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A Quantitative Study of Internal and External Interactions of Homodimeric Glucocorticoid Receptor Using Fluorescence Cross-Correlation Spectroscopy in a Live Cell

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Glucocorticoid receptor (GR α) is a well-known ligand-dependent transcription-regulatory protein. The classic view is that unliganded GR α resides in the cytoplasm, relocates to the nucleus after ligand binding, and then associates with a specific DNA sequence, namely a glucocorticoid response element (GRE), to activate a specific gene as a homodimer. It is still a puzzle, however, whether GR α forms the homodimer in the cytoplasm or in the nucleus before DNA binding or after that. To quantify the homodimerization of GR α , we constructed the spectrally different fluorescent protein tagged hGR α and applied fluorescence cross-correlation spectroscopy. First, the dissociation constant (K_d) of mCherry₂-fused hGR α or EGFP-fused hGR α was determined *in vitro*. Then, K_d of wild-type hGR α was found to be 3.00 μ M in the nucleus, which was higher than that *in vitro*. K_d of a DNA-binding-deficient mutant was 3.51 μ M in the nucleus. This similarity indicated that GR α homodimerization was not necessary for DNA binding but could take place on GRE by means of GRE as a scaffold. Moreover, cytoplasmic homodimerization was also observed using GR α mutated in the nuclear localization signal. These findings support the existence of a dynamic monomer pathway and regulation of GR α function both in the cytoplasm and nucleus.

Understanding the interactions and dynamic properties of biomolecules in living cells is of paramount importance in life sciences. Glucocorticoid receptor α (GR α) is a natural-steroid- and synthetic-steroid-regulated transcription factor, a member of the nuclear receptor superfamily that regulates a variety of physiological functions via several mechanisms. It is widely thought that unliganded GR α is primarily located in the cytoplasm as part of a multiprotein complex with chaperones and immunophilins^{1–3}. After ligand binding, GR α is translocated to the nucleus, where it works either as a homodimer that binds to positive or negative glucocorticoid response elements (GRE) located in the promoter regions of target genes, or as a monomer that cooperates with other transcription factors to induce transcription^{4–7}. In addition, the homodimer of GR α can act as a repressor in association with a negative GRE, and as a monomer can tether other transcription factors such as NF- κ B^{8,9}. A number of *in vitro* studies suggest that GR α homodimerizes after ligand binding^{10–14}. It has been demonstrated that two molecules of the DNA-binding domain of GR α bind to a GRE in a cooperative manner, where binding of the first molecule accelerates binding of the second molecule^{10,15,16}. It was also reported, however, that the preformed homodimer of the GR α preferentially binds to the GRE rather than sequential binding of the monomer^{17–21}. It is still unclear whether binding of GR α to the GRE is followed by simple sequential or cooperative binding of the second monomer. Several recent studies have shown homodimerization of GR α *in vivo*^{17,20,22}. Moreover, GR α homodimerization in the cytoplasm before translocation to the nucleus has been reported^{17,20,23}. However, it

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is still a matter of debate whether GR α homodimerizes in the cytoplasm or in the nucleus *in vivo* and what the function of homodimer formation in the cytoplasm is. Thus, there are still many questions about GR α function and formation. They can be answered by analyzing the affinity properties of GR α and/or formation of a complex with associated molecules in a live cell.

To find out when and where GR α homodimerizes, we used fluorescence cross-correlation spectroscopy (FCCS) to determine the binding affinity of transiently expressed enhanced green fluorescence protein (EGFP)-fused GR α , mCherry tandem dimer (mCherry₂) protein-fused GR α , and appropriate GR α mutants, in each case in the nucleus and cytoplasm before and after addition of ligands. FCCS is a well-investigated method for determination of direct associations between spectrally different fluorescence labeled proteins in femtoliter confocal volumes^{24–30}. The femtoliter confocal volume allows us to easily resolve the measurement positions in the nucleus and cytoplasm. The parameters obtained by this method are the concentrations of the labeled particles (free and bound particles) and their diffusion constants as well as the molecular sizes of their complexes³¹. FCCS has various intracellular applications, including determination of dissociation constants (K_d) of fluorescently labeled proteins^{30, 32–36}.

In our experiments here, a positive cross-correlation was obtained in wild-type (WT) GR α after addition of dexamethasone (Dex) as a synthetic ligand. Then, K_d values of homodimerization of full-length WT GR α and its mutants were determined and compared in living cells. Using this approach, we were able to evaluate GR α homodimerization in the cytoplasm and in the nucleus *in situ*. Our findings support the presence of a GR α homodimer in both the cytoplasm and nucleus before association with a GRE. The diffusion properties of WT GR α and mutants in the nucleus and cytoplasm in the presence and absence of Dex were also compared using a distribution of the diffusion constants.

Results

Analysis of hGR α homodimerization *in vitro* using FCCS. K_d of homodimerization of WT hGR α *in vitro* was determined by means of a single-cell measurement system combined with fluorescence correlation spectroscopy (FCS) and a microwell: the FCS-microwell system¹⁴. The microwell system was upgraded to FCCS (FCCS-microwell system) to determine K_d of the homodimerization of GR α . U2OS cells, which do not have endogenous hGR α (Figs S2A and S15A), were transiently cotransfected with a plasmid expressing WT hGR α fused to a tandem dimer of mCherry (mCherry₂) and EGFP (Fig. S1A and B). The tandem, mCherry₂, was used instead of monomer mCherry³⁰ because of a stronger signal of relative cross amplitude (RCA) in living cells (Fig. S3). The RCA provides a relative signal of an interaction calculated by a division of the cross-correlated amplitude by one of the autocorrelation amplitudes^{26, 37}. The RCA of EGFP-mCherry₂ was less than one, because the confocal volumes between the green and red channel were incompletely overlapped³⁰ and a photobleaching of fluorescent proteins may be affected. However, the fluorescent intensity was not dramatically decreased in our experiments.

EGFP-hGR α and mCherry₂-hGR α were localized to the cytoplasm in the absence of Dex (Fig. 1(a)) but localized to the nucleus in the presence of Dex (Fig. 1(b)). After cell lysis, the autocorrelation and cross-correlation functions were measured in the microwell (Fig. 1(c) and (d)). The RCA of the interaction between EGFP-hGR α and mCherry₂-hGR α show similar tendencies against concentration ratio of mCherry₂-hGR α and EGFP-hGR α (Fig. S4A), and was significantly higher than that of the negative control of EGFP and mCherry₂, suggesting that FCCS could detect the GR α homodimerization (Fig. S4B). The concentrations of homodimeric GR α [Dimer] and monomeric GR α [Monomer] were calculated in the FCCS analysis (Supplemental information). To determine K_d values of GR α homodimerization, a scatter plot was generated from the square of the concentration of monomeric GR α [Monomer]² and the concentration of the homodimeric GR α [Dimer], and linear regression calculation was carried out to find the best-fit line through each scatter plot by equation (15). K_d was calculated from the slope of the regression line^{30, 32}. K_d of the homodimerization of WT hGR α was found to be 416 ± 57.4 and 139 ± 9.27 nM in the absence and presence of Dex, respectively (Fig. 1(e) and (f)). This K_d value was in good agreement with the data in our previous report determined by brightness analysis using the FCS-microwell system¹⁴. This consistency suggested that K_d values for GR α homodimerization can be determined using FCCS. Moreover, C421G (Figs 2(h) and S1C and D), a DNA-binding-deficient mutant³⁸, and A458T (Figs 2(h) and S1E and F), a homodimerization-deficient mutant³⁹, were analyzed using the FCCS-microwell system. The A458T mutant and C421G mutant were also localized to the nucleus in the presence of Dex (Fig. 2(a) and (b)). The autocorrelation and cross-correlation functions were then examined after cell lysis (Fig. 2(c) and (d)). K_d of the homodimerization of the C421G mutant and A458T mutant was found to be 244 ± 23.8 and 379 ± 49.6 nM in the presence of Dex, respectively (Fig. 2(e) and (f)).

A summary of the K_d values of GR α homodimerization *in vitro* is shown in Fig. 2(g). There is a significant difference between the WT in the absence and presence of Dex, suggesting that the hGR α homodimerization was induced by Dex. Moreover, K_d of homodimerization of the C421G mutant was significantly lower than that of the WT in the absence of Dex, and was significantly higher than K_d of the WT in the presence of Dex. This finding suggested that GR α homodimerization was not necessary for DNA binding but that DNA has a role of scaffolds for GR α homodimerization. The A458T mutant showed similar K_d value to that of the WT in the absence of Dex. These results indicated that FCCS can determine K_d of hGR α homodimerization.

