion-expression vector. The mutant gene product, N1Q/H22Y/R45K-mEGF, was expressed in Escherichia coli HB101 by induction at late log phase of a 1-liter culture of freshly transformed bacteria with 200 μM isopropyl-β-D-thiogalactopyranoside (final concentration) for 2 h at 30°C. Expressed mutant mEGF was isolated from the osmotic shock fluid and purified to homogeneity as described (22), with the exception that Sep-Pak C18 cartridges were substituted for the LiChroprep RP-C8 column used in the initial purification step. Approximately 10–12 ml of osmotic shock fluid was loaded onto each Sep-Pak cartridge pre-equilibrated with 95:5 water:AcCN buffered with 0.1% triethylamine/acetate, pH 6.0. Each cartridge was washed with 3 ml of equilibration buffer and eluted with 4 ml of 50:50 water:AcCN buffered with 0.1% triethylamine/acetate, pH 6.0. All eluates were pooled, lyophilized, redissolved in 20 mM HEPS, pH 7.4, and subjected to semipreparative reverse-phase HPLC purification (22). Purified N1Q/H22Y/R45K-mEGF was stored in water at −70°C.

Preparation of N1pE/H22Y/R45K-mEGF from N1Q/H22Y/R45K-mEGF—Purified N1Q/H22Y/R45K-mEGF (~250 μg) was incubated under argon in 0.30 ml of 5% (v/v) aqueous acetic acid at 42°C for 22 h with continuous stirring, and the reaction was terminated by addition of 0.60 ml of water and injected onto a 4.6 × 220-mm Brownlee C8 AquaPore RP-300 (7 μm) HPLC column equilibrated with 98:2 AcCN/water to obtain a 4.6 × 220-mm Brownlee C8 AquaPore RP-300 (7 μm) HPLC column equilibrated with 98:2 AcCN/water (10 ml).

Characterization of N1Q/H22Y/R45K-mEGF and N1pE/H22Y/R45K-mEGF—Competition binding assays were performed as described (22). EGF receptor autoradiography assays on suspensions of A431 membrane vesicles were performed as described by Rousseau et al. (22). In a 50-μl reaction, 50 pmol of [125I]EGF was incubated with 1 mg of A431 membrane vesicles in a total volume of 0.6 ml of incubation buffer containing 20 mM NaCl, 0.1% sodium azide, 0.1% Triton X-100, 0.1% SDS, 10% glycerol, 10 mM NaCl, 10 mM NaCl, pH 7.4, and 10 mM MgCl2. The second and third RIPA buffer resuspensions were followed by sonication (10 min at 50% power, 50% duty cycle), using a Branson cell disruptor 200 sonifier. After each RIPA buffer extraction, the suspension was centrifuged at 120,000 × g for 30 min at 4°C, and the resulting supernatant was collected. The pooled supernatants were applied to 4 ml of 1:1 suspension of hydrated WGL resin. The resin was incubated and washed, and the cross-linked EGF-receptor complex, together with any other bound glycoproteins, was eluted four times with N,N′,N′-triacetylchitotriose and dialyzed against 4 × 3.5 liters of water as described (14). Dimerization of the 125I-N1pE/H22Y/R45K-mEGF-EGF Complex—Aliquots of dicylated WGL eluate were repeatedly added to each of two 1 ml Reacti-Vials (Pierce) and dried using a Speed Vac concentrator (Savant). The dried residue in each vial was dissolved in 0.20 ml of freshly prepared digest buffer (8 M urea, 100 mM Tris-HCl, pH 8.5, 1 mM EDTA, 20 mM methylamine) and reduced and carboxamidomethylated as described (14, 26). Each sample was diluted with 20 ml of water and digested with endoproteinase LysC by stirring at 33°C with a total of 2.7 μg of LysC divided into three aliquots added over a period of 23 h. Digestion was terminated by addition of 5-fold molar excesses of aprotinin, leupeptin, and N-tosyl-L-lysylchloromethyl ketone.

Immunoprecipitation and Purification of 125I-N1pE/H22Y/R45K-mEGF-linked EGF Receptor Fragment—The combined LysC digests, diluted with 1.5 volumes of immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 0.02% SDS, 150 mM NaCl), were rechromatographed on a 50 ml column of protein A-Sepharose (Pharmacia) equilibrated with 0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl, and 1 M NaCl, and the resulting eluate was concentrated and concentrated, as described (14).

