Promotion of Fibroblast Adhesion by Triple-helical Peptide Models of Type I Collagen-derived Sequences*

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The dissection of the activities mediated by type I collagen requires an approach by which the influence of triple-helical conformation can be evaluated. The \( \alpha_1 \beta_1 \) and \( \alpha_2 \beta_2 \) integrin binding sites within type I collagen are dependent upon triple-helical conformation and contained within residues 124–822 from \( \alpha_1(1) \). Seven \( \alpha_1(1) \)-derived triple-helical peptides (THPs) were synthesized based on charge clustering (\( \alpha_1(1)496–507 \), \( \alpha_1(1)385–396 \), \( \alpha_1(1)406–417 \), \( \alpha_1(1)415–423 \), \( \alpha_1(1)448–456 \), \( \alpha_1(1)496–507 \), and \( \alpha_1(1)526–537 \)). Three additional THPs were synthesized based on charge clustering (\( \alpha_1(1)185–96 \), \( \alpha_1(1)433–441 \), and \( \alpha_1(1)772–786 \)) based on previously described or proposed activities (Kleinman, H. K., McGoodwin, E. B., Martin, G. R., Klebe, R. J., Fietzek, P. P., and Wooley, D. E. (1978) J. Biol. Chem. 253, 5642–5646; Staatz, W. D., Fok, K. F., Zutter, M. M., Adams, S. P., Rodriguez, B. A., and Santoro, S. A. (1991)) .

The peptide analog (\( \alpha_1(1)1772–786 \)) exhibited considerably lower levels of cell adhesion promotion even at peptide concentrations as high as 100 \( \mu \)M. This sequence was important for cell recognition, as the \( \alpha_1(1)1772–786 \) THP had greatly reduced cell adhesion activity compared to the wild-type sequence. Triple-helical peptides were designed based on charge clustering (\( \alpha_1(1)496–507 \), \( \alpha_1(1)385–396 \), \( \alpha_1(1)406–417 \), \( \alpha_1(1)415–423 \), \( \alpha_1(1)448–456 \), \( \alpha_1(1)496–507 \), and \( \alpha_1(1)526–537 \)). Three additional THPs were synthesized based on charge clustering (\( \alpha_1(1)185–96 \), \( \alpha_1(1)433–441 \), and \( \alpha_1(1)772–786 \)) based on previously described or proposed activities (Kleinman, H. K., McGoodwin, E. B., Martin, G. R., Klebe, R. J., Fietzek, P. P., and Wooley, D. E. (1978) J. Biol. Chem. 253, 5642–5646; Staatz, W. D., Fok, K. F., Zutter, M. M., Adams, S. P., Rodriguez, B. A., and Santoro, S. A. (1991)).

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Collagens are important structural components of the extracellular matrix (ECM). They are distinguished by their triple-helical conformation composed of three chains with a repeating Gly-X-Y sequence motif. In addition to providing the structure of connective tissue, collagens can mediate intracellular communication. Cell-collagen interactions play a role in a number of processes including cell migration (1, 2), collagen catabolism (3, 4), and the aggregation of platelets (5–13). One approach for developing novel therapies for diseases linked to ECM interactions, such as tumor cell metastasis, atherosclerosis, inflammation, and thrombosis, or to better understand normal processes such as wound healing, is to identify cellular recognition sites within collagen molecules and dissect the structure-activity relationship for receptor-ligand binding (14).

Type I collagen, the most abundant protein in higher vertebrates, is composed of two identical \( \alpha_1 \) chains and an \( \alpha_2 \) chain. Cytogenon bromide (CB) fragments of the \( \alpha_1 \) chain have been used to locate integrin-mediated cell binding sites within type I collagen. The \( \alpha_1(1)CB3 \) fragment (residues 403–551) and the \( \alpha_1(1)CB8 \) fragment (residues 124–402) contain binding sites for \( \alpha_1 \beta_1 \) integrin (2, 15). \( \alpha_1(1)CB3 \) also contains a binding site for the hepatocyte \( \alpha_2 \beta_1 \) integrin (15), while \( \alpha_1(1)CB8 \) (residues 552–822) and \( \alpha_1(1)CB8 \) contain binding sites for the platelet \( \alpha_1 \beta_1 \) integrin (12, 13, 16).