FCCS analysis of WT hGR α in the nucleus and cytoplasm. To study the GR α homodimerization in living cells, FCCS was performed in living U2OS cells. The cells were transiently cotransfected with plasmid constructs expressing EGFP-hGR α and mCherry₂-hGR α (Fig. S1A and B). The fusion proteins mCherry₂-hGR α and EGFP-hGR α were initially localized to the cytoplasm in the absence of Dex (Fig. 3(d), inset), but after the addition of Dex, both mCherry₂-hGR α and EGFP-hGR α were translocated to the nucleus during 20 min (Fig. 3(e),

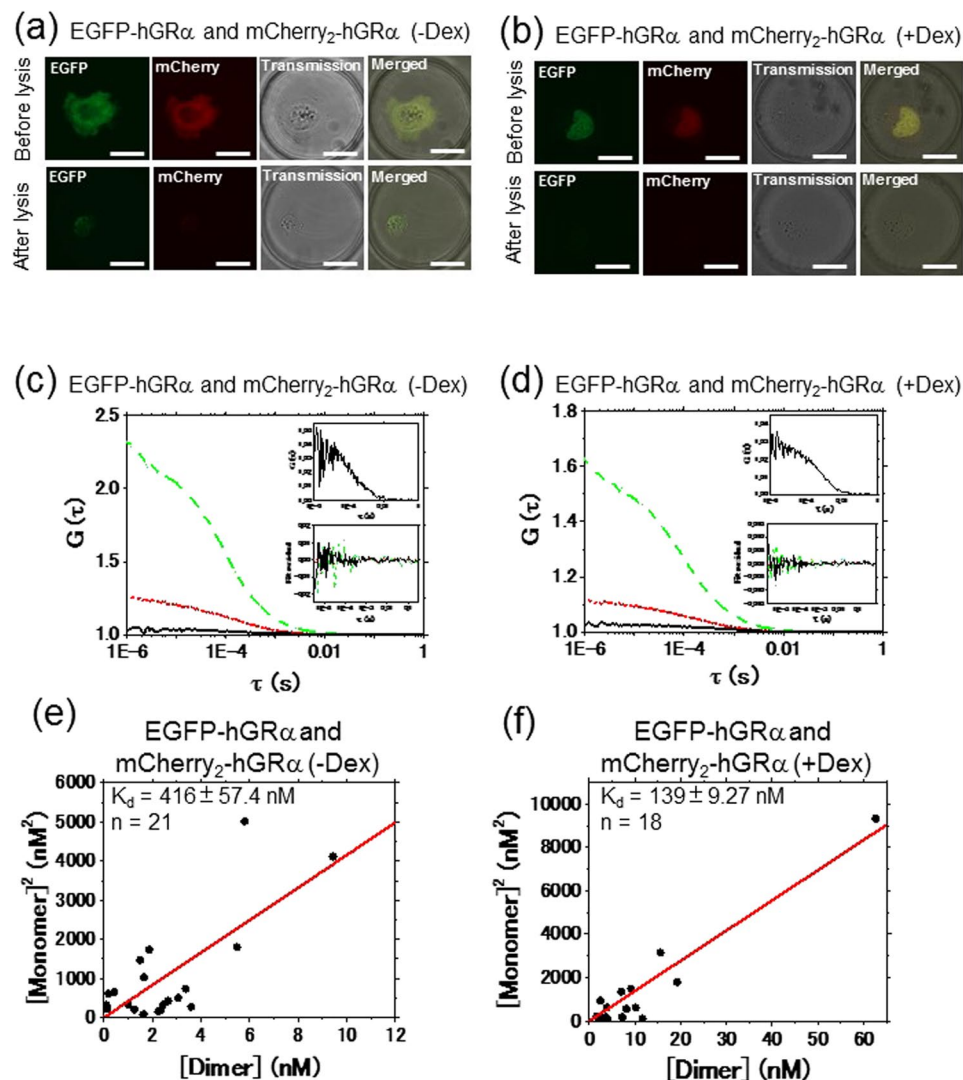


Figure 1. *In vitro* K_d analysis of EGFP-GR α and mCherry₂-GR α using the FCCS-microwell system. Typical auto- and cross-correlation curves constructed by measurements in microwells after lysis of U2OS cells coexpressing EGFP-hGR α and mCherry₂-hGR α in the absence or presence of Dex. The green dashed line, red dotted line, and black solid line denote the autocorrelation of the green channel [$G_G(\tau)$], autocorrelation of the red channel [$G_R(\tau)$], and cross-correlation [$G_C(\tau)$], respectively. The insets show an enlarged graph of the cross-correlation curve and fitting residuals of autocorrelation and cross-correlation curves. LSM images of U2OS cells coexpressing EGFP-hGR α and mCherry₂-hGR α before and after cell lysis in the absence (a) and presence (b) of Dex. The scale bar is 20 μ m. FCCS was performed in a microwell after cell lysis in the absence (c) and presence (d) of Dex. (e, f) Results of K_d determination using a scatter plot and linear regression. The plots represent the square of the concentration of the monomeric hGR α versus the concentration of the dimer of hGR α . The solid red line shows the linear fit. The slope indicates K_d . (e) mCherry₂-hGR α and EGFP-hGR α in the absence of Dex. (f) mCherry₂-hGR α and EGFP-hGR α in the presence of Dex.

inset). As in another report^{40,41}, the transcription-regulatory activity of GR α was retained after tagging with such fluorescent proteins.

Typical autocorrelation and cross-correlation curves of FCCS conducted in the cytoplasm and nucleus are shown in Fig. 3. As a negative control, U2OS cells were cotransfected independently with mCherry₂- and EGFP-encoding plasmids, and FCCS was carried out in the absence (Fig. 3(a)) and presence of Dex (Fig. 3(b)). The cross-correlation amplitude was not observed in either case, pointing to no unknown interaction between EGFP and mCherry₂. As a positive control, U2OS cells were cotransfected with p50-mCherry₂/nuclear localization signal (NLS)-encoding and p50-EGFP/NLS-encoding plasmids (Fig. S1M and N), which are coexpressed in the nucleus as the proteins of interest (Fig. 3(c), inset). The p50 protein is a subunit of NF- κ B, and proteins of this family associate as a homo- (p50-p50) and heterodimer (p50-p65). Endogenous expression of p50 was not detected in U2OS cells (Figs S2B and S15B). A high cross-correlation amplitude was observed (Fig. 3(c)) between the p50-mCherry₂/NLS and p50-EGFP/NLS in FCCS measurement. Low cross-correlation amplitude of mCherry₂-hGR α and EGFP-hGR α was observed in the absence of Dex (Fig. 3(d)) in the cytoplasm. In contrast, a

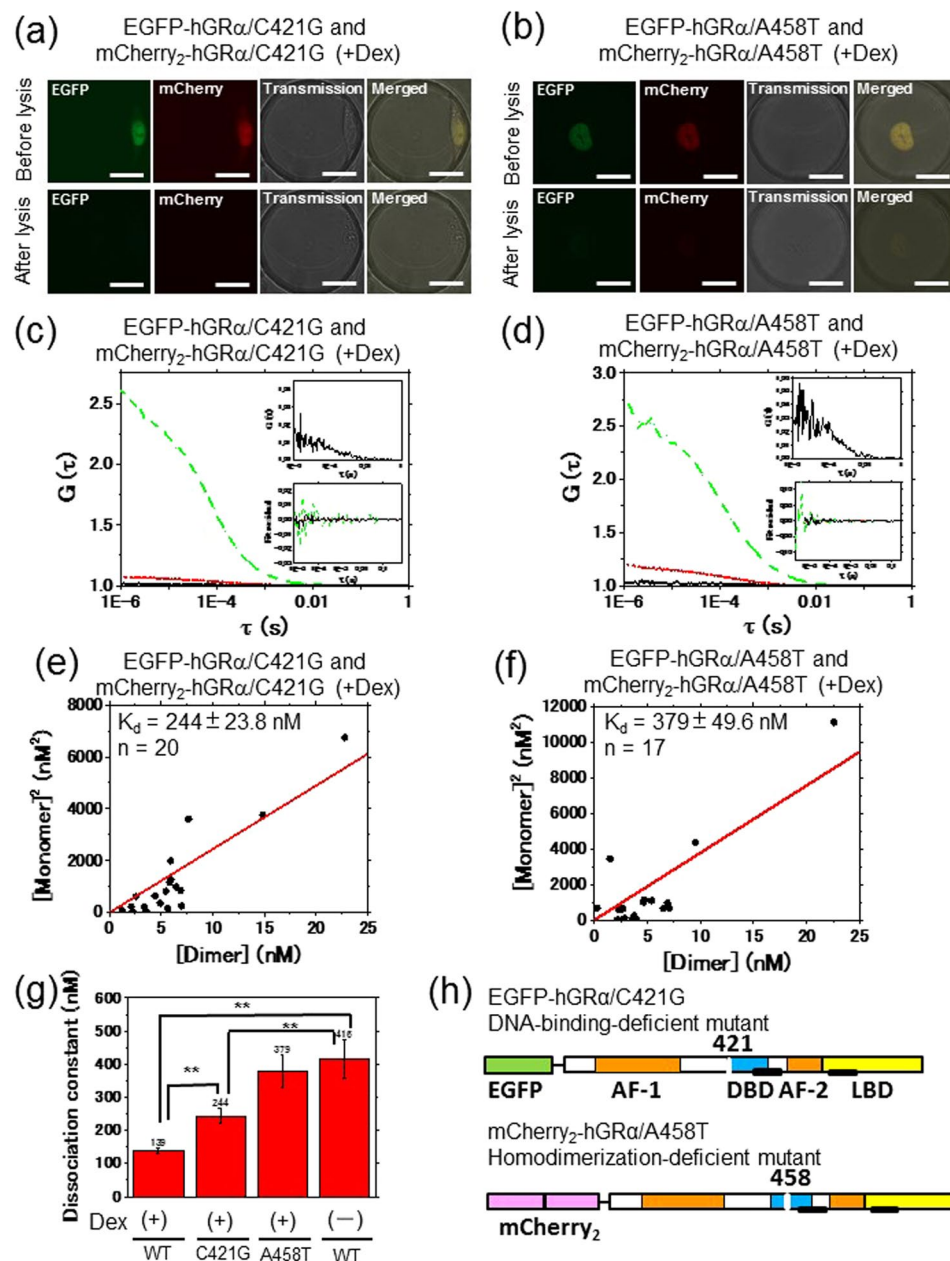


Figure 2. Determination of *in vitro* K_d of GR α mutants using the FCCS-microwell system. Typical auto- and cross-correlation curves constructed by measurements in microwells after lysis of U2OS cells coexpressing EGFP-hGR α mutants and mCherry₂-hGR α mutants in the presence of Dex. The green dashed line, red dotted line, and black solid line denote the autocorrelation of the green channel [$G_G(\tau)$], autocorrelation of the red channel [$G_R(\tau)$], and cross-correlation [$G_C(\tau)$], respectively. The insets show an enlarged graph of the cross-correlation curve and fitting residuals of autocorrelation and cross-correlation curves. LSM images of U2OS cells coexpressing EGFP-hGR α mutants and mCherry₂-hGR α mutants before and after cell lysis in a microwell for the C421G mutant (**a**) and A458T mutant (**b**). The scale bar is 20 μ m. FCCS was performed in a microwell after cell lysis for the C421G mutant (**c**) and A458T mutant (**d**) in the presence of Dex. (**e**, **f**) Results of K_d determination using a scatter plot and linear regression. The plots represent the square of the concentration of the monomeric hGR α versus the concentration of the dimer of hGR α . The solid red line shows the linear fit. The slope indicates K_d . (**e**) The C421G mutant in the presence of Dex. (**f**) The A458T mutant in the presence of Dex. (**g**) A summary of *in vitro* K_d values determined using the FCCS-microwell system. WT: wild type, C421G: the C421G mutant, A458T: the A458T mutant. Statistical analysis was based on ANOVA (** $p < 0.01$). (**h**) A schematic diagram of mCherry₂- and EGFP-fused constructs of mutated hGR, C421G (DNA-binding-deficient mutant) and A458T (homodimerization-deficient mutant).

