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Exploring the Structural Requirements of Collagen-Binding Peptides

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Abstract

Collagen synthesis and tissue remodeling are involved in many diseases; therefore collagen specific binding agents have been developed to study collagen changes in various tissues. Based on a recently reported collagen binding peptide, which contains unnatural Biphenylalanine (Bip) amino acid residue, constructs with various structure variations were synthesized to explore the contributions of unnatural Bip residue, conformational restraint, and amino acid sequence in collagen recognition. Their binding efficiency to collagens was evaluated in vitro using pure collagens. The results indicate that the C-terminal unnatural Bip residue, rather than the peptide sequence or conformational restraint, dominated the collagen I binding. Subsequent tissue binding study showed that the selected peptide didn't offer preferential selectivity over collagen I in tissue, suggesting that a simple in vitro binding assay cannot adequately model the complex biological environment.

Keywords

Collagen; Biphenylalanine; targeting peptides; binding affinity; fibrosis

INTRODUCTION

Collagen synthesis and tissue remodeling are involved in many extracellular matrix (ECM)-related processes and diseases such as angiogenesis, ischemic cardiomyopathy, and atherosclerosis^{1–7}. Accumulation of excess collagen within the interstitium causes pathologic hypertrophy and ventricular dysfunction in hypertensive patients^{5,6}. Increasing collagen deposition and modifications of collagen phenotypes alter the compliance of the myocardium after infarcts, resulting in dilatation of the heart, possible heart failure and even sudden cardiac death^{5,6}. Recently, we have used a protein probe, CNA35, to delineate collagen of pathological fibrosis from the normal myofibrils in ischemic cardiovascular tissues⁸. In conjunction with a myocyte targeting probe, detailed architecture of cardiovascular fibrosis could be clearly visualized. This myocyte targeting probe is a novel peptide-based molecule comprising three Biphenylalanine (Bip) residues that shows distinct binding selectivity to cardiac troponin I (cTnI)⁹.

Several collagen-binding proteins, including CNA35 used in our prior study, have been described^{8,10–13}. With an appropriate fluorescent label, collagen deposition within ECM could be clearly visualized in histological sections. However for *in vivo* imaging, these cell binding proteins all suffer from limited diffusion into the extravascular matrix due to their large molecular sizes. To efficiently reach the extravascular interstitium, it is essential for

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collagen-recognizing agents to have relatively low molecular masses¹⁴. With this prerequisite in mind, a few peptide-based collagen-targeting agents have been identified by phage display^{14–16}. They are often modified by adding unnatural amino acids or structurally restrained to impart specific biological features and/or enhance construct durability^{14–16}. Nonetheless, the effects of these modifications on binding characteristics are understudied. Therefore, we selected a lead collagen binding peptide (C1)¹⁴ and explored the contribution of conformational restraint, unnatural amino acid residue incorporation, and amino acid sequence modifications on collagen recognition.

MATERIALS AND METHODS

Materials

All reagents used were of analytical or HPLC grade. Dichloromethane (DCM), N-Methylpyrrolidone (NMP) and Methanol (MeOH) were purchased from Fisher (Fair Lawn, NJ, USA). *N,N*-Dimethylformamide (DMF), Diethylether, Acetonitrile (MeCN), Diisopropylethylamine (DIPEA), Piperidine and Collagen type I were purchased from Sigma-Aldrich (St. Louis, MO, USA). Triisopropylsilane (TIS), Triethylamine (TEA) and 1,2-ethanedithiol (EDT) and Kaiser test kit purchased from Sigma-Aldrich (Milwaukee, WI, USA). HOBt and HBTU were purchased from Applied Biosystems (Foster City, CA, USA). Fmoc protected amino acids were purchased from AnaSpec (Fremont, CA, USA). Fmoc-L-3,3-Biphenylalanine was purchased from Synthetech (Albany, OR, USA). Fmoc-Rink Amide MBHA-resin was purchased from Novabiochem (La Jolla, CA, USA). Collagen Type II, III and IV were purchased from Rockland (Gilbertsville, PA, USA). Bicinchroninic acid (BCA) protein assay kit was purchased from Novagen, EMD Bioscience (Gibbstown, NJ, USA). Citrate buffer, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody, 3,3-diaminobenzidine tetrahydrochloride (DAB) and DAPI medium were purchased from Vector Laboratories (Burlingame, CA). Mayer's hematoxylin and 3% hydrogen peroxide were purchased from Dako (Carpinteria, CA). Primary antibodies of collagen I and III were purchased from Abcam (Cambridge, CA).

