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The interaction of Hsp104 with yeast prion Sup35 as analyzed by fluorescence cross-correlation spectroscopy

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ABSTRACT

Prions are self-propagating amyloids. Yeast prion [*PSI*$^+$] is a protein-based heritable element, in which amyloid aggregates of the Sup35 protein are transmitted to daughter cells. Hsp104, an ATP-dependent disaggregase, and other chaperones are essential to maintain [*PSI*$^+$]. Although previous reports have demonstrated the physical interactions of Hsp104 and Sup35 amyloids, the mechanism how Hsp104 interacts with Sup35 amyloids remains to be elucidated. Here we investigated the interaction between Hsp104 and Sup35 in the lysates of [*PSI*$^+$] cells using fluorescence cross-correlation spectroscopy (FCCS), which can analyze the codiffusion events of different fluorophores. FCCS analysis showed a strong interaction between Hsp104 and Sup35 in [*PSI*$^+$] lysates, but not in [*psi*$^-$] lysates, suggesting that Hsp104 recognizes the amyloid aggregates of Sup35. Although the interaction was retained in ATP-depleted [*PSI*$^+$] lysates, addition of ATP or guanidine hydrochloride, which is an inhibitor of Hsp104, to [*PSI*$^+$] lysates weakened the interaction.

1. Introduction

Prions are propagating protein conformations comprised of ordered protein aggregates called amyloids [1]. Although the concept of prions were originally developed from mammalian neurodegenerative diseases *e.g.*, scraipie, the budding yeast *Saccharomyces cerevisiae* also has prions, including [*PSI*$^+$] and [*URE3*], and thus provides a tractable model system with which to understand for the study of prion biology [2–5]. The yeast prions are protein-based heritable elements, in which amlyoid aggregates of prion proteins are transmitted to daughter cells. Maintenance of yeast prions-involves two distinct steps: the growth and the multiplication of the amyloids [6]. Stable maintenance of prions relies on a delicate balance between the growth and multiplication phases (*e.g.*, [7]). In the growth phase, pre-existing amyloids elongate in a self-catalyzed manner (*e.g.*, [8–10]). In addition to the amyloid elongation, the multiplication phase, which is dependent on several chaperones, including Hsp104, is required for the transmission of all known yeast prions [11].

Among the chaperones, Hsp104 is the only indispensable chaperone for the transmission of yeast prion amyloids from mother to daughter cells [7,12–14]. Hsp104, a chaperone for thermotolerance in yeast [15,16], is an ATP-dependent disaggregase in collaboration with the Hsp70/Hsp40 family [17]. In addition to its role in thermotolerance, Hsp104 is critical for the remodeling of the yeast prion amyloids in cells [7,12–14]. The deletion, inactivation, or overexpression of Hsp104 cures [*PSI*$^+$] [11]. With respect to inactivation, the addition of a millimolar amounts of guanidine hydrochloride (GdnHCl) leads to the eventual elimination of [*PSI*$^+$] through the inhibition of Hsp104 [18–23].

After the original discovery that Hsp104 is required for the propagation of [*PSI*$^+$] in yeast genetics [11], several reports showed a physical interaction between Hsp104 and Sup35, the [*PSI*$^+$] determinant. Specifically, Hsp104 colocalizes with Sup35 aggregates in [*PSI*$^+$] cells when Hsp104 and Sup35 are fused with fluorescent proteins, *e.g.*, GFP [24]. Biochemical approaches have also revealed a direct interaction of Hsp104 with Sup35 [25–29], or the involvement of Hsp104 in the amyloids elongation of Sup35 [30–32]. However, the molecular details underlying this ATP-dependent

Abbreviations: FCCS, fluorescence cross-correlation spectroscopy; RCA, relative cross-correlation amplitude; Sup35NM, N and M domains of Sup35.

