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Detection of substrate binding of a collagen-specific molecular chaperone HSP47 in solution using fluorescence correlation spectroscopy

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ABSTRACT

Heat shock protein 47 kDa (HSP47), an ER-resident and collagen-specific molecular chaperone, recognizes collagenous hydrophobic amino acid sequences (Gly-Pro-Hyp) and assists in secretion of correctly folded collagen. Elevated collagen production is correlated with HSP47 expression in various diseases, including fibrosis and keloid. HSP47 knockdown ameliorates liver fibrosis by inhibiting collagen secretion, and inhibition of the interaction of HSP47 with procollagen also prevents collagen secretion. Therefore, a high-throughput system for screening of drugs capable of inhibiting the interaction between HSP47 and collagen would aid the development of novel therapies for fibrotic diseases. In this study, we established a straightforward method for rapidly and quantitatively measuring the interaction between HSP47 and collagen in solution using fluorescence correlation spectroscopy (FCS). The diffusion rate of HSP47 labeled with Alexa Fluor 488 (HSP47-AF), a green fluorescent dye, decreased upon addition of type I or III collagen, whereas that of dye-labeled protein disulfide isomerase (PDI) or bovine serum albumin (BSA) did not, indicating that specific binding of HSP47 to collagen could be detected using FCS. Using this method, we calculated the dissociation constant of the interaction between HSP47 and collagen. The binding ratio between HSP47-AF and collagen did not change in the presence of sodium chloride, confirming that the interaction was hydrophobic in nature. In addition, we observed dissociation of collagen from HSP47 at low pH and re-association after recovery to neutral pH. These observations indicate that this system is appropriate for detecting the interaction between HSP47 and collagen, and could be applied to high-throughput screening for drugs capable of suppressing and/or curing fibrosis.

1. Introduction

HSP47, a heat shock protein with molecular mass of 47 kDa, is an ER-resident and collagen-specific molecular chaperone [1]. HSP47 belongs to the serine protease inhibitor (serpin) superfamily, and is also known as Serpin H1. It binds to collagen and promotes its triple-helical folding in the ER. HSP47 predominantly recognizes collagenous amino acid sequences, i.e., glycine-Xaa-Yaa (Gly-Xaa-Yaa) repeats, in which Xaa and Yaa are often proline (Pro) and

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hydroxyproline (Hyp), respectively. Hydroxylation of Pro residues is required to stabilize the triple-helical structure of collagen [2]. Since the surface of the collagen triple-helix region becomes hydrophobic, HSP47 must bind to the triple-helical form of procollagen to prevent its lateral aggregation in the ER [3]. The amino acid residues in HSP47 responsible for hydrophobic collagen binding have been identified [4].

The binding of HSP47 to collagen has been analyzed using several methods, including biochemical pull-down assay including immunoprecipitation [5], surface plasmon resonance (SPR) [6], fluorescence quenching measurement [7], Förster/fluorescence resonance energy transfer (FRET) [8], and bimolecular fluorescence complementation (BiFC) [8]. Those studies revealed that HSP47 binds to purified mature type I–V collagen, gelatin (denatured collagen), and peptides containing Gly-Pro-Pro repeats. The equilibrium dissociation constants (K_d) between HSP47 and collagen were determined using SPR and fluorescence quenching analysis [6,7].

HSP47 is translocated into the ER *via* a signal sequence at the N-terminus and recycled from the Golgi to the ER *via* an ER-retention signal sequence (RDEL) at the C-terminus. During this cycle, dissociation between HSP47 and collagen at low pH promotes both the retention of HSP47 in the ER and appropriate trafficking of triple-helical collagen [1,9].

Various fibrotic diseases, including liver cirrhosis and idiopathic pulmonary fibrosis, are characterized by abnormal chronic collagen accumulation in tissues [9–11]. Accordingly, it has been hypothesized that suppression of HSP47 activity would slow the progression of these diseases. Indeed, in mice, knockdown of HSP47 ameliorates liver fibrosis by inhibiting collagen secretion [12]. Therefore, the establishment of effective and high-throughput screening methods for discovering compounds capable of suppressing HSP47 would be clinically valuable.

Here, we demonstrate that the interaction between HSP47 and collagen in solution can be quantitatively and conveniently detected using fluorescence correlation spectroscopy (FCS) [13,14]. This FCS system could be applied to high-throughput screening of reagents to identify molecules that inhibit the interaction between HSP47 and collagen, and could thus slow or curing of fibrotic disease.

