Efficient approach to synthesis of two-chain asymmetric cysteine analogs of receptor-binding region of transforming growth factor-α

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Received 28 October 1991, accepted for publication 26 January 1992

The putative receptor-binding region of human transforming growth factor-α (TGFα) has been shown to be contributed by two fragments: an A-chain (residue 12–18) and a 17-residue carboxyl fragment (residue 34–50) that includes a disulfide-containing C-loop (residue 34–43). An approach to the synthesis of two-chain analogs containing an intermolecular disulfide linked A-chain and the 17-residue carboxyl fragment (C-fragment) possessing receptor-binding activity is described. The synthesis was achieved by the solid-phase method using the Boc-benzyl protecting group strategy. The single Cys of the A-chain was activated as a mixed disulfide with 2-thiopyridine to form the intermolecular disulfide bond with Cys41 or Cys46 of the C-fragment on the resin support. Prior to this reaction, the acetamido (Acm) protecting group of Cys41 or Cys46 was removed by Hg(OAc)₂ on the resin support. The peptide and side chain protecting groups including the S-methylbenzyl moiety of the Cys34 and Cys43 were concomitantly cleaved by high HF. The intramolecular disulfide with two unprotected Cys was formed in the presence of an intermolecular disulfide. This intramolecular disulfide bond formation was usually not feasible under the traditionally-held scheme at basic pH since disulfide interchange would occur faster than intramolecular oxidation. To prevent the disulfide interchange, a new method was devised. The intramolecular disulfide bond oxidation was mediated by dimethylsulfoxide at an acidic pH, at which the disulfide interchange reaction was suppressed. The desired product was obtained with a 60–70% yield. In contrast, the conventional scheme of using I₂ to form the intermolecular disulfide between the Cys(Acm) of the A-chain and C-fragment with the preformed intramolecular disulfide bond in solution phase did not result in any product. The purified two-chain analogs were found to be unstable and rearranged to the homo-dimers. This reaction was greatly accelerated in I₂, which explained the difficulty associated with the conventional scheme. When assayed against A431 and NRK clone 49F cells, both the A-chain and the C-fragment did not exhibit any biological activity independently, but the two-chain analogs showed low receptor-binding activity with an IC₅₀ at 0.3 mM level. Unexpectedly, dimeric C-fragment, which resulted from the rearrangement reaction, also showed receptor-binding activity. Our results demonstrate that the two-chain analogs exhibit low but distinct biological activity and provide evidence that the putative TGFα receptor binding region may be discontinuous. In addition, we also provide an efficient approach to further explore the two-chain receptor-binding analogs of TGFα.

Key words: disulfide formation, asymmetric; disulfide formation, dimethylsulfoxide-mediated; epidermal growth factor, receptor-binding region; solid-phase peptide synthesis; transforming growth factor-α

Human transforming growth factor type α (TGFα) is a member of the epidermal growth factor (EGF) family (1–10). This family of growth factors, which binds to and activates a common EGF receptor-tyrosine protein kinase, is important for the maintenance and growth of both normal and malignant cells. TGFα and EGFs are produced by a diverse range of cells and organisms (11–21) and because of common occurrence and mitogenic functions, they are important therapeutic targets for wound healing and antitumor agents.

TGFα contains the essential salient features of the EGF family. It has 50 amino acids and three disulfide bonds. The study of structure-function relationships of TGFα and EGF has been frustrated, however, by the lack of small peptide analogs that contain high biological activity (27–29). Part of the reason could be the lack of definitive proof for the exact location of the receptor-binding region of TGFα.

