The Significance of Valine 33 as a Ligand-specific Epitope of Transforming Growth Factor α*

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Although binding of epidermal growth factor (EGF) and transforming growth factor α (TGFα) to the EGF receptor (EGFR) is mutually competitive, their binding is not identical, and their biological activities are not always equivalent. To probe for ligand-specific interactions, we have synthesized analogues of TGFα with modifications to the residue lying between the fourth and fifth cysteines (the ‘hinge’). Although this residue lies in a structurally conserved region of the protein, it is not conserved within the EGFR ligand family. Our results show that in TGFα there is a preference for a bulky hydrophobic hinge residue; this contrasts with EGF, for which a hydrogen bond donor functionality is preferred. Sequence analysis of the human EGFR ligands revealed that the nature of the hinge residue correlated with the sequence in the B-loop β-sheet. As this region is an important determinant in recognition of TGFα by the chicken EGFR, we assessed the mitogenicity of the TGFα hinge mutants, as well as the other EGFR ligands, using chicken embryo fibroblasts. The preference of the chicken EGFR for TGFα hinge mutants with hydrophobic side chains paralleled that of the human EGFR. Betacellulin and heparin-binding EGF-like growth factor also possess an hydrophobic hinge; both were at least as potent as TGFα for chicken embryo fibroblasts. EGF and amphiregulin, both with hydrogen bond donor functionalities at their hinge, displayed markedly decreased in potency by comparison with TGFα. We propose that EGFR ligands can be subclassified into TGFα-like and EGF-like and that this is of functional significance, identifying a potential mechanism whereby EGFR can discriminate between its ligands.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated. Tissue culture materials were from Life

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1. The abbreviations used are: EGFR, epidermal growth factor receptor; TGFα, transforming growth factor α; EGF, epidermal growth factor; AR, amphiregulin; HB-EGF, hepatic binding epidermal growth factor-like growth factor; BTC, betacellulin; CEF, chicken embryo fibroblast; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.
The Role of Val-33 in TGF-α

Production of Hinge Mutants—Mutation of Val-33 in TGF-α was achieved using either site-directed mutagenesis of M13/TGF-2 or by polymerase chain reaction-directed mutagenesis of the HindIII-EcoRI fragment of BB23 using a two-step amplification procedure (23) and Vent polymerase. For the polymerase chain reaction-directed mutagenesis, the antisense primers across the mutation site (TAAACAGAGTTGCAATACCGATGCCGGTTTGT) incorporated base changes at Val-33 (ATA) for Ile (GAT), Gly (GCC), and Asn (GTT); the primers used at the ends of the TGF-α DNA incorporated BamHI restriction sites were 5’-sense, cggcatcAGTGGTTGATCCAC, and 3’-antisense, tggacCTT-ATAAGCTAGCA.

Expression and Purification of Recombinant TGF-αs and mEGF—TGF-α, mEGF, and the mutant growth factors fused to the α-factor secretion vector, pWYG9/EGF, encoding murine EGF was a gift from Dr J. Clare, Wellcome Research Laboratories, Beckenham, Kent, UK. Recombinant human HB-EGF, BTC and AR were from R & D Systems, Abingdon, Oxford; the activity of the HB-EGF was standardized against human EGF in a competitive binding assay prior to use in mitogenesis assays.

Production of TGF-α Yeast Expression Vector—TGF-αs was expressed in Saccharomyces cerevisiae using the yeast expression vector pWYG9/EGF (20) in which a synthetic TGF-α mini-gene obtained from clone BB23 replaced that of mEGF. The TGF-α expression vector was constructed using the following steps: (i) the 179-base pair HindIII-EcoRI fragment containing the TGF-α mini-gene was cloned into the HindIII and EcoRI sites of M13mp18 (21); (ii) site-directed mutagenesis (22) was used to remove the Met codon from the 5’-end of TGF-α and to insert an XhoI restriction site and codons for Lys and Arg (generation of a Kex II proteolytic cleavage site) adjacent to the first valine of hTGF-α forming M13/TGF-2; (iii) the 715-base pair AatI-BglII fragment from pWYG9/EGF containing the yeast gal7 promoter and a-factor prepro leader sequence fused to mEGF was inserted into the AatI and BamHI sites of pUC18 to form pUC/EGF/1; (iv) the 173-base pair XhoI-EcoRI fragment from M13/TGF/2 was inserted into the XhoI-EcoRI sites of pUC/1 to replace the mEGF coding sequence and form pUC/CTF/2; (v) the 715-base pair AatI-BamHI fragment from pUC/CTF/2 was inserted into the AatI-BglII sites of pWYG9/EGF to form pWYG9/TF/2. As the TGF-α mini-gene contained a KpnI site not present in mEGF, this was used to ensure correct replacement of mEGF by hTGF-α.