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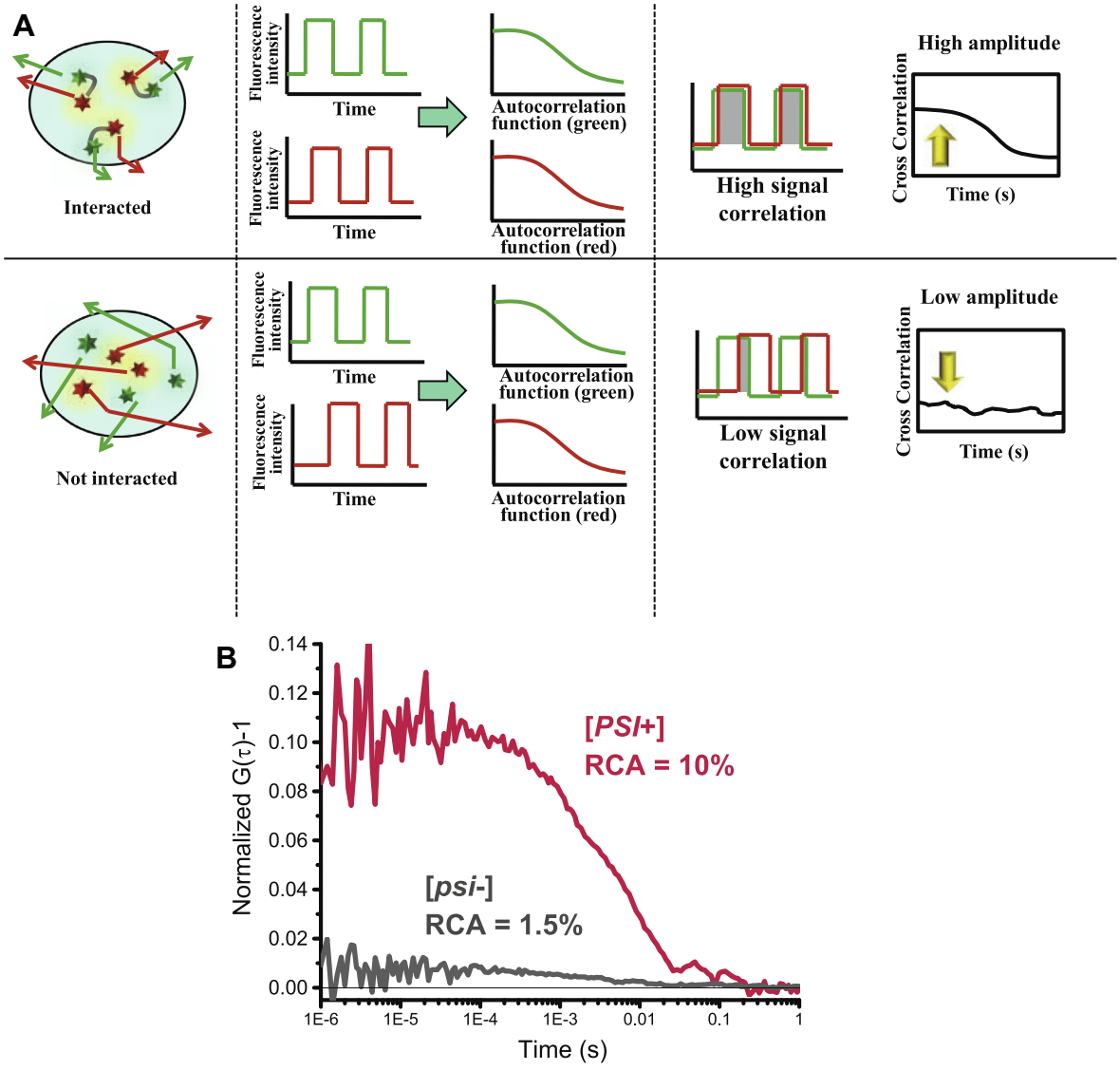

