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Heat shock-induced interactions among nuclear HSFs detected by fluorescence cross-correlation spectroscopy

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ABSTRACT

The cellular response to stress is primarily controlled in cells via transcriptional activation by heat shock factor 1 (HSF1). HSF1 is well-known to form homotrimers for activation upon heat shock and subsequently bind to target DNAs, such as heat-shock elements, by forming stress granules. A previous study demonstrated that nuclear HSF1 and HSF2 molecules in live cells interacted with target DNAs on the stress granules. However, the process underlying the binding interactions of HSF family in cells upon heat shock remains unclear. This study demonstrate for the first time that the interaction kinetics among nuclear HSF1, HSF2, and HSF4 upon heat shock can be detected directly in live cells using dual color fluorescence cross-correlation spectroscopy (FCCS). FCCS analyses indicated that the binding between HSFs was dramatically changed by heat shock. Interestingly, the recovery kinetics of interaction between HSF1 molecules after heat shock could be represented by changes in the relative interaction amplitude and mobility.

1. Introduction

The molecular response of cells to cellular stress begins with the activation of heat shock transcription factors (HSFs), which exist as three mammalian isoforms (i.e., HSF1, HSF2, and HSF4) [1–3]. HSFs activated by cellular stress bind to heat-shock element (HSE) within the promoters of their target genes to increase the synthesis of ubiquitous heat shock proteins (HSPs) [4,5]. Among the three mammalian HSFs, HSF1 is activated by classical stresses, such as heat shock and chemical stress, and is closely associated with the stress-induced expression of heat shock genes. The activation of HSF1 is a molecular process that involves trimerization, the acquisition of DNA-binding activity, and phosphorylation [3]. Unlike HSF1, HSF2 lacks intrinsic classical stress responsiveness and has been proposed to be related to the specific HSP expression observed during developmental processes [2]. HSF4, which lacks the carboxyl-terminal hydrophobic repeat (HR-C) domain necessary for the suppression of HSF trimer formation, shows constitutive DNA binding activity. The stress-responsiveness of HSF4 remains unknown [6,2,7].

Recent studies proposed that the interplay between HSFs can regulate their transcriptional function and play an important role in activating their target genes. For instance, HSF1 was demonstrated to physically interact with HSF2 on the stress granules and be necessary for HSF2-mediated transcriptional enhancement by heat shock [8,9]. Recently, the interaction between HSF2 and the oligomerization domain of HSF4a was also reported [10]. These studies reflect that HSF family members can commonly regulate various cellular stresses via their combinational interaction, even though little is known about the details of the molecular interaction and the related regulation mechanism.

In this study, we quantified the molecular interactions among the three HSFs in the nuclei of live cells before and after heat shock using highly sensitive fluorescence correlation spectroscopy (FCS) [11–13] and dual color fluorescence cross-correlation spectroscopy (FCCS) [14], which allow for the detection of molecular interactions as well as changes in molecular diffusion according to the physiological conditions of cell [15–17]. For the first time, this approach reveals the binding interactions among nuclear HSF1, HSF2, and HSF4 at the positions of the nucleoplasmic pool, excluding heat

Abbreviations: HSF, heat shock transcription factor; HS, heat shock; FCCS, fluorescence cross-correlation spectroscopy; RCA, relative cross-correlation amplitude.

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shock stress granules. The findings demonstrate molecular interplay via homo- and hetero-complex formation upon heat shock stress.

2. Materials and Methods

2.1. Plasmid constructions

Green fluorescent protein (GFP) plasmid DNA (pEGFP-N1, Clontech) and pmCherry-C1 were used to generate the GFP or mCherry fusion proteins human HSF1-GFP, HSF1-mCherry, HSF2-mCherry and HSF4-mCherry by cloning polymerase chain reaction (PCR)-amplified DNA fragments. HSF1-GFP and HSF1-mCherry were generated by cloning the PCR-amplified full-length HSF1 region into the *KpnI*/*HindIII* sites of pEGFP-C1 or pmCherrey-C1 using the forward primer 5′- CCCAAGCTTGGATGGATCTGCCCGTGGGCC-3′ and reverse primer 5′- GGGGTACCCTAGGAGACAGTGGGGTCCTTG-3′. HSF2- and HSF4-mCherry were also generated using PCR-amplified full-length HSF2 and HSF4 as well as the forward primer 5′-GGAATTCCATGAAGCAGAGTTCGAACGTGCC-3′ and reverse primer 5′-GGGGTACCTTAGCTATCTAAAAGTGGCATA-3′ for HSF2 and forward primer 5′-CCCAAGCTTGGATGGTGCAG-GAAGCGCCAGC-3′ and reverse primer 5′-GGGGTACCTTAGGGG-GAGGGACTGGCTTC-3′ for HSF4. All constructs were verified by DNA sequencing.

