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Collagen−Protein Interactions Mapped by Phototriggered Thiol Introduction

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The elucidation of protein−protein interactions is one of the most important subjects in the postgenome era. Ideally, determination of the three-dimensional structure of complexes of proteins is required, but such structural analyses are not adaptable to all proteins.

Collagen, characterized by a long triple-helical structure, is a major component of extracellular matrices.[1] Collagen regulates cell functions by binding to receptors on the cell surface and by interacting with secreted proteins such as growth factors or cytokines.[2] To date, various proteins have been reported to specifically bind to collagen.[3] Mapping of the protein-binding sites on collagen is needed as attention shifts to the functional implications of the interaction between collagen and proteins.[3] However, for the most part, binding sites have not been mapped at the amino acid level due to the intractable properties of collagen arising from a low solubility and its peculiar triple-helical structure. Conventionally, electron microscopic observation and CNBr fragments of collagen are used for the determination of protein-binding sites.[4] However, these are insufficient to determine the binding epitopes recognized by proteins. Herein, we describe a new strategy for mapping the protein-binding sites on collagen with higher resolution by using a site-specific photo-cross-linking strategy. A Cys residue is introduced into the collagen-binding protein by site-directed mutagenesis (Figure 1b, step a). A photoreactive cross-linker, APDP (Figure 1a),[5] can be introduced into cysteine −SH groups on proteins (step b). The complex of APDP-modified protein and collagen (step c) is cross-linked by ultraviolet (UV) irradiation (step d). Cleaving the cross-link by reduction, an −SH group is newly generated on collagen (step e). This −SH is useful for labeling the sites of cross-linking, which will be touched upon later (step f).

The strategy described here is demonstrated by employing pigment epithelium-derived factor (PEDF) as a model for the collagen-binding protein.[6] PEDF is known as an anti-angiogenic/neurotrophic factor,[7] and its collagen-binding site has been identified.[6b] Cys substitutions were made where the collagen-binding site is localized (F383), and on the opposite surface of the site (Y211) by site-directed mutagenesis (Figure 2a). These Cys −SH groups were functionalized with APDP.

We first tested the binding of APDP-modified PEDF (APDP-PEDF) to immobilized collagen I. APDP-PEDF F383C (APDP-F383C) and APDP-PEDF Y211C (APDP-Y211C) bound to collagen I, whereas APDP-PEDF D299N/F383C (APDP-D299N/F383C), mutated in the collagen-binding site, did not (Figure 2b). Next, the APDP-PEDF proteins were mixed with collagen I, and a photoinduced cross-linking reaction was carried out using UV irradiation. The resulting cross-linked products were directly analyzed by SDS−PAGE under nonreducing conditions. A photo-cross-linking reaction using APDP-F383C resulted in the detection of a band corresponding to the cross-linked product, depending on

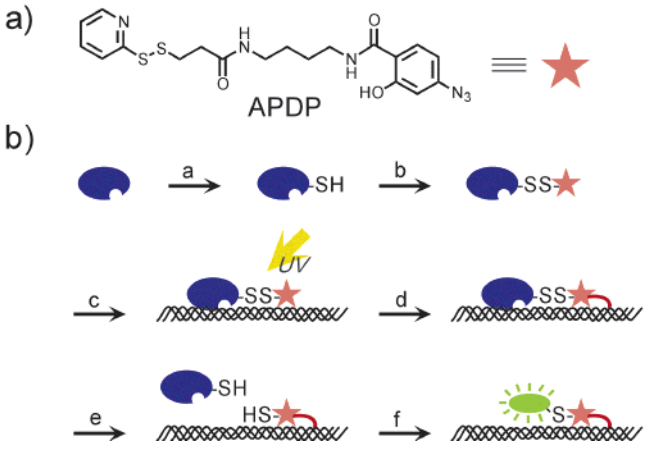

Figure 1. (a) Structure of APDP. (b) Steps in the photo-cross-linking and fluorescent labeling of cross-linked sites in collagen.

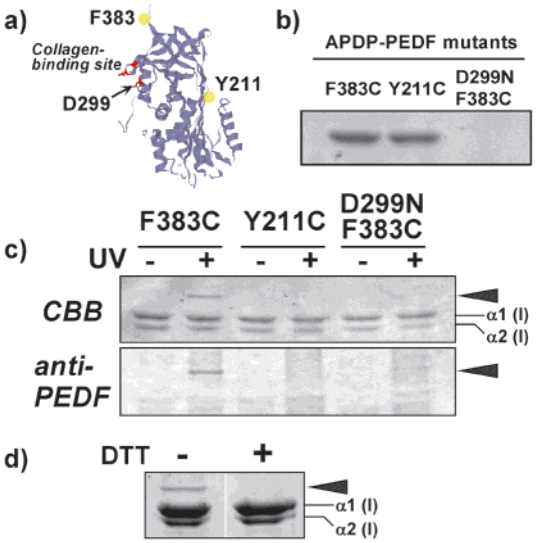

Figure 2. (a) Structure of PEDF showing the positions mutated to Cys and its collagen-binding site. (b) Binding profile of the PEDF mutants modified with APDP (APDP-PEDF). (c) Photo-cross-linking of APDP-PEDF to collagen I. (d) Reduction of cross-linked product with DTT. Arrowheads indicate the band corresponding to the photo-cross-linked product.

