Constrained Peptide Analogues of Transforming Growth Factor-α Residues Cysteine 21-32 Are Mitogenically Active

USE OF PROLINE MIMETICS TO ENHANCE BIOLOGICAL POTENCY*

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Two proline mimetics, the enantiomers of 2-aza-bicyclo[2,2,1]heptane-3-carboxylic acid, have been incorporated in place of Pro30 into synthetic peptides based on the B-loop β-sheet sequence of human transforming growth factor-α (TGF-α) (residues Cys21-Cys32). The peptides were further modified by inclusion of an N-terminal phenylalanine and constrained by formation of an intramolecular disulfide bond. While no mitogenic response was observed in the parental NR6 cell line, the peptides stimulated DNA synthesis in NR6/HER cells (NR6 fibroblasts transfected with the human epidermal growth factor receptor). Induction of DNA synthesis was dose dependent, with EC50 values in the range 130–330 μM; in the presence of low doses of TGF-α, the mitogenic effect of the peptides was additive, up to the plateau response achieved by maximal doses of TGF-α alone. These effects are consistent with the peptides acting via the same mechanism as TGF-α. Analysis of the structure of the peptides by NMR indicated that the presence of the mimetics significantly increased the propensity of the peptide=proline bond to adopt the cis conformation. These data confirm the role of the β-sheet in receptor activation, and emphasize the importance of presentation of peptides in an appropriate conformation for recognition.

Epidermal growth factor (EGF)1 and transforming growth factor-α (TGF-α) belong to a family of EGF-like growth factors (1) that stimulate a variety of epithelial and mesenchymal cells. The biological effects of these growth factors are mediated via the transmembrane epidermal growth factor receptor (EGF-R) (2), a type 1 receptor tyrosine kinase (3). The EGF-R and its ligands are of considerable interest not only in normal physiological processes such as wound healing (4) and embryogenesis (5) but also through their involvement in the pathology of hyperproliferative (6) and neoplastic (7) diseases. Thus, the identification of small bioactive molecules may have several clinical applications.

NMR studies have identified the major structural element in EGF and TGF-α as a double stranded anti-parallel β-sheet (8) which includes the 10 residues between the third and fourth cysteines of the growth factors. Mutational analysis of Ile23 (9) and Leu26 (10) in EGF has implicated the β-sheet in the ligand: receptor interaction. In TGF-α, we have recently identified this same region as one which interacts with the chicken EGF-R (11). These observations confirm our previous studies using an epitope mapping technique which identified a putative receptor binding cavity in TGF-α (12). The cavity was formed from the B-loop β-sheet, as well as the C-loop and flexible C-terminal tail of TGF-α. The side chain of Phe21, a known receptor contact residue, was not identified by the mapping technique, but was found to occupy a central position within this cavity.

Previous studies have shown that synthetic peptide fragments of EGF (13–16) and TGF-α (17–19) bind only weakly to EGF-R, nevertheless, only peptides corresponding to the B-loop segment of EGF have been consistently shown to bind to the EGF-R and invoke a weak mitogenic response. While low activity of synthetic peptide fragments may reflect the complex interaction of the ligand with EGF-R, it may also be due to failure of these peptides to adopt a conformation similar to that of the native ligand. Hence, some workers have used peptidomimetics (20) or cyclization (21) to apply additional conformational constraints.

In the present study, we have generated constrained peptides based on the β-sheet segment of TGF-α and have exploited the presence of proline in this peptide. This amino acid has a pyrrolidine structure and an N-alkylated amide bond which gives rise to cis and trans rotamers of comparable energy (22). In view of these structural implications, it represented an attractive site for modification and development of TGF-α analogue peptides.

EXPERIMENTAL PROCEDURES

The abbreviations used are: EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; TGF-α, transforming growth factor alpha; ABHC, 2-aza-bicyclo[2,2,1]heptane-3-carboxylic acid; TOCSY, total correlated spectroscopy; ROESY, rotating frame nuclear Overhauser spectroscopy; PDGF, platelet-derived growth factor; ACM, acetamidomethyl; HPLC, high performance liquid chromatography; Fmoc, N-(9-fluorenylethoxycarbonyl); Oe, rotating frame nuclear Overhauser enhancement.