Targeting Peptides Synthesis

All targeting peptides were synthesized by solid-phase peptide synthesis (SPPS) using the standard Fmoc strategy on an automatic synthesizer (ABI-433A, Applied Biosystems). Rink amide MBHA resin, 250 μmol , with substitution level of 0.7 $\mu\text{mol}/\text{mg}$ was used as the support for peptide amide synthesis. Four fold molar excess, relative to the resin loading, of each Fmoc protected amino acids were coupled sequentially to the resin support using the HBTU/HOBt coupling strategy.

After completion of the peptide chain elongation, Fluorescein 5-isothiocyanate (FITC) was coupled to the last *N*-terminal amino acid, Beta Alanine (*BAla*). Three equivalents of FITC (750 μmol , 292 mg), relative to the resin loading, were dissolved in 4 mL NMP followed by adding 1 mL DIPEA. This solution was vortexed thoroughly for 2–3 min then added to Rink amide MBHA resin bound peptide in a manual SPPS reaction vessel and agitated gently overnight under N_2 at RT in the dark. The reagents were drained and washed five times with NMP. The completion of FITC coupling was confirmed by Kaiser Test.

Thereafter, all protecting groups were removed and the peptides were cleaved from the resin support using a deprotection-scavenger cocktail that consists of 94% TFA:2.5% H_2O :1.0% TIS:2.5% EDT. The dry peptidyl resin was mixed with the deprotection-scavenger cocktail (10–20 mL cocktail/gm peptidyl resin) in a manual SPPS reaction vessel at room temperature in the dark with gentle agitation under N_2 for three hours. The cleavage cocktail containing collagen targeting peptides were filtered and reduced to ~1 mL in volume.

Addition of cold diethyl ether provided peptide amide precipitates of **A1–A3** in good yields. Cyclic targeting peptides **C1**, **C2** and **C3** were synthesized by dissolving 10 mg of purified acyclic peptides **A1**, **A2** and **A3** in 500 μ L solution of DMSO in 25 mM sodium acetate buffer solution, pH 5. The mixtures agitated gently for 24 hr at RT in the dark.

Targeting peptides **A1–A3** and **C1–C3** were dissolved in ~6 mL of MeCN:H₂O (50:50 v/v) and purified by reversed phase high performance liquid chromatography (RP-HPLC) using Nova-Pak® HR C18 Waters preparative Column, 6 μ m, 60 Å, 19 mm ID $\times$ 300 mm L, (Waters, Milford, MA, USA) on a Varian-ProStar 210 Chromatography system (Varian, Palo Alto, CA, USA). Targeting peptides, **A1–A3** and **C1–C3**, were purified by eluting the column at 8 ml/min with linear gradient from 0% B to 50% B in 60 minutes. Detection was carried out at 220 nm and 280 nm using a Varian-ProStar L-345 UV-Vis detector (Varian, Palo Alto, CA, USA). The purity of targeting peptides was analyzed by analytical RP-HPLC using C-18 Vydac column, 5 μ m, 4.6 mm ID $\times$ 150 mm L (GRACE, Deerfield, IL, USA) on Varian 920-LC Liquid Chromatography system coupled to a UV-Vis/Fluorescence diodarray detector, and equipped with Galaxie Chromatography Data system™ (version 1.9, Varian, Palo Alto, CA, USA). The identity of the purified peptides was confirmed by ESI-MS using Thermo Finnigan LCQ Fleet mass spectrometer and the raw data were analyzed using Xcalibur software (ThermoFisher Scientific, West Palm Beach, FL, USA). The purity of the purified targeting peptide fractions were confirmed by LC-MS on C-18 Vydac column, 5 μ m, 4.6 mm ID $\times$ 150 mm L, (GRACE, Deerfield, IL, USA) using Accela HPLC coupled with PDA detector and autosampler (ThermoFisher Scientific, West Palm Beach, FL, USA). Fractions with the same purity were collected together and lyophilized to yield yellowish powders with > 97% purity. ESI-MS showed the molecular ions (m/z) of each acyclic (**A1–A3**) and cyclic (**C1–C3**) targeting peptides. The conjugates were stored at 4 °C in the dark.