Fig. 1. FCCS measurements of the interaction between Sup35NM-mKate2 and Hsp104-GFP. (A) A scheme illustrating the detection of an interaction between two fluorescent molecules by FCCS. When two molecules, which are individually labeled with two different fluorophores interact, overlap between the two fluorescence fluctuation is increased, resulting in a high amplitude cross-correlation function. By contrast, when two molecules do not interact, overlap is decreased, resulting in a low amplitude cross-correlation function. (B) Typical cross-correlation functions between Sup35NM-mKate2 and Hsp104-GFP in [*PSI*$^+$] lysates (*red*) or [*psi*$^-$] lysates (*gray*) are shown. Quantified RCA values are shown in the inset. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

interaction with Sup35 are poorly understood. Therefore, we investigated the interaction between Sup35 and Hsp104 in yeast lysates using fluorescence cross-correlation spectroscopy (FCCS). FCCS is an advanced fluorescence correlation spectroscopy (FCS) [7,33–35], which allows for a comparison between two fluorescent colors to extract codiffusion events that reflect interactions between differently labeled molecules [36–38].

2. Materials and methods

2.1. Plasmids

The yeast plasmids YCp-GAL1p-SUP35NM-mKate2 [*LEU2*] and YCp-GAL1p-mKate2, expressing the Sup35NM domain conjugated with the mKate2 and mKate2 monomer, respectively, under the control of the galactose inducible *GAL1* promoter, were constructed as previously described [39]. mKate2 cDNA was amplified by PCR from an mKate2-N plasmid (Evrogen, Moscow, Russia).

2.2. Yeast strains and media

The strains used in this study were established from a 74-D694 strain (*MAT***a** *ade1-14 leu2-3112 his3-Δ200 trp1-289 ura3-52* [*PSI*$^+$] or [*psi*$^-$]) [34]. GFP was inserted into the carboxyl terminus of the chromosomal Hsp104 gene. The strains harboring YCp-GAL1p-SUP35NM-mKate2 or YCp-GAL1p-mKate2 were maintained in a synthetic complete medium (using Difco yeast nitrogen base) lacking leucine (SC-Leu). Standard rich medium (YPD) was used to maintain a wild-type strain as a control. SRaf-Leu media contained 2% raffinose (w/v) instead of glucose. To induce expression from the *GAL1* promoter, galactose was added to a final concentration of 2% (w/v). Yeast strains were grown at 30 °C.

2.3. Cell lysis

Cells were suspended in the following buffer: 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, Complete™ protease inhibitor cocktail EDTA-free (Roche, Basel,