Production of hinge mutants—mutagenesis of Val-33 was constructed using the following steps: (i) the 179-base pair HindIII-EcoRI fragment containing the TGF-α mini-gene was cloned into the HindIII and EcoRI sites of M13mp18 (21); (ii) site-directed mutagenesis (22) was used to remove the Met codon from the 5’-end of TGF-α and to insert an XhoI restriction site and codons for Lys and Arg (generation of a Kex II proteolytic cleavage site) adjacent to the first valine of hTGF-α forming M13/TGF-2; (iii) the 715-base pair AatI-BglII fragment from pWYG9/EGF containing the yeast gal7 promoter and a-factor prepro leader sequence fused to mEGF was inserted into the AatI and BamHI sites of pUC18 to form pUC/EGF/1; (iv) the 173-base pair XhoI-EcoRI fragment from M13/TGF/2 was inserted into the XhoI-EcoRI sites of pUC/1 to replace the mEGF coding sequence and form pUC/CTF/2; (v) the 715-base pair AatI-BamHI fragment from pUC/CTF/2 was inserted into the AatI-BglII sites of pWYG9/EGF to form pWYG9/TF/2. As the TGF-α mini-gene contained a KpnI site not present in mEGF, this was used to ensure correct replacement of mEGF by hTGF-α.
hydrogen peroxide in 0.1 M sodium acetate buffer with 0.15 M 3,3,5,5'-tetramethylbenzidine dihydrochloride as chromogen. The reaction was halted by the addition of 2M H2SO4 (50 μl/well), and absorbance was read at 450 nm. The TGFα in the samples was estimated by reference to a TGFα standard curve in the range of 64–500 ng/ml.

Cell Culture—Cells were routinely cultured in Dulbecco’s modified Eagle’s medium, containing 10% (v/v) heat inactivated fetal bovine serum, 50 IU/ml penicillin, 50 IU/ml streptomycin, 1 mM L-glutamine, 1 mM sodium pyruvate, and 1 x nonessential amino acids. The NR6/HER medium was supplemented with 100 μg/ml of genetin sulfate to maintain the selection of the EGFR encoding plasmid.

Mitogenesis Assays—Mitogenic activity of recombinant growth factors was determined by measuring the stimulation of incorporation of [125I]iododeoxyuridine into DNA of NR6/HER cells or primary cultures of chicken embryo fibroblasts (CEFs) as described previously (16). In the case of the CEFs, responsiveness to growth factor stimulation tended to vary between individual primary cultures (identified as CEF1, etc) and declined in passage; assays were routinely performed using primary cultures between passages 3 and 10. EC50 values were determined by nonlinear curve fitting to the logistic function:

\[ f(x) = \frac{d}{1 + \left(\frac{x}{c}\right)^d} \]

125I-Labeled mEGF Competitive Binding Assays—Relative binding affinities were determined by measuring the ability of recombinant growth factors to displace [125I]mEGF binding to EGFR on HN5 cells (25). IC50 values were determined by nonlinear curve fitting as above.

Receptor Tyrosine Phosphorylation—EGF receptor phosphorylation was determined using HN5 cells in 96-well plates at 50% confluence. Cells were incubated with 50 μl of sample diluted in Dulbecco’s modified Eagle’s medium for 10 min at 22°C. The samples were then removed, and cells were washed twice in phosphate-buffered saline before solubilization in 50 μl of 1 x sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 3% (v/v) glycerol, 0.002% (w/v) bromphenol blue, 5% (v/v) mercaptoethanol) containing phosphatase and protease inhibitors (1 mM NaF, 1 mM Na3VO4, 70 μM phenylmethylsulfonyl fluoride) and heating at 95°C for 3 min. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using a biotinylated antiphosphotyrosine antibody (PY20) as described previously (26). The phosphorylated band at 170 kDa was confirmed as EGFR using a polyclonal anti-EGFR antibody.