Collagen Microplate Preparation

Type I collagen was supplied as aqueous solution in 20 mM acetic acid with protein concentration of ~4.0–4.5 mg/mL. Type II, III, and IV collagens were provided as aqueous solutions in 500 mM acetic acid with protein concentration of 1 mg/mL. Collagen II and IV were free from other collagens with less than 1% cross reactivity while type III collagen contained 10% type I collagen, according to the manufacturer. Type I collagen contained 5% contaminants composed mainly of type III collagen. Collagens were used as provided.

Aliquots (~100 μ g) of various collagen phenotypes (I, II, III, IV) solutions were dispensed in the wells of 96 well non-binding surface assay plates (Corning, NY, USA). The plates were incubated at 37°C for 6–8 hrs to allow collagens to gel. The ungelled collagens and excess liquids were removed by extensive rinsing of the coated wells with cold 1X PBS (4 times $\times$ 150 μ L $\times$ 3–5 min) on a plate shaker. The collagen coated plates were then dried overnight at 2–8 °C to obtain a thin layer of collagen fibrils on the bottom of the wells.

Coated Collagen Concentration Assay

The bicinchronic acid (BCA) working reagent was prepared by mixing 4 mL BCA solution with 80 μ L 4% cupric sulfate (50:1, v/v). Aliquots (25 μ L) of BSA-standards in serial dilutions (0, 25, 125, 250, 500, 1000 μ g/mL) with 1X PBS buffer were dispensed into wells of a 96 well black-wall, clear-bottom plate (Corning, NY, USA) followed by 200 μ L BCA working reagent and mixed on a plate shaker for 30 seconds. The mixtures were incubated at 37 °C for 30 min, and then cooled down to RT. The absorbance of the solutions was measured at 560 nm using SpectraMax.M2° spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Control experiments were performed simultaneously by replacing the mixture samples with blank standards. The actual changes of absorbance were corrected

by subtracting the absorbance of the blank BSA standards from the absorbance of the measured samples. The standard curve was generated by plotting the corrected absorbance versus the known weight of the BSA standards using the best fit curve. The concentrations of the collagen phenotypes in the coated wells were assayed by interpolating the corrected absorbance within the linear range of the standard curve. On average, 20–22 μg of collagen was loaded to each well.

Preparation of microplate with Collagen I and III

Solutions of types I and III collagen were mixed in serial weight ratios (100:0, 90:10, 50:50, 30:70, 10:90 μg) into 96 well plates. The plates were prepared as described above. To validate the coating composition, diluted primary antibody to type I collagen (100 μL , 20 $\mu\text{g}/\text{mL}$) was added to each well. The plate was covered with an adhesive plastic and incubated overnight at 4 °C and then washed four times with 1X PBS. To each well, 100 μL of FITC-conjugated secondary antibody diluted in blocking buffer was added. The plate was again covered with an adhesive plastic and incubated for 2 hr at r RT then washed four times with 1X PBS. Plates were imaged using an IVIS-200 optical imaging system (Xenogen/Caliper, Mountain View, CA). Acquired images were analyzed using Living Image 3.1 software, and the fluorescence levels were quantified using identical sized regions of interest (ROI) place at the relevant wells.