Several distinct sequences derived from type I collagen CB fragments have been identified as cell adhesion sites. The adhesion of Chinese hamster ovary cells to type I collagen is inhibited by the 757–791 sequence located within the \( \alpha_1(1)CB7 \) fragment (17). A peptide incorporating residues \( \alpha_1(1)769–783 \) supports human fibroblast adhesion and migration and inhibits human fibroblast and human T-lymphocyte attachment to type I collagen (18, 19). By using short synthetic peptides, an \( \alpha_2 \beta_1 \) integrin binding site could be identified containing the minimal sequence AspGlu-Glu-Ala (20). This sequence corresponded to residues 435–438 within the \( \alpha_1(1)CB3 \) fragment. The \( \alpha_1(1)434–438 \) peptide inhibits adhesion of platelets and breast carcinoma cells to type I collagen in a concentration-dependent manner (20) and, not surprisingly, was not effective at inhibiting \( \alpha_2 \beta_1 \)-mediated chondrosarcoma cell adhesion to type II collagen (21).

There may be two types of active sequences within type I collagen. The activity of some sequences may require triple-cycd(5.4)undec-7-ene; Dde, 1-(4,4-dimethyl-2,6-dioxo cyclohex-1-yliden)-ethyl; DIAE, N,N-diisopropylleucilamine; DIPCDI, N,N-diisopropylcarbodiimide; DMF, N,N-dimethylformamide; DMPAMP, 4-(2,4-dimethoxyphenylaminomethyl)phenoxo; ESMs, electrospray mass spectrometry; FABMS, fast atom bombardment mass spectrometry; Fmoc, N-(9-fluorenyl)methoxycarbonyl; GPP*, N-Tris(Gly-Pro-Hyp)-Ahx-Lys-Lys-Tyr-Gly; HBTU, 2-(1H-benzotriazole-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate; HOBT, 1-hydroxybenzotriazole; mAB, monoclonal antibody; MMP, matrix metalloproteinase; NHDF, normal human dermal fibroblasts; PBS, phosphate-buffered saline solution; RP-HPLC, reversed-phase high performance liquid chromatography; SSP, single-stranded peptide; Tbu, tert-butyl; TFA, trifluoroacetic acid; THP, triple-helical peptide.
helicity, while others might be nonfunctional when contained in native, triple-helical conformation but revealed in the denatured state (33). Only single-stranded peptides (SSPs) have been utilized in prior studies on the cellular activities of type I collagen sequences. However, the triple-helical conformation of collagen has been shown to be important (if not crucial) for influencing cell adhesion (2, 7, 13, 22–25), cell spreading (13, 28, 29). Conversely, cell adhesion to denatured, but not native, collagen can be inhibited by linear peptides containing Arg-Gly-Asp sequences (15, 30–33). Denatured collagen Arg-Gly-Asp sites are bound by the α5β1 (15) or α6β1 integrin (30). To fully understand the role of collagen in ECM remodeling requires delineation of native collagen active sites from denatured collagen sites.

In the present study, we have examined potential cellular recognition sites within type I collagen and studied the significance of triple-helical conformation. Sequences were derived from α1(I)CB fragments that possess integrin binding sites. To utilize relatively short sequences (≤15 residues) and yet ensure triple-helical conformation under biological assay conditions, we applied a methodology developed specifically for the assembly of collagen-model, triple-helical peptides (THPs) (34, 35). A total of 11 THPs have been synthesized. Cellular recognition was studied by assaying normal human dermal fibroblast adhesion to these THPs. The influence of triple-helicity was examined by comparing the activity of a SSP and THP containing the same collagen-derived sequence. The potential involvement of integrins in mediating cell adhesion to a specific THP was determined. The branched peptide-resin was synthesized by using Fmoc-Gly-Sar resin (substitution level 0.46 mmol/g) using Fmoc methylation