2.2. LSM imaging, FCS, and FCCS measurements and data analysis

All of the FCS and FCCS measurements based on LSM observation were taken at 25C with an LSM 510 combined with a ConfoCor2 system (Carl Zeiss) as described in our previous studies [13,15,17]. GFP was excited at the 488 nm laser line, and mCherry was excited at the 543 nm laser line. The confocal pinhole diameter was adjusted to 70 mm for 488 nm and 78 nm for 543 nm. The emitted light was collimated and then split by a NFT570 dichroic mirror and detected at 505–530 nm by the green channel for GFP and at 600–650 nm by the red channel for mCherry. The fluorescence auto-correlation functions (FAFs) of the red and green channels, $G_r(\tau)$ and $G_g(\tau)$, and the corresponding fluorescence cross-correlation function (FCF), $G_c(\tau)$, were calculated using the following:

$$G_x(\tau) = 1 + \frac{\langle \delta I_i(t) \cdot \delta I_j(t+\tau) \rangle}{\langle I_i(t) \rangle \langle I_j(t) \rangle} \quad (1)$$

where τ denotes the time delay (lag time) in μsec, I_i is the fluorescence intensity of the red channel ($i = r$) or green channel ($i = g$), and $G_r(\tau)$, $G_g(\tau)$, and $G_c(\tau)$ denote the auto-correlation functions of the red ($i = j = x = r$), green ($i = j = x = g$), and cross ($i = r, j = g$, $x = c$), respectively. The acquired $G_x(\tau)$ were fitted by a model equation:

$$G_x(\tau) = 1 + \frac{1}{N} \sum_i F_i \left(1 + \frac{\tau}{\tau_i}\right)^{-1} \left(1 + \frac{\tau}{s^2 \tau_i}\right)^{-1/2}, \quad (2)$$

where F_i and τ_i are the fraction and diffusion time of component i, respectively. 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 structure parameter representing the ratio $s = z_0/w_0$. The structure parameter was calibrated using the known diffusion coefficient of Rhodamine 6G (Rh6G, $D = 280\ \mu m^2/s$) [12,13]. The photochemical term represented by the triplet was omitted in Eq. (2) for simplicity. The diffusion time of component i, τ_i, is related to the translational diffusion coefficient, D, of component i by

$$\tau_i = \frac{w^2}{4D_i} \quad (3)$$

The diffusion coefficients of proteins were calculated from the diffusion coefficient of Rh6G and measured diffusion times of Rh6G and proteins tagging GFP or mCherry as follows:

$$\frac{D}{D_{Rh6G}} = \frac{\tau_{Rh6G}}{\tau} \quad (4)$$

where D and τ denote the diffusion coefficients and the diffusion times of protein molecules, respectively. The single diffusion coefficient obtained from one-component analyses ($i = 1$ in Eq. (2)) was described by D, and the two diffusion coefficients from two-component analyses ($i = 2$ in Eq. (2)) were described by D_{fast} and D_{slow}. For live HeLa cells, all measurements were carried out in the nucleoplasm. All acquired $G_x(\tau)$s of GFP, mCherry, and tandem GFP-mCherry from the nuclei were fitted with a one-component model and no change was detected in the D values, irrespective of heat shock (data not shown). The acquired $G_x(\tau)$s of the fluorescently tagged HSF1 from the nuclei of cells prior to heat shock were fitted by a one-component model. For heat shocked cells, the $G_x(\tau)$s were fitted by a one- or two-component model. To quantitatively evaluate the binding interaction, the amplitude of the cross-correlation function was normalized by the amplitude of the autocorrelation function of the RFP molecule to calculate the relative cross-correlation amplitude (RCA = [Gc(0)-1]/[Gr(0)-1]). To obtain the stationary fluctuating signals with minimal photobleaching, we selected the cells that expressed each fluorescent protein at a low level. In addition, the excitation of fluorescent proteins in the nuclei was reduced as much possible to minimize the effect of photobleaching on the FCS and FCCS analyses. All functions were sequentially measured five times for 10 s at one point. Fluorescent proteins showed slow bleaching during the measurement of heat-shocked cells at some positions in the nuclei, even though the minimal excitation was used. Bleaching can be induced when the detection volume contains stress granules. Therefore, the bleached measurements of cells after heat shock were excluded from analysis. Moreover, the FCCS measurement of some cells that show simultaneous photobleaching and decay of the count-rate in both channels were excluded because this phenomenon gives rise to an artificial cross-correlation amplitude and an apparent component of slow mobility.