Materials—Recombinant human EGF was purchased from Life Technologies, Inc., and recombinant human TGF-α was purchased from British Biotechnology, UK. NR6 and NR6/HER (NR6 fibroblasts trans-
Biolactive TGF-α Peptides

A

B

C

FIG. 1. Structure of TGF-α and derivation of TGF-α analogue peptides. a, the primary structure of TGF-α showing the three-looped motif with the residues incorporated into N-pheTGF-α[21–32] highlighted; b, the primary structure of N-pheTGF-α[21–32] highlighting Pro⁶; c, the three-dimensional structure of TGF-α visualized using RasMol v2.4 and NMR coordinates obtained from Dr. T. P. Kline (39). In contrast to the simple appearance of the linear peptides, the spectra of the cyclic peptides were substantially more complex (Fig. 3b). For each peptide, up to four resonances per proton were detectable. From these resonances, spin systems similar to the major (trans-prolyl) conformation in the spectra of the linear peptides could be conveniently located with little change in their relative proton chemical shifts. Similarly, resonances corresponding to the cis-prolyl conformations were also evident but the disulfide constraint induced greater resonance heterogeneity and discrete resonances were apparent for most backbone protons. The remaining peaks were suggestive of a separate family of conformations, with cis- and trans-pro subfamilies. Assignment of spin-systems for each of the conformations was hampered by the degree of degeneracy between chemical shifts, making unambiguous assignment of particular rOes difficult. Furthermore, the rOe intensity was poor due to the large number of different chemical shifts displayed by each proton, consequently very few long range (nonsequential) rOes were observed. Although a tentative sequential assignment of the major conformation of each peptide was possible, the number of rOe-derived constraints available was insufficient to restrict conformational space and so produce a reliable picture of the three-dimensional structure of TGF-α.

RESULTS

Initial studies focused on TGF-α residues Cys²¹-Cys²⁲ which were constrained by introduction of an intramolecular disulfide bond. Although these two cysteines are not disulfide-bonded to each other in the intact growth factor (Fig. 1a), molecular modeling using NMR-derived conformations of TGF-α revealed that they were sufficiently close (5 Å) to form an undistorted disulfide bond without significant changes in the conformation of the backbone. The peptide was further modified by introduction of an N-terminal phenylalanine to mimic the presence of Phe⁵, a known receptor contact residue whose side chain lies in close proximity to the prolyl amide bond (Fig. 1a). This was consistent with cis-trans isomerization, which was estimated for each compound and shown to be greatest for linear N-pheTGF-α[21–32] (Table I). In contrast to the simple appearance of the linear peptides, the spectra of the cyclic peptides were substantially more complex (Fig. 3b). For each peptide, up to four resonances per proton were detectable. From these resonances, spin systems similar to the major (trans-prolyl) conformation in the spectra of the linear peptides could be conveniently located with little change in their relative proton chemical shifts. Similarly, resonances corresponding to the cis-prolyl conformations were also evident but the disulfide constraint induced greater resonance heterogeneity and discrete resonances were apparent for most backbone protons. The remaining peaks were suggestive of a separate family of conformations, with cis- and trans-pro subfamilies. Assignment of spin-systems for each of the conformations was hampered by the degree of degeneracy between chemical shifts, making unambiguous assignment of particular rOes difficult. Furthermore, the rOe intensity was poor due to the large number of different chemical shifts displayed by each proton, consequently very few long range (nonsequential) rOes were observed. Although a tentative sequential assignment of the major conformation of each peptide was possible, the number of rOe-derived constraints available was insufficient to restrict conformational space and so produce a reliable picture of the three-dimensional structure of TGF-α.
of the peptide conformation.

The extent of the resonance heterogeneity in spectra of the cyclic compounds was unexpected. We considered two possible explanations for this: slow conformational exchange, perhaps as a result of disulfide bond isomerization, or the presence of an impurity arising, for example, from racemization of a chiral center. Although our careful analytical procedures had indicated that the peptides were homogeneous, disulfide bond isomerization is unlikely to lead to separate resonances in view of previous reports which indicate that this is rapid in all but the most severely constrained peptides (30, 31). This led us to consider the possibility of racemization. During Fmoc peptide synthesis, racemization of the C-terminal residue during coupling to certain resins is a minor problem (32). However, it has also been observed that when this residue is cysteine, further racemization can occur during chain elongation (33). Further inspection of the NMR spectra of the linear compounds revealed two or three weak cross-peaks (identified as X in Fig. 3a) which could not be readily explained by cis-trans isomerization at the prolyl residue. Comparison of their chemical shifts and rOes (where visible) identified them with the two C-terminal residues. For the cyclic compounds, we conclude that the second family of resonances apparent in the NMR spectra represent trans- and cis-conformers of the TGF-α peptide containing a C-terminal D-cysteine residue. These are more noticeable in the spectra of the cyclic compounds because the presence of a disulfide constraint leads to a greater perturbation in the local environment. Although separation of the D-cysteine containing peptides was not possible, we estimated that our syntheses all contained about 20% D-cysteine. Therefore, different ratios of D- and L-cysteine could not be the sole explanation for any differences in biological activity between compounds.