Binding Affinity to Type I Collagen

The binding-affinity of acyclic **A1–A3** and cyclic **C1–C3** fluorescent targeting peptides to type I collagen was determined fluorometrically using a plate-reader. A 30 μL aliquot of 10 μM fluorescent targeting peptide, in 1X-PBS (pH 7.4), was dispensed into type I collagen coated-wells of non-binding surface assay plate, and the total volumes were brought to 100 μL using the same buffer. The plate was incubated on a plate shaker for 2 hrs at RT. The supernatants that contained unbound peptides were collected. Each well was further washed with cold 50 μL 1X-PBS buffer, and then the washing solution was collected and combined with the supernatants. The fluorescence intensities of the combined solution were monitored on a 96 well black-wall, clear-bottom plate using SpectraMax.M2^e fluorescence spectrophotometer at λ_{ex} of 492 nm and λ_{em} of 518 nm at 27 °C. Control experiments were performed in parallel by dispensing 30 μL of 10 μM peptides **A1–A3** and **C1–C3** directly into the wells of an uncoated 96 well non-binding surface assay plates, and the total volume was brought to 150 μL using the same buffer. The amount of the bound targeting peptides **A1–A3** and **C1–C3** to each collagen phenotype was quantified by subtracting the unbound supernatant fluorescence from the total fluorescence. The resulted net fluorescence value of **C1** to type I collagen was set as 100% binding. The binding percentages of peptides **A1–A3** and **C2–C3** were calculated relative to that 100% binding of **C1**. Same procedure was applied to study **C1** binding with collagen I and III mixtures.

Immunohistochemical Staining of Collagens

Collected C57BL/6 mouse skeletal muscle was fixed in 10% natural buffered formalin for 18 hours, embedded in paraffin wax, sliced into 4 μm sections, and mounted on positively charged slides. Unstained tissue sections were deparaffinized with xylene for 13 minutes and rehydrated with ethanol and water for 12 minutes. Heat mediated antigen retrieval was performed with citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) using a steamer at 98 °C for 20 minutes and cooled down to room temperature for another 20 minutes. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes. Protein was blocked with 5% normal horse serum in PBS for 5 minutes. Tissue sections were incubated with primary antibodies of collagen I or III in blocking solution at a 1/100 and 1/200 dilutions, respectively, for 30 minutes at RT followed by HRP conjugated goat

anti-rabbit IgG secondary antibody for 15 minutes at RT. Slides were developed with DAB substrate for 5 minutes and counterstained with Mayer's hematoxylin for 30 second, washed and dehydrated with ethanol, cleared with xylene and mounted.

Histofluorescence Staining with C1 probe

Deparaffinized mouse skeletal muscle sections were blocked with 5% BSA for 30 minutes, followed by incubation with 10 μ M solution of **C1** fluorescent probe (~500 μ L/section) for 1 hour in the dark using a humidified chamber at RT. Tissue sections were rinsed extensively (~5 minutes) with PBS for three times, counterstained with DAPI (DNA Satin), coverslipped with mounting medium, and stored in the dark. The FITC fluorescent signal of the tissue sections was assessed using fluorescent microscopy.

RESULTS AND DISCUSSION

A few known collagen-binding peptides contain unnatural amino acids and/or conformational restraint^{14–16}. Among them, one construct termed **C1** in this study (Figure 1) was identified from phage library and has been used to image myocardial scar tissue in animals^{14,15}. **C1** is primarily composed of 16 amino acid residues, with an unnatural Bip at the C-terminus, cyclized via a disulfide bond between two cysteine residues at positions 4 and 13. Previously, we demonstrated the critical role of Bip residue in cTnI recognition⁹. To investigate the structural effect of the lead Bip containing peptide **C1**, a series of structurally related peptides were prepared (Figure 1). To examine the sequence preferences and the contribution of the Bip residue, peptides **C2** and **C3** were designed by reordering the amino acid sequence (**C2**) and eliminating Bip from the C-terminus (**C3**). Acyclic analogs of all targeting peptides (**A1–A3**) were also prepared to investigate the effect of cyclization on binding affinity. To evade any possible interference, the FITC fluorochrome was placed at the N-terminus, three amino acid residues apart from the cyclization motif for all targeting peptides. The molecular masses of all prepared peptides were validated by mass spectral analysis (Supplement 1).