Two important clues contribute to the understanding of the putative receptor-binding portion of TGFα. First,
there is a substantial body of point-substituted synthetic analogs of TGFx or EGF with biological activity. Second, both 1NMR structures of EGF and TGFx have been determined and found to be similar in the overall backbone structures (22–26). The conclusion based on the NMR studies suggests that the B loop, with its dominant β-sheet structure, acts as a scaffolding that brings A- and C-loops together, and forms the receptor-binding region. The receptor-contact residues (Fig. 1) are likely to comprise three discontinuous fragments: residue 12–18 of the A-chain; the tripeptide Arg24-Cys-Glu, of the C-loop; and the dipeptide, Asp47-Leu, of the external COOH fragment, also of the C-loop. These results indicate that the receptor-binding region of TGFx is likely to comprise discontinuous fragments spatially orientated by the disulfide-bonded structure, and the β-sheet structure of the B-loop. The putative receptor-binding region is hypothesized to be composed of three disconnected regions: the A-chain (H-T-Q-F-C-F-H) consisting of residue 12–18 and the C-fragment consisting of residue 34–50 which includes the residue Arg47, Asp47, and Leu48. It would, therefore, be useful to prepare receptor-binding analogs by combining both regions with an interchain disulfide linkage. This will require the synthesis of a two-chain asymmetric cystine TGFx analog (Fig. 1) such as the structures of compound 1 and 2.

There are several approaches to form the interchain disulfide chain. A convenient approach is to use non-specific oxidation in which all sulfhydryls are concomitantly subjected to oxidation to arrive at the desired product. In the second approach, Cys of both chains are selectively protected by Acm or trityl (30–32) and are subjected to oxidation to arrive at the desired product. In this paper, we describe the synthesis of both hetero- and homo-disulfide products. To provide specific disulfide formation, the third approach (33) uses thiol disulfide exchange reaction in which one chain is activated and the other chain contains a free sulfhydryl. In this paper, we describe the synthesis of the two-chain TGFx receptor-binding region analogs by the latter two approaches. Moreover, we use dimethylsulfoxide (DMSO) as a new disulfide and oxidation reagent which overcomes several limitations of the conventional approaches.

FIGURE 1
Occurrence of A-chain and C-fragment that forms the putative receptor-binding region of TGFx.

Asymmetric cysteine analogs of TGFx

EXPERIMENTAL PROCEDURES

Material and methods
Methylbenzhydramine hydrochloride resin (MBHA resin) was purchased from Advanced Chemtech. PAM resin and Boc amino acids were purchased from Bachem Inc. USA; CH2Cl2 from Fisher Scientific, DMF from Baxter, trifluoroacetic acid from Halocarbon, and N,N'-dicyclohexylcarbodiimide was from Fluka Chemical 1-hydroxybenzotriazole, p-cresol, N,N-diisopropylethylamine, dimethylsulfide, and 2,2'-thiopyridine were obtained from Aldrich Chemical.

Amino acid hydrolysis of the free peptide was performed in 5.7 N HCl heating at 110°C for 24 h. The hydrolysates were analyzed on a Beckman 6300 high performance amino acid analyzer. Analytical and preparative HPLC were performed on Shimadzu and Waters' HPLC. Mass spectrometric analysis was determined by electrospray ionization.

Synthesis of C fragment analogs of TGFx
Two C-fragments (residue 34–50): Cys(MeBzl)-His(Dnp)-Ser(Bzl)-Gly-Tyr(BrZ)-Val-Gly-Ala-Arg(Tos)-Cys(MeBzl)-Glu(OBz1)-His(Dnp)-Cys(Acm)-Asp(OBz1)-Leu-Leu-Ala-OCH2-Pam resin and Cys(MeBzl)-His(Dnp)-Ser(Bzl)-Gly-Tyr(BrZ)-Val-Gly-Cys(Acm)-Arg(Tos)-Cys(MeBzl)-Hgl(OBz1)-His(Dnp)-Ala-Asp(OBz1)-Leu-Leu-Ala-OCH2-Pam resin, were synthesized separately with an automatic peptide synthesizer (ABI 430A) on Boc-Ala-OCH2-Pam resin (0.43 g, substitution 0.8 mmol/g) (34–38). Standard protocols of double coupling with performed symmetric anhydrides (39) in DMF were used except for Arg(Tos), Asn, and Glu which were coupled as hydroxybenzotriazole esters (40). The yield of 1.5 g was 98% based on theoretical yield.

