The Interaction of an Epidermal Growth Factor/Transforming Growth Factor α Tail Chimera with the Human Epidermal Growth Factor Receptor Reveals Unexpected Complexities*

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It has been assumed that substitution of homologous regions of transforming growth factor α (TGF-α) into epidermal growth factor (EGF) can be used to probe ligand-receptor recognition without detrimental effects on ligand characteristics for the human EGF receptor (EGFR). We show that a chimera of murine (m) EGF in which the carboxyl-terminal tail is substituted for that of TGF-α (mEGF/TGF-α(44–50)) results in complex features that belie this initial simplistic assumption. Comparison of EGF and mEGF/TGF-α(44–50) in equilibrium binding assays showed that although the relative binding affinity of the chimera was reduced 80–200-fold, it was more potent than EGF in mitogenesis assays using NR6/HER cells. This superagonist activity could not be attributed to differences in ligand processing or to binding to other members of the c-erbB family. It appeared to be due, in part, to choice of an EGFR-overexpressing target cell where high receptor number compensated for the low affinity of the ligand; it also appeared to be related to the ability of the chimera to activate the EGFR tyrosine kinase. Thus, when EGF autophosphorylation was measured, mEGF/TGF-α(44–50) was more potent than EGF, despite its low affinity. When tested using chicken embryo fibroblasts, substitution of the TGF-α carboxyl-terminal tail into mEGF failed to enhance its binding affinity for chicken EGFRs; however, the chimera was intermediate in potency between TGF-α and mEGF in mitogenesis assays. Our results suggest a contextual requirement for EGFR recognition which is ligand-specific. Further, the unpredictable responses to chimeric ligands underline the complex nature of the processes of ligand recognition, receptor activation, and the ensuing cellular response.

The epidermal growth factor receptor (EGFR) ligand family, of which EGF and transforming growth factor α (TGF-α) are the best characterized members, bind to their cognate receptor in a mutually competitive fashion and have association constants in the range of 10⁹–10¹⁰ M⁻¹ (1). The structural homology of these proteins arises from the presence of six highly conserved cysteine residues and two glycine residues (2). Of the remaining residues, only four show significant homologies: the invariant Arg⁴¹ and Tyr⁵⁷ and the semiconserved Leu⁴⁷ and Tyr⁵⁸ (EGF numbering). Mutational and chemical analyses of Arg⁴¹ have demonstrated that this is probably the most important receptor contact residue, contributing at least 3 orders of magnitude to the overall binding free energy (3). Leu⁴⁷, which lies in the flexible C-tail of the growth factor, is also important; both site-directed mutagenesis (4) and controlled proteolysis (5) have shown that loss of Leu⁴⁷ decreases receptor binding affinity by around 2 orders of magnitude. The requirement of aromaticity at positions 13 (6) and 37 (7, 8) is less stringent; however, these residues also appear to contribute to receptor binding.

The fact that the EGF ligand family exhibits only a limited pattern of common surface residues suggests that their binding may not be identical. This possibility is readily apparent for the chicken EGFR, which displays differential affinity for EGF and TGF-α (9). In the case of the human EGFR, which displays comparable affinities for EGF and TGF-α (1, 9), the ability of the monoclonal antibody 13A9 (10) to prevent binding of TGF-α, but not EGF, to the human EGFR suggests that their binding is not identical. This is further supported by the observation that insertional mutagenesis into domain II of EGFR reduces binding of TGF-α but not EGF (11).

We have explored the basis for the differential recognition of EGF and TGF-α and have shown that the B-loop β-sheet is a major distinguishing feature that contributes to ligand recognition by the chicken EGFR (12). Further, the “hinge residue” that lies at the end of the β-sheet is a ligand-specific residue for the human EGFR (13). From a consideration of the nature of the hinge residue and its relation to the sequence of the B-loop β-sheet, we have postulated that the EGF family can be subdivided into EGF-like and TGF-α-like and that this is of functional significance in receptor recognition (13). Thus, the receptor binding surface comprises a discontinuous epitope involving residues from the structural core of the EGF motif as well as the C-tail.