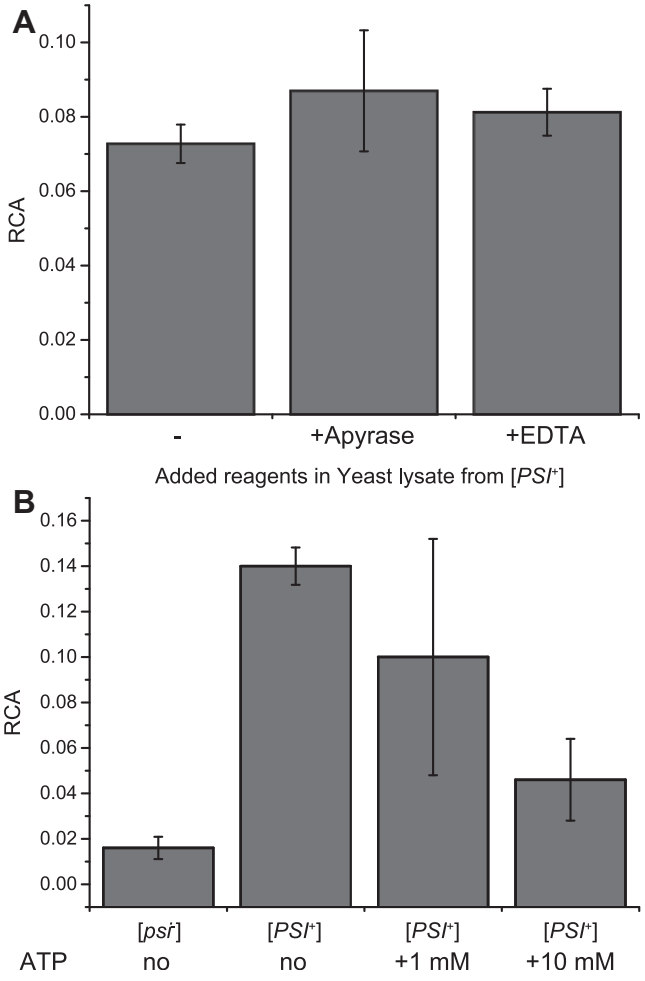

Fig. 2. The role of adenine nucleotides on the Sup35NM–Hsp104 interaction. (A) The effects of apyrase or EDTA on interactions between Sup35NM-mKate2 and Hsp104-GFP in [*PSI*$^+$] lysates, as analyzed by FCCS. RCA values in lysates including apyrase or EDTA were compared with those taken in [*PSI*$^+$] lysates without those additives (mean ± S.D., $n = 3$). (B) The effects of ATP on Sup35NM–Hsp104 interactions in [*PSI*$^+$] lysates. RCA values taken after the addition of ATP, at 1 or 10 mM final concentration, into [*PSI*$^+$] lysates are shown (mean ± S.D., $n = 3$).

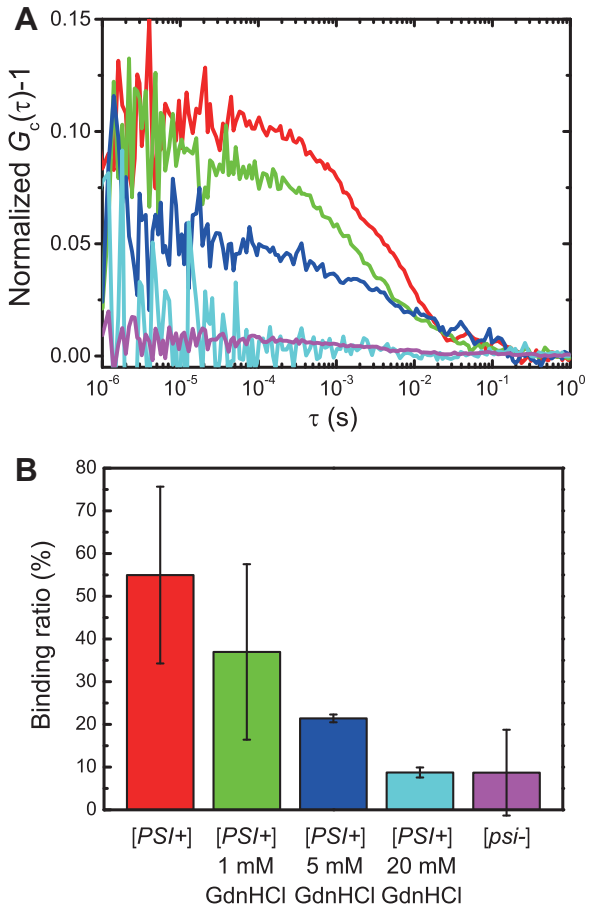

Fig. 3. The effect of guanidine hydrochloride on the Sup35NM–Hsp104 interaction. (A) Representative normalized cross-correlation functions between Sup35NM-mKate2 and Hsp104-GFP in [*PSI*$^+$] lysates (*red*), in the presence of 1 mM (*green*), 5 mM (*blue*) and 20 mM (*cyan*) GdnHCl, and in [*psi*$^-$] lysates (*purple*). (B) The quantification of data shown in (A). Colors correspond to those in (A). The RCA value of a GFP-mCherry tandem dimer was normalized to 100% (mean ± S.D., $n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Switzerland). Then, cells in suspension were lysed with glass beads (Sigma–Aldrich) during 5 cycles of vortexing for 1 min and incubation at 4 °C for 2 min. The supernatants were recovered by centrifugation at 15,780*g* for 10 min.