2. Materials and methods

2.1. Fluorescence labeling of proteins

Purified HSP47 in which Cys138 was replaced with alanine (C138A) as described previously [15], human protein disulfide isomerase (PDI) as described previously [16], and bovine serum albumin (BSA; purchased from Sigma-Aldrich, St. Louis, MO, USA) were incubated with Alexa Fluor 488 carboxylic acid and succinimidyl ester (A-20000, Thermo Fisher Scientific, Waltham, MA, USA), and covalently fluorescently labeled. For the labeling reactions, the molar ratios of protein to dye were 1:20, 1:10, and 1:5 for HSP47, BSA, and PDI, respectively. Proteins and dye were incubated overnight at 4 °C. To stop the reaction, a 10% volume of 1.5 M hydroxylamine was added, and the samples were incubated for 1 h. Labeled proteins were purified and diluted in a 50 mM HEPES-KOH (pH 7.5) or 200 mM phosphate buffer at various pH values using a gel-filtration micro-spin column (#CS-900, Princeton Separations, Adelphia, NJ, USA).

2.2. Fluorescence correlation spectroscopy (FCS)

FCS measurements were performed using a ConfoCor 2 system combined with an LSM 510 (Carl Zeiss, Jena, Germany) through a C-Apochromat 40 × /1.2 NA Korr water-immersion objective (Carl Zeiss). A confocal pinhole diameter was adjusted to 70 μm. Alexa Fluor 488 was excited at 488 nm, and emission signals were detected using a 505 nm long-pass filter. Measurements were performed in a cover-glass chamber (#155411, Thermo Fisher Scientific) in the absence or presence of acetic acid-solubilized type I and III collagen (Nitta Gelatin, Osaka, Japan). Obtained fluorescence autocorrelation function (ACF), $G(\tau)$, from which the lag time (τ), was analyzed using a two-component diffusion model including the triplet state was given by Eq. (1):

$$G(\tau) = 1 + \frac{1}{N}\left[\frac{T}{1-T}\exp\left(-\frac{\tau}{\tau_{\text{triplet}}}\right)\right] \times \left[\frac{1-F}{\left(1+\frac{\tau}{\tau_{\text{free}}}\right)^{-1}\left(1+\frac{\tau}{s^2\tau_{\text{free}}}\right)^{-\frac{1}{2}}} + \frac{F}{\left(1+\frac{\tau}{\tau_{\text{bound}}}\right)^{-1}\left(1+\frac{\tau}{s^2\tau_{\text{bound}}}\right)^{-\frac{1}{2}}}\right] \quad (1)$$

where τ_{free} and τ_{bound} are the diffusion times of free and bound molecules, respectively; F denotes bound ratio of Alexa Fluor 488–-labeled HSP47; N is the average number of fluorescent molecules in the detection volume defined by the beam waist w_0 and the axial radius z_0; s is a structure parameter representing the ratio of w_0 and z_0; T is the triplet fraction; and τ_{triplet} is the relaxation time of the triplet state. $G(\tau)$s of samples were measured for 60 s. Following pinhole adjustment, the diffusion time (τ_{Rh6G}) and structure parameter (s) were determined before measurements using a 0.1 μM Rhodamine 6G (Rh6G) solution as a standard. The diffusion coefficient and molecular weight were determined using those of Rhodamine 6G according to a previous study [17].

2.3. Calculation of K_d using FCS

Determination of the equilibrium K_d using FCS was performed using a modification of a previously reported procedure [13]. The fraction of the slow component was determined from curve-fitting analysis of ACF using a two-component diffusion model. After normalization, the binding fraction (F) and collagen concentration ([C]) were plotted. The plots were fitted using the Origin 2016 software (OriginLab) using Eq. (2):

$$F = \frac{[C]^n}{{K_d}^n + [C]^n} \quad (2)$$

where n is the Hill coefficient.

2.4. Analysis of pH-dependent interaction between HSP47 and collagen

The binding ratio was obtained from a two-component diffusion model (Eq. (2)). Half-binding pH was calculated according to the Boltzmann function (Eq. (3)) using the Origin 2016 software:

$$B(x) = 1 + \left[1 + \exp\left(\frac{x - x_0}{dx}\right)\right]^{-1} \quad (3)$$

where $B(x)$ is the binding ratio, x is a pH parameter, x_0 is the half-binding pH, and dx is the constant of the sigmoidal curve.

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