Although structural analysis by two-dimensional ¹H NMR shows that the C-tail is flexible in the solution conformations of either EGF (14) or TGF-α (15), recent NMR analyses have shown that the conformation of the C-tail of TGF-α becomes more ordered upon receptor binding (16). Although substitution of the C-tail of TGF-α into human EGF has been reported to restore high affinity binding of hEGF to the chicken EGFR (17), its effects on binding to the human EGFR have not been evaluated. In view of the possibility of nonidentical binding of EGF and TGF-α, we examined the consequences on binding and activation of the human EGFR when a similar substitution...
was introduced into mEGF. Our results demonstrate that binding of the C-tail of the growth factor to EGFFR exhibits ligand-specific features; the substitution has unexpected effects on receptor binding and tyrosine kinase activation which have not been documented previously for such a chimeric ligand. This work has been presented in abstract form (18).

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma (Poole, Dorset, United Kingdom (UK)), unless otherwise stated. Tissue culture materials and recombinant hEGF were from Life Technologies, Inc. (Paisley, Renfrewshire, UK). Recombinant betacellulin was purchased from R&D Systems Europe Ltd. (Abingdon, Oxon, UK); its purity was cited as >97% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining and its biological activity (ED₅₀) against EGF responsive mouse Balb/c 3T3 fibroblasts was in the range of 0.1–0.3 ng/ml. Yeast extract, Bacto-peptone, and yeast nitrogen base were from Difco Laboratories Ltd. (West of England). DNA was isolated from skin obtained from 14-day-old chicken embryos. The SKOV3 ovarian carcinoma cell line was a gift from Prof. B. Gusterson, Institute of Medical Genetics (Thame, Oxfordshire, UK). The HN5 human squamous cell carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). HN5 cells were maintained in a defined medium (23) containing 1.34% yeast nitrogen base and 2% calf serum as the carbon source. Growth factor expression was induced for 48 h after the addition of galactose. Growth factors were purified from medium by Sep-Pak extraction, Mono Q anion exchange chromatography, and C₁₈ reversed phase chromatography as described previously (13). Purified proteins were subjected to laser desorption mass analysis and protein concentrations determined by absorbance at 280 nm where the λ₂₈₀ of a 1 mg/ml solution of the growth factor in water was calculated as ([(A × 5,650) + (n × 1,351/n)/mw]) (3) where A = absorbance at 280 nm, n = number of tryptophan and tyrosine residues, respectively, and mw = molecular weight of the peptide as determined by laser desorption mass analysis.

Receptor Binding Assays—Competitive binding assays with ¹²⁵I-labeled mEGF were performed using NR6/hEGF, or CEF3 as described previously (12, 13). Direct binding assays were also performed with NR6, NR6/hHER, SKOV3, CB3, and CB4 cells using ¹²⁵I-labeled betacellulin, ¹²⁵I-labeled mEGF, and ¹²⁵I-labeled mEGF/TGF-β₃ (specific activities in the range of 0.74–2.62 MBq/µg). In these assays cells were seeded in 24-well plates and used when 90% confluent; labeled growth factor (200 µl/well) was added to the cells in phosphate-buffered saline containing 1% (w/v) bovine serum albumin and 0.02% (w/v) sodium azide (to inhibit receptor internalization) and binding allowed to proceed for 5 h at 22 °C. Nonspecific binding was determined using a 100-fold excess of the equivalent unlabeled ligand.

Mitogenesis Assays—The ability of mEGF and mEGF/TGF-β₃ to induce DNA synthesis in confluent and quiescent NR6/hHER, HFF5, or CEF3 was determined by measuring incorporation of the thymidine analog 5-[¹²⁵I]UdR into DNA as described previously (12, 13). Processing of mEGF and mEGF/TGF-β₃ by NR6/hHER Cells—Confluent and quiescent NR6/hHER cells were prepared in 96-well trays exactly as in mitogenesis assays. ¹²⁵I-Labeled mEGF or ¹²⁵I-labeled mEGF/TGF-β₃ (specific activity 2.6–2.62 MBq/µg) was added to the cells in complete mitogenesis assay buffer to give final concentrations in the range of 0.015–1.0 nM. After incubation for 8 h at 37 °C, the medium was removed and passed over an Econopac 10DG desalting column to separate intact growth factor from degradation products. Control experiments demonstrated that >90% of labeled growth factor was voided from the column prior to exposure to cells; furthermore, C₁₈ reversed phase chromatography confirmed that the labeled EGFR or mEGF/TGF-β₃ contained in the void volume after exposure to NR6/HER cells for 8 h was chromatographically indistinguishable from that which had not been exposed to cells (in all cases one major (85–90%) and one minor peak (10–15%) were obtained representing the mono- and di-idio forms of the labeled growth factor (data not shown)). Thus, label contained in the void volume was taken as a measure of intact growth factor left in medium after exposure to cells.