2.4. FCCS measurements and data analysis

FCCS measurements were performed using a combination system comprising both an LSM 510 META and a ConfoCor3 with a C-Apochromat 40× 1.2NA water immersion objective lens (Carl Zeiss, Jena, Germany) [36–38]. GFP was excited using the 488 nm laser (22.7 μW) and mKate2 was excited using the 594 nm laser (13.8 μW). Fluorescence signals from GFP were collected through a 505–540 nm band pass filter and signals from mKate2 were collected through a 655–710 nm band pass filter. Measurements were performed for 150 s. The auto- or cross-correlation functions were given by:

$$G_{cross}(\tau) = 1 + \frac{\langle I_G(t) \cdot I_R(t+\tau)\rangle}{\langle I_G(t)\rangle \cdot \langle I_R(t)\rangle} \quad (1)$$

where τ indicates the time delay, and $I_G(t)$ and $I_R(t)$ represent the fluorescence intensities of the GFP and mKate2 channels, respectively. $G_G(\tau)$, $G_R(\tau)$, and $G_{cross}(\tau)$ denote the autocorrelation functions of GFP, mKate2, and the cross-correlation function, respectively.

Curve fitting analysis was performed on AIM Version 4.2 software (Carl Zeiss). The acquired auto- or cross-correlation functions were fitted with a one-, two-, or three-component model as follows:

$$G(\tau) = \frac{1}{N}\sum_j F_j \left[1 + \frac{\tau}{\tau_j}\right]^{-1} \left[1 + \frac{\tau}{S^2\tau_j}\right]^{-\frac{1}{2}} + 1 \quad (2)$$

F_j and τ_j indicates the fraction and diffusion time, respectively, of component j; N is the average number of fluorescent particles in the excitation-detection volume defined by radius w_0 and length $2z_0$, and S is the structural parameter representing the ratio $S = z_0/w_0$. The average number of GFP particles (N_G) and mKate2 particles (N_R) was defined as follows:

$$N_G = \frac{1}{G_G(0) - 1} \quad N_R = \frac{1}{G_R(0) - 1} \quad (3)$$

The number of particles that has both GFP and mKate2 fluorescences (N_{cross}) can be calculated as follows:

$$N_{cross} = \frac{G_{cross}(0) - 1}{(G_G(0) - 1)(G_R(0) - 1)} \quad (4)$$

Here, when N_G, N_R are constant, $G_{cross}(0)$ is directly proportional to N_{cross}. For the quantitative evaluation of cross-correlations among various samples, the relative cross-correlation amplitude, RCA [36], was calculated as the number of cross-correlated particles divided by the number of red-fluorescent particles. The binding ratio was calculated by dividing the measured RCA values by the RCA value of a recombinant mCherry-GFP tandem dimer tagged with polyhistidine, which was expressed in BL21(DE3) *E. coli* cells and purified by an Ni–NTA column.

3. Results and Discussion

To elucidate the binding manner between Sup35 and Hsp104, we employed fluorescence cross-correlation spectroscopy (FCCS) analysis [36–38], which can quantitatively evaluate protein–protein interactions in solution with single molecule sensitivity (Fig. 1A). We constructed yeast strains chromosomally expressing Hsp104-GFP and transiently expressing the N and M domains of Sup35 (Sup35NM) fused with a red fluorescent protein, mKate2, under the control of the galactose-inducible *GAL1* promoter in [*PSI*$^+$] or [*psi*$^-$] cells. Lysates from [*PSI*$^+$] or [*psi*$^-$] cells were analyzed by FCCS. In [*PSI*$^+$] lysates, the normalized cross-correlation function between Hsp104 and Sup35NM showed high amplitude (*red* in Fig. 1B). By contrast, a flat and low amplitude cross-correlation function was observed in [*psi*$^-$] lysates (*black* in Fig. 1B). Next, to quantify the strength of the interaction, we calculated the value of the relative cross-correlation amplitude, RCA, which exhibits the relative strength of a molecular interaction [36–38]. The RCA value in [*PSI*$^+$] lysates was 10%, which is significantly higher than that in [*psi*$^-$] lysates (1.5%, Fig. 1B). This result indicated that the interaction between Hsp104 and Sup35NM in [*PSI*$^+$] lysates was stronger than it was in [*psi*$^-$] lysates. Since [*PSI*$^+$] lysates contain amyloid oligomers of Sup35NM (*e.g.*, [33,35]), the FCCS result suggested that Hsp104 recognizes these amyloid oligomers of Sup35NM.