EXPERIMENTAL PROCEDURES

Materials—All standard peptide synthesis chemicals and solvents were analytical reagent grade or better and purchased from Applied Biosystems, Inc. (Foster City, CA) or Fisher Scientific (Pittsburgh, PA). Fmoc-Gly-Sar resin (substitution level 0.7 or 0.65 mmol/g) and Fmoc-Gly-Pro-Hyp were purchased from Bachem Biosciences Inc. (Philadel-phia, PA). Fmoc-DMPAMP resin (substitution level = 0.46 mmol/g) was from Novabiochem (La Jolla, CA) and Fmoc-Met, Fmoc-Hyp(Bu), and Fmoc-Ahx from Advanced ChemTech (Louisville, KY). All other Fmoc-amino acid derivatives were from Novabiochem or Perseptive Biosystems (Framingham, MA). All amino acids are of L-configuration. Fmoc-amino acid derivatives were from Novabiochem or Perseptive Biosystems (Framingham, MA). All amino acids are of L-configuration. Fmoc-Ahx from Advanced ChemTech (Louisville, KY). All other Fmoc-amino acid derivatives were from Novabiochem or Perseptive Biosystems (Framingham, MA). All amino acids are of L-configuration.

Preparation of (N-Tris(Fmoc-Ahx)-Lys-Lys-Tyr(Bu)-Gly-Sar resin—0.97 g of Fmoc-Tyr(Bu) (2.1 mmol), 1.96 g of Fmoc-Lys(Dde) (2.1 mmol), and 1.96 g of Fmoc-Lys(Dde) (2.1 mmol) were successively coupled to 1.0 g of Fmoc-Gly-Sar resin. The coupling was repeated using the same procedure. The resin was deprotected and cleaved with water-TFA (1:19) and was modified for other THPs according to their side-chain requirements.

The flow rate was 1.0 ml/min. Eluants were 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The elution gradient was 0–100% B in 70 min with elution at the described eluents. The purity of the THP was determined using HPLC (42). Analysis by FABMS gave (M + H)+ = 834.4 Da by ESMS and (M + H)+ = 834.6 Da by FABMS (theoretical (M + H)+ = 834.5 Da). Triple-Helical Peptide Synthesis and Purification—Incorporation of individual amino acids onto the branched peptide resin was performed initially by Fmoc solid-phase methodology on a Gilson AMS 422 Automated Multiple Peptide Synthesizer. The cycles for the Gilson AMS 422 were modified from those described previously (38) by using double couplings with 6-fold excess of Fmoc-amino acids. Addition of the 6 final repeats of Gly-Pro-Hyp was done manually in a shaker vessel. The couplings of the tripeptides to 0.11 g of peptide resin (substitution = 0.26 mmol/g) were performed with 57 mg of Fmoc-Gly-Pro-Hyp (0.12 mmol), 17 mg of HOBT (0.112 mmol), 32 mg of HBTU (0.1 mmol), and 30 µl of DIEA (0.212 mmol) in DMF for 2–3 h. Deprotection of the Fmoc-peptide resin was performed with DBU-piperidine-DMF (1:48) for 30 min, and the resin was subsequently washed three times with DMF.

The large-scale synthesis of peptide α1(I)772–786 THP was on an Applied Biosystems 431A Peptide Synthesizer. Peptide assembly, including final stepwise incorporation of individual Fmoc-Gly, Fmoc-Pro, and Fmoc-Hyp(Bu) residues, was performed by Fmoc solid-phase methodology as described (34) with several modifications. Coupling utilized 0.45 M HBTU, 0.50 M HOBT, and 0.95 M DIEA in DMF for 1 h, while Fmoc removal was achieved with 0.1 M HOBT in piperidine-1-methyl-2-pyrrolidinone (1:4) for 24 min and 6 min. The following cleavage procedure was applied for the large-scale synthesis of α1(I)772–786 THP and was modified for other THPs according to their side-chain protection (39). 203 mg of the peptide resin was Fmoc-deprotected with DBU-piperidine-DMF (1:48). Side-chain deprotection and cleavage from the resin were performed with water/thioanisole/TFA (1:1:18). The crude α1(I)772–786 THP was precipitated with methyl tert-butyl ether, lyophi-

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### Table I

| α1(I) Location | CB Fragment | Sequence |
|----------------|-------------|----------|
| 85–96          | 4 + 5       | Gly-Met-Lys-Gly-His-Arg-Gly-Phe-Ser-Gly-Leu-Asp |
| 256–270        | 8           | Gly-Glu-Hyp-Gly-Ala-Hyp-Gly-Asn-Lys-Gly-Asp-Thr-Gly-Glu-Hyp |
| 385–396        | 8           | Gly-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Gl...
Fmoc-Hyp(tBu) using an automated peptide synthesizer. The THP was purified by a two-step method developed recently (40, 54). Edman degradation analysis of the purified α1(I)772–786 THP gave the sequence (Gly-Pro-Hyp)₆-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Hyp. Laser desorption mass spectrometry analysis gave (M+K)⁺ = 9868.7 Da (theoretical (M+K)⁺ = 9865 Da). During the purification of α1(I)772–786 THP, we isolated a peptide which, by Edman degradation analysis, had the sequence (Gly-Pro-Hyp)₆-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val (40). This peptide was missing the first three amino acids next to the branch (α1(I) residues 784–786) and was thus designated α1(I)772–783 THP.