EGFR Tyrosine Phosphorylation Assays—The ability of mEGF and mEGF/TGF-β₃ to induce autophosphorylation of EGFR in HN5 cells was determined as described previously (13). The bands on the Western blots were quantified by densitometry.

RESULTS

Both mEGF and mEGF/TGF-β₃ were efficiently expressed by S. cerevisiae and were readily purified to yield single peaks by C₁₈ reversed phase chromatography. In the case of mEGF, laser desorption mass analysis of the purified protein identified a single peak of mass 5774.8 closely corresponding to the predicted mass (M + 1) of mEGF 1–51 (5771.6). The A₂₆₀ of a 1 mg/ml solution was then calculated to be 3.1, and this was used to quantify the purified protein. For mEGF/TGF-β₃ the purified protein comprised a major peak of estimated mass 5249.4 in agreement with the predicted mass of the full-length chimera (5250.0). On the basis of these results, the A₂₆₀ of a 1 mg/ml solution was calculated to be 1.27. In the chimera preparations, a minor peak of mass 5195.4 was also detected; this was most similar to the predicted mass (M + 1) of mEGF/TGF-β₃, in which the carboxyl-terminal alanine was lost and the methionine at position 21 was oxidized to a sulfoxide (M + 1 = 5194.9). Oxidation of Met¹ⁱ has been observed previously in preparations of recombinant mEGF, but this modification was reported to have no effect on biological activity (24). Reanalysis of the chimera preparations by analytical C₁₈ reversed phase chromatography indicated that the contaminant represented about 8% of the total growth factor protein.

FIG. 1. Diagrammatic representation of mEGF and the mEGF/TGF-α₄₄–₅₀ chimera. In the chimera the carboxyl-terminal 7 residues from TGF-α are shown in boldface.

Complex Interactions of an EGF/TGF-α Chimera
nM) was much poorer than mEGF (IC50 = 56 nM) in its ability to compete with 125I-labeled EGF for binding to human EGFRs expressed by the genetically modified NR6/HER cell line (Fig. 3). In similar experiments with the human squamous carcinoma cell line HN5, IC50 values were 85.9 ± 9.1 nM for the chimera compared with 0.42 ± 0.05 nM for mEGF (data not shown).

When the mitogenic potencies of mEGF and mEGF/TGF-α44–50 were measured using NR6/HER cells (Fig. 3A), we were surprised to find that the chimera was a superagonist compared with mEGF (EC50 = 16.4 ± 13 and 120.2 ± 6.2 pm, respectively) i.e. the chimera was approximately 7-fold more potent than mEGF. This shift in mitogenic potency was far in excess of that expected from its receptor binding activity; therefore, we also assayed the mitogenic activity of the chimera using HFFs, which naturally express human EGFRs. In this case, the mitogenic activity of the chimera (EC50 = 4,150 ± 750 pm versus 56 ± 10 pm for mEGF) closely paralleled its low receptor binding affinity (Fig. 3B).

As a human EGF/TGF-α44–50 chimera has been reported to restore the activity of hEGF for the chicken EGFR (17), we tested the mEGF/TGF-α44–50 chimera for binding to chicken EGFRs in competitive binding assays using CEFs and in mitogenesis assays. Fig. 4A shows the greater ability of TGF-α to compete with 125I-labeled EGF for binding to chicken EGFRs compared with mEGF; in the same assay, the relative affinity of the chimera (IC50 = 1.8 ± 0.2 nM) closely paralleled that of mEGF (IC50 = 2.8 ± 0.3 nM). In mitogenesis assays (Fig. 4B), TGF-α was also more potent (EC50 = 0.13 ± 0.02 nM) than mEGF (EC50 = 0.95 ± 0.12 nM); the chimera was found to be of intermediate activity (EC50 = 0.36 ± 0.05 nM).