Synthesis of A chain corresponding to the residue 12–18 of A-loop (Ac-His-Thr-Gln-Phe-Cys-Phe-His-NH2)
The A-chain (residue 12–18) was synthesized manually on p-methylbenzhydramine resin (2.55 g, substitution 0.6 mmol/g) (41). Double couplings were performed with symmetric anhydrides for Cys and Phe in CH2Cl2. His(Dnp), Gln and Thr(Bzl) were coupled as hydroxybenzotriazole esters. At the completion of the synthesis, the resin was acetylated with 45% acetic anhydride in DMF for 10 min. The resin was washed thoroughly by DMF and CH2Cl2. 3.56 g (theory: 4.35 g) of peptide resin was obtained (theory: 4.35 g) to give 82% yield.

The resin (0.36 g) was treated four times with 10% thiophenol in DMF for 8 h to remove the N'-Dnp protecting group of His42 and then with 50% (v/v) CF3COOH/CH2Cl2 for 20 min to remove the N'-Boc group. The dried peptide resin was treated with the low/high HF method (43, 44). After HF, the crude peptide was washed with cold ether three times, extracted into 100 mL of 50% acetic acid, diluted to
500 mL, and lyophilized. The lyophilized powder was purified by preparative C-18 reverse-phase chromatography.

To remove the Cys(Acm), Hg(OAc)$_2$ was used (45). 4.82 mg Hg(OAc)$_2$ (15.16 pmol) was added to 7.82 mg of purified heptapeptide in 0.41 mL H$_2$O (pH was adjusted to 4) while stirring at room temperature. Using C-18 reverse-phase HPLC to monitor the deprotection process, the reaction was completed within 1 h. The solution was diluted to 7 mL, and mercaptoethanol was added while stirring for 2 h. The mixture was purified by C18 reverse-phase HPLC by aqueous CH$_3$CN containing 0.0445% TFA with a linear gradient from 10% CH$_3$CN to 22% in 1 h at 2.5 mL/min. The major peak was pooled under a stream of N$_2$ and lyophilized to obtain 4.2 mg of product in 58% yield. Amino acid analysis gave the expected molar ratio of the title compound. Mass spectrometric analysis gave (M + H)$^+$ 1030.9 and (M + 2H)$^2+$ 1031.2 (calc. 1032.1).

Preparation of Ac-His-Thr-Glu-Phe-Cys(SPyr)-Phe-His-NH$_2$

Mixed disulfide formation on the peptide. Hg(OAc)$_2$ (44.36 mg, 0.139 mmol) in 0.44 mL of 3% HOAc was added to the purified heptapeptide (71.98 mg, 0.139 mmol) in 0.44 mL of 3% HOAc for 4 h at room temperature. The mixture was diluted with water, H$_2$S was bubbled into the reaction solution to precipitate Hg(II) salt. After 10 min, the black solution was filtered through a layer of celite and sodium acetate (0.6 g) was added to the filtrate, followed by 2-pyridyl disulfide (154 mg, 0.7 mmol) in 3.5 mL propanol and 2 mL of 0.1M sodium acetate buffer. The solution turned turbid and another 7 mL propanol and 20 mL H$_2$O was added to clarify the solution. The reaction was completed within 15 min based on C18 reverse-phase HPLC monitoring. After 1 h, the solution was adjusted to pH 3, filtered and purified by C-18 reverse phase HPLC by aqueous CH$_3$CN containing 0.045% TFA with a linear gradient from 10% CH$_3$CN to 44% in 45 min. The major peak appeared in the 33% of B buffer and was pooled. After lyophilization, 72.6 mg of the desired product (70% of peptide content) was obtained to give a 71% yield. The amino acid analysis gave the expected molar ratio and the mass spectrometric analysis gave (M + H)$^+$ 1068.8 and (M + 2H)$^2+$ 1069.2 (calc. 1069.3).