3. Results

To evaluate the interaction properties among HSF1, HSF2, and HSF4 in live cells, we used FCS and FCCS analyses, which are highly sensitive methods for the quantification of molecular diffusion and interaction in live cells as well as in aqueous solutions (Fig. 1a and b). In this study, we focused on the binding manner among the heat shock factors only in the nucleoplasm, although the mobility and interaction of three HSFs can be characterized in the compartments of live cells, such as the cytosol, the nucleolplasm, and the nucleolus. To detect and compare the interaction between HSFs before and after heat shock (HS), a procedure consisting of LSM and FCCS measurement with three steps was employed as described in Fig. 1c. To set the appropriate timing for the FCCS measurement, HeLa cells expressing HSF1-GFP were observed by LSM before and after HS treatment. HSF1-GFP was diffusely localized in the nuclei of HeLa cells, and the cells did not show nuclear stress granules of HSF1-GFP in the nucleus prior to HS (Fig. 1c inset upper). After HS, HSF1-GFP formed stress granules and showed a strong fluorescence intensity in the nucleus, which indicated that the HSF1-GFPs were activated by HS. The result of nuclear granule formation agrees well

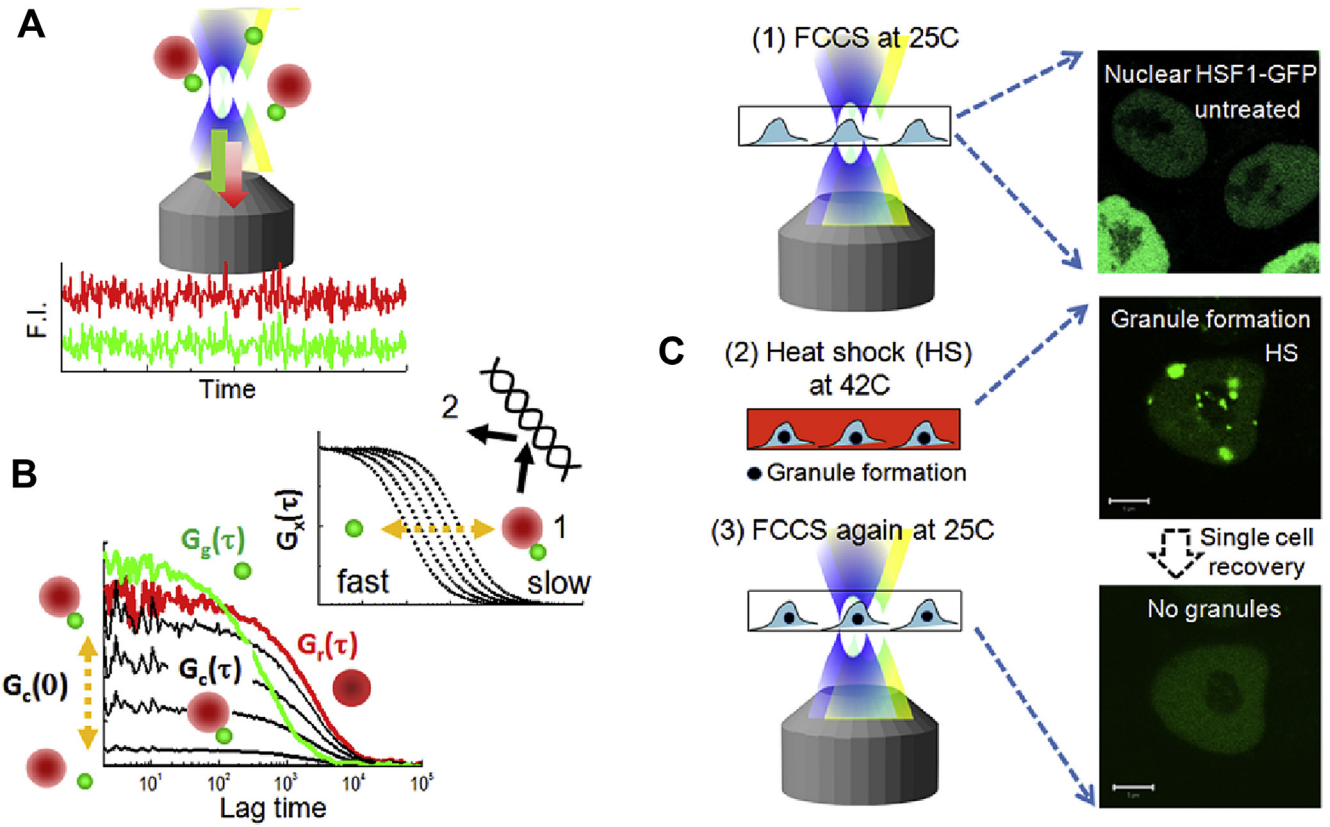

Fig. 1. Schematic diagram of dual color fluorescence cross-correlation analysis on nuclear HSFs in live cells. (A) FCCS is based on the temporal fluctuation analysis of the fluorescence intensities of two different molecules (green and red) detected from two excitation volumes (blue and yellow) defined by an objective and confocal detection setup. (B) FCCS provides an FCF (G_c) and two auto-correlation functions (FAF, G_g and G_r), respectively. The G_c curves shown in black allow the measurement of the direct interaction or co-diffusion of two specific molecules that transition through the two open volumes, which is represented by the relative cross-correlation amplitude (RCA, see Materials and methods). The RCA value corresponds to the number fraction of the bound molecules to unbound free molecule. $G_c(0)$ shifts to up or down according to the binding strength (yellow arrow). The auto-correlation functions shift to the