In view of the anomalous mitogenic response of the NR6/HER cells, we examined whether this may have arisen from differences in ligand processing. Induction of mitogenesis depends on prolonged exposure of cells to growth factor concentration during an 8-h time window. Although we found that NR6/HER cells consumed more EGF than mEGF/TGF-α44–50 in 8 h (Fig. 5A), the difference between the concentrations of EGF and chimera was insufficient to account for the 2–3 log shift in mitogenic response over that predicted from the receptor binding assays. Fig. 5B shows that when the mitogenic response was plotted as a function of the measured intact ligand concentration at 8 h, the chimera was still more potent than mEGF.

To explore further the basis for the superagonistic activity of mEGF/TGF-α44–50, we measured its ability to stimulate autophosphorylation of EGFR. When used at equimolar concentrations, the chimera was more potent than mEGF at inducing tyrosine phosphorylation of EGFR in HN5 squamous carcinoma cells, which overexpress the EGFR (Fig. 6, A and B). This indicated that the chimera had the ability to activate EGFR in excess of that anticipated from its receptor binding affinity.

Since betacellulin has been reported to bind to c-erbB4 as well as EGFR (26), we wanted to investigate whether the superagonist activity of the chimera could be attributed to alterations in its binding specificity. This was determined by comparing the activity of 125I-labeled mEGF/TGF-α44–50 with that of mEGF and betacellulin in direct binding assays using a range of cell lines expressing different members of the c-erbB family (Fig. 7A). As expected, 125I-labeled mEGF bound to NR6/HER cells as well as to SKOV3 cells (which express EGFR and overexpress c-erbB2); it did not bind to the parental NR6 cell line, which does not express EGFRs, nor did it bind to CB3 or CB4 cells that have been transfected to express either c-erbB3 or c-erbB4 and lack EGFR. In contrast, 125I-labeled betacellulin bound to all five cell lines (albeit weakly to NR6...

![Fig. 2. Comparison of the receptor binding activities of mEGF (open symbols) and mEGF/TGF-α44–50 (filled symbols). Relative receptor binding affinities were determined in competitive binding assays with 125I-labeled mEGF using NR6/HER as described under "Experimental Procedures." Data points shown are from two independent experiments (○/□ and ▲/▼).](image)

![Fig. 3. Comparison of the mitogenic activities of mEGF (open symbols) and mEGF/TGF-α44–50 (filled symbols). Mitogenic activities were determined by measuring incorporation of the thymidine analog [125I]dUdR into DNA of NR6/HER (panel A) or HFF (panel B) cells as described under "Experimental Procedures." Data points shown are from two independent experiments (○/□ and ▲/▼).](image)
and CB3 cells), confirming the broader specificity of betacellulin, which recognizes EGFR (present in NR6/HER and SKOV3 cells) as well as c-erbB4 (present in CB4 cells). The superior binding of 125I-labeled betacellulin to NR6/HER cells compared with 125I-labeled EGF, presumably reflects expression of c-erbB4 (or c-erbB3) by NR6 cells, as evidenced by binding of 125I-labeled betacellulin to the parental cell line. The pattern of binding of 125I-labeled mEGF/TGF-α44–50 closely paralleled that of mEGF but was reduced in magnitude consistent with its lower relative binding affinity determined in competitive binding assays. Failure of the chimera to bind to c-erbB4 was confirmed in a competitive binding assay with 125I-labeled betacellulin; even when used at a 1,000-fold higher concentration over unlabeled betacellulin, the mEGF/TGF-α44–50 failed to compete for binding to c-erbB4 (Fig. 7B). These data suggest that the binding specificity of the chimera is exclusively toward EGFR.

DISCUSSION

Substitution of homologous regions of EGF and TGF-α has been used previously to explore the receptor binding regions of EGF and TGF-α by exploiting the differential affinities for the chicken EGFR for these growth factors (12, 17). In our previous studies where we substituted the B-loop β-sheet of TGF-α into mEGF, the relative receptor binding affinity of the chimera for the human EGFR was unchanged by comparison with mEGF; however, its affinity for the chicken EGFR was enhanced by 1 order of magnitude. Hence, our finding that substitution of the C-tail of TGF-α into mEGF caused a reduction in binding affinity for the human EGFR was unexpected, particularly as this region of the growth factor is not a part of the structural core of the molecule. However, our data are consistent with several previous lines of evidence which suggest that binding of EGF and TGF-α is nonidentical. In a previous study, Kramer et al. (17) produced a panel of hEGF/TGF-α chimeras and reported that substitution of the C-tail of TGF-α into mEGF caused a reduction in binding affinity for the human EGFR was unexpected, particularly as this region of the growth factor is not a part of the structural core of the molecule. However, our data are consistent with several previous lines of evidence which suggest that binding of EGF and TGF-α is nonidentical.