Sequence of the EGF-linked EGF Receptor Fragment—Methanol (1.5 volumes) was added to the concentrated sample, which was then applied to a Protein A-Sepharose support (Beckman) in small aliquots, as described (14). Sequencing of the samples was performed on an Applied Biosystems/Perkin-Elmer Procise 400 fluorescence gel-permeation chromatograph and in-line microbore chromatograph equipped with a phenylthiohydantoin-amino acid separation program provided by the manufacturer. Data acquisition and analysis were carried out with Applied Biosystems 610A software run on a Macintosh computer.

RESULTS AND DISCUSSION

Leu47 of EGF is highly conserved among mammalian species and has been shown to be a critical residue for the high affinity recognition of EGF by the receptor (24, 27). It would, therefore, be of great interest to identify residues of the receptor close to Leu47 in bound EGF. Residues near Leu47, but not forming part of the same binding surface, would, therefore, be good candidates for cross-linking to identify residues in this functionally important part of the receptor. Molecular modeling of mEGF using the published coordinates from the solution structures solved by high field NMR (4, 5) suggested that Arg46 is such a residue. In addition, previous studies had shown that substitution of Lys for Arg46 had no effect on EGF binding to the receptor (28); therefore, Arg46 was mutated to Lys. To avoid undesirable side reaction of the cross-linker with His42, His42 was mutated to Tyr, the corresponding residue in hEGF (15). To limit the site of cross-linking to Lys128, Asn128 was mutated to Gin, and methods were developed to cyclize the resulting Gin128 to pGlu and to purify hormone with cyclized amino terminus. N1Q/H22Y/R45K-mEGF was expressed as a periplasmic pro-
to SDS-PAGE under conditions in which free \(125\text{I}-\text{N1pE/H22Y/R45K-mEGF}\) was not electrophoresed off the gel, showed that the cyclization of the amino-terminal Gln of the EGF mutant for each (data not shown), confirming the mutations and the cyclization of Gin\(^1\) to pGlu. Comparisons of \(\text{N1pE/H22Y/R45K-mEGF}\) with wild-type mEGF in competition binding and auto-cyclization of Gln1 to pGlu, blocking Edman degradation of the \(125\text{I}-\text{N1pE/H22Y/R45K-mEGF}\) linked receptor before (lane 1) and after (lane 2) LysC digestion. Lanes 3–6, \(125\text{I}-\text{N1pE/H22Y/R45K-mEGF}\) linked receptor before (lanes 5 and 6) and after (lanes 3 and 4) LysC digestion. Complete digestion of cross-linked receptor was achieved, resulting in two distinct radiolabeled bands. Comparison of \(125\text{I}-\text{N1pE/H22Y/R45K-mEGF}\) control sample lanes with cross-linked receptor lanes identifies the more slowly migrating (upper) band, which appears just below the 6 kDa marker, as the \(125\text{I}-\text{N1pE/H22Y/R45K-mEGF}\) linked EGF receptor fragment.

LysC, resulting in a single major \(125\text{I}-\text{N1pE/H22Y/R45K-mEGF}\) receptor fragment (Fig. 2).

Because the only Lys in \(\text{N1pE/H22Y/R45K-mEGF}\) is modified by SSFSB, digestion with LysC was expected to yield intact, reduced, and carboxyamidomethylated \(\text{N1pE/H22Y/R45K-mEGF}\) linked to a fragment of the receptor. We therefore used a polyclonal antiserum to mEGF to immunoprecipitate this adduct from the digest. Purification of the cross-linked fragment from other components of the immunoprecipitate was carried out by electrophoresis in SDS-Tricine gels. The location of the adduct was determined by obtaining a phosphorimage of the wet gel (Fig. 2), and the cross-linked fragment was recovered by elution of the excised gel band and was applied to sequencing membranes.

The purification step with the greatest loss is immunoprecipitation (data not shown), most likely due to the polyclonal antiserum used for immunoprecipitation, which was raised to native, wild-type mEGF. It is likely that only a small fraction of the IgG's in the antiserum recognized the reduced, denatured, and carboxyamidomethylated \(125\text{I}-\text{N1pE/H22Y/R45K-mEGF}\) linked to a fragment of the receptor.