The peptides were tested for their ability to induce DNA synthesis in murine fibroblasts stably transfected with the human EGF-R (NR6/HER cells). None of the linear precursor peptides exhibited any biological activity. In contrast, when constrained by introduction of an intramolecular disulfide bond, the two peptides containing the proline analogues, N-pheTGF-α[21–32]-L-ABHC and N-pheTGF-α[21–32]-D-ABHC stimulated DNA synthesis with EC₅₀ values of 130 and 330 μM, respectively (Fig. 4a); the equivalent L-Pro- or D-Pro-containing cyclic peptides were inactive. When the N-pheTGF-α[21–32]-L-ABHC and N-pheTGF-α[21–32]-D-ABHC peptides were assayed alone, incorporation of label into DNA approached that obtained with a maximal dose of TGF-α. In presence of TGF-α, N-pheTGF-α[21–32]-L-ABHC produced an additive response (data not shown), but this did not exceed the maximal level of stimulation achieved by TGF-α alone (Fig. 4b, left side).

Even though N-pheTGF-α[21–32]-L-ABHC elicited a mitogenic response, no detectable increase in EGF-R phosphorylation was observed when the peptide was tested on the parental NR6 cell line, HN5 (data not shown). Therefore, to confirm that the peptide was acting via the EGF-R it was tested on the parental NR6 cell line which lacks EGF-R. Fig. 4b shows that whereas both N-pheTGF-α[21–32]-L-ABHC and TGF-α were mitogenic for NR6/HER cells, neither stimulated DNA synthesis in NR6 cells, even though these cells were responsive to PDGF.
DISCUSSION

In recent years, generation of synthetic peptides with improved properties for probing ligand:receptor interactions has been greatly facilitated by use of modifications such as introduction of unnatural amino acids and other conformational constraints to mimic receptor bound “bioactive” conformations. By exploiting our knowledge of the three-dimensional structure of TGF-α and by use of unnatural amino acids, we report for the first time the ability of an analogue peptide based on the B-loop β-sheet segment of TGF-α to exhibit EGF-R activity. In relative terms, the peptide still exhibits low activity by comparison with TGF-α (−10⁶ times less active). However, given that residues from the C-domain (Arg42 and Leu48 in TGF-α) contribute about half of the binding free energy of the growth factor with EGF-R (34–37), we might reasonably expect that a B-loop peptide would have an affinity 10⁵ times less than TGF-α (10¹⁰ M⁻¹) and a correspondingly low mitogenic activity.

The specificity of the peptides for the EGF-R was evident from their ability to stimulate NR6/HER but not NR6 cells. Furthermore, in the presence of varying doses of TGF-α, induction of mitogenesis by the peptides never exceeded the plateau level of stimulation caused by maximal doses of TGF-α alone. Failure to observe EGF-R autophosphorylation in response to the peptides may reflect the fact that there is not an absolute requirement for receptor phosphorylation to initiate mitogenic signaling (38). Indeed, EGF mutants with modified C-terminal tails also have impaired ability to activate EGF-R autophosphorylation, even though they are still mitogenically active (35). For the TGF-α B-loop analogue peptides, which completely lack this region of the growth factor, it is perhaps not surprising that their ability to activate the kinase is low.

**TABLE I**

| Peptide                  | cis-| % |
|--------------------------|----|---|
| Linear N-pheTGF-α[21–32] | 6  |
| Linear N-pheTGF-α[21–32]_Pro | 30 |
| Linear N-pheTGF-α[21–32]_ABHC |   |
| Linear N-pheTGF-α[21–32]_L-ABHC | 48 |

**Fig. 3.** TOCSY spectra showing the NH region of (a) linear ACM-blocked and (b) cyclic N-pheTGF-α[21–32]_ABHC recorded in 90%H₂O/D₂O.In (a), spin systems have been identified according to residue using the single-letter code prime (') characters denote a secondary conformation arising from the presence of proline cis and trans isomers, while additional peaks arising from racemization of Cys³² are identified as X. The partial assignment in (b) highlights similarities with (a).