The triple-helical conformation of α1(I)772–786 THP and α1(I)772–783 THP was evaluated by CD spectroscopy. At low temperatures, α1(I)772–786 THP exhibited typical features of a collagen-like conformation (Fig. 2), such as a large negative molar ellipticity ([θ] at λ = 200 nm and a positive [θ] at λ = 225 nm (55). As the temperature was increased, [θ]₂₂₀ increased whereas [θ]₂₂₅ decreased (Fig. 2). The negative [θ]₂₂₅ at high temperatures indicates a melted triple-helix. The α1(I)772–783 THP had similar CD spectral characteristics (data not shown). The thermal stabilities of α1(I)772–786 THP and α1(I)772–783 THP were studied by measuring [θ]₂₂₅ as a function of temperature. When the temperature was increased from 10 °C to 80 °C, α1(I)772–786 THP showed a structural transition (triple-helix ⇔ coil) with a midpoint (Tm) of 43 °C (Fig. 3). The α1(I)772–783 THP also showed a transition over this temperature range, with Tm = 36 °C (Fig. 3). Both THPs had sufficiently stable triple-helical structures to allow for cellular assaying.

Adhesion of Normal Human Dermal Fibroblasts to α1(I)772–786 THP and Analogs—Biological studies proceeded with the following peptides: α1(I)772–786 THP, α1(I)772–783 THP, α1(I)772–786 SSP, and GPP*. The THP is composed of a carboxyl-terminal branch generated from 2 Lys residues, one α1(I)772–786 sequence per chain and six Gly-Pro-Hyp repeats per chain. GPP* is composed of the carboxyl-terminal branch and eight Gly-Pro-Hyp repeats per chain.

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![Fig. 2. CD spectra of purified α1(I)772–786 THP in acetic acid/water (1:99) at 10 °C and 80 °C. Spectra were recorded at α1(I)772–786 THP = 0.13 mM.](image)

![Fig. 3. Thermal transition curves for purified α1(I)772–786 THP (solid line) and α1(I)772–783 THP (dashed line) in acetic acid/water (1:99) at α1(I)772–786 THP = 0.13 mM and α1(I)772–783 THP = 0.11 mM. Molar ellipticities ([θ]) were recorded at λ = 225 nm while the temperature was increased from 10 °C to 80 °C.](image)

![Fig. 4. Structures of α1(I)772–786 THP, α1(I)772–783 THP, α1(I)772–786 SSP, and GPP*. The THP is composed of a carboxyl-terminal branch generated from 2 Lys residues, one α1(I)772–786 sequence per chain and six Gly-Pro-Hyp repeats per chain. GPP* is composed of the carboxyl-terminal branch and eight Gly-Pro-Hyp repeats per chain.](image)

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had low levels (<10%) of adhesion-promoting activity over the concentration range of 0.2–10 μM (Fig. 5).

To study the significance of collagen triple-helical structure on cellular recognition, we compared the NHDF adhesion-promoting ability of the α1(I)772–786 THP to the α1(I)772–786 SSP (Fig. 6). NHDFs showed a profound preference for binding and adhesion to the THP compared with the SSP. Half-maximal fibroblast cellular adhesion occurred at THP concentrations of 1.6 μM, while less than 10% cell adhesion was seen for the SSP up to a concentration of 10 μM. At a peptide concentration of 100 μM, cell adhesion to the SSP was only ~40% of the level promoted by the THP (data not shown). There was no NHDF adhesion to the generic THP containing 8 repeats of Gly-Pro-Hyp (GPP*) (Fig. 6).