The collagen selectivity of **C1** was evaluated with type I, II, III, and IV collagens since these four comprise over 90% of the collagen in connective tissues^{17–19}. To measure peptide binding, a previously described microplate binding assay was used in the study^{14,20–22}. A thin layer of collagen fibrils was first applied to the bottoms of wells of a 96-well plate. On average, each well contained 20–22 μ g of collagen. After extensive washing, the amount of bound collagen was quantified by the BCA protein assay using a standard curve constructed with a BSA protein standard (Supplement 2). **C1** (10 μ M) was then added to each well and incubated for 2 h. The unbound peptide was removed, followed by extensive washing. Fluorescence measurement revealed that **C1** exhibited relatively high affinity for collagen I. In contrast, its binding affinities for collagen II, III, and IV relative to that for collagen I were 61, 51, and 48%, respectively (Figure 2A).

Binding of acyclic **A1–A3** and cyclic **C1–C3** peptides to type I collagen were further compared using **C1** as a reference (Figure 2B). The results indicated that **C1** has the highest affinity for type I collagen. The binding of **C2**, the amino acid residues of which had been rearranged while the cyclic structure and biphenylalanine residue were retained, to type I collagen was 94% of that of **C1**. **C3**, which did not possess the unnatural amino acid residue biphenylalanine in its C-terminus, exhibited lower affinity (30% relative to that of **C1**) for type I collagen. This striking decrease in binding highlighted the importance of the unnatural amino acid residue biphenylalanine in collagen recognition.

The critical effect of the biphenylalanine residue in collagen targeting was further examined using acyclic peptides. The binding affinities of **A1**, **A2**, and **A3** for type 1 collagen were

74%, 74%, and 22%, respectively, of that of **C1**. The minor structural modification of introducing biphenylalanine was sufficient to increase the binding affinity of the acyclic targeting peptides for type I collagen. The influence of the biphenylalanine residue on collagen binding also overrode the effect of amino acid sequences as observed with **A1** and **A2**, both of which bound to type I collagen with similar affinity (~74%). In all cases, cyclic targeting peptides **C1–C3** exhibited superior binding to type I collagen than acyclic peptides **A1–A3**, but the benefit of cyclization was much smaller than that of incorporation of the biphenylalanine residue. These observations indicated that the biphenylalanine residue has the greatest effect on type I collagen binding.

To simulate the actual collagen microenvironment in the ECM, we investigated the binding of **C1** to co-existing collagen I and III. Thin layers of type I and type III collagen fibrils were applied in serial weight ratios (100:0, 90:10, 50:50, 30:70, 10:90) to the bottoms of the wells of a 96-well plate. The composition of the coated layers was further validated by immunomaging using a primary antibody to type I isoform and a fluorescein-labeled secondary antibody (Figure 3A). The highest fluorescence signal was observed with 100% type I isoform, whereas the intensity of the signals decreased proportionally with increasing amounts of type III collagen in the coating fibrils. Thereafter, **C1** was tested with these mixed collagens. Among all compositions, **C1** exhibited the highest affinity for pure type I collagen, which was defined as 100% binding (Figure 3B). Relative to that with unmixed type I collagen, a 58% reduction in binding was observed after introducing only 10% type III to the collagen matrix. Further decreases in binding were observed by increasing the weight ratio of type III collagen in the collagen fibrils to 50, 70, and 90% (64, 66, and 68% reductions relative to that with pure type I collagen, respectively). Although the binding affinity of **C1** decreased progressively along with the ratios of type I to type III isoforms, this correlation was not linearly proportional. These results indicate that the binding affinity of **C1** for specific collagen matrices is affected by the interaction between type I and III collagen. It is known that the interactions among collagen phenotypes, in addition to the composition of the collagen matrix, greatly affects the specific properties of certain tissues^{17,18}.