On the resin. A-chain peptide resin (130 mg, 42.8 pmol) with N$_2$-Dnp deprotected from His was shaken with 0.1 M Hg(OAc)$_2$ in DMF for 4 h, drained, washed with DMF for four times, and then washed with 10% mercaptoethanol in DMF solution overnight. After the resin was thoroughly washed by DMF to remove mercaptoethanol completely, it was reacted with 2-pyridyl disulfide (18.83 mg, 85.6 pmol) in DMF solution containing 30% propanol and 5 drops of 1 M NaHCO$_3$. The reaction was monitored by the change of UV absorption at 343 nm. After 22 h, the reaction was completed. The resin was washed with DMF 3 times and dried in a desiccator under high vacuum overnight. The resin was treated with 9 mL HF and 1 mL anisole at 0° for 1 h (44). No thiol or sulfide scavenger should be added to the HF cleavage step. After the HF cleavage, the anisole was removed with CH$_3$CN, and the peptide was extracted into 10% deaerated HOAc, diluted, and purified by C18 reverse-phase HPLC. The major peak was pooled and was identical with the title compound obtained from the method for the peptide (above).

Preparation of the two-chain analogs 1 and 2

C loop (residue 34–50) resin (60 mg, 16.1 pmol) was treated with 10% thiophenol in DMF solution for 8 h four times to remove the Dnp protecting group of His. After the resin was washed thoroughly with DMF, the Cys(Acm) was removed by adding 0.1 M Hg(OAc)$_2$ in DMF solution and stirring for 6 h. The resin was drained, washed with DMF again and shaken in 10% mercaptoethanol DMF solution overnight to remove Hg(II) salt. The resin was drained again, washed with deaerated DMF to remove mercaptoethanol. Cys(Spyr)-heptapeptide (40.62 mg, 38 pmol) was added to the resin in 2.43 mL of DMF-propanol and 40 µL DMEA. After the reaction, 24.74 mg of the starting material was recovered, therefore the actual amount used was 15.85 mg. This reaction was monitored by HPLC and the change of UV absorption at 343 nm due to the release of 2-thiopyridone. After 1.5 h, A$_{343}$ was 0.0749, and 22 h later, A$_{343}$ increased to 0.2576 and became steady. Using HPLC, Cys(Spyr)-heptapeptide was 98%, and 2-thiopyridone was 2%, 1.5 h after the reaction started. 22 h later, Cys(Spyr)-heptapeptide was 84%, and the thiopyridone became 16% and steady. The resin was drained and Cys(Spyr)-heptapeptide (24.74 mg) was recovered. TFA was then used to remove the Boc groups. The resin was washed with DMF and dried in a desiccator under high vacuum for 1 h. The resin was treated with HF (9 ml) in the presence of anisole (1 mL) at 0° for 50 min. After the removal of HF, the crude peptide was extracted into 50 mL of 10% acetic acid. It was diluted into 5% acetic acid solution, and then 11 mL DMSO was added before adjusting the pH to 4 with 1.6 mL of NH$_4$OH for disulfide oxidation. The oxidation process was monitored by HPLC. After 8 h, preparative C18-reverse phase HPLC was used for purification using aqueous acetonitrile containing 0.045% TFA with a linear gradient from 5% CN$_3$CN to 25% CH$_3$CN in 50 min. The major product was 43.4 mg and was purified again by a preparative column with a linear gradient from 15% B to 35% in 5 min and the desired product eluted at B conc. of 32.62% to give 56% yield. Amino acid analysis revealed: Asp(1):1.1, Thr(1):0.8, Ser(1):0.9, Glu(2):1.8, Gly(2):2.1, Ala(2):2.0, Val(1):1.0, Leu(2):2.2, Tyr(1):0.8, Phe(2):1.7, His(4):3.5, Arg(1):1.1. Mass
Spectrometry analysis: theory, 2791.1, found (M + 4H)+, 2789.6, (M + 5H)+, 2791.0 (M + 3H)+, 2789.4. The major side product was the homo-dimer. Amino acid analysis showed that Asp(1): 1, Ser(1): 1, Glu(1): 1, Gly(2): 2, Ala(2): 2, Val(1): 1, Leu(2): 2, Tyr(1): 0.9, His(2): 2, Arg(1): 1. Mass spectrometric analysis theory: 3662.1 found: (M + 3H)3+ 3661.5, (M + 4H)4+ 3661.0, (M + 5H)5+ 3661.5, (M + 6H)6+ 3662.4.

