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Analysis of intranuclear binding process of glucocorticoid receptor using fluorescence correlation spectroscopy

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Abstract The diffusion properties of EGFP-hGRα and mutants C421G, A458T and I566 in living cells were analyzed. The wild type and mutants C421G and A458T translocated from the cytoplasm to the nucleus after addition of Dex; however, the Brownian motions of the proteins were different. The diffusion constant of wild-type GRα after addition of Dex slowed to 15.6% of that in the absence of Dex, whereas those of A458T and C421G slowed to 34.8% and 61.7%, respectively. This is the first report that dimer formation is less important than the binding activity of GRα to GRE in the living cell.

Keywords: Glucocorticoid response element; Diffusion constant; Glucocorticoid receptor; Mutant; Dexamethasone; RU486

1. Introduction

Understanding the interactions and dynamic properties of biomolecules and biomolecular networks in living cells is of central importance in life science. Direct observation of the actions of transcription factors in the living cell can provide important insights into gene regulatory mechanisms. We analyzed the diffusion of green fluorescence protein fused human glucocorticoid receptor α (EGFP-hGRα) before and after addition of ligands in the nuclei of living cells using fluorescence correlation spectroscopy (FCS).

The glucocorticoid receptor (GR) is a transcriptional regulatory protein that controls broad physiological gene networks, and has pathological effects in a range of diseases; therefore the protein offers an excellent target for therapeutic intervention [1,2]. GR is associated with several proteins in the absence of ligands in the cytoplasm. The humans GR receptor can be found in two isoforms GRα, and GRβ. GRα consists of 777 amino acids, binds hormones and activates glucorticoid-responsive genes. Upon ligand binding, GRα is driven into the nucleus, and regulates transactivation by association with specific genomic glucocorticoid response elements (GRE).

Various functional domains and many cofactors of GRα have been identified by biochemical and molecular biological methods [3], and dynamics of GRα in the living cell has been analyzed using fluorescence recovery after photobleaching (FRAP) [4–6].

Large studies of FRAP using GFP-tagged proteins have been employed to understand diffusion kinetics of nuclear factors. However, even the sensitivity of conventional fluorescence techniques, including FRAP expanded to the single molecule level, can detect only slow dynamics [7].

FCS has been used to analyze the microenvironment of the cell membrane [8], endoplasmic reticulum [9] and nucleus and nucleolus [10], though the changes in the diffusion (Brownian) motion of fluorophores could not be detected by FRAP because of rapid movement. We have detected a slow-moving component of EGFP-hGRα in the nucleus with the addition of dexamethasone (Dex). To elucidate this slow component, we constructed three hGRα mutants: EGFP-hGRα/C421G (C421G) which cannot associate with GRE [11], EGFP-hGRα/A458T (A458T), with no dimerization ability [12], and EGFP-hGRα/I566 (I566), in which the ligand binding domain (LBD) was deleted to reduce association with some cofactors [1].

2. Materials and methods

2.1. Chemicals and plasmids

Dexamethasone (Dex) and RU486 were purchased from Sigma (St. Louis, MO, USA). These chemicals were used as ethanol solutions.

Plasmids encoding the hGRα fused with green fluorescent protein (pCMX-hGRα-GFP) were kindly provided by Dr. H. Tanaka [13] and Dr. Y. Nomura [14]. To obtain brighter fluorescence, pEGFP-hGRα was constructed by PCR amplification of hGRα fragments and ligation into pEGFP-C1 (Clontech, Palo Alto, CA, USA). EGFP-hGRα/I566 (I566) and EGFP-hGRα/A458T (A458T) plasmids were constructed by PCR amplification, EGFP-hGRα/C421G (C421G) plasmid was constructed by a two step PCR procedure [15], with primers containing mutations using pEGFP-hGRα as a template. After purification of PCR products, the digested fragments inserted into pEGFP-hGRα were cut with restriction enzymes. The pairs of primers and restriction enzymes for I566, A458T and C421G are shown below:

I566; forward: 5′-AATGATTGCATCATCGATAAAATTCGAAGA-3′,
reverse: 5′-CACTTGGATCCCTCATAACATGTTGAGCGTA-GTC-3′,
restriction enzymes: *Cla*I and *Bam*HI.
A458T; forward: 5′-GGGTCCCCAGGTAAAGAGACGAA-3′,
reverse: 5′-TTTATCGATGATGCAATCATTCCTTCCA-G*T*AC-ATAGGT-3′,
restriction enzymes: *Esp*3I and *Cla*I.

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Abbreviations: FCS, fluorescence correlation spectroscopy; FAF, fluorescence autocorrelation function; EGFP, enhanced green fluorescent protein; Dex, dexamethasone; LBD, ligand binding domain; DBD, DNA binding domain; GR, glucocorticoid receptor; GRE, glucocorticoid response element

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C421G; forward-1: 5′-GGGTCCCCAGGTAAAGAGACGAA-3′,
reverse-1: 5′-CAGAGCACACCAGGCCGAGTTTGGGAGG-3′,
and
forward-2: 5′-ACCTCCCAAACTCGGCCTGGTGTGCTCTG-3′,
reverse-2: 5′-CAGAGGTTTCTTGTGAGACTCCTGTAGTG-3′
restriction enzymes: *Esp*3I and *Cla*I.

All of the above PCRs were performed using KOD-Plus- (TOYOBO, Tokyo, Japan) according to the manufacturer's instructions.

2.2. Transient transfection

HeLa cells were transfected using the lipofection reagent FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany) with 0.1 μg/well pEGFP-C1, pEGFP-hGRα, pEGFP-hGRα/I566, pEGFP-hGRα/A458T or pEGFP-hGRα/C421G according to the manufacturer's instructions.