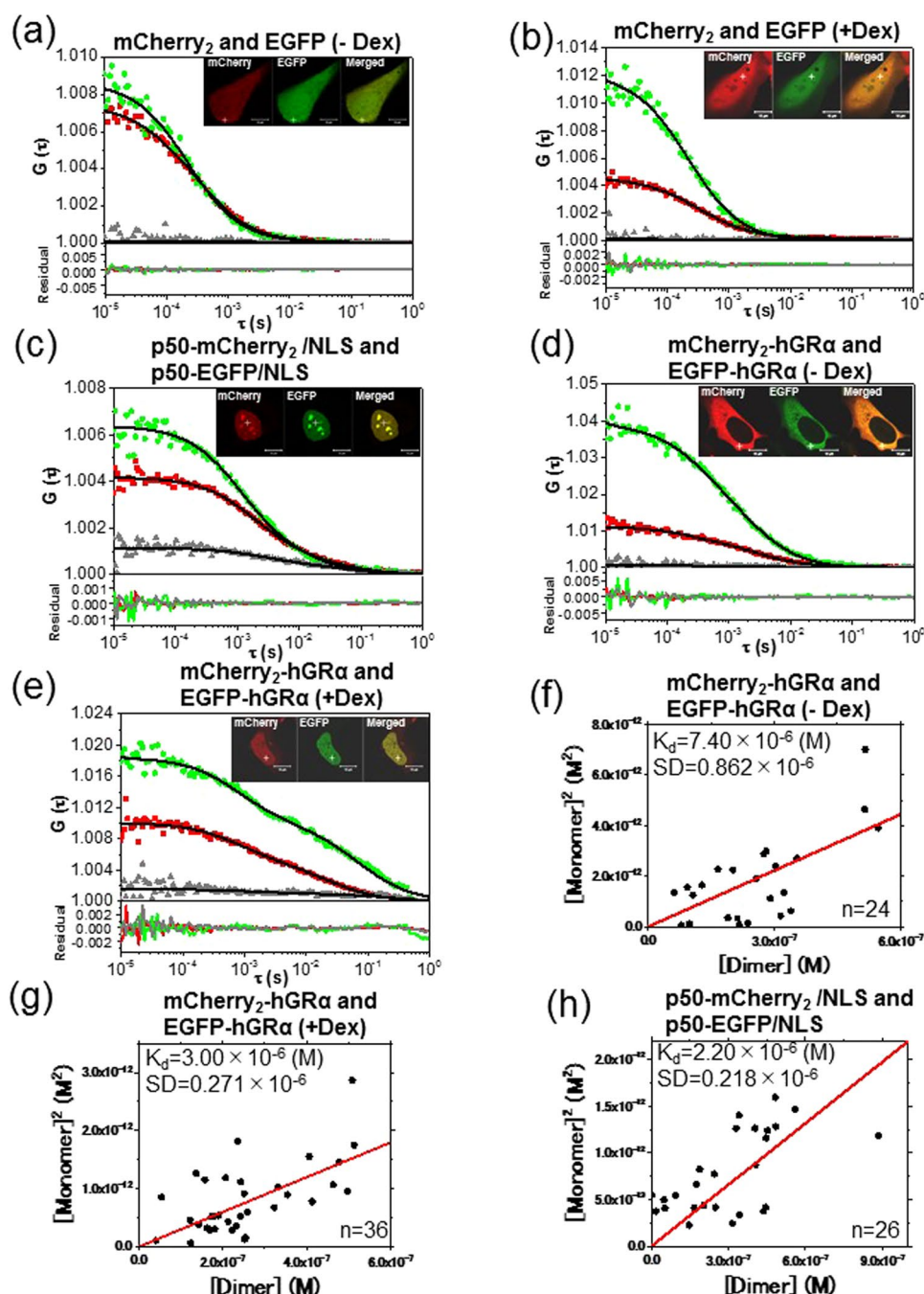


Figure 3. FCCS and K_d analysis of mCherry₂-GR α and EGFP-GR α . Typical auto- and cross-correlation curves constructed by measurements in U2OS cells coexpressing the pairs of chimeric fusion proteins before and after addition of the ligand. The filled green diamonds, red squares, and gray triangles denote the autocorrelation of the green channel [$G_G(\tau)$], autocorrelation of the red channel [$G_R(\tau)$], and the cross-correlation curve [$G_C(\tau)$], respectively, with their fits (solid black line) and residuals. The insets show LSM images of U2OS cells coexpressing the pairs of chimeric fusion proteins. Measurement positions of FCCS are indicated by the white crosshairs. The scale bars are 10 μ m. FCCS was performed in U2OS cells expressing mCherry₂ and EGFP as a negative control (a) before and (b) after addition of Dex, showing a flat cross-correlation amplitude. (c) A U2OS cell coexpressing p50-mCherry₂/NLS and p50-EGFP/NLS as a positive control. (d) A U2OS cell coexpressing mCherry₂/hGR α and EGFP/hGR α in the cytoplasm before addition of Dex. (e) A U2OS cell coexpressing mCherry₂/hGR α and EGFP/hGR α in the nucleus 20 min after addition of 100 nM Dex. (f,g,h) Results of K_d determination using a scatter plot and linear regression. The plots represent the square of the concentration of the monomeric hGR α versus the concentration of the dimer of hGR α . The solid lines show the linear fit. The slope indicates K_d . (f) mCherry₂-hGR α and EGFP-hGR α before addition of Dex. (g) mCherry₂-hGR α and EGFP-hGR α after addition of Dex. (h) p50-mCherry₂/NLS and p50-EGFP/NLS.

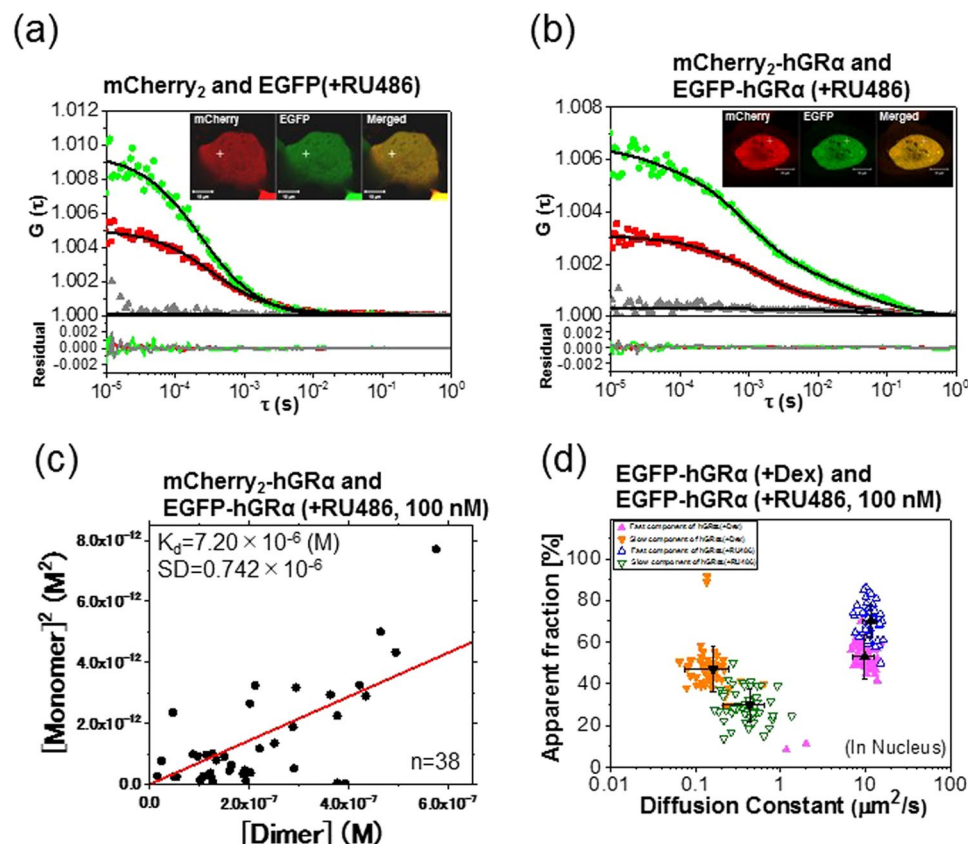


Figure 4. The effect of RU486 on GR α analyzed by FCCS. Typical auto- and cross-correlation curves obtained from U2OS cells coexpressing the pairs of chimeric fusion proteins before and after addition of the ligand. The filled green diamonds, red squares, and gray triangles denote autocorrelation of the green channel [$G_G(\tau)$], autocorrelation of the red channel [$G_R(\tau)$], and the cross-correlation curve [$G_C(\tau)$], respectively, with their fits (solid black lines) and residuals. The insets show LSM images of U2OS cells coexpressing the pairs of chimeric fusion proteins. Measurement positions of FCCS are indicated by the white crosshairs. The scale bars are 10 μ m. FCCS was performed in U2OS cells expressing (a) mCherry₂ and EGFP as a negative control, after addition of RU486 and showing a flat cross-correlation amplitude; (b) U2OS cells coexpressing mCherry₂-hGR α and EGFP-hGR α in the nucleus 20 min after addition of 100 nM RU486. (c) The K_d plot represents the square of the concentrations of the monomeric hGR α versus the concentration of the dimer of hGR α after addition of RU486. The solid line shows the linear fit. (d) The scatter plots represent the diffusion constants versus their fractions from fitting analysis of FCCS data with a two-component model. Black symbols indicate the average of the diffusion constants of the fast and slow components. The data are presented as mean $\pm$ SD. Fast and slow components are shown with different colors and symbols. EGFP-hGR α after addition of Dex (filled symbols) and RU486 (open symbols).