left or to the right according to the size change of the probe molecule (inset, 1) or according to the transient interactions of probe molecule with immobile structures such as DNA (inset, 2). For clarity, the shift in the correlation functions was simplified by using a one-component model. The FI and subscripts of g, r, and c stand for the averaged fluorescence intensity, green, red, and cross, respectively. (C) The procedures of the FCS or FCCS measurement on nuclear HSF molecules in live HeLa cells before and after heat shock are shown schematically. (1) First, HeLa cells expressing HSFs tagging GFP or co-expressing HSFs tagging GFP and mCherry, all of which lacked stress granules, were observed by LSM, and then the positions in the nucleoplasm were measured by FCCS. The inset shows the representative LSM image of HeLa cells expressing HSF1-GFP (upper). (2) The glassed chamber containing cells was then moved to the incubator to receive HS at 42C for 30 min, which induces stress granules of HSF1 in the nucleus (black balls). (3) The chamber containing heat shocked cells was again set up on the microscopy maintained at 25C, and single cells maintaining stress granules were then selected and measured continuously by FCCS until the stress granules completely vanished. Representative LSM images of the nucleus of a HeLa cells expressing HSF1-GFP after HS and after the recovery are shown (center and lower). Scale bar, 5 μm.

with previous studies [18–20]. The nuclear granules were maintained for tens of minutes, even after resetting the heat shocked cells on the microscope at 25C, and gradually diminished with time (Fig. 1c inset center and lower) [18,20]. The result demonstrated that FCCS could be used to detect changes in the interaction between HSFs during the delayed recovery time after HS, even though our procedure could not detect changes in the molecular state of HSF molecules upon and soon after HS. Therefore, FCCS measurements were carried out and compared before and after HS by considering the nuclear granule of HSF1 as a marker of heat shock activation. Notably, several seconds of FCCS measurement combined LSM observation is sufficient to detect the mobility and interaction of target molecules [13–15].