UV irradiation (Figure 2c). On the other hand, both APDP-Y211C and APDP-D299N/F383C failed to cross-link with collagen I (Figure 2c). These results indicated that cross-linking was introduced specifically, depending on the collagen-binding activity of APDP-PEDF and the structural proximity of the photo-cross-linker. The treatment of the photo-cross-linked product with DTT resulted in the disappearance of the corresponding band, showing the existence of an intact disulfide bridge (Figure 2d).

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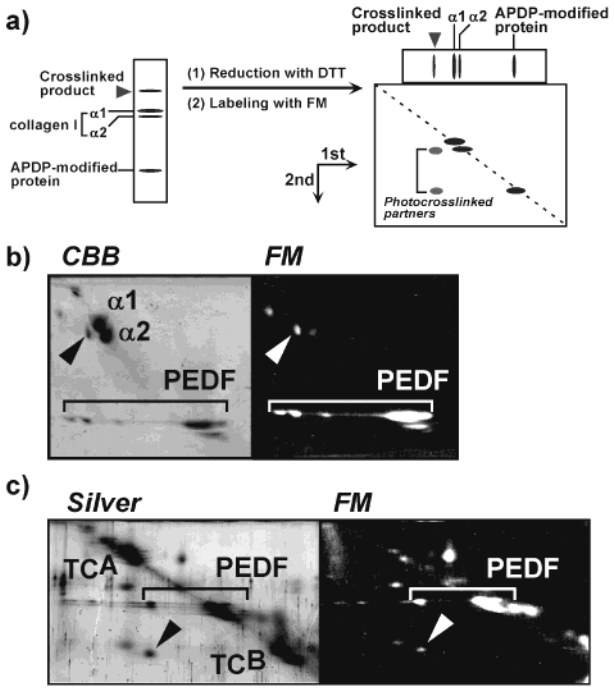

Figure 3. (a) Schematic representation of the two-dimensional (2D) diagonal electrophoresis system used to detect the cross-linked partners as fluorescent spots. (b) Analysis of the cross-linking product by 2D electrophoresis. Detection of spots under UV light (right) and the same gel stained with CBB (left). (c) Analysis of MMP-1 digests of the cross-linked product by 2D electrophoresis. Fluorogram as seen under UV light (right) and proteins on the same gel visualized by silver staining (left). Arrowheads indicate the spot corresponding to the photo-cross-linked partner of APDP-PEDF F383C.

It is emphasized that unique −SH groups can be introduced into the collagen molecule via cleavage of the disulfide bridge in the photo-cross-linked product, and that most of the collagenous triple helices conveniently have no Cys residue. This thiol-transfer procedure is a key step because −SH groups are useful for identifying the sites of cross-linking in collagen. To demonstrate this strategy, we analyzed the cross-linked products using a combination of fluorescein-5-maleimide (FM) modification (Figure 1b, step f) and two-dimensional (2D) diagonal electrophoresis (Figure 3a). Photo-cross-linked products were subjected to SDS−PAGE in the first dimension under nonreducing conditions, and then a reduction with DTT was performed in the gel (Figure 3a). After the labeling of −SH groups on the proteins with FM, SDS−PAGE in the second dimension was carried out (Figure 3a). With this system, one can detect photo-cross-linked partners as fluorescent spots on the gel under UV light (Figure 3a). As shown in Figure 3b, the cross-linked partner of APDP-F383C was detected as a fluorescent spot (right panel), and this spot was judged to be derived from the α2 chain of collagen I by subsequent CBB staining of the gel (left).

Next, we treated photo-cross-linked products with MMP-1 to map roughly the sites of cross-linking in collagen I. MMP-1 cleaves collagen I at a site three-fourths of the distance from the N-terminal, resulting in three-fourths and one-fourth length fragments, termed TC^A and TC^B, respectively.[8] The MMP-1 digests were analyzed in a similar manner. The cross-linking at the TC^B fragment of the α2 chain was judged to be predominant on the basis of 2D electrophoresis (Figure 3c). This observation indicates that PEDF binds to the C-terminal one-fourth of collagen I. In principle, the use of bacterial collagenase instead of MMP-1 would provide information on the peptide sequence since bacterial collagenase generates smaller fragments of collagen that can be analyzed by mass spectrometry and/or the Edman method.

Although we did use APDP containing a long, flexible chain (spacer arm length of 21 Å), considering the advantage of flexibility in the photo-cross-linking efficiency, *N*-[(2-pyridyldithio)ethyl]-4-azidosalicylamide (PEAS)[9] containing a shorter spacer arm (14 Å) is also applicable in our strategy (see Supporting Information), which permits us to introduce −SH groups more closely to the protein-binding sites.

In summary, we have developed a new strategy for mapping the protein-binding sites in collagen using a photoreactive cross-linker. This strategy enables an −SH group to be introduced into collagen in the vicinity of the protein-binding sites. The advantage of this system is that the sites of cross-linking are easily identified by fluorescence derivation of the −SH group transferred into collagen with the 2D electrophoresis system. This phototriggered thiol introduction will be also useful for determining the binding sites for other collagen-binding proteins, such as von Willebrand factor,[10] discoidin domain receptors,[2b] etc. This strategy should provide novel insight into the functions of collagen as a multifaceted protein.

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Supporting Information Available: Experimental procedures, use of PEAS, considerations for the possibility of nonspecific cross-linking and the quantitative measurement of cross-linking (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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