cross-correlation amplitude was observed in the presence of Dex (Fig. 3(e)) in the nucleus. For quantitative analysis, K_d values of the GR α homodimerization in U2OS cells were computed in the absence and presence of Dex. The RCA of the interaction between EGFP-hGR α and mCherry₂-hGR α in the living cells show similar tendencies against concentration ratio of mCherry₂-hGR α and EGFP-hGR α (Fig. S5A and B), and was significantly higher than that of the coexpression of EGFP and mCherry₂, indicating that FCCS could detect the GR α homodimerization in the living cells as well as *in vitro* (Fig. S5C). K_d of p50-mCherry₂/NLS and p50-EGFP/NLS in the nucleus was found to be 2.20 μ M (Fig. 3(h)). This result was consistent with another report on the micromolar range of K_d for p50 homodimerization *in vitro*⁴². K_d values of mCherry₂-hGR α and EGFP-hGR α were found to be 7.40 μ M in the absence of Dex in the cytoplasm (Fig. 3(f)) and 3.00 μ M in the presence of Dex in the nucleus (Fig. 3(g)). K_d was significantly ($p < 0.01$) lower in the presence of Dex than in its absence. These quantitative results suggested that WT GR α has a tendency toward homodimerization in the presence of Dex and toward monomerization in the absence of Dex.

To create a model of inhibition of GR α homodimerization, cells coexpressing mCherry₂-hGR α and EGFP-hGR α were incubated with RU486 (mifepristone), which is an inhibitor of transcription-regulatory activity^{43,44}. In the inset of Fig. 4(b), mCherry₂-hGR α and EGFP-hGR α relocated to the nucleus after the addition of RU486, as with Dex. A negligible cross-correlation amplitude was occasionally observed (Fig. 4(b)), but the obtained K_d value was 7.20 μ M (Fig. 4(c)), which was the same as that of the WT in the absence of Dex (7.40 μ M) in the cytoplasm, suggesting that WT GR α had a tendency toward the monomer form during treatment with

RU486. However, at the high concentration (1 μM) of RU486, the homodimerization of GR α in the presence of RU486 has been reported after a number and brightness analysis *in vivo*²². K_d value was also determined at 1 μM RU486 treatment. Low cross-correlation amplitude was observed (Fig. S6A) and K_d value was 8.59 μM (Fig. S6B), which was similar to that in the presence of 100 nM RU486. The diffusion constant of EGFP-hGR α was determined by the autocorrelation function fitted to the two-component model, which also provided the dynamic properties of GR α before and after the addition of Dex. Figure S7 shows scatter plots of diffusion constants versus apparent fraction percentages of EGFP-hGR α in the absence and presence of Dex. The diffusion constant of the slow component decreased in the presence of Dex compared with its absence (Fig. S7). Overall, these results indicated a slowdown of GR α mobility in the presence of Dex for the complex formation with associated molecules and for interaction with DNA. Furthermore, the effect of the antagonist (RU486) on the diffusion of GR α was assessed in FCCS experiments (Fig. 4(d)). The diffusion constant of the fast component of WT GR α was not affected by the presence of RU486, in contrast to Dex. In addition, the diffusion constant of the slow component in the presence of RU486 became larger than that during Dex treatment, suggesting that the molecule became fast moving (Fig. 4(d), downward triangle). These results indicated dissociation of the initial complex and/or an unstable complex formation of GR α with the GRE in the presence of RU486, in agreement with our previous report².

FCCS analysis of the mutants of hGR α in the nucleus. To confirm the homodimerization of GR α in living cells, U2OS cells were transiently cotransfected with mCherry₂-hGR α /C421G and EGFP-hGR α /C421G (Fig. S1C and D), mCherry₂-hGR α /A458T and EGFP-hGR α /A458T (Fig. S1E and F), and with mCherry₂-hGR α /C421G-A458T and EGFP-hGR α /C421G-A458T (Fig. S1G and H). According to the insets of Fig. 5(a), (b), and (c), C421G, A458T, and C421G-A458T were translocated to the nucleus after the addition of Dex as WT GR α . It should be noted that there was no difference in the static laser scanning microscopy (LSM) imaging method between the WT and mutants. The cross-correlation amplitude was observed in the C421G mutant (Fig. 5(a)) and A458T mutant (Fig. 5(b)) after the addition of Dex. In contrast, the C421G-A458T (Fig. 5(c)) mutant showed low cross-correlation amplitude in the nucleus after the addition of Dex. K_d values of mCherry₂- and EGFP-fused C421G, A458T, and C421G-A458T were calculated from each slope: 3.51, 6.11, and 5.84 μM , respectively (Fig. 5(d)–(f)). These results suggested that the tendencies of the A458T and C421G-A458T mutants toward a monomer form were stronger than that of the WT (3.00 μM). In contrast, the tendency of C421G to homodimerization was similar to that of the WT. The scaffold effect of DNA for GR homodimerization was not significantly observed *in vivo*, which was observed *in vitro* experiments. Next, the diffusion properties of GR α mutants were analyzed in the nucleus of a live cell. The autocorrelation functions of EGFP-hGR α /C421G, EGFP-hGR α /A458T, and EGFP-hGR α /C421G-A458T mutants in the nucleus were analyzed by two-component fitting. Comparative analysis of the diffusion constants of WT GR α and its mutants are shown in scatter plots in Fig. S8. The fast component of each mutant was not affected by the addition of Dex. In contrast, the diffusion constants of the slow component increased after the addition of Dex, in comparison with the WT (Fig. S8).

FCCS analysis of NLS region-mutated hGR α in the cytoplasm. To test whether the GR α homodimerizes in the cytoplasm, we constructed mCherry₂- and EGFP-fused nuclear localization signal 1-mutated (ΔNLS) hGR α that did not relocate to the nucleus, and A458T- ΔNLS mutants that neither formed homodimers nor relocated to the nucleus. U2OS cells were transiently cotransfected with plasmids encoding mCherry₂-hGR α / ΔNLS and EGFP-hGR α / ΔNLS (Fig. S1I and J) and mCherry₂-hGR α /A458T- ΔNLS and EGFP-hGR α /A458T- ΔNLS (Fig. S1K and L). As a positive control, FCCS was conducted on p50-mCherry₂ and p50-EGFP (Fig. S1O and P), which were coexpressed in the cytoplasm (Fig. 6(a), inset). K_d values were found to be 1.77 μM for p50-mCherry₂ and p50-EGFP in the cytoplasm (Fig. 6(e)). According to the insets of Fig. 6(c) and (d), the mCherry₂- and EGFP-fused ΔNLS mutant and the A458T- ΔNLS mutant were localized to the cytoplasm in the presence of Dex. FCCS was performed in the cytoplasm in the absence (Fig. 6(b)) and presence of Dex (Fig. 6(c)). Unexpectedly, a cross-correlation amplitude was observed in both Fig. 6(b) and Fig. 6(c). K_d values of mCherry₂-hGR α / ΔNLS and EGFP-hGR α / ΔNLS in the absence and presence of Dex were found to be 2.28 μM (Fig. 6(f)) and 2.19 μM (Fig. 6(g)), respectively. Our results suggested that in the condition without Dex stimulation, GR α / ΔNLS had a lesser tendency toward monomerization but also formed a homodimer in the cytoplasm, whereas the proportion of GR α homodimers tended to increase after the addition of Dex. To confirm the GR α homodimerization in the cytoplasm, FCCS of the homodimerization-deficient mutant, mCherry₂-hGR α /A458T- ΔNLS , and EGFP-hGR α /A458T- ΔNLS was performed in the presence of Dex (Fig. 6(d)). Very low cross-correlation amplitude was only observed. K_d was found to be 8.52 μM (Fig. 6(h)), which was higher than that of EGFP-hGR α / ΔNLS and mCherry₂-hGR α / ΔNLS in the absence and presence of Dex. Figure 7(a) and (b) show summaries of the obtained K_d values in living cells. This evidence pointed to the presence of the homodimer of GR α in the cytoplasm. The diffusion properties of these mutants were analyzed in the cytoplasm of living cells. Comparative analysis of the diffusion constants of the WT and mutants is shown in scatter plots in Fig. S9. In the cytoplasm, the fast and slow components of each mutant were not affected by the addition of Dex. This observation was suggestive of formation of the complex between the NLS-mutated GR α and other cytoplasmic proteins, same as WT GR α . Taken together, these results supported the hypothesis that GR α can homodimerize in the cytoplasm in the presence of Dex.