To identify binding properties between HSF1s in live cells, HeLa cells co-expressing HSF1-GFP and mCherry-HSF1 were measured according to the procedure shown in Fig. 1c. Both HSF1-GFP and mCherry-HSF1 showed the same nuclear localization and diffuse pattern prior to HS (Fig. 2a). Each nucleus of heat-shocked cells showed that HSF1 formed stress granules, and the granules of HSF1-GFP and mCherry-HSF1 strongly co-localized, which demonstrated that co-expressed HSF1-GFP and mCherry-HSF1 were equally activated by heat shock (Fig. 2b). FCCS measurements were also carried out on the nuclei of cells for two conditions. For untreated cells (Fig. 2c), a one-component model fit each auto-correlation curve measured in the nucleoplasm well, and the mobility of HSF1 could be presented by a single diffusion coefficient ($D = 11.1\ \mu m^2/s$). The D value of nuclear HSF1-GFP is slightly smaller than that of the tandem GFP dimer ($D = 15\ \mu m^2/s$) [13]. The amplitude of the fluorescence cross-correlation ($G_c(0)$) of HSF1-GFP and mCherry-HSF1 in the nucleoplasm and the correspondent RCA value were very low, which indicated that the interaction between nuclear HSF1s was not detected or very weak. The heat shocked cells that showed stress granules, a marker of heat shock activation, were selected, and FCCS measurements were carried out on the nucleoplasmic positions, excluding nuclear stress granules. After each FCCS measurement, the decrease in nuclear granules was confirmed to show that the granules were formed by heat shock as described in Fig. 1. Interestingly, $G_c(0)$ of the nuclear HSF1s and the corresponding RCA value dramatically increased in cells containing stress granules (Fig. 2d). This result indicates nuclear HSF1 molecules strongly bound each other and formed stable homo-complexes after heat shock. Moreover, the auto-correlation