**Fig. 4.** Mitogenic activity of cyclic N-pheTGF-α[21–32] peptides. a, the activity of N-pheTGF-α[21–32] (●), N-pheTGF-α[21–32]_Pro (○), N-pheTGF-α[21–32]_ABHC (△), and N-pheTGF-α[21–32]_L-ABHC (□), was measured by incorporation of the thymidine analogue, 125-I-UdR, into DNA in NR6/HER cells during S-phase as described under “Experimental Procedures.” In this experiment, maximal incorporation of label in the presence of TGF-α was 7.5 × 10⁴ cpm. Similar results were obtained in three separate assays. b, comparison of the mitogenic activity of N-pheTGF-α[21–32]_ABHC (0.5 mg/ml), TGF-α (6 ng/ml, a maximally stimulating dose) and PDGF (2.5 IU/ml) on NR6/HER cells (hatched bars) or NR6 cells (cross-hatched bars). Basal incorporation of label was 600 and 2040 cpm in the NR6/HER and NR6 cells, respectively. Results are mean of triplicate determinations.
We believe previous studies failed to generate bioactive peptides because they used the entire TGF-α B-loop sequence constrained only by a disulfide bond in the native pairing (i.e. between cysteines two and four). To yield mitogenic compounds, we found it necessary both to reduce the size of the peptide and to introduce some additional conformational constraints. Our choice of sequence, with a non-native disulfide bond between cysteines three and four, was based on the observation that the N-domain is more properly described as being formed by three segments (A, J (joining), and B) rather than by the conventional two loop designation (i.e. A-loop and B-loop, see Fig. 1). The A-segment is comprised of the part of the A-loop between the first and second cysteines, the J-segment is common to the A- and B-loops and lies between the second and third cysteines, while the B-segment forms the β-sheet in the B-loop between the third and fourth cysteines (Fig. 5a). This definition emphasizes the importance of the disulfide bonds which act as nodes from which the three segments are presented (Fig. 5b). These nodes lie sufficiently close together so that any one of the three segments can be independently modelled by linking its ends to create a cycle. In the case of the TGF-α peptides, we studied the B-segment which was closed by a non-native disulfide bond.

Our data indicate that both cyclization and substitution with prolyl mimetics were important for activity. It seems likely that the beneficial effect of the cyclic constraint arises from stabilization of peptide conformations more suitable for binding to the EGF-R. In native TGF-α, residues 21–32 form a β-sheet (8,39) and this has been implicated in receptor binding (11, 12). However, in our TGF-α peptides, the disulfide constraint appeared unable to maintain an anti-parallel alignment of the peptide main chain as no nonsequential ROEs characteristic of this structural element were evident in their NMR spectra. Nevertheless, a restrictive effect of the disulfide was apparent, where it induced additional resonance heterogeneity in the cyclic compared to the linear compounds. The contribution of the bicyclic proline analogues to peptide activity may also be related to their effect on peptide conformation, mostly likely prolyl amide cis-trans isomerization. Differences in the degree of isomerization were evident in the linear compounds (Table I) and the highest proportion of the cis isomer was observed in the precursor of the most active cyclic compound. Although NMR spectra of the cyclic compounds showed clear evidence for the cis prolyl isomer, quantitation of the ratio of trans/cis was precluded by the complexity of these spectra.

One problem which became evident from the NMR studies was the apparent racemization of the C-terminal cysteine. Although not frequently reported, this problem may be quite common in peptides with C-terminal cysteine, being particularly difficult to detect using conventional analytical quality control; mass spectroscopy cannot identify this impurity, and it represents the most stringent test for HPLC since it leads to inversion of the hydrogen and the carboxyl group at the C terminus where it has minimal effects on conformation. For our peptides, the degree of racemization was similar, as would be expected since they were synthesized under identical conditions and differed only in the presence of the individual proline analogues. Thus, it is unlikely that racemization per se could account for the differing activities of the peptides. It remains to be determined whether biological activity resides with one or both isomers.

In conclusion, we have shown that appropriately constrained B-segment fragments of TGF-α are mitogenically active on cells expressing EGF-R. The relative activity of the peptides correlated with the proportion of the cis-proline isomer in their linear precursors and this may have contributed to their biological potency after cyclization. Our observation that the ABHC proline mimetics can be readily incorporated into peptides and used to enhance the population of cis-propeptidyl bonds may have wider synthetic applications in the development of other bioactive peptides.

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