Discussion

As mentioned in the introduction, there are a number of controversial issues regarding homodimerization of GR α . Generally, steroid receptors regulate transcription via two main pathways. In the first one, two molecules of steroid receptors bind to DNA in a cooperative manner; thus, binding of the first molecule accelerates the binding of the second molecule sequentially, forming a homodimer via a dimerization interface (*monomer pathway*). In the other

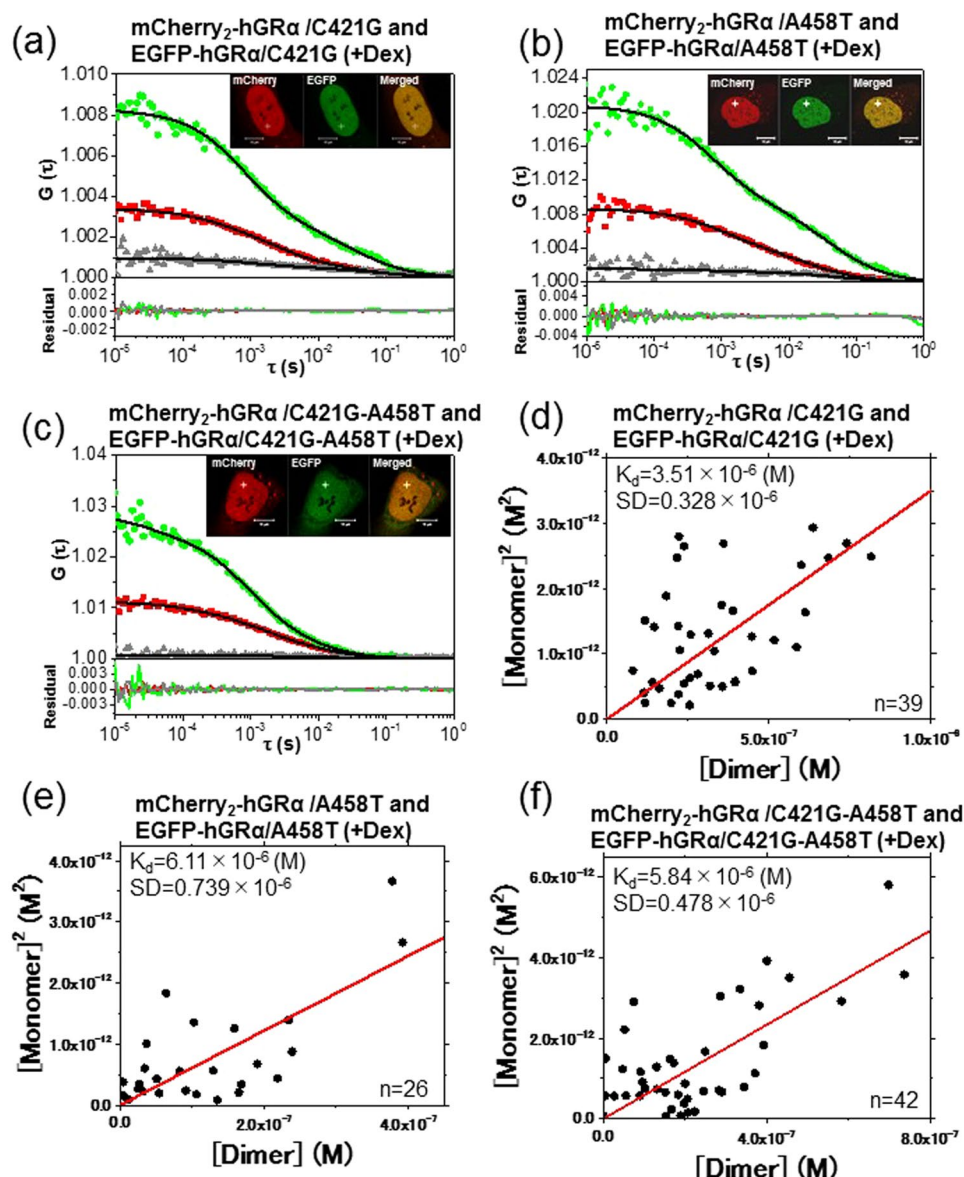


Figure 5. FCCS and K_d analysis of GR α mutants. Typical auto- and cross-correlation curves obtained from U2OS cells coexpressing the pairs of chimeric fusion proteins after addition of the ligand. The filled green diamonds, red squares, and gray triangles denote autocorrelation of the green channel [$G_G(\tau)$], autocorrelation of the red channel [$G_R(\tau)$], and cross-correlation curve [$G_C(\tau)$], respectively, with their fits (solid black lines) and residuals. The insets show LSM images of U2OS cells coexpressing the pairs of chimeric fusion proteins. FCCS analyses were performed in the nucleus, which is indicated by the white crosshairs. The scale bars are 10 μ m. FCCS was conducted on U2OS cells coexpressing (a) mCherry₂-hGR α /C421G and EGFP-hGR α /C421G, (b) mCherry₂-hGR α /A458T and EGFP-hGR α /A458T, or (c) mCherry₂-hGR α /C421G-A458T and EGFP-hGR α /C421G-A458T 20 min after addition of 100 nM Dex. (d,e,f) Results of K_d determination using scatter plots and linear regression. The plots represent the square of the concentration of the monomeric hGR α versus the concentrations of the dimer of hGR α . The solid lines show the linear fit. The slope indicates K_d . (d) mCherry₂-hGR α /C421G and EGFP-hGR α /C421G after addition of Dex. (e) mCherry₂-hGR α /A458T and EGFP-hGR α /A458T after addition of Dex. (f) mCherry₂-hGR α /C421G-A458T and EGFP-hGR α /C421G-A458T after addition of Dex.

pathway, preformed homodimers of steroid receptors bind to DNA (*dimer pathway*)^{22,45–48}. In the case of GR α , it is still a subject of debate when and where the homodimerization takes place and whether it proceeds through the monomer pathway^{10,15,16,49} or the dimer pathway^{11,17–21,50}. Our observations and other studies indicate that the transition time from the cytoplasm to the nucleus ranges from 10 to 60 min after the addition of Dex^{2,51,52}. We can hypothesize a dynamic monomer pathway where GR α is in equilibrium between monomeric and homodimeric forms in the cytoplasm as well as in the nucleus during this rather long transition time, and where

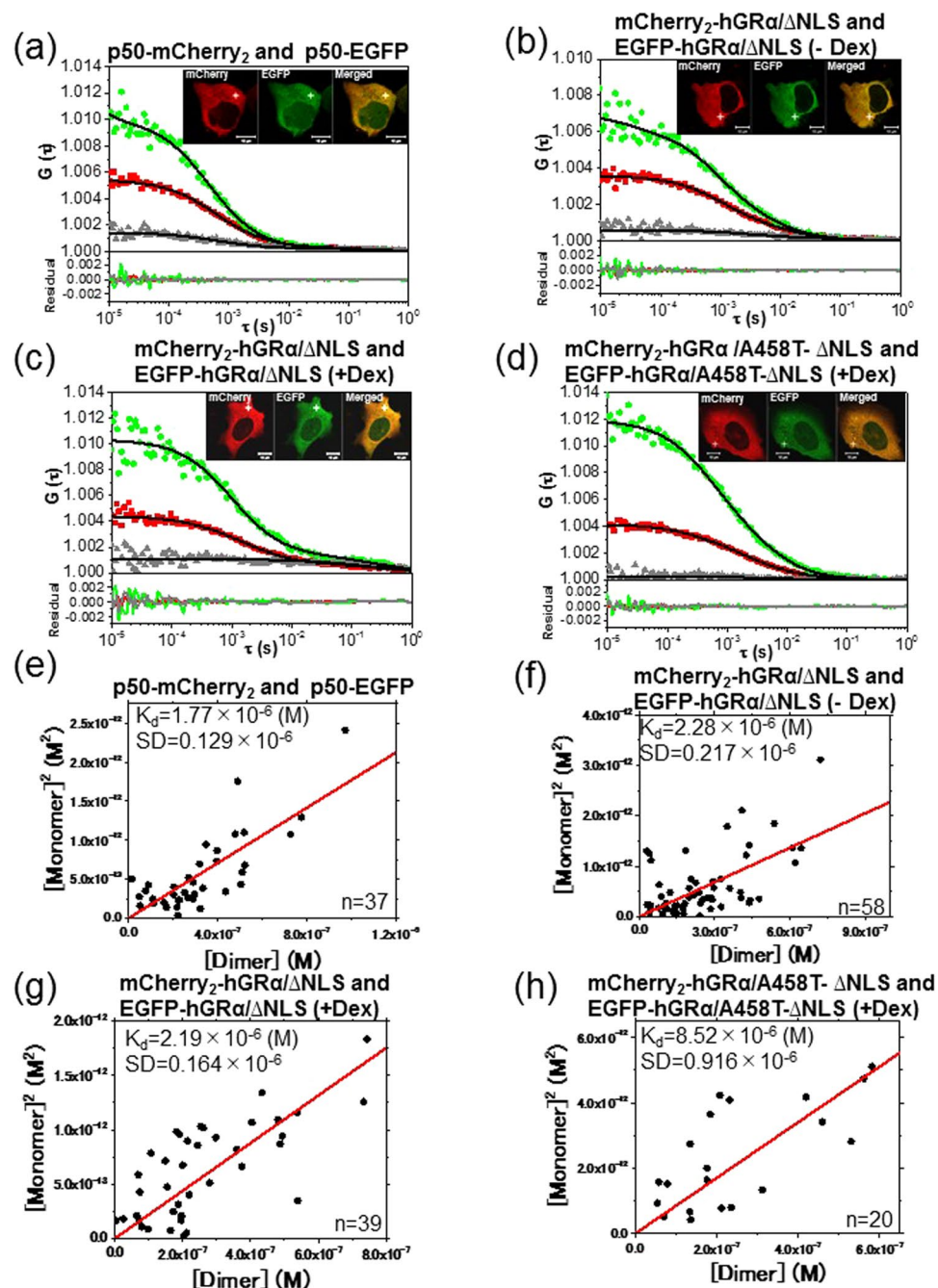


Figure 6. The effect of NLS mutation on formation of the GR α dimer. Typical auto- and cross-correlation curves obtained from U2OS cells coexpressing the pairs of chimeric fusion proteins before and after addition of the ligand. The filled green diamonds, red squares, and gray triangles denote autocorrelation of the green channel [$G_G(\tau)$], autocorrelation of the red channel [$G_R(\tau)$], and the cross-correlation curve [$G_C(\tau)$], respectively, with their fits (solid black lines) and residuals. The insets show LSM images of the U2OS cells coexpressing the pairs of chimeric fusion proteins. FCCS analyses were carried out in the cytoplasm, which is indicated by the white crosshairs. The scale bars are 10 μ m. FCCS was performed using U2OS cells coexpressing (a) p50-mCherry₂ and p50-EGFP as a positive control, (b) mCherry₂-hGR α /ΔNLS and EGFP-hGR α /ΔNLS before addition of Dex, (c) mCherry₂-hGR α /ΔNLS and EGFP-hGR α /ΔNLS 20 min after addition of 100 nM Dex, or (d) mCherry₂-hGR α /A458T-ΔNLS and EGFP-hGR α /A458T-ΔNLS 20 min after addition of 100 nM Dex. (e–h) Results of K_d determination using scatter plots and linear regression. The plots represent the square of the concentration of the monomeric hGR α versus the concentrations of hGR α dimer. The solid line shows the linear fit. The slope indicates the K_d . (e) p50-mCherry₂ and p50-EGFP. (f) mCherry₂-hGR α /ΔNLS and EGFP-hGR α /ΔNLS before addition of Dex. (g) mCherry₂-hGR α /ΔNLS and EGFP-hGR α /ΔNLS after addition of Dex. (h) mCherry₂-hGR α /A458T-ΔNLS and EGFP-hGR α /A458T-ΔNLS after addition of Dex.

