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Abstract of Doctoral Thesis

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学 位 論 文 題 名
Title of Doctoral Thesis

Study of Homodimer Glucocorticoid Receptor interaction Using Fluorescence Cross-Correlation Spectroscopy in the Living Cell
(蛍光相互相関分光法を用いた生細胞におけるグルココルチコイド受容体
2 量体化機構の研究)

Human glucocorticoid receptor α (hGR α) is a well-known ligand dependent transcriptional regulatory protein. The classical view is that unliganded GR α is predominantly localize in cytoplasm, upon ligand binding the GR α is translocated to the nucleus, and then associate with the glucocorticoid response element (GRE) to transactivate the specific gene. Moreover, the GR α could transrepress the specific gene binding with GRE as a monomer. Although the dimerization of GR α is strongly related to its transcriptional activity, it is puzzle whether GR α forms dimer in the nucleus before and after DNA binding. Furthermore, it is still a matter of debate whether GR α homodimerized in the cytoplasm or in the nucleus *in vivo*, and what the function of dimer formation in the cytoplasm is. Thus, many unanswered questions still exist regarding GR α . These can be answered by determination of the affinity properties of GR α and/or associated molecules in the living cell. Quantitative characterization and understanding the dynamic properties of biomolecules in live cells are one of the most important challenges in modern biology. All biomolecules are involved in some type of interaction as they perform their functions, and quantification of dissociation constants (K_d s) is an important parameter for understanding biomolecular interactions.

To address the question of when and where GR α is homodimerized, the binding affinities of transiently expressed green fluorescence protein (EGFP), mCherry tandem dimer (mCherry₂) protein-fused GR α and appropriate GR α mutants were determined before and after addition of ligands in the nucleus and cytoplasm in living cells using two-laser-beam fluorescence cross-correlation spectroscopy (FCCS). FCCS is a well-investigated method for observing direct associations between differently fluorescent-labeled proteins in femtoliter confocal volumes, and it enables us to directly and noninvasively observe the interactions between proteins in living cells. The femtoliter confocal volume allows us to resolve the measurement positions in the nucleus and cytoplasm. The parameters obtained using 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 complexes. The concentrations of the fluorescently labeled proteins were calculated from the autocorrelation functions in the FCCS analysis. These parameters inform us of the molecular interactions *in situ*. FCCS has various intracellular applications, including determination of the dissociation constants (K_d) of fluorescent-labeled proteins.

To understand the homodimerization of GR α dissociation constants were determined. The K_d value of mCherry₂ and EGFP fused wild type hGR α was determined to be 4.42 μ M. On the other hand, the K_d of the DNA binding deficiency (C421G) and dimerization deficiency (A458T) and mutants were 4.88 μ M and 9.91 μ M in the nucleus of living cell, respectively. As a result, we confirmed that DNA binding is not necessary for dimerization. In order to understand cytoplasmic dimerization we determined the K_d value for mCherry₂ and EGFP fused Δ NLS (nuclear localization signal) and A458T- Δ NLS mutants in living cell. The estimated K_d was 3.24 μ M and 6.84 μ M, respectively. This finding indicated the cytoplasmic dimerization of GR α . However, the micromolar range of K_d value is higher than the expected nanomolar range, highly validate that the GR α initially shows partial dimerization in cytoplasm and later it translocate to nucleus as a monomer and it may further dimerize inside the nucleus. These findings support the dynamic monomer pathway and regulation of GR α in cytoplasm and nucleus.

Members of the Rel/NF κ B family of transcription factors play a vital role in the regulation of rapid cellular responses. Among the most abundant and best understood of these dimers are the p50/p65 heterodimers and the homodimers of p50 and p65. We used two-laser beam FCCS to quantify the heterodimerization of p50/p65 and homodimerization of p50 and p65 as control experiment. For that purpose, IPT (immunoglobulin-like plexin transcription factor) domain of p50 and p65 were labeled with either mCherry₂ or EGFP fluorescent protein (p50-mCherry₂ and p50 EGFP or p65-mCherry₂ and p65-EGFP). The K_d of p50/p65 heterodimer, p50/p50 and p65/p65 homodimers were determined to be 0.46 μ M, 1.78 μ M and 2.59 μ M respectively in the cytoplasm.

In this work two-laser-beam of FCCS was used instead of single-beam FCCS because the apparatus is commercially available and it provides more flexible laser power tuning for each fluorescence probe. This gives us adequate fluorescence intensity to reduce pseudo positive cross-correlation signals that can be caused by the tail of fluorescence spectrum of EGFP. The technical importance of this work is that flexible laser beam power using two lasers can be applied for any combination of proteins, homo- or heterodimers, for analysis of protein dynamics in living cells. Other than FCCS, there are few methods to determine the K_d values of biomolecules in living cells.

Overall, these findings also contribute to the mechanistic understanding of GR α and it could be assumed ***Dynamic monomer pathway***. This is the first report of such a process is detected, and the results could be important for determining the signaling transduction process of living cells in the view point of dynamically property of biomolecules. Moreover, we found the different binding affinities both in cytoplasm and nucleus of living cell. Thus the results presented in this report should be helpful to understand the interactions of NF κ B dimers quantitatively in living cell on the basis of their dissociation constants.