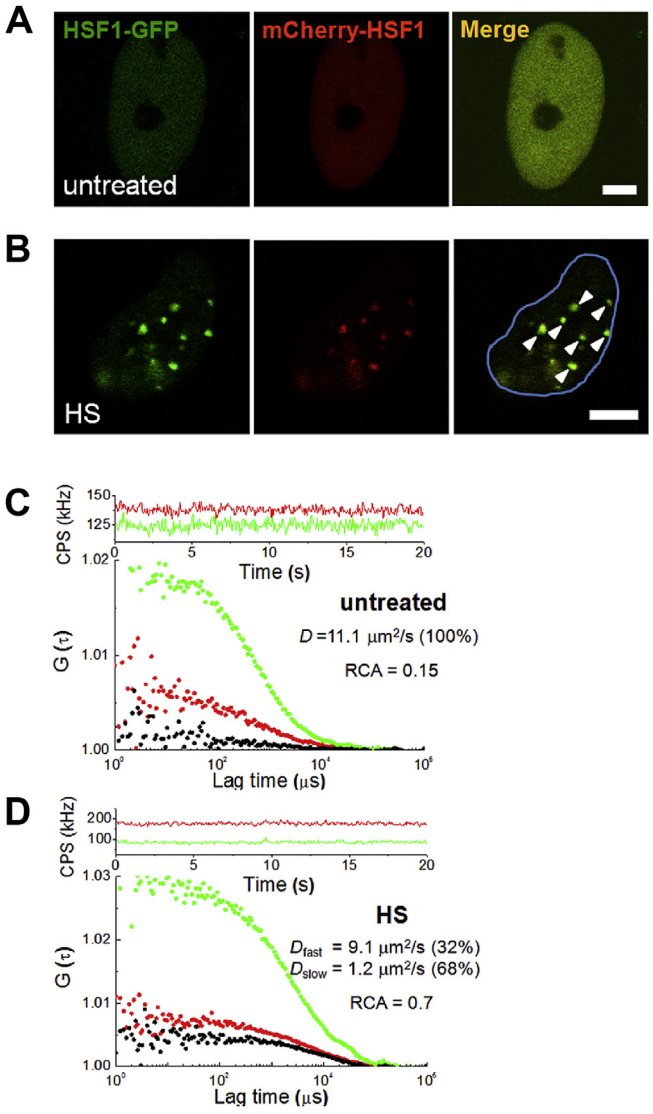

Fig. 2. LSM observation and FCCS measurements on the nucleoplasm of HeLa cells co-expressing HSF1-GFP and mCherry-HSF1. Representative confocal LSM images of HeLa cells co-expressing HSF1-GFP and mCherry-HSF1 at 24 h after transfection are shown. (A) and (B) show HeLa cell nuclei before and after HS, respectively. Stress granules in the nucleus of heat-shocked cell in (B) are indicated by the arrowhead. Solid line indicates the nucleus. The LSM images in (A) and (B) were taken of cells that lowly expressed the proteins for FCCS measurement. Positions in the nucleoplasm part excluding stress granules were selected and measured by FCCS. Scale bar, 5 μm (C and D) Representative fluorescence correlation functions measured in the nucleoplasm of HeLa cells co-expressing HSF1-GFP and mCherry-HSF1 are shown. Cells were measured by FCCS prior to HS (C) and after HS at 42C for 30 min. The average fluorescence intensities (count rate, CPS in kHz) during 20 s, corresponding FAFs of HSF1-GFP (green, G_g) and mCherry-HSF1 (red, G_r), and FCFs (black, G_c) are shown.

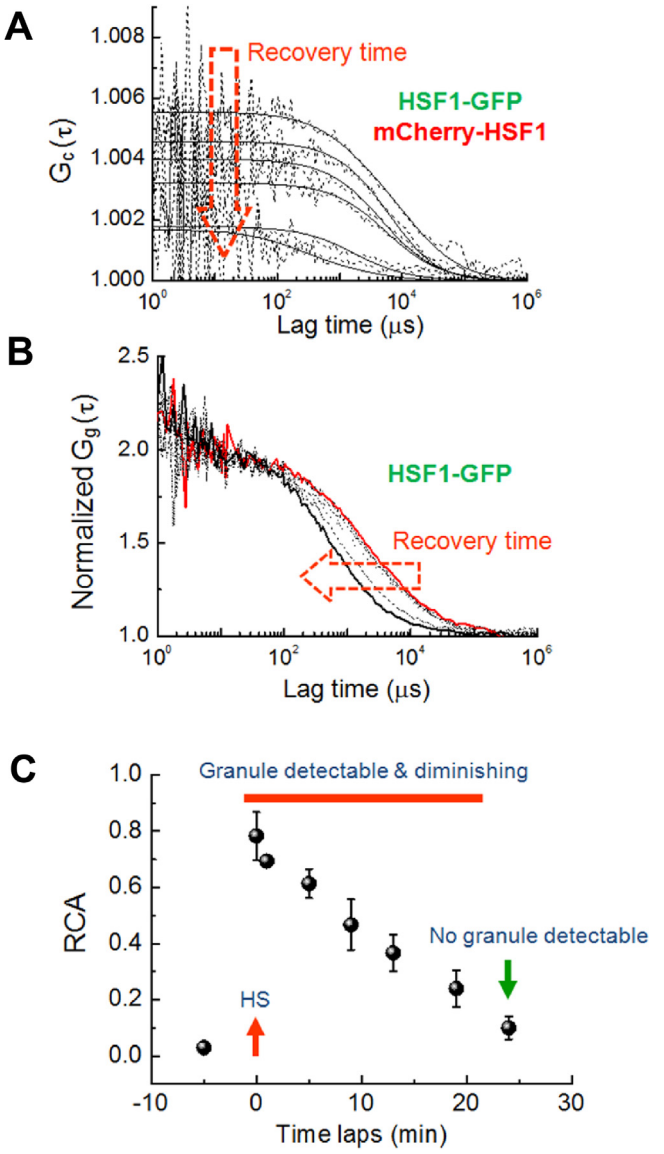

Fig. 3. Kinetics of HSF1 binding interaction during recovery after heat shock. Heat-shocked HeLa cells co-expressing HSF1-GFP and mCherry-HSF1 were examined by the microscopy maintained at 25C, and a single cell with nuclear granules of HSF1-GFP and mCherry-HSF1 was then selected and measured by LSM and FCCS as the nuclear granule gradually diminished (recovery process). (A) Time-lapse FCFs, (G_cs), obtained from six consecutive FCCS measurements on the nucleus of a single cell during the recovery process are shown (see also C). FCCS measurements were carried out on the positions excluding nuclear granules of HSF1-GFP and mCherry-HSF1. (B) The normalized FAFs (G_gs) of HSF1-GFP corresponding to the cross correlation functions in (A) are shown. (C) Change in the mean value of relative cross-correlation amplitude (RCA), a degree of interaction amplitude, during the recovery process are summarized. (see also Materials and Methods). The data point at minus 5 min represents the mean RCA value obtained from HeLa cells prior to HS.