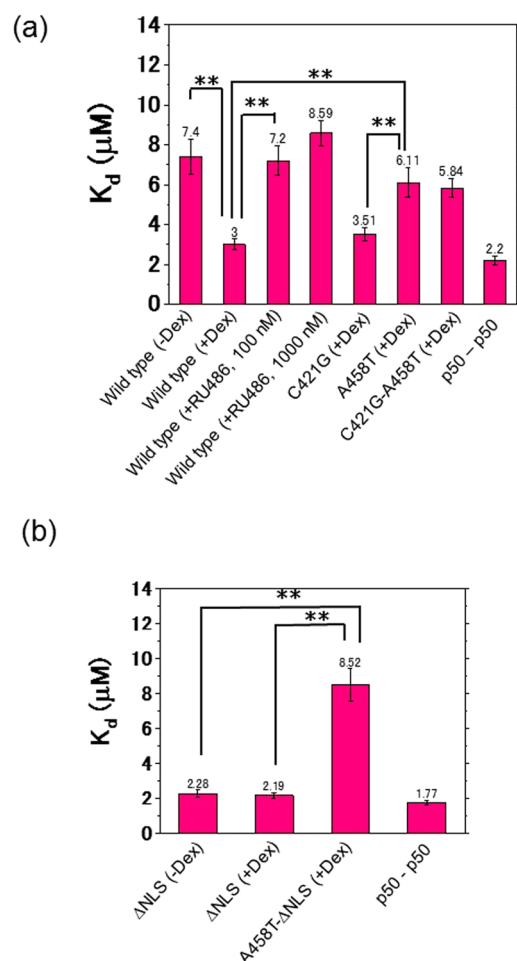


Figure 7. A summary of the dissociation constants (K_d) of the WT and mutants before and after addition of ligands. The bars indicate the dissociation constants, K_d . **(a)** Before addition of Dex, the dissociation constant (K_d) of the WT was 7.40 μM in the cytoplasm, and after addition of Dex the K_d values of the WT, C421G, A458T, and C421G-A458T were 3.00, 3.51, 6.11, and 5.84 μM , respectively, in the nucleus. K_d of the control p50-p50 in the nucleus was 2.20 μM . **(b)** Before addition of Dex, K_d of ΔNLS was 2.28 μM and after addition of Dex, K_d values of the ΔNLS and A458T- ΔNLS mutants in the cytoplasm were 2.19 and 8.52 μM , respectively. K_d of the p50-p50 dimer in the cytoplasm was 1.77 μM . The data are presented as mean $\pm$ SD. Statistical analysis was based on ANOVA (* $p < 0.05$, ** $p < 0.01$).

GR α relocates to the nucleus as a monomer and forms the GR α homodimer before DNA binding in the nucleus (Fig. 8).

K_d of homodimerization of the WT and mutants of GR α *in vitro* was confirmed using the FCCS-microwell system. The *in vitro* K_d of WT GR α was determined to be 416 and 139 nM in the absence and presence of Dex, respectively (Fig. 1(e) and (f)). This result is in good agreement with K_d obtained in the brightness analysis using the FCS-microwell system¹⁴. Moreover, K_d for homodimerization of the C421G mutant and A458T mutant *in vitro* was 244 and 379 nM, respectively, in the presence of Dex (Fig. 2(e) and (f)). The tendencies of K_d values of the WT relative to the mutants *in vitro* were similar to those in living cells (Figs 2(g) and 7(a)). On the other hand, the absolute values of K_d *in vitro* were lower than those in living cells. This finding suggests that there are some mechanisms that keep the monomer form of hGR α in living cells.

Next, K_d values of hGR α homodimerization were determined by FCCS in living cells. K_d of WT GR α was 7.40 μM in the cytoplasm in the absence of Dex (Fig. 3(f)) and 3.00 μM in the nucleus in the presence of Dex (Fig. 3(g)), indicating that GR α has a tendency toward homodimerization in the nucleus (Fig. 8(a), vii, viii). By contrast, K_d of the GR α homodimerization was in the nanomolar range *in vitro* (Fig. 2(g)). Our findings indicate that GR α forms a homodimer in the nucleus in the presence of Dex; however, judging by the rather high value of K_d (3.00 μM), GR α in equilibrium is distributed between the monomeric form and homodimeric form (Fig. 8(a), v–vii). The presence of both monomeric and homodimeric forms at the rather high value of K_d than expression level of hGR α in the living cells (300 nM to 2300 nM for WT hGR α) may enable formation of complex with other nuclear receptors such as mineralocorticoid receptor. K_d of the A458T mutant (homodimerization-deficient mutant³⁹) was 6.11 μM in the presence of Dex (Fig. 5(e)), which is higher than that of WT GR α in the presence of Dex (3.00 μM) and lower than that of WT GR α in the absence of Dex (7.40 μM). The tendency of the A458T

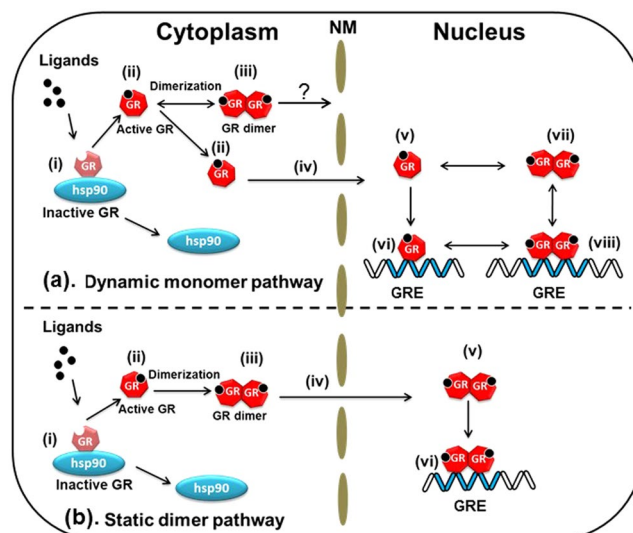


Figure 8. The proposed model for the pathways of glucocorticoid receptors. (a) The dynamic monomer pathway: (i) hGR α is localized to the cytoplasm as a complex or in free form in the uninduced state. (ii) hGR α is activated after ligand binding. Activated hGR α is in equilibrium between a monomer and dimer (iii) but transport of dimeric hGR α is unclear. (iv) Activated monomer hGR α relocates into the nucleus and is in both the free state (v) and monomer form, which can bind to a GRE as an unstable complex (vi). (vii) hGR α further dimerizes in the nucleus. (viii) The preformed dimer of hGR α associates with the GRE and other transcription factors. The dimer and monomer are not only distributed in the cytoplasm but also in the nucleus even after ligand binding; however, transport of hGR α is carried out in the monomeric form of hGR α . The concentration of hGR α in the nucleus can be controlled by changing the K_d of hGR α and GRE in the nucleus. (b) The static dimer pathway: (i) hGR α is localized to the cytoplasm as a complex or in free form in the uninduced state. (ii) hGR α is activated after ligand binding. Activated hGR α exists in the cytoplasm as a dimer (iii). (iv) In the dimer form, hGR α is translocated. The preformed dimer (v) of hGR α associates with a GRE and transcription factors (vi). The dimer of hGR α is distributed in both the cytoplasm and nucleus, but the monomer is found only in the cytoplasm. hGR α is transported in the dimer form. The concentration of hGR α in the nucleus can be controlled by the activity and functions of the NPC. hGR α : human glucocorticoid receptor α , hsp90: heat shock protein 90, GREs: glucocorticoid response elements, NM: nuclear membrane, NPC: nuclear pore complex

mutant toward the monomer was stronger than that of WT GR α in the presence of Dex (Fig. 7(a)). These results indicate that the A458T mutation in GR α impairs the homodimerization in living cells, but some part of the A458T mutant forms a homodimer. This finding is consistent with the literature data, which suggest that the A458T mutant can form a homodimer⁵³.

In the present study, effects of RU486 on the process of hGR α homodimerization were also examined. It has been demonstrated that RU486 works as an antagonist of transcription-regulatory activity^{43,44}, but some studies revealed its partial agonist behavior for transcription of GR α ^{22,54–56}. K_d of WT GR α was 7.20 μ M and 8.59 μ M in the presence of 100 nM and 1000 nM RU486, respectively (Figs 4(c) and S6); these values are the similar to that of the WT in the cytoplasm in the absence of Dex (7.40 μ M). This observation suggests that a lack of homodimer of GR α inhibits the transcriptional-regulatory activity. In contrast, homodimerization of GR α in the presence of RU486 has been reported after a number and brightness analysis *in vivo*²². This discrepancy is unclear, but may be due to the different cell line with the expression of GR α in the living cells.