2.3. FCS measurement and analysis

FCS measurement was performed with a LSM510-ConfoCor2 combination system (Carl Zeiss, Jena, Germany) before and every 10 min after addition of 100 nM of the indicated ligands. Each set of FCS measurements was carried out five times with a duration of 15 s. Fluorescence autocorrelation functions (FAFs, G(τ)) were acquired and fitted with the FCS Fit program by one-, two-, or three-component models as follows:

$$G(\tau) = \frac{\langle I(t)\rangle\langle I(t+\tau)\rangle}{\langle I(t)\rangle^2} = \frac{1 - F_{\text{triplet}} + F_{\text{triplet}}\exp(-\tau/\tau_{\text{triplet}})}{N(1-F_{\text{triplet}})} \times \sum_i \frac{F_i}{(1+\tau/\tau_i)\sqrt{1+\tau/s^2\tau_i}} + 1 \quad (1)$$

where F_{triplet} is the average fraction of triplet state molecules, τ_{triplet} is the triplet relaxation time, F_i and τ_i are the fraction and diffusion time of component i, respectively, N is the number of fluorescence molecules in the detection volume element defined by radius w_0 and length $2z_0$, and s is the structural parameter representing the ratio, $s = z_0/w_0$. In this paper, the fluorescence autocorrelation function was shown as normalized by N for comparisons of τ.

$$\text{Normalized } G(\tau) = N(G(\tau) - 1) \quad (2)$$

Diffusion constants of samples were obtained from the ratio with the diffusion constant of R6G and diffusion time τ_{R6G} and τ_{sample} [8].

3. Results and discussion

3.1. Mobility of EGFP in the living cell

As shown in Fig. 1A, EGFP was distributed without distinction in the cytoplasm and nucleus even if Dex was added. Similar FAFs were obtained for the nucleus and cytoplasm (Fig. 1B). Most FAFs in the nucleus and cytoplasm could be fitted with a one-component model and the averages of the diffusion constants were calculated to be 20.4 ± 3.2 μm²/s and 19.3 ± 0.9 μm²/s, respectively. This indicated that EGFP did not interact with any proteins or diffuse as free-moving molecules whether in the nucleus or cytoplasm. Moreover, this mobility of EGFP was not affected by addition of ligands (Fig. 1B, C). FAFs in PBS buffer solution could be fitted with the one-component model and the average diffusion constant was calculated to be 72.8 ± 2.8 μm²/s (Fig. 1B), the same as in previous reports [8].

3.2. The effect of Dex on the localization and diffusion of EGFP-hGRα

To observe the localization of EGFP-hGRα in HeLa cells, confocal LSM images were taken before and after addition of Dex. EGFP-hGRα was mainly localized in the cytoplasm in the absence of Dex. However, the subcellular localization of EGFP-hGRα was changed to the nucleus by exposure to Dex, a transactivation agonist, within 30 min (Fig. 2A). We also measured FAFs of EGFP-hGRα in the nuclei of the living cells. As shown in Fig. 2B, normalized FAFs were clearly shifted to the right by the presence of Dex in the nucleus. This shift indicated that a slow-moving EGFP-hGRα appeared. Under this concentration of Dex, the slow-moving fraction reached a plateau in 20 min (data not shown). On the other hand, FAFs obtained from the cytoplasm did not differ in the presence and absence of Dex (Fig. 2C).

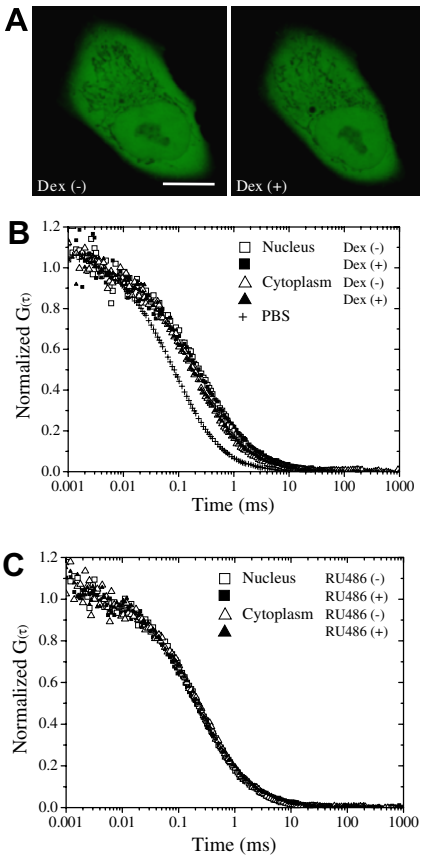

Fig. 1. LSM images and normalized FAFs in a HeLa cell expressing EGFP. (A) A HeLa cell expressing EGFP was imaged before (left) and 60 min after (right) addition of Dex to 100 nM. The scale bar represents 10 μm. (B) FAFs were acquired in PBS buffer (cross) or at the nucleus (square) and cytoplasm (triangle) in HeLa cells expressing EGFP. FCS measurement performed before (open symbols) and 60 min after (closed symbols) addition of 100 nM Dex (C) or RU486.

Most FAFs in the nuclei could be fitted with a two-component model. The average diffusion constants and fractions are summarized in Table 1, which shows that the fractions of each component were not much changed (Table 1, F1 and F2). On the other hand, addition of Dex reduced the diffusion constants of the second component to 15.6% of that in the absence of Dex (Fig. 7). This result could indicate that this slow-moving component originated from the formation of a complex with transcription cofactors and/or interaction with DNA of the activated hGRα. It is noted that the fast component of wild-type GRα decreased in the presence of Dex, this may indicate that an initial complex of GRα and a cofactor could be detected.