curves of G_g (τ) and G_r (τ) were largely shifted to the right and showed a slow mobility ($D_{slow} = 1.2\ \mu m^2/s$), which is 10-fold slower than that of HSF1-GFP prior to heat shock and was newly induced (see also Fig. 3b). Because the change in molecular weight via the trimerization of HSF1 induces only a 1.4-fold decrease in the diffusion coefficient, which induces a small shift in the auto-correlation function to the right. This result suggests that a homo-complex of fluorescently tagged HSF1s after heat shock also interacts with other molecules, such as immobile target DNAs, which considerably slows the diffusion of the HSF1 homo-complex and therefore induces a large shift of the corresponding function to the right. Hetero-complex or homo-oligomers were formed much more frequently than HSF1 homo-trimers, which also slows the mobility of HSF1 and cannot be avoided.

After confirming the dramatic change in the interaction between fluorescently tagged HSF1s due to heat shock stress, the binding kinetics between nuclear HSF1s were traced with single cell nuclei during the recovery after heat shock (Figs. 1c and 3). Consistent with the large increase of interaction between HSF1 after heat shock, single HeLa cell showed strong interactions

between HSF1-GFP and mCherry-HSF1 soon after heat shock. Furthermore, the interaction ($G_c(0)$) gradually decreased thereafter for a recovery time of 25 min (Fig. 3a, dotted arrow), during which the fluorescence intensity of granules gradually decreased and then vanished (data not shown). Moreover, the significantly slowed diffusional mobility of HSF1 soon after heat shock gradually changed into fast mobility according to the decrease in the interaction (Fig. 3b), which indicated that the binding interaction and slowed diffusion of the HSF1 homo-complex were coupled. The change in the relative interaction amplitude (RCA value) between nuclear HSF1s during the recovery process in single cells is summarized in Fig. 3c. The result suggests that the kinetics of the interaction between HSF1s Previous studies suggested that the HSF1 stress granules and the activation of the heat shock response were coupled. Moreover, the transcription of heat shock proteins (HSPs), such as hsp70, was demonstrated to rapidly increase upon heat shock, followed by a linear decrease during recovery [19,20].

Recently, the interplay between HSF2 and HSF1 has recently been reported; they interact on the stress granules formed after heat shock [8,9]. In addition, molecular interactions between HSF2 and HSF4a have also been suggested [10]. The studies suggest that the functional interplay between HSF family members can be orchestrated via the direct formation of a hetero-trimer complex between them followed by the interaction with target DNAs. However, detailed information on this binding interplay in live cells and the mechanistic relationship between the interplay and heat shock response remain unclear [2,3]. In addition to detecting the interaction between nuclear HSF1s, we examined the hetero-complex formation between HSF1-GFP and others, such as nuclear HSF2-mCherry and HSF4a-mCherry before and after heat shock (Fig. 4). Interestingly, HSF1 interacted very weakly with HSF2 and HSF4 in untreated cells (Fig. 4, under green line), but strongly bound them after heat shock (Fig. 4, under red line). Conversely,

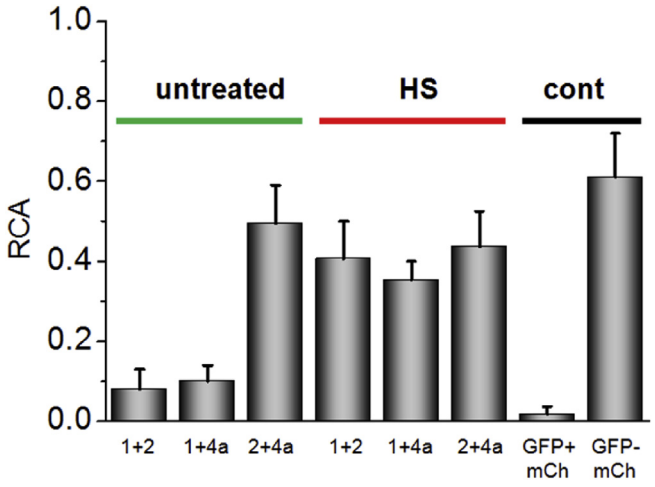

Fig. 4. Binding interaction among nuclear HSF1, HSF2, and HSF4a detected in the nuclei of live HeLa cells. HeLa cells were co-transfected with two different colors of HSFs (HSF1, HSF2, HSF4a) tagged with GFP and mCherry (mCh). As a control, HeLa cells expressing linked GFP-mCherry (GFP-mCh) in tandem and co-expressing GFP and mCherry (GFP + mCh) were used. Cells with low expression were selected, and the nuclear HSFs were measured by FCCS before and after HS at 42C for 30 min. A summary of RCA values is shown (refer Materials and Methods). RCA values for GFP-mCh and GFP + mCh was not changed by heat shock, respectively, and therefore all values of RCA before and after heat shock was summarized together and represented by average control value (cont). The mean RCA values with standard deviation were obtained from analyses of the nuclei of HeLa cells (n = 5) at each condition. HSF1, HSF2, and HSF4a are respectively represented by 1, 2, and 4a. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

HSF2 was tightly bound to HSF4a even without heat shock, and the binding interaction decreased slightly after heat shock. HeLa cells co-expressing monomer GFP and mcherry or expressing GFP in tandem linked with mCherry were used as negative and positive controls, respectively (Fig. 4, under black line). The diffusional mobility and RCA values of the controls did not differ before and after heat shock (data not shown). The result demonstrates that three HSF family members specifically interact with each other upon heat shock stress. The average strength of the hetero-complex formation between HSF1 and HSF2 (RCA = 0.4) or between HSF1 and HSF4a (mean RCA = 0.38) was much lower than that of the homo-complex between HSF1s (Fig. 3c, mean RCA = 0.8). The result suggests that the formation of a HSF1 homo-complex is much more favorable in the heat shock response than the formation of a hetero-complex with HSF2 or with HSF4a.

4. Discussion

Previous studies using LSM observations on mammalian culture cells expressing GFP-tagged HSF demonstrated that the temporal heat shock response is well described by the rapid and reversible dynamics of stress granules formed by nuclear HSF1 in live cell upon heat shock [18–22]. All of the studies focused only on the formation and dynamics of nuclear granules upon heat shock. A previous study using FRAP and FCS analyses on HSF-GFP in *Drosophilia* polytene nuclei demonstrated that the mobility of the nucleoplasmic HSF-GFP and exchange kinetics of the protein at specific chromosomal foci changed after HS [11]. Our study using dual-color FCCS successfully demonstrated that nuclear HSF1s was highly mobile and interacted very weakly with itself prior to HS. However, HSF1s strongly bound each other after HS in the nucleoplasmic part, excluding the stress granules. The ability of HSF1 to form homotrimers and its increased interaction with target DNA after HS can explain these results well [2,3,23]. Most recently, heterotrimer formation between HSF1 and HSF2, a process that lacks intrinsic stress responsiveness, was proposed to be able to integrate transcriptional activation in response to distinct stress [8,9,24]. In the previous study, HSF2 was found to be recruited to stress-inducible promoters by forming heterotrimers with HSF1 on nuclear stress granules, as observed by fluorescence lifetime imaging (i.e., FLIM) in live cells. The method was not demonstrated for detecting binding interaction between HSF1 and HSF2 in the nucleoplasmic region, excluding the stress granules that contain target DNA of satellite III [9]. Our result demonstrated that nuclear HSF1 could form hetero-complexes after HS with both nuclear HSF2 and HSF4a in the nuleoplasmic region. Moreover, the formation of a hetero-complex between HSF2 and HSF4a was detected even prior to HS, which is consistent with in vitro analyses of their constitutive interaction [10]. Combined with previous studies, our result suggests that monomers of HSF1 and hetero-complexes of HSF2 and HSF4, all of which are mobile, are present in the nucleus even before HS, and they can be rearranged after heat shock to form various types of hetero-complexes composed of HSF1-HSF2, HSF1-HSF4, or HSF1-HSF2-HSF4 in addition to HSF1 homo-trimers.

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