Table I lists the data from Edman sequencing of the two preparations, identifying residues in seven of the first eight sequencing cycles, with no identifiable residue in the second cycle of each run. Sequence analysis of the receptor fragment-\(125\text{I}-\text{N1pE/H22Y/R45K-mEGF}\) adduct was greatly simplified by the cyclization of the amino-terminal Gin of the mEGF mutant to pGlu, blocking Edman degradation of the \(125\text{I}-\text{N1pE/H22Y/R45K-mEGF}\), so that only residues of the cross-linked receptor fragment were detected. Alignment of the experimentally determined sequence with the derived sequence of the EGF receptor (8) identifies the receptor fragment as a LysC fragment beginning with Thr\(^{\text{464}}\), i.e. cleaved after Lys\(^{\text{463}}\). The absence of an identifiable signal in cycle two, which corresponds to Lys\(^{\text{465}}\) of the receptor sequence, combined with the observation that this site is not cleaved by LysC, argues strongly that Lys\(^{\text{465}}\) is the point of attachment of the receptor to Lys\(^{\text{465}}\) of bound \(\text{N1pE/H22Y/R45K-mEGF}\).
Based on internal homologies, the extracytoplasmic ligand binding domain of the receptor has been divided into four subdomains: the amino-terminal subdomain I, a Cys-rich subdomain II, subdomain III, which has notable sequence homology with subdomain I, and subdomain IV, a second Cys-rich subdomain that has notable homology with subdomain II (29).

We have shown by affinity labeling and protein sequencing that the α-amino terminus of bound mEGF is proximal to Tyr311 in subdomain I of the receptor (14). Considerable indirect evidence has accumulated that subdomain III plays a major role in EGF binding. Epitopes for three monoclonal antibodies that block EGF binding were localized to residues between Ala351 and Asp364 in subdomain III (30), although subsequent studies showed that these residues were not directly involved in EGF binding (31). Furthermore, domain swapping experiments between human and chicken EGF receptors implicated both the amino and carboxyl halves of subdomain III, as well as subdomain I, in EGF binding (32, 33).

A second natural ligand for the EGF receptor is TGFα, a peptide that has the same overall three-dimensional fold as EGF (7), with some sequence homology, including the placement of the three disulfide bridges (34). While EGF and TGFα compete for binding, and therefore share the same, or overlapping binding sites on the receptor, the biological responses to the two factors are not identical (3). For this to be the case, the receptor must discriminate between bound EGF and bound TGFα. In a recent study, van Zoelen and co-workers (35) found that mutation of Arg45 → Ala in hEGF significantly increased its affinity for the chicken EGF receptor, relative to wild-type hEGF. The significance of this finding rests on the earlier observation that while hEGF and hTGFα bind with similar affinity to the human EGF receptor, the chicken EGF receptor binds them with different affinities (36).

The residue of hTGFα that corresponds to Arg45 of EGF is Ala (34). Therefore, while both hEGF and hTGFα bind to the human EGF receptor with high affinity, the ability of the chicken receptor to distinguish between their binding and the finding that the Arg45 → Ala mutation essentially abolished this differential affinity (35) suggest that residues near Lys45 in the three-dimensional structure of the human receptor may be responsible for the ability of the receptor to discriminate between EGF and TGFα.

Lys45, which we have identified in this study as proximal to Lys39 of bound N1pE/H22Y/R45K-mEGF, is near the interface of subdomains III and IV of the extracytoplasmic domain of the receptor. The present study provides the first direct evidence for the juxtaposition of specific residues in subdomain III with a specific site on the bound hormone. Furthermore, our approach of affinity cross-linking with SSFSB site-directed mutants of mEGF, each bearing a single lysyl residue and having Asn1 mutated to Gln for subsequent cyclization to pGlu, provides a general strategy for a more complete mapping of the EGF binding site, and by extension, the binding sites of other small polypeptide hormones that can be similarly manipulated.

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REFERENCES

1. Carpenter, G., and Cohen, S. (1990) J. Biol. Chem. 265, 7709–7712

2. Fry, M. J., Panayiogou, G., Book, G. W., and Waterfield, M. D. (1993) Protein Sci. 2, 1785–1797

3. Derynck, R. (1992) Adv. Cancer Res. 58, 27–52

4. Montelione, G. T., Wuthrich, K., Burgess, A. W., Nice, E. C., Wagner, G., Gibson, K., and Scheraga, H. A. (1989) J. Mol. Biol. 213, 236–249