In a previous study, Kramer et al. (17) produced a panel of hEGF/TGF-α chimeras and reported that substitution of the C-tail of TGF-α into hEGF was responsible for conferring high affinity binding toward the chicken EGFR. We could not confirm this; in our own experiments using CEFs, we found the activity of mEGF/TGF-α44–50 in receptor binding assays to be low, comparable to that of wild type mEGF. However, the earlier studies assumed that interchange of segments of EGF and TGF-α would have no effect on the receptor binding affinity of the chimeras for the human EGFR, and their quantification depended on standardization in a receptor binding assay using human EGFRs. As our present results demonstrate, such an
assumption is invalid for an mEGF/TGF-α tail chimera and is likely to be so for an hEGF/TGF-α tail chimera. Thus, the previous study overestimated the concentration of the chimera by 80–200-fold; this would have been sufficient to give the apparent increase in binding affinity when tested on the chicken EGFR.

Although the affinity of mEGF/TGF-α44–50 for the human EGFR might be explained by perturbations in the structure of the growth factor in the free or receptor-bound state, its low affinity is apparently at odds with its mitogenic potency for NR6/HER cells. Previous studies with a Leu47→Val mutant of mEGF have attempted to explain the higher than expected mitogenic potency of the mutant EGF by differences in ligand processing (27). An identical explanation has been proposed to account for differences in cell response between mEGF and TGF-α (28). Our data for the mEGF/TGF-α44–50 chimera could not be explained in this way; after correction for differences in processing of mEGF or mEGF/TGF-α44–50 by NR6/HER cells, the chimera was still much more potent than mEGF. This finding is not specific to the mEGF/TGF-α44–50 chimera. In other related studies using an hEGF Leu47→Ala mutant (29), we have also found discrepant results for mitogenic potency which could not be attributed to ligand processing. In these studies, mitogenic potency was found to correlate with ligand affinity when tested on cells expressing around 10^4 receptors (e.g., HFFs); however, when tested on cells that express more than 10^5 receptors (e.g., NR6/HER fibroblasts or NRK52E cells) the mutant growth factor was equipotent with EGF. We are currently using mathematical models describing formation of dimeric ligand-receptor complexes to understand the influence of ligands of differing efficacy on cells with a range of EGFR densities and its relationship to the production of activated receptors.

Although we consider the enhanced mitogenic potency of the mEGF/TGF-α44–50 chimera to be related to the high receptor number of NR6/HER cells, the chimera differed from any other mutant that we have examined previously in that it was more potent than wild type EGF or TGF-α. The reason for the superagonist activity appears to reside in its greater ability to stimulate the EGFR tyrosine kinase, not just in excess of its receptor binding capacity, but over and above that attained by EGF. This contrasts with other mutant growth factors that we have produced where receptor-ligand affinity paralleled receptor phosphorylation (13).

One possible reason for enhanced activity of the chimera was that it possessed a broader receptor specificity, as has been shown previously for betacellulin. However, the mEGF/TGF-α44–50 chimera showed no demonstrable binding activity to receptors other than EGFR. We therefore suggest that the...
potent activity of the chimera in phosphorylation assays may be due to unusual receptor binding kinetics that influence the formation and stability of activated receptors (either homodimers or heterodimers); alternatively, the chimeric nature of the ligand may result in production of higher receptor oligomers that are able to maintain the complex in an activated state with a longer half-life. We are currently performing detailed kinetic analyses to examine these possibilities.

In conclusion, we have demonstrated that the properties of chimeric EGFR ligands are not always predictable, as had been assumed previously (17). The mEGF/TGF-a4–50 chimera exhibited unusually low affinity for EGFR yet was highly mitogenic for some cell lines. The basis for this discrepancy could not be attributed to any single factor. Thus, although differences in ligand depletion made a small contribution to the overall response, it could not provide a complete explanation for the superagonist activity of the chimera. From the present studies and other related work, we propose that the mitogenic effect of low affinity ligands can be enhanced in cells such as NR6/HER which express high EGFR numbers. This belief is supported by the observation that the chimera can activate the EGFR tyrosine kinase more effectively than EGF.

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