Biological uses
The mitogen assay of synthetic analogs was performed on normal rat kidney cells in a modified Eagle's medium with 10% heat-inactivated calf serum for 24 h and maintained in a medium containing 0.2% calf serum for 3 days before the assay as described (1). Incorporation of [3H]thymidine was counted after 24 h exposure. Dose-response curves of the radioreceptor were obtained and performed as described (1). Inhibition of [125I]-EGF binding to the EGF-receptor was determined by subconfluent monolayers of formalin-fixed A-431 cells after 1 h incubation at 22°C with either synthetic TGFα or natural mouse EGF.

RESULTS AND DISCUSSION

Background and rationale for a new scheme
Our original scheme for the synthesis of the two-chain, two-disulfide analogs 1 and 2 is shown in Fig. 2. The single cysteinyl moiety of the A-chain was protected with the Acm protecting group (46, 47). The three cysteines of the C-fragment were blocked selectively by two types of protecting groups. The Cys at position 41 (1) or Cys 46 (2) of the C-fragment was protected with Acm while cysteines at position 34 and 43 were protected with the p-methylbenzyl group. In such a scheme, the intramolecular disulfide bond at positions 34 and 43 after HF treatment was formed without difficulty by air oxidation while the remaining Cys(Acm) was then used to form the intermolecular disulfide with the A-chain by I2 oxidation (Fig. 3). However, no intermolecular disulfide product was obtained despite many attempts and changes of protocol. The major product was the dimeric C-fragments (Fig. 7).

The difficulty associated with the intermolecular disulfide could be attributed to the instability of the intermolecular disulfide which rearranged to the dimeric products under the reaction condition (see below for further explanation). In view of the difficulty, a new scheme was devised to form the intermolecular disulfide bond prior to the formation of the intramolecular disulfide bond using the same scheme of the protecting groups. To achieve this scheme, the thiol group of the A-chain had to be activated and we chose to use the mixed disulfide of 2-thiopyridine. However, the key to success was the formation of the intramolecular disulfide mediated by dimethylsulfoxide at low pH which minimized the disulfide interchange reaction.

Synthesis of the thiol-activated A-chain
The synthesis of Cys(SPyr)-containing and thiol-activated A-chain was accomplished in two ways (Figs. 4 and 5). In the first approach (Fig. 4) the A-chain was synthesized by the solid-phase method and purified. The Acm protecting group of Cys was removed to allow the sulfur-sulfur bond formation of Cys(SPyr). In the second approach (Fig. 5) all the manipulations including the sulfur-sulfur bond formation of Cys(SPyr) were performed on the solid support to allow the attainment of the final product after the HF cleavage from the resin. The advantage of the first approach was that since some of the byproducts were removed during the intermediate purification step, the desired product appearing as a clear major peak that facilitated both purification and identification (Fig. 7). The advantage of the second approach was that the inherent advantage of the solid-phase scheme was exploited to the fullest without manipulation of the intermediates. However, the final product was obtained as
The deblocking of Cys(Acm) with \( \text{Hg(OAc)}_2 \) was accomplished in 4 h and the \( \text{Hg}^{2+} \) salt was removed with 10% mercaptoethanol in DMF in 18 h. Although the model study with resin anchored dipeptide containing Cys(Acm) showed that the reaction was facile and complete removal of Cys(Acm) could be achieved in 0.5 h (45), the long reaction time for the deblocking of A-chain served only a prudent measure. I2 or other electrophilic reagents could also be used to effect the same deblocking reaction. The second step of the mixed disulfide reaction with pyridyl disulfide was carried out in a mixed solvent containing 30% propanol. The reaction was slow as monitored by the UV absorption at 343 nm due to the release of thiopyridone and required 22 h for completion. HF cleavage of the peptide resin in the absence of any thiol or sulfide scavengers produced the desired product in 50% yield (43, 44). A side product which accounted for 45% of the major product was identified as the homo-dimer which was formed during the activation step.