Our results also answered the question whether binding of GR α to DNA is necessary for homodimerization. The C421G mutant, which cannot associate with a GRE, does not have a transcription-regulatory activity³⁸. K_d of the C421G-A458T double mutant of GR α was 5.84 μ M in the presence of Dex (Fig. 5(f)); this value was higher than that of the C421G mutant but was the same as that of A458T mutant in the presence of Dex. Some studies suggested that GR α homodimerizes only after DNA binding^{10,16,49}. In contrast, earlier studies had revealed GR α homodimerization in solution, independently of DNA binding^{14,23}. Our results support GR α homodimerization before DNA binding (Fig. 8(a), vii) according to FCCS measurement of the DNA-binding-deficient mutant (C421G) and a double mutant with DNA-binding and dimerization deficiencies (C421G-A458T; Fig. 7(a)). To reconcile these discrepancies, however, a more dynamic view of hGR α is needed.

K_d of the A458T mutant and C421G-A458T mutant was higher than that of C421G and lower than those of the WT (without Dex) (Fig. 7(a)). Thus, these results suggest that C421G-A458T and A458T has a tendency to be in the monomeric form but also in the homodimeric form. Moreover, different diffusion properties were observed between A458T and C421G-A458T (Fig. S8E). The diffusion constant of the slow component of the C421G-A458T mutant was greater (faster) than that of A458T in the presence of Dex (Fig. S8E). This finding suggests that C421G-A458T cannot bind to a GRE but A458T can do so as a monomer (Fig. 8(a), v $\rightarrow$ vi). This result seems to support the finding that monomers of WT GR α and of the A458T mutant have a weak

transcription-regulatory activity in a reporter assay involving a palindromic GRE sequence⁵⁷. These data suggest that an initial and/or unstable complex of the A458T GR α mutant with the GRE forms in the presence of Dex (Fig. 8(a), v→vi), in line with our previous reports^{2,51}.

To test whether GR α homodimerizes in the cytoplasm, the K_d values of Δ NLS and A458T- Δ NLS mutants of GR α in the presence of Dex were determined by FCCS. These mutants were expected to be incapable of translocation to the nucleus or formation of homodimers in the presence of Dex. We found the K_d values of Δ NLS to be 2.28 μ M in the absence of Dex (Fig. 6(f)) and 2.19 μ M in its presence (Fig. 6(g)). These data are suggestive of the presence of a preformed homodimer in the unliganded state in the cytoplasm because K_d was lower than that of the WT GR α in the absence of Dex. It is possible that the mutation of NLS elicits a conformational change and/or a big change in electrostatic properties of the GR α moieties that facilitates formation of homodimers. In contrast, K_d of the A458T- Δ NLS mutant was estimated to be 8.52 μ M (Fig. 6(h)). These findings reinforce the idea that cytoplasmic homodimerization of GR α takes place at the initial stage of stimulation (Fig. 8(a), ii→iii) in agreement with other studies^{17,23}. As expected, the tendency of the A458T- Δ NLS mutant toward the monomeric state is stronger than that of the Δ NLS mutant after the addition of Dex. However, the results do not support the notion that GR α relocates into the nucleus in homodimeric form because K_d is still in the micromolar range, i.e., much higher than the *in vitro* K_d values. It can thus be reasonably assumed that the K_d value should be in the nanomolar concentration range if all of GR α form a homodimer after ligand binding. Therefore, our results do not support the translocation of GR α from the cytoplasm to the nucleus as a homodimer (Fig. 8(a), iii and iv) although a recent study showed the translocation of GR α in the homodimer form¹⁷. Further experiments, such as single-molecule tracking or multipoint FCCS need to be carried out to uncover the details of quaternary structure during the transport through the nuclear pore; we would like to do these experiments in a future study.

There may be a static dimer pathway, where GR α is transported from the cytoplasm to the nucleus in the homodimeric form. Thus, the concentration of GR α in the nucleus can be controlled by activity of the nuclear pore complex (Fig. 8(b), NPC). In contrast, our results support the existence of a dynamic monomer pathway, in which the concentration of GR α in the nucleus can be controlled by changes in the binding affinity between GR α and a GRE (Fig. 8(a)). Our findings appear to be substantiated by a report on a mineralocorticoid receptor (MR) indicating that only homodimers that form in the nucleus (after activation by ligand binding) can be transcriptionally active, whereas homodimers in the cytoplasm do not have the ability to relocate to the nucleus or regulate gene expression⁴⁵.

In conclusion, our quantitative data show homodimerization of hGR α in the nucleus and cytoplasm of living cells. To our knowledge, this is the first report of quantitative differences between homodimerization of WT GR α and homodimerization of its mutants on the basis of dissociation constants. The evidence obtained in this study suggests that DNA binding is not necessary for GR α homodimerization in the nucleus *in vivo*. Our findings should advance the understanding of the homodimerization, DNA binding, and dynamics of GR α in living cells.

Materials and Methods

Chemicals and antibodies. Dexamethasone (Dex) and RU486 were purchased from Sigma-Aldrich. McCoy's 5A modified medium and charcoal-stripped fetal bovine serum were purchased from GIBCO (Invitrogen). A mouse monoclonal anti-GR antibody (ab9568) was acquired from Abcam; a monoclonal anti-GFP (mouse IgG1-K) antibody (GF200) from Nacalai Tesque, Inc.; anti-actin clone C4 (mouse monoclonal) antibody from Millipore; an anti-NF- κ Bp50 (D-6) sc-166588 mouse monoclonal IgG1 antibody from Santa Cruz Biotechnology, and the alkaline phosphatase-conjugated anti-mouse antibody was purchased from Biosource™.

Plasmids. All schematic representations of the plasmids are shown in Fig. S1. The plasmids encoding human glucocorticoid receptor α (hGR α) fused with EGFP, its mutants A458T and C421G were described elsewhere². The pEGFP-hGR α /C421G-A458T was constructed by insertion of the fragment amplified from DNA with the A458T mutation² into pEGFP-hGR α /C421G as a vector with restriction enzymes Esp3I and ClaI. For Δ NLS mutation (K494A, K495A, and K496A), a first-step PCR was performed using the following primers:

Forward-1: 5'-gggtcccaggtaaagagacgaa-3' and
Reverse-1: 5'-ccttttatggcggtgttttcgagcttc-3' and
Forward-2: 5'-cgaaaaacagccgccataaaaggaattcag-3' and
Reverse-2: 5'-agaacatccaggagtactgcagtaggg-3'

with pEGFP-hGR α and pEGFP-hGR α /A458T as a template. Then the first-step PCR products were mixed as a template, and second-step PCR was performed with the above forward-1 and reverse-2 primers. The second-step PCR product was digested with Esp3I and PstI, and ligated into pEGFP-hGR α as a vector that was digested with the same restriction enzymes.

To construct the mCherry tandem dimer (mCherry₂)-fused hGR α , the fragment encoding EGFP was swapped for the fragment encoding mCherry₂ by digestion with AgeI and Bsp1407I and ligation with the "Mighty mix" DNA ligation kit (Takara, Japan). To construct the mCherry₂-fused hGR α mutants (C421G, A458T, C421G-A458T, Δ NLS, and A458T- Δ NLS), the hGR α in pmCherry₂-hGR α was swapped for the hGR α containing each mutation with ScaI and Bsp1407I and ligation with the "Mighty mix" DNA ligation kit.

As a positive control, we used the well-known p50 protein, a subunit of NF- κ B. We constructed a plasmid encoding the IPT (immunoglobulin-like plexin transcription factor) domain of p50 fused with the N terminus of mCherry₂ or EGFP (Fig. S10). For localization of p50 to the nucleus, p50-mCherry₂/NLS and p50-EGFP/NLS were constructed. The SV40 large T antigen NLS (Pro-Lys-Lys-Lys-Arg-Lys-Gly) fused with the C-terminal mCherry₂ or EGFP and the p50 fragment flanked by NheI and AgeI sites were inserted into the N-terminal

pmCherry₂/NLS or pEGFP/NLS, then digested at the same restriction sites. As a negative control, we used a plasmid encoding mCherry₂ and EGFP.

Cell culture and transient transfection. U2OS cells were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C in McCoy's 5A modified medium supplemented with 10% charcoal-stripped fetal bovine serum, 100 U/mL penicillin G and 100 µg/mL streptomycin. For FCCS, U2OS cells were plated on a Lab-Tek® 8-well chamber cover glass (Nunc™) and cotransfected with different fusion constructs where the ratio of the amounts of the two plasmids was kept at 2:1 (200 ng/well pmCherry₂-hGRα and 100 ng/well pEGFP-hGRα) using Optifect™ (Invitrogen). After 16 hrs of transfection, Dex or RU486 was added to each well at a final concentration of 100 nM with further incubation for 20 min at 37 °C.

Western blotting. One day before transient transfection, U2OS cells (10⁵/well) were seeded on a 6-well Nunclon™Δ chamber (Nalge Nunc International). Cells were transiently transfected with the transfection reagent (mock) alone or with 1 µg/well pEGFP-hGRα, its mutants, or p50-EGFP using Lipofectamine™ 2000. After 4 hrs of transfection, the medium was replaced with a fresh one. Twenty-four hours after transfection, cells were washed with ice-cold PBS, trypsinized, collected in PBS containing trypsin inhibitor 4-[2-aminoethyl]benzenesulfonyl fluoride hydrochloride (Sigma-Aldrich), and centrifuged. The cell pellets were lysed in CelLytic™ M lysis buffer (Sigma-Aldrich) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich). The homogenates were centrifuged (15000 rpm, 4 °C) for 10 min, and the cell lysates were collected. The lysates were solubilized with 2 × Laemmli sample buffer (Nacalai Tesque), heat denatured at 65 °C for 15 min, electrophoresed in a precast 7.5% polyacrylamide gel (ePAGEL, ATTO), and then transferred onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked overnight in 5% skim milk and washed three times in PBST buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4, 0.05% Tween 20) at room temperature and incubated with the primary antibodies: anti-GR, anti-GFP, anti-actin, and anti-NF-κB p50 (1:1000 dilution in “Can Get Signal” Solution I; TOYOBO) for 1 hr at room temperature. After three washes in PBST, the membranes were incubated with an alkaline phosphatase-conjugated anti-mouse IgG antibody (secondary antibody, 1:1000 dilution in “Can Get Signal” solution II; TOYOBO) for 1 hr at room temperature. Then, the membranes were washed three times with PBST and reacted with an alkaline phosphatase substrate (SIGMA FAST™ BCIP®/NBT) solution.