Inhibition of Cell Adhesion to α1(I)772–786 THP by Anti-integrin mAbs—The inhibition of NHDF cell adhesion to α1(I)772–786 THP was compared for different anti-integrin mAbs (Table II). Initial experiments compared inhibition of adhesion by normal mouse IgG and the anti-β1 and anti-α2 mAbs at varying THP coating concentrations. Inhibition of adhesion was seen for the anti-β1 mAb at [THP] = 2.0, 5.1, and 10 μM. The anti-α2 mAb results were somewhat inconclusive. Studies at [THP] = 10 μM suggested that the anti-α2 and anti-α5 may inhibit adhesion to the α1(I)772–786 THP and that the anti-α5 integrin subunit mAb was not an inhibitor.

Inhibition of Cell Adhesion to Type I Collagen—To determine the ability of α1(I)772–786 SSP and α1(I)772–786 THP to inhibit cell adhesion to type I collagen, NHDFs were incubated with increasing peptide concentrations in the range of 0.5–10 μM. Neither the α1(I)772–786 SSP nor the α1(I)772–786 THP inhibited NHDF adhesion to type I collagen in a concentration-dependent fashion (data not shown). In contrast to the SSP and THP, type I collagen inhibited cell adhesion in a concentration-dependent fashion (data not shown).

**DISCUSSION**

The dissection of the various biological activities mediated by type I collagen requires an approach by which the influence of triple-helical conformation can be evaluated. More specifically, the mechanisms of collagen catabolism may require two types of cellular recognition sites, those that are dependent upon triple-helical conformation and those that are revealed upon denaturation of the triple-helix. Our initial interest is in identifying sites that are recognized in triple-helical conformation. The α1(1),α2(1), and α1(II) integrin binding sites are dependent upon triple-helical conformation (56) and contained within α1(1)CB3, α1(1)CB7, and α1(1)CB8 (encompassing residues 124–822 from α1(1)) (2, 12, 13, 15, 16). From the 124–822-residue region, we selected sequences that contained “clusters” of charged residues. Charged residues are often found in clusters in type I collagen (57), and cellular activities have been ascribed previously to collagen-derived synthetic peptides that have clustered charged residues (35, 44, 46–48). Seven THPs were synthesized based on charge clustering ([α1(1)]256–270, [α1(1)]385–396, [α1(1)]406–417, [α1(1)]415–423, [α1(1)]448–456, [α1(1)]496–507, and [α1(1)]526–537). Three additional THPs were synthesized ([α1(1)]85–96, [α1(1)]433–441, and [α1(1)]772–786) based on previously described or proposed activities (17, 20, 49).

The 10 THPs were screened for cell adhesion activity without prior purification of the peptides. Edman degradation sequence analysis indicated that each crude THP contained a substantial amount of the desired peptide. Thus, the crude THPs were adequate for screening purposes. Of the 10 THPs, α1(I)772–786 THP had the greatest cell adhesion-promoting activity. The other 9 THPs exhibited low levels of activity (<10%), similar to the generic triple-helical peptide GPP*°. Although the other THPs may represent active sequences, only the α1(I)772–786 THP was pursued in this study.

The large scale synthesis and purification of the α1(I)772-
Fibroblast Adhesion to Triple-helical Peptides

786 THP proceeded as described for other THPs (34), with two important modifications. First, the (Gly-Pro-Hyp)6 region of the THP was assembled stepwise using individual Fmoc-amino acids, not Fmoc-Gly-Pro-Hyp tripeptide blocks. Stepwise assembly had not been possible previously using Fmoc-Hyp, but was successful with the Fmoc-Hyp( tBu) is ovative used here. We believe that tBu side-chain protection of Hyp minimizes interstrand hydrogen bonding. Interstrand hydrogen bonding can be detrimental for efficient peptide assembly (for a recent review, see Ref. 58). Second, a recently developed two-step RP-HPLC method was used for the purification of α1(I)772–786 THP (40) which also allowed for the isolation of the deletion peptide α1(I)772–783 THP.

The α1(I)772–786 THP was highly active, with half-maximal cell adhesion occurring at a peptide concentration of 1.6 μM. Trip helicity was essential for activity of this sequence, as the non-triple-helical peptide analog (α1(I)772–786 SSP) exhibited considerably lower levels (<40%) of cell adhesion even at peptide concentrations as high as 100 μM. The triple-helical dependence for cell binding to the α1(I)772–786 sequence is even more pronounced than for the α1(I)V1263–1277 sequence described previously (35). Within the α1(I)772–786 sequence itself, residues 784–786 (Gly-Leu-Hyp) were important for cellular recognition, as the α1(I)772–783 THP had greatly reduced cell adhesion activity compared with α1(I)772–786 THP.