Hsp104 is a ring-forming AAA$^+$ machine that functions in an ATP-dependent manner. To analyze the ATP-dependence of the Sup35NM–Hsp104 interaction in [*PSI*$^+$] lysates, we added apyrase, an enzyme that hydrolyzes ATP or ADP to AMP, to scavenge residual ATP/ADP, or EDTA, a chelating agent of Mg^{2+}, as an inhibitor of magnesium-ATPase. The RCA values were not changed by the addition of apyrase or EDTA (Fig. 2A), suggesting that residual ATP or ADP in [*PSI*$^+$] lysates did not affect the interaction between Sup35NM and Hsp104. Then, we tested the addition of ATP to [*PSI*$^+$] lysates. The RCA values from [*PSI*$^+$] were gradually decreased in the presence of increasing ATP concentrations (Fig. 2B), suggesting that the Sup35NM–Hsp104 interaction was weakened when Hsp104 was provided with ATP.

To address how the adenine nucleotide-binding properties of Hsp104 influence the Sup35NM–Hsp104 interaction in [*PSI*$^+$] lysates, GdnHCl, which has been known to cure yeast prions [18–23], was added to the lysate. After confirming a strong cross-correlation function between Sup35NM-mKate2 and Hsp104-GFP in [*PSI*$^+$] lysates (Fig. 3A and B, *red*), we added 1, 5, or 20 mM GdnHCl to the lysate. The Sup35NM–Hsp104 interaction decreased with increasing concentrations of GdnHCl (Fig. 3A and B, *green*, *blue* and *cyan*). At 20 mM GdnHCl, the signal amplitude was almost the same as that observed in [*psi*$^-$] lysates (Fig. 3A and B, *purple*), indicating that amyloid-dependent interactions were almost abolished under those conditions.

Our FCCS results clearly show that Hsp104 interacts with the diffuse amyloid states of Sup35NM in lysates of [*PSI*$^+$] cells. The physical interaction between Hsp104 and Sup35NM has already been detected by several means [25–29], including fluorescence microscopy-based colocalization analyses and a pull-down assay using histidine-tagged Sup35. Although these previous studies provided evidence for a Sup35NM–Hsp104 interaction, dynamic information was lacking due to limitations of the methods employed. In this context, FCCS has an advantage over those static approaches since the principle of FCCS is based on the diffusion analysis of fluorescence molecules in solution.

We found that the addition of ATP weakened the Sup35NM–Hsp104 interaction. Since Bösl et al. have shown that ATP hydrolysis stimulates the release of the substrate protein from Hsp104 [40], ATP binding and hydrolysis in our system would also weaken the Sup35NM–Hsp104 interaction.

The mechanism by which the addition of GdnHCl cures yeast prions has been extensively investigated [18–23,34,41]. We found that the addition of GdnHCl abolished the Sup35NM–Hsp104 interaction in [*PSI*$^+$] lysates. The abolition was not related to the denaturing properties of GdnHCl, since several reports have shown that GdnHCl specifically alters the ATPase and nucleotide-binding properties of Hsp104 [42,43]. Based on a previous observation that the binding of GdnHCl to Hsp104 is nucleotide-dependent [42], Hsp104 in lysates, under our experimental conditions, would bind nucleotides, probably ADP. Grimminger et al. have shown that GdnHCl binding to Hsp104 increases its affinity for ADP [42], a low affinity state for substrate proteins [40]. As a consequence, GdnHCl would abolish the Sup35NM–Hsp104 interaction, providing mechanistic insights into the role of Hsp104 in the maintenance of yeast prions.

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