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The variety of complexes formed by EcR and Usp nuclear receptors in the nuclei of living cells

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ABSTRACT

The heterodimer of the ecdysone receptor (EcR) and ultraspiracle (Usp), members of the nuclear receptor superfamily, is considered to be functional receptor for the ecdysteroids that coordinate essential biological processes in insects. In this work we have applied a bimolecular fluorescence complementation (BiFC) method to directly analyze the formation of the EcR/Usp complex. The BiFC experiments were carried out in mammalian cells which are routinely used for heterologous studies of the EcR/Usp complex, including experiments on EcR-based artificial molecular gene switches. BiFC analysis, supported by flow cytometry, revealed that EcR–Usp interaction is nuclei-restricted. If expressed separately, Usp and EcR are able to form nuclear complexes in the absence of the cognate dimerization partner. We have observed that Muristerone A that is widely used for the induction of ecdysteroid-dependent transcription in mammalian cells, does not significantly change the number of EcR/Usp and EcR/EcR complexes, and it does not influence their subcellular localization.

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1. Introduction

Nuclear hormone receptors play key roles in the regulation of the development and metabolic homeostasis in metazoa. The activity of many known nuclear receptors is controlled by binding small lipophilic ligands, including hormones. According to the traditional model, ligands diffuse through the cellular plasma membrane and bind to the cognate hormone receptor, which selectively affects the transcription of specific genes (Laudet and Gronemeyer, 2002). In recent years receptor-specific mechanisms have been described that regulate the transcriptional activity of some nuclear receptors through the regulation of their subcellular distribution (Hager et al., 2000, 1998). It is now generally accepted that nuclear receptors shuttle between the cytoplasm and the nucleus (Black et al., 2001; Bunn et al., 2001; Prufer and Barsony, 2002; Shank and Paschal, 2005). Interestingly, the actual localization of a particular receptor molecule is controlled not only by its interactions with nuclear import and export machinery, predefined by receptor-specific nuclear import and export signal sequences (NLS and NES respectively), but also by homo- or heterodimerization with other nuclear receptor molecules (Katagiri et al., 2000; Prufer and Barsony, 2002).

Representatives of the nuclear receptor superfamily have been identified in almost all classes of metazoans, and the availability of complete genome sequences has revealed some interesting data regarding nuclear receptors (Escriva et al., 2004). In contrast to the complexity of human hormone signaling pathways, *Drosophila melanogaster* has only one lipophilic hormone acting as a nuclear receptor ligand, the steroid hormone 20-hydroxyecdysone (20E) (King-Jones and Thummel, 2005). The 20E hormone, which is considered to be the principal determinant of developmental timing, controls diverse biological processes, including morphogenetic, apoptotic, physiological, reproductive and behavioral responses. As with vertebrate steroid hormones, 20E exerts its influence via a member of the nuclear receptor superfamily, a product of the *EcR* gene (EcR) (Riddiford et al., 2000). Although EcR can bind a ligand (i.e., 20E) on its own (Christopherson et al., 1992; Grebe et al., 2004), this binding is greatly increased by the product of the *ultraspiracle* gene (Usp) (Koelle et al., 1991; Oro et al., 1990; Yao et al., 1993), which is another member of the nuclear receptor

Abbreviations: 20E, 20-hydroxyecdysone; BiFC, bimolecular fluorescence complementation; CFP, cyan fluorescence protein; EcR, product of *EcR* gene; FRAP, fluorescence recovery after photobleaching; LBD, ligand binