Mapping the binding sites of MMPs on type II and III collagens using triple-helical peptide Toolkits

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Abstract

Libraries of triple-helical collagen-like peptides (Collagen Toolkits) have helped to define collagen II and III binding specificities of numerous collagen-binding proteins. Here I describe a simple solid phase binding assay utilising a biotin-streptavidin system to screen the Collagen Toolkits for binding of two distinct matrix metalloproteinases (MMPs) implicated in cancer: the collagenolytic MMP-1 (collagenase 1) and the non-collagenolytic MMP-3 (stromelysin 1). The screening revealed markedly disparate binding footprints of these MMPs on collagens II and III, in line with their distinct biological activities. Analogous screening of other potentially collagen-binding proteases may shed light on their inherent tissue retention capabilities and their pro- or anti-metastatic potential.

Key words Collagen Toolkit, collagen binding, triple-helical peptide, collagenase, stromelysin, solid phase binding assay

1 Introduction

Triple-helical fibrillar collagens (types I, II, III, V and XI) are the most abundant extracellular matrix (ECM) proteins providing tissues and organs with form, stability and connectivity. ECM turnover is slow during tissue homeostasis, but relatively rapid in development, organ morphogenesis, wound healing, and in tissue-destructive diseases, such as cancer [1]. Matrix metalloproteinases (MMPs) collectively cleave most, if not all ECM components [2]. Among them are collagenases: MMP-1, −8 and −13, and stromelysins: MMP-3, −10 and −11, all secreted as inactive zymogens comprising an N-terminal pro-domain followed by a catalytic (Cat) domain connected to the C-terminal hemopexin (Hpx) domain via a flexible linker (hinge region). Although stromelysins do not cleave triple-helical collagens, at least MMP-3 can bind to them [3, 4]. The most striking evidence of MMP-3 binding to collagenous matrix is in colorectal cancer [5]. Such binding could interfere with collagen fibrillogenesis and, thus, enhance collagenolysis by rendering newly secreted collagen

molecules more exposed to collagenases [6]. MMP-3 is also well known as a potent activator of MMP collagenases [7]. Other proteases may reside in tissues through binding of various insoluble ECM assemblies, to directly or indirectly regulate tissue destruction and cancer metastasis. Methods defining ECM binding specificities of such proteases might reveal targets for future treatments.

Libraries of triple-helical peptides (THPs) developed in Farndale lab (University of Cambridge, UK) and known as Collagen Toolkits [8] proved useful for mapping the footprints of many collagen-binding proteins, including MMP-1 [9], -3 [6] and -13 [10], on collagens II and III. These peptide Toolkits embrace the entire helical domains of homotypic (composed of three identical chains) collagens II and III (Fig. 1A). Toolkit II contains 56 THPs and Toolkit III contains 57 THPs (Fig. 1B). Consecutive THPs in each Toolkit span 27 amino acids of *guest* (human) collagen sequence, with 9-residue overlap between neighbouring THPs. The *guest* sequences are flanked by *host* sequences: 5 Gly-Pro-Pro repeats and Gly-Pro-Cys knots that impart the triple-helical structure (Fig. 1).

The Toolkits are commonly screened for binding of various proteins using a 96-well solid phase binding assay format combined with various detection methods. This chapter focuses on mapping the binding of the prototypic interstitial collagenase 1 (MMP-1) and the non-collagenolytic stromelysin 1 (MMP-3) on collagens II and III using a simple, antibody-free detection. Active site (E200A) mutants of both full-length enzymes (as well as their pro-forms and individual domains) are minimally biotinylated and their binding to specific Toolkit peptides is measured colorimetrically using streptavidin conjugated with horseradish peroxidase (HRP) and a chromogenic HRP substrate (Figs. 2 and 3).

2 Materials

2.1 MMPs

Recombinant proMMP-1(E200A) and proMMP-3(E200A), their mature (full-length) forms and individual Cat and Hpx domains were expressed and purified as described previously [11]. Various MMPs may now also be commercially available.

2.2 Biotinylation of MMPs

- Sephadex G-25M PD-10 desalting gravity columns (GE Healthcare)
- 50 mM N-Cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer, pH 8.8, supplemented with 200 mM NaCl and 10 mM CaCl2.
- EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific)

2.3 Collagen Toolkits

Collagen Toolkits II and III are available from the University of Cambridge spinout company CambCol Laboratories (Littleport, Cambridgeshire, UK), as pre-coated 96-well plates and as individual peptides. The synthesis of Toolkit peptides has been described in the literature [12, 13]. All *guest* sequences of Toolkits II and III are listed in Fig. 1B.

2.4 Solid phase binding assay

- 5 μg/ml Toolkit peptide stock solutions (56 from Toolkit II and 57 from Toolkit III) in 10 mM acetic acid and Costar® High Binding 96-well microtiter plates (Corning, UK) (or pre-coated plates from CambCol Laboratories, *see above*)
- TNC buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM CaCl2, 0.02 % NaN3) supplemented with 0.05 % Tween 20 (TNC-T)
- bovine serum albumin (Sigma)
- Streptavidin-horseradish peroxidase conjugate (R&D, UK)
- 3,3',5,5'- tetramethylbenzidine (TMB) 2-Component Microwell Peroxidase Substrate Kit™ (KPL, UK)
- 6 % sulphuric acid solution
- Colorimetric microplate reader

3 Methods

3.1 Biotinylation of MMPs

For a detailed protocol on optimal biotinylation of a proteolytic enzyme, please, refer to another chapter in Methods in Molecular Biology by Santamaria (2020) [14]. See also Note 1.