Thin sections of mouse skeletal muscles were then stained with **C1** to confirm collagen binding in tissue samples, and the binding of **C1** was compared with that of collagen type I and III antibodies. Collagen I antibody showed abundant brown positive staining in the organized ECM but not inside cells (Figure 4A). In contrast, images of collagen III antibody-stained tissues exhibited only limited spotty staining (Figure 4B). Unexpectedly, when skeletal muscle tissues were stained with fluorescein-labeled **C1**, only faint fluorescent signals were observed inside cells (Figure 4D). This peptide failed to highlight the existing collagens within the ECM that were positively stained with collagen I or III antibody. A similar outcome was observed from fluorescence images of smooth muscle tissues and heart tissues stained with **C1** (data not shown).

In this study, peptide constructs derived from the lead **C1** were designed to elucidate the underlying mechanism of its collagen-binding affinity. The results could not establish an explicit role of the peptide sequences and conformation constraints in collagen binding. The C-terminal unnatural biphenylalanine residue demonstrated a modest contribution to collagen binding. Although in vitro collagen binding assays^{14,16,20–22} in the literature indicated that the relative binding affinity of peptides could be conveniently demonstrated, subsequent histological staining couldn't substantiate selective binding to the extracellular collagen since the fluorescence staining was different from those of collagen I and III antibodies. This is suggesting that this simple in vitro binding assay cannot adequately model the complex biological environment.

Without a good positive control, the obtained relative affinity data might confound the interpretation of the in vivo imaging results. Therefore, the molecular detail behind the in vivo enhancement on MR imaging, which was believed to reflect collagen targeting^{14,15}, remains to be proven. While **C1** could not show selective binding to extracellular collagens, it was interesting to observe weak binding between **C1** and intracellular components. This unexpected observation could lead to the development of new cellular binding agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations List

cTnI	cardiac troponin I
Bip	Biphenylalanine
FITC	Fluorescein 5-isothiocyanate
βAla	beta alanine
BCA	Bicinchronic acid
ECM	Extracellular matrix
RP-HPLC	reversed phase high performance liquid chromatography
MS	mass spectra
UV	ultraviolet
TNBS	2,4,6-trinitrobenzenesulfonic acid

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Figure 1. Collagen-targeting peptides. **(A)** General structure of the fluorescent collagen-targeting peptide **C1**. **(B)** List of the developed fluorescent collagen-targeting peptides **A1–C3** and their amino acid sequences.

Figure 2.

Differential binding profiles of fluorescent collagen-targeting peptides: (A) **C1** (10 μ M) bound to various collagen phenotypes (I, II, III, IV); (B) **A1–C3** (10 μ M) to type I collagen. The fluorescence intensities were monitored using a fluorescence spectrophotometer at $\lambda_{\text{ex}} = 492$ nm and $\lambda_{\text{em}} = 518$ nm at 27 °C. The amount of bound targeting peptide to each collagen phenotype was quantified by subtracting the unbound supernatant fluorescence from the total fluorescence. The binding of **C1** to collagen was set as 100%.

Figure 3.

(A) Immunofluorescence of type I and III collagen differential coating. Image acquired using Ex/Em of green fluorescence protein (GFP) with IVIS-200. Radiance scale of the color bar represents the counts. (B) Differential binding profiles of the fluorescent collagen-targeting peptide **C1** (10 μ M) to mixtures of serial weight ratios (100:0, 90:10, 50:50, 30:70, 10:90; in μ g) of type I to type III collagen. The fluorescence intensities were monitored using a fluorescence spectrophotometer at $\lambda_{\text{ex}} = 492$ nm and $\lambda_{\text{em}} = 518$ nm at 27 °C. The amount of bound **C1** for each ratio of type I collagen to type III collagen was quantified by subtracting the unbound supernatant fluorescence from the total fluorescence. The binding of **C1** to unmixed type I collagen was set as 100%.

Figure 4.

Representative images of mouse tissue sections of skeletal muscles stained with (A) collagen type I antibody, (B) collagen type III antibody, (C) H & E, and (D) C1. Although type I collagen could be detected in greater abundance than type III collagen in the ECM, C1 failed to label collagen fibrils or to distinguish collagen from noncollagen matrices.