Adhesion of NHDF to the α1(I)772–786 THP is inhibited by an anti-β1 integrin subunit mAb. It thus appears that an integrin mediates NHDF binding to α1(I)772–786 THP. Our preliminary results were inconclusive, suggesting the α2β1, α5β1, or α3β1 integrin may be involved. It has been shown that fibroblasts use the α2β1 integrin for collagen, but not laminin, binding (59). Binding of the α2β1 integrin to the α1(I)CB7 fragment (residues 552–822) is conformationally dependent (13), consistent with the conformationally dependent binding of NHDF to α1(I)772–786 THP. Alternatively, fibronectin has been proposed as a “bridge” for ovarian cell binding to the α1(I)757–791 sequence (17). MMP-1 cleavage of the 775–776 bonds or mutation of α1(I)Gln774 and Ala777 to Pro dramatically alters fibronectin binding to type I collagen (17, 60). The α2β1 integrin may mediate chondrosarcoma cell binding to denatured type II collagen via a fibronectin bridge (21), and thus an integrin may serve a similar function for cell binding via a fibronectin bridge to the α1(I)772–786 region of type I collagen. Further investigations are ongoing to definitively determine the integrin(s) utilized for cellular recognition of the α1(I)772–786 THP.

One curious result is the ability of the α1(I)772–786 THP to promote cell adhesion in a concentration-dependent fashion, but not inhibit cell adhesion to type I collagen. In retrospect, it would have been somewhat surprising if the α1(I)772–786 THP did inhibit NHDF binding to type I collagen due to the multiple integrin binding sites within type I collagen. It is also possible that there are interactions between the THP and type I collagen. We have previously demonstrated aggregation of THPs (35), but have not examined the association of THPs and collagen.

Fibroblast interaction with collagen has tremendous implications for understanding the regulation of collagen metabolism and hence processes such as wound healing. Degradation of collagen may proceed (i) intracellularly following phagocytosis or (ii) extracellularly by MMPs (3, 4, 61). Fibroblasts both phagocytize type I collagen (3, 4) and produce MMP-1 (62). For fibroblasts, the two mechanisms of collagen catabolism may be inversely correlated (4). For example, interleukin 1α inhibits phagocytosis and enhances pro-MMP-1 release, while transforming growth factor-β has the opposite effect (4). There is evidence that suggests that the α1(I)772–786 sequence mediates both proposed mechanisms of collagen turnover. We have demonstrated that fibroblasts bind to the triple-helical α1(I)772–786 sequence. Internalization of type I collagen by fibroblasts is reduced after collagen is cleaved at the 775–776 bonds (3). Thus, fibroblast phagocytosis of type I collagen appears to include at least part of the 772–786 region. Several members of the MMP family (MMP-1, MMP-2, and MMP-8) hydrolyze the triple-helical region of type I collagen at position 775 in the collagen chains (27, 63). Thus, extracellular degradation of type I collagen occurs within the 772–786 region. This region may also regulate MMP production. Prior studies have shown that a SSP incorporating residues α1(I)769–783 supports human fibroblast adhesion and induces the production of MMP-1 (18, 19, 64). Although the induction mechanism is unknown, it may be related to an integrin-mediated binding to type I collagen which results in tyrosine phosphorylation of pp125FAK (65) and induction of MMP-1 mRNA levels (66). If the α2β1 integrin is indeed the cell surface adhesion molecule that binds α1(I)772–786 THP, we would be able to study a discreet cell signaling mechanism that influences collagen metabolism. Also, our α1(I)772–786 THP may have even greater activity for promoting cell signaling and MMP production than the α1(I)769–783 SSP, as cell adhesion to triple-helical collagen results in considerable up-regulation of protein synthesis compared with denatured collagen (67).

We view the studies presented here as an encouraging start to understanding the variety of biological activities mediated by type I collagen. As stated by Tuckwell et al. (21), “crucially, the demonstration of conformation dependence suggests that linear peptides may be unsuitable (for studying) cell-collagen interactions and implies that more sophisticated methods may be necessary for future studies.” Our triple-helical peptide approach appears to be a logical one for the identification of conformationally dependent collagen-mediated functions.

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