RESULTS

As Val-33 lies in a structurally conserved region of TGFα, the first series of Val-33 substitutions were chosen because they were known to be functional in other members of the growth factor family (i.e. Val → Ile, Val → Asn, and Val → Lys). These TGFαs were all efficiently expressed by the recombinant yeast cells, and each was readily purified by ion exchange and reversed phase chromatography. The latter procedure ensured that any incorrectly folded growth factor was separated from the purified product (27); in each case, purification yielded a single major peak (e.g. Fig. 2a) with molecular masses where confirmed by laser desorption mass analysis (e.g. Fig. 2b).

Conservative substitution of Val-33 → Ile was found to have no effect on the ability of the recombinant TGFα to compete in receptor binding assays (Fig. 3a and Table I) and slightly

![Image](https://example.com/image.png)
exhibited activities similar to that of wild type TGFα (Fig. 4). All TGFα paralleled the observed receptor binding and mitogenic activities of TGFα in HN5 squamous carcinoma cells closely to stimulate DNA synthesis in quiescent NR6/HER cells as described under “Experimental Procedures.”

| Substitution | IC50  | EC50 |
|--------------|-------|------|
| Val (wild type) | 1.9 ± 0.3 | 0.82 ± 0.18 |
| Ile | 1.8 ± 0.3 | 0.37 ± 0.08 |
| Leu | 1.7 ± 0.3 | 2.7 ± 0.6 |
| Met | 2.7 ± 0.5 | 1.7 ± 0.4 |
| Asn | 7.5 ± 1.2 | 8.6 ± 1.8 |
| Lys | 1200 ± 400 | 90 ± 18 |
| Gly | >10 | 29 ± 6 |

Effect of mutation of the hinge residue on the receptor binding and mitogenic activity of TGFα

The chicken EGFR displays markedly different affinities for mEGF and TGFα, as the means ± S.E. (n = 4). Assays using chicken embryo fibroblasts (CEF) and AR were tested for their ability to stimulate DNA synthesis in CEFs (CEF3, passage 5–6) as described under “Experimental Procedures.” Data are means ± S.E. (n = 4).

**FIG. 5. Comparison of the mitogenic activities of TGFα hinge mutants toward chicken embryo fibroblasts.** Purified wild type TGFα (●), mEGF (○), and TGFα hinge mutants Val-33 → Ile (△), Val-33 → Asn (○), and Val-33 → Lys (★) were tested for their ability to stimulate DNA synthesis in CEFs (CEF3, passage 5–6) as described under “Experimental Procedures.” Data are means ± S.E. (n = 4).
is tolerated at the hinge. Thus, although this residue lies within the core of the protein, there is no intrinsic structural requirement for any particular side chain. This is confirmed by NMR-derived structures of many EGF modules (29) and in a study of several EGF hinge mutants (17), where only proline substitution was shown by NMR spectroscopy to affect the ligand structure. Therefore, changes in activity arising from substitutions of the hinge residue in TGFα or EGF cannot readily be dismissed as the result of conformational perturbations.

We show that substitution of Val-33 with other bulky hydrophobic residues did not significantly alter the ability of TGFα to bind or activate either the human or the chicken EGFR, whereas substitution with Asn, as found in EGF, or Lys, as found in AR, produced ligands with reduced affinity. Our observations contrast with similar work by Niyogi and co-workers (17) or Koide et al. (30), which showed that in EGF, hydrogen bond donor residues were preferred over hydrophobic residues. We also demonstrate that in most cases, receptor binding affinity and mitogenicity are similarly affected. For the Lys-33 mutant, however, ligand affinity was very poor compared with mitogenic potency, but this is in agreement with other mutagenesis studies. For example, in a study of Tyr-37 mutations in EGF, the mutants exhibited differing receptor-binding affinities and abilities to activate the EGFR tyrosine kinase, but most mutants stimulated thymidine incorporation similarly to wild type (31). Likewise, mutations of Leu-47 dramatically reduced receptor binding affinity but had a more modest influence on mitogenic activity (32). This must in part reflect the complexity of the bioassays where the measurements are dependent on numerous kinetically controlled cellular events. Such complex factors may also underlie the slightly superagonistic activity of the Ile-33 mutant, which exhibited equivalent affinity to wild type TGFα but which showed elevated tyrosine kinase activity.