**Intermolecular disulfide bond formation on resin**

The C-fragment was prepared by the solid-phase method. The protection scheme for the three Cys involved a-methylbenzyl for Cys34 and Cys43 but Acm for Cys41. The two key steps of the disulfide formation involved the interdisulfide formation between Cys41 and the A-chain, and the intradisulfide formation between Cys34 and Cys43 (Fig. 6). The Acm group of Cys41 was removed on the resin support by Hg(OAc)2 and the thiol-activated A-chain was coupled to the resulting free thiol on the resin support in a mixed solvent of DMF and propanol. The reaction was monitored by UV absorption at 343 nm due to the release of 2-thiopyridone. After 22 h, the reaction was judged to be complete as the level of UV-absorption became steady.

**Dimethylsulfoxide (DMSO) mediated formation of intramolecular disulfide bond at low pH**

The key to the new approach is the use of DMSO-mediated oxidation of the intramolecular disulfide bond (48) in the presence of the intermolecular disulfide bond (Fig. 6). DMSO is a mild oxidant selective for disulfide bond formation in peptides. Facile disulfide bond formation by DMSO in aqueous buffered solutions is found to proceed in a wide range of pH. Since the disulfide bond interchange reaction occurs at neutral and basic pH, the oxidation of the intramolecular disulfide bond can be performed at the acidic pH to minimize the disulfide bond interchange reaction. Furthermore, the rate of the disulfide formation is dependent on the concentration of DMSO and the rate of the intramolecular disulfide bond formation can be conveniently adjusted with an increased concentration of DMSO. We have found that a 20% DMSO at about 3 M concentration would render most disulfide oxidation complete within 2 h (48).

The side-chain protecting groups and the peptide
Asymmetric cysteine analogs of TGFα

Ac-His-Thr-Glu-Phe-Cys(SPyr)-Phe-His-NH₂

FIGURE 6
Scheme used for preparation of the two-chain analogs.

were cleaved by the high HF procedure (43, 44) without the use of any thiol or sulfide scavengers to preserve the integrity of the intermolecular disulfide bond. The intermolecular disulfide bond between Cys34 and Cys41 was oxidized by the presence of 20% DMSO in the reaction mixture. The low pH oxidation greatly minimized the disulfide exchange reaction and allowed the selective intramolecular reaction to occur. After HF, the crude C-fragment was extracted into deaerated aqueous acetic acid under argon, and the solution was adjusted to pH 4 containing 20% DMSO. The oxidation was completed in 8 h as monitored by HPLC. This reaction could not be achieved by electrophilic agent such as I₂ at acidic pH (vide infra) or air oxidation at basic pH because of the disulfide interchange reaction. The two-chain analogs were purified by C18-reverse phase HPLC (Fig. 7) without difficulty, and the purified products gave the expected molar ratio of amino acids and molecular mass. This scheme of reaction was performed on four two-chain disulfide analogs (Fig. 3) and the yield, based on the C-fragment, ranged from 60–70%. The major side products of the reaction were the homomeric dimers.