5. Kohda, D., and Inagaki, F. (1992) Biochemistry 31, 11928–11939

6. Hommel, U., Dudgeon, T. J., Fallon, A., Edwards, R. M., and Campbell, I. D. (1991) Biochemistry 30, 8881–8888

7. Hommel, U., Harvey, T. S., Driscoll, P. C., and Campbell, I. D. (1992) J. Mol. Biol. 227, 271–282

8. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tatum, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. W., Whittle, N., Waterfield, M. D., and Seeburg, P. H. (1984) Nature 309, 418–425

9. Carpenter, G., and Wahl, M. I. (1990) in Handbook of Experimental Pharmacology (Sporn, M. B., and Roberts, A. B., eds) Vol. 95, pp. 69–71, Springer-Verlag, Berlin

10. Staros, J. V., and Guyer, C. A. (1995) Ciba Found. Symp. 197, 269–282

11. Kramer, R. H., Lenferink, A. E. G., van Bueren-Koorneef, I. L., van der Meer, A., van der Pol, M. L. M., and van Zoelen, E. J. J. (1994) J. Biol. Chem. 269, 8078–8071

12. Savage, C. R. Jr., Inagami, T., and Cohen, S. (1972) J. Biol. Chem. 247, 7612–7621

13. Woltjer, R. L., Wedas-Henderson, L., Papayanopoulous, I. A., and Staros, J. V. (1990) Biochemistry 33, 7341–7346

14. Woltjer, R. L., Lukas, T. J., and Staros, J. V. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7801–7805

15. Gregory, H. (1975) Nature 257, 325–327

16. Anjaneyulu, P. S. R., and Staros, J. V. (1987) Int. J. Pept. Protein Res. 30, 117–124

17. Savage, C. R., Jr., and Cohen, S. (1972) J. Biol. Chem. 247, 7609–7611

18. Carpenter, G., and Cohen, S. (1976) J. Biol. Chem. 251, 1592–1597

19. Rousseau, D. L. Jr., and Staros, J. V. (1992) Biochemistry 31, 5928–5934

20. Rousseau, D. L. Jr., Staros, J. V., and Beechem, J. M. (1995) Biochemistry 34, 14508–14518

21. Cohen, S., Ushiro, H., Stoscheck, C., and Chinkers, M. (1982) J. Biol. Chem. 257, 1523–1531

22. Kunkei, T. A., Jr., Zaid, D. J., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382

23. Rousseau, D. L., Geyer, C. A., Beth, A. H., Papayanopoulous, I. A., Wang, B., Wu, R., Mrzczkowski, B., and Staros, J. V. (1993) Biochemistry 32, 7983–7993

24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

25. Ray, P., Moy, F. J., Montelione, G. T., Liu, J. F., Narang, S. A., Scheraga, H. A., and Wu, R. (1988) Biochemistry 27, 7289–7295

26. Stone, K. L., Lopresti, J. B., Crawford, J. B., DeAngelis, R., and Williams, K. R. (1989) in A Practical Guide to Protein and Peptide Purification for Microsequencing (Matsudastra, P. T., ed) pp. 31–74, Academic Press, San Diego, CA

27. Matsuura, R. K., Yette, M. L., Stevens, A., and Niyogi, S. K. (1991) J. Cell. Biochem. 46, 242–249

28. Stockman, S. R., Tatadi, D. K., and Niyogi, S. K. (1992) J. Cell. Biochem. 50, 35–42

29. Lax, I., Burgess, W., Beloff, F., Ullrich, A., Schlessinger, J. (1988) Mol. Cell. Biol. 8, 1831–1834

30. 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

31. Brown, P. M., Debanne, M. T., Grothe, S., Bergman, D., Caron, M., Kay, C., and O'Connor-McCourt, M. D. (1989) Eur. J. Biochem. 225, 223–233

32. Lax, I., Beloff, F., Howk, R., Ullrich, A., and Givol, D. (1990) EMBO J. 9, 421–427

33. Lax, I., Fischer, R. N., Nog, C., Segre, J., Ullrich, A., and Givol, D. (1991) EMBO J. 1, 337–345

34. Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y., and Goeddel, D. V. (1984) Cell 38, 287–297

35. van de Poll, M. M. L. M., Lenferink, A. E. G., van Vugt, M. L. M., and van Zoelen, E. J. J. (1991) J. Biol. Chem. 270, 22337–22343

36. Lax, I., Jutisson, A., Howk, R., Sap, J., Beloff, F., Winkler, M., Ullrich, A., Vennstrom, B., Schlessinger, J., and Givol, D. (1988) Mol. Cell. Biol. 8, 1970–1978