1. If MMP stock solutions contain primary amines other than those on the protein of interest, they require buffer-exchanging into an amine-free buffer for efficient protein biotinylation. That can be done using Sephadex G-25M PD-10 columns equilibrated with the CHES buffer detailed above, following manufacturer's protocol.
2. 10 mM EZ-Link Sulfo-NHS-LC-Biotin solution in distilled water is then added at 1:2 protein:biotin molar ratio and incubated for 1 h at room temperature.
3. Biotinylated protein is next passed over another PD-10 column equilibrated in TNC buffer to remove excess biotin.

3.2 Toolkit screening

All Collagen Toolkit binding assays are carried out in triplicates (3 wells per condition; Fig. 2) and compared analyses are always developed simultaneously. All assays should be repeated at least 3 times (see representative results in Fig. 3). See also Notes 2 and 3.

1. Unless pre-coated plates are purchased, 96-well microtiter plates can be manually coated with 5 μg/ml THP solutions in 10 mM acetic acid (30-50 μl per well) and incubated overnight at 4 °C.
2. The plates are then washed three times with TNC-T (3 x 200 μl per well) and blocked with 3 % bovine serum albumin in TNC-T (200 μl per well) for 2 h at room temperature.
3. After three washes as above, biotinylated proteins at 1 μM concentration are next added (30-50 μl per well) and incubated for 1-2 h at room temperature.

4. After another three washes as above, streptavidin-HRP is added at the manufacturer's recommended dilution and incubated for 1 h at room temperature.
5. The plates are developed using a chromogenic TMB substrate for a fixed time (1-3 min), according to manufacturer's instructions (50 μl per well) and stopped by addition of 6 % sulphuric acid (200 μl per well).
6. The binding levels are read using a microplate reader set to measure absorption at 450 nm wavelength.
7. Numerical binding values can be plotted as bar graphs (mean +/- standard deviation) (Fig. 3).

4 Notes

1. It is advisable to test that the active version of the enzyme of interest retains full enzymatic activity once biotinylated at the level equivalent to that of the inactive enzyme used in the binding assays. The biotinylation neither affected the collagenolytic activity of wild-type MMP-1 nor the collagen unwinding activity [11] of MMP-1(E200A).
2. If a collagen-binding protease of interest might exhibit collagenolytic activity, it is best to use a catalytically inactive mutant of that protease, whose collagen binding affinity is, however, unaffected (such as the E200A mutant of MMP-1), since the enzyme may otherwise destroy its binding site and fail to report binding.
3. Plates coated with Toolkit peptides should be stored at low temperatures to prevent irreversible denaturation of THPs into gelatin and should be screened as fresh as possible. Assays should not be performed at temperatures exceeding 25 °C.

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Figure legends

Fig. 1 Collagen Toolkits. (**A**) Schematic representation of the redundancy in Toolkit peptides that reduces the resolution of the binding site mapping to 9-residues (level of inter-peptide overlap). Variable g*uest* sequences are represented with black bars and *host* sequences (the same for every peptide) are shown for one peptide only (GPP, Gly-Pro-Pro repeats; GPC, Gly-Pro-Cys *knots*). (**B**) *Guest* sequences of all peptides in each Toolkit. The 27 residues in each peptide are divided into *chunks* of 9 to visualise how the last *chunk* of a preceding peptide becomes the first *chunk* of the consecutive one (top left, broken arrow).

Fig. 2 Solid phase binding assay detection system. 96-well plates are coated with Toolkit peptides in triplicates (each Toolkit is represented by at least 2 plates). Minimally biotinylated MMPs are added to the wells and the wells are washed. Bound MMPs are detected using streptavidin-horseradish peroxidase (HRP) conjugate and a chromogenic HRP substrate. Cat, catalytic domain; Hpx, hemopexin domain.

Fig. 3 Representative results of Toolkit II and III screening with pro-, full-length and single-domain derivatives of MMP-1 (**A**) and -3 (**B**) (EA, E200A mutation in the active site). GPP, triple-helical peptides composed solely of Gly-Pro-Pro repeats (control). Each bar represents mean binding level (absorption at 450 nm wavelength) and error bars indicate standard deviation. Full-length, mature forms of both MMP-1(E200A) and MMP-3(E200A) show most potent binding to distinct Toolkit peptides (black bars). Only MMP-1(E200A) (the active site mutant of collagenase 1) can recognise the single scissile bond of mammalian collagenases that resides in peptide 44 (roughly 3/4 of the way from the N-terminus of the fibril-forming collagen molecule) (**A**). MMP-3(E200A) (the active site mutant of a non-collagenolytic stromelysin 1) exhibits much broader collagen binding specificity (**B**). Pro, pro-domain; Cat, catalytic domain; Hpx, hemopexin domain.