Instability of inter-disulfide bond and formation of homo-dimeric C-fragment
We were intrigued by the difficulties associated with the conventional scheme of forming the two-chain analogs (see Fig. 2). Several laboratories have shown successfully that such a scheme works well for heteromeric disulfide formation (30–33). One probable explanation for our difficulty may be the presence of four histidines, two on each chain. The imidazole side chains could be correctly positioned to catalyze the intermolecular disulfide bond disproportionation and subsequent rearrangement into homo-dimers. Purified two-chain analogs 1 and 2 were tested for their stability at pH 3–4. 30% of the homo-dimeric product of C-fragment from 1 was observed in 5 days. The rate of rearrangement of 2 was 3-fold faster than 1. However, the rate of rearrangement was accelerated nearly 20-fold in the presence of I₂ and iodide ion. These results show that the two-chain analogs are unstable under the scheme of I₂ oxidation and would rearrange rapidly under the reaction condition to the more stable homo-dimeric products.

Biological activity
The primary in vitro assays for TGFα have been the mitogenic and radioreceptor assays using the normal rat kidney fibroblasts (NRK) and the human epidermal carcinoma A431 cells which are rich in EGF receptors, respectively. For all synthetic analogs, the receptor-binding assay in concentrations as high as 1 mM was used for screening biological activity and those analogs

FIGURE 7
Analytical C₁₈ reverse-phase HPLC. (A) reactants for Fig. 2, purified A-chain containing Acm (peak 1) co-injected with the purified C-fragment also containing Acm (peak 2). Crude reaction products of activated A-chain with 2-thiopyridine prepared by the solution method (B, see Fig. 4) and by the solid-phase method (C, see Fig. 5). The desired product (peak 3 and 6) is found in the mixture with the homo-dimers (4, 7, 8). The 2-thiopyridone is often the most prominent peak in these spectra (peaks 5, 9, and 12). Crude (D) and purified (E) product of the disulfide-linked A-chain and C-fragment. The desired product is indicated by peaks 10 and 13. Peak 11 is the homo-dimer.

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that displayed activity higher than 1 mM were considered inactive (Table 1). Both the A-chain and the C-fragment were found to be inactive based on the above criterion. The two-chain analogs 1 and 2 were found to contain low receptor-binding activity with an IC50 in the range 0.1–0.3 mM which was about 10 000-fold less active than TGFx. There was essentially no differences between the acetylated or non-acetylated analogs of 1 and 2. Unexpectedly, the homo-dimeric analogs of the C-fragment 3 and 4 were found to also contain low receptor-binding activity with an IC50 ranging from 0.3–0.4 mM. More interestingly, their mitogenicity with an ED50 at 0.03–0.1 mM was fold more active than the two-chain analogs.

CONCLUSION

Our results describe a new approach for the formation of two-chain, two-disulfide peptides for structure-function relationship studies. The salient features of our approach are as follow:

1. This approach fully exploits the attendant advantages of the solid-phase scheme. The activation of the A-chain to form the sulfur-sulfur bond of mixed disulfide with thiopyridine, the deprotection of the Cys(Acm) groups, and the formation of the intermolecular disulfide bond can be performed on the solid-phase scheme.

2. Contrary to conventional thinking, the disulfide bond is relatively stable in the high pH condition in the absence of thiol or sulfide scavengers. This advantage can be exploited to allow the formation of the intermolecular disulfide bond between two chains on the resin support to increase yield and recycling of the excess reagent.

3. The formation of an intramolecular disulfide bond from two unprotected Cys in the presence of the intermolecular disulfide bond can be accomplished selectively and efficiently by DMSO-mediated oxidation at an acidic pH.

We have also shown that the two-chain analogs possess low biological activity. The low biological activity of analogs 1 and 2 could result from the conformation and orientation of the A-chain posed by the intermolecular disulfide bond not being optimal. However, this approach will allow further exploration of two-chain analogs to define the receptor-binding region of TGFx.

ACKNOWLEDGMENT

This work was supported by PHS Grant number CA36544, awarded by the National Cancer Institute, and DHHS. We thank Dr. B. Chait of the Rockefeller University Mass Spectrometry Center for the mass spectrometric analyses, Dr. Paul Haser for his comments, and Ms. Jenny Park for the preparation of the manuscript.

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