Examination of the sequences of known human EGFR ligands reveals that they can be divided into two families, EGF-like or TGFα-like, based upon the nature of their hinge residue. This classification also correlates with a particular pattern of residues in the α-sheet, being characterized most conveniently by the presence or the absence of a proline; TGFα, HB-EGF, and BTC are TGFα-like ligands, whereas EGF and AR are EGF-like. This evidence implicates the B-loop α-sheet as a recognition element in EGF ligands, a role supported by recent work with EGF/TGFα B-loop chimeras (16). It is also consistent with our observation that the EGFR ligands fall into two groups when tested on chicken EGFR: the high affinity TGFα-like ligands and the two low affinity EGF-like ligands. Examination of known EGFR ligands from different species (Table II) indicates that with the exception of rat and mouse HB-EGF, they can all be grouped according to the same classification. Presumably, the difference in the rodent HB-EGFs reflects some variation within the EGFR from this species; it would be of interest to determine whether rodent HB-EGF has a lower affinity for the human EGFR, as does mEGF for the chicken EGFR (15). Differences in behavior of rodent HB-EGF have already been reported in studies of cellular sensitivities to diphtheria toxin. Whereas human or monkey cells are extremely sensitive to diphtheria toxin, rodent cells are not, even though the HB-EGF precursor (the diphtheria toxin receptor) shows about 80% sequence identity between human and mouse. Recent studies with chimeric HB-EGFs have indicated that the most critical residues for toxin binding lie between residues 122–148 (33, 34); significantly the hinge residue lies within this region (residue 133).

It is well known that EGF and TGFα compete for binding to EGFR, suggesting a common ligand binding site. However, it need not be the case that the recognition of both EGF and TGFα-like ligands is through homologous residues or that EGF and TGFα bind in identical conformations. For example, identical interactions for EGF and TGFα could be achieved by different residues providing the same critical functional groups in a common receptor binding pocket. However, we have been unable to identify any spatially related functionality in the region of the β-sheet and the hinge residue, so it is unlikely that this can account for the differing hinge preferences we have observed. We therefore conclude that the B-loop β-sheet region provides a mechanism for discrimination between EGF and TGFα and that this part of the growth factor composition recognizes different receptor residues depending on its composition.

When combined with other information, our evidence suggests a model for the binding of ligands to the EGFR. The picture that is emerging is of both common and specific points of interaction between the EGFR and its ligands. In common among EGFR ligands are residues such as Arg-42 and Leu-48, which contribute a large proportion of the binding free energy, as well as Phe-15, Phe-17, and His-18, which are semi-conserved and together may form a receptor binding patch (8). It is noteworthy that this pattern of conservation is largely preserved in the heregulins and viral growth factors and thus this epitope might be a common feature of all c-erbB ligands. Ligand-specific interactions involving the B-loop β-sheet may provide a mechanism for discrimination between individual growth factors. In this ligand-binding model, the N-domain of EGF and TGFα may be accommodated in exclusive subsites, or they may occupy a common subsite utilizing distinct or overlapping functional epitopes, as is the case for the human growth hormone receptor (35). Either mechanism would be consistent with the differential inhibition of binding of EGF and TGFα by the 13A9 monoclonal antibody (9). Ligand-specific interactions may then result in the transduction of different signals or signals of differing intensity or duration and consequent differences in cellular response.

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The Role of Val-33 in TGFα

### Table II

| Growth factor | Sequence |
|--------------|----------|
| Human EGF    | GVCYKIEALDKYACNVV |
| Guinea pig EGF | GVCYKIESLNTYACNVI |
| Murine EGF   | GVCYKIESLDSTYCNCVI |
| Rat EGF      | GECYKIESVDRVVCNGP |
| Human AR     | GECYKIELHYELAVCGQQ |
| Murine SDGF  | GECYRIINLEVTCNCHQ |
| Rat SDGF     | GECYRIINLEVTCNCHQ |
| Eproginul    | GQCYILVDRKFRKPCGEV |
| VEGF         | GICIHARDIGMDOEL |
| Human BTC    | GRCRFVVAEQTPSCVDK |
| Murine BTC   | GRCRFVVDQPTSCICKEK |
| Human TGFα   | GTCRFVILEQPACVCHS |
| Porcine TGFα | GTCRFVILEQPACVCHS |
| Bovine TGFα  | GTCRFVILEQPACVCHS |
| Rat TGFα     | GTCRFVILKPEQACVCHS |
| Human HB-EGF | GECYKVELRAPSICICH |
| Monkey HB-EGF| GECYKVELRAPSICICH |
| Murine HB-EGF| GECYQLEERPTSCICLGP |
| Rat HB-EGF   | GECYRKLWRPSICLGP |
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