Microscopy and FCCS. Live-cell fluorescence imaging and FCCS measurements were performed by a LSM 510-ConfoCor3 (Carl Zeiss), which contained Ar⁺ laser and He-Ne laser, a water immersion objective (C-Apochromat, 40x, 1.2NA; Carl Zeiss), and two avalanche photodiodes. This setup was used both for FCCS and LSM imaging. The pinhole diameter was adjusted to 70 µm. EGFP and mCherry were excited by the 488-nm (15 µW) and 594-nm (8 µW) lasers, respectively. The emission signals were split by a dichroic mirror (600-nm beam splitter) and detected at 505–540 nm for EGFP and at 615–680 nm for mCherry. FCCS was performed 10 times with duration of 5 s before and 20 min after addition of the indicated ligands.

Data analysis. FCCS data were analyzed by AIM software (Carl Zeiss). The autocorrelation functions from the green and red channels, $G_G(\tau)$ and $G_R(\tau)$, and the cross-correlation function, $G_C(\tau)$, were computed as follows:

$$G_G(\tau) = 1 + \frac{\langle \delta I_G(t) \cdot \delta I_G(t + \tau) \rangle}{\langle I_G(t) \rangle \cdot \langle I_G(t) \rangle} \quad (1)$$

$$G_R(\tau) = 1 + \frac{\langle \delta I_R(t) \cdot \delta I_R(t + \tau) \rangle}{\langle I_R(t) \rangle \cdot \langle I_R(t) \rangle} \quad (2)$$

$$G_C(\tau) = 1 + \frac{\langle \delta I_G(t) \cdot \delta I_R(t + \tau) \rangle}{\langle I_G(t) \rangle \cdot \langle I_R(t) \rangle} \quad (3)$$

where τ denotes the delay time; I_G and I_R are the fluorescent intensity of the green and red channels, respectively; and $G_G(\tau)$, $G_R(\tau)$, and $G_C(\tau)$ denote the autocorrelation functions of green, red channels and cross-correlation function, respectively. The acquired auto- and cross-correlation functions were fitted to a two-component model⁵⁸:

$$G(\tau) = 1 + \frac{1 - F_{\text{triplet}} + F_{\text{triplet}} \exp(-\tau/\tau_{\text{triplet}})}{N(1 - F_{\text{triplet}})} \times \left(\left(\frac{F_{\text{fast}}}{1 + \tau/\tau_{\text{fast}}} \right) \sqrt{\frac{1}{1 + \tau/s^2\tau_{\text{fast}}}} + \left(\frac{F_{\text{slow}}}{1 + \tau/\tau_{\text{slow}}} \right) \sqrt{\frac{1}{1 + \tau/s^2\tau_{\text{slow}}}} \right) \quad (4)$$

where F_{triplet} is the average fraction of triplet state molecules, τ_{triplet} is the triplet relaxation time, F_{fast} and F_{slow} are the fractions of the fast and slow components, respectively, and τ_{fast} and τ_{slow} are the diffusion times of the fast and slow components, respectively. For cross-correlation fitting, the triplet was not used. N is the average number of fluorescent particles in the excitation-detection volume defined by ω_1 and ω_2 which are a radius of the short and long axis of the confocal volume, and s is the structural parameter representing the ratio $s = \omega_2/\omega_1$. The values of $\omega_{1,i}$ ($i = G$ or R) are calculated from the diffusion coefficients of rhodamine 6 G and Alexa 594 used as standard dyes, respectively.

$$\omega_{1,i} = \sqrt{4D \cdot \tau_{Di}} \quad (5)$$

The volume elements V are calculated according to

$$V_i = \pi^{3/2} \cdot \omega_{1,i}^2 \cdot \omega_{2,i} \quad (6)$$

$$V_C = \left(\frac{\pi}{2}\right)^{3/2} (\omega_{1,G}^2 + \omega_{1,R}^2)(\omega_{2,G}^2 + \omega_{2,R}^2)^{1/2} \quad (7)$$

The apparent total numbers of autocorrelation particles N_G and N_R and of complex cross-correlated particles N_C are given in the case which brightness of fluorescent protein is homogenous by

$$N_G = \frac{1}{G_G(0) - 1} \quad (8)$$

$$N_R = \frac{1}{G_R(0) - 1} \quad (9)$$

$$N_C = \frac{G_C(0) - 1}{(G_R(0) - 1) \cdot (G_G(0) - 1)} \quad (10)$$

When N_G and N_R are constant, $G_C(0)$ is directly proportional to N_C . The backgrounds of the resulting number of particles were corrected by subtracting autofluorescence intensity of mock-transfected U2OS cells, as follows⁵⁹:

$$N_{i,corrected} = N_{i,measured} \cdot \left[1 - \frac{I_{i,background}}{I_{i,measured}}\right]^2 \quad (11)$$

Then,

$$N_{C,corrected} = (G_C(0) - 1) \cdot N_{G,corrected} \cdot N_{R,corrected} \quad (12)$$

Diffusion constants of the samples were calculated from the ratio of the diffusion constant of rhodamine 6 G (D_{Rh6G} ; $414 \mu\text{m}^2/\text{s}$) and diffusion time τ_{R6G} and τ_{Sample} ⁶⁰.

The apparent concentration of each fluorescent protein was calculated with A (Avogadro's number) as shown below:

$$[C_{i,corrected}] = \frac{N_{i,corrected}}{V_i \cdot A} \quad (13)$$

$$[C_{C,corrected}] = \frac{N_{C,corrected}}{V_C \cdot A} \quad (14)$$

In actual measurement of EGFP-hGR α and mCh₂-hGR α , monomer, homo-color dimer and hetero-color dimer were present in the living cells and lysate. The particle brightness of homo-color dimer was twice higher than that of monomer and hetero-color dimer. The square of average brightness of monomer, hetero-color dimer and homo-color dimer was contributed to the amplitude of autocorrelation functions. Therefore, their concentrations were calculated using relative values of particle brightness of EGFP-hGR α and mCh₂-hGR α against EGFP and mCherry₂ co-expression sample (See supplemental information).

Determination of K_d . The dissociation constant K_d was determined using the following equations:

$$K_d = \frac{[M]^2}{[D]} = \frac{([G] + [R])^2}{[GG] + [RR] + [RG]} \quad (15)$$

$[M]$ and $[D]$ show the concentration of monomeric hGR α and dimeric hGR α , respectively. In the cells, EGFP-hGR α and mCh₂-hGR α were expressed. Therefore, $[M]$ and $[D]$ was transformed to $[G] + [R]$ and $[GG] + [RR] + [RG]$, respectively. G and R denotes the EGFP-hGR α and mCh₂-hGR α . The concentration of hetero-color dimer, $[RG]$ was calculated from the cross-correlation amplitude. Monomers and hetero-color dimers, $[G] + [RG]$ and $[R] + [RG]$ and homo-color dimers, $[GG]$ and $[RR]$ were calculated using relative values of particle brightness of EGFP-hGR α and mCh₂-hGR α against EGFP and mCherry₂ co-expression sample (See supplemental information), because particle brightness of homo-color dimer is twice higher than monomeric GR and hetero-color dimer. Taken together with concentration of hetero-color dimer $[RG]$ calculated from the cross-correlation amplitude, concentrations of monomers ($[G]$ and $[R]$), homo-color dimers ($[GG]$ and $[RR]$) and hetero-color dimer $[RG]$ were separately determined. According to the simulation result, the measured K_d values were completely matched to the given K_d values by the K_d calculation method with the concentration of homo-color dimer, but were not matched without its consideration (Fig. S14). The relative cross amplitudes

(RCA) *in vitro* and *in vivo* were significantly higher than that of coexpression of EGFP and mCherry₂ as a negative control. Moreover, fold change of RCA values against negative control was over 5.6 for *in vitro* experiments and 17 for *in vivo* experiments (Figs S4 and S5), suggesting that the background cross-correlation amplitude, such as cross-talk signal is not dramatically affected to cross-correlation amplitude of interactions of EGFP-hGR α and mCh₂-hGR α . Some data points in which concentrations of monomer ([G] or [R]) or homo-color dimer ([GG] or [RR]) show minus values due to experimental errors were excluded from K_d determination (Figs S11 and S12). Then a scatter plot of the products of concentrations of monomeric GR ([M] = [G] + [R]) versus the concentration of the dimeric GR ([D] = [GG] + [RR] + [RG]) was generated with a line of best fit, and the K_d was calculated from the slope of the regression line^{30,32}. All data points were strongly correlated between the square of the concentration of monomeric GR and the concentration of dimeric GR (Fig. S13).

Determination of *in vitro* K_d by FCCS-microwell system. U2OS cells were cotransfected with 2 μ g pmCherry₂-hGR α and 1 μ g pEGFP-hGR α using ViaFect™ (Promega). The culture method for microwell and extraction method of hGR α from the nucleus were described previously¹⁴. The optical setup for FCCS was the same as for *in vivo* FCCS. The power of 488-nm and 594-nm lasers was 15 and 12 μ W, respectively. The data analysis and computation of K_d values of hGR α were the same as for *in vivo* FCCS.

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Author Contributions

M.K. conceived of the project and supervised all research. M.T., S.O., and M.K. wrote the main manuscript text. M.K. and S.M. designed the experimental procedures, M.T., S.O., and S.M. performed the experiments. J.Y. and M.K. derived the equations and performed simulation. All the authors reviewed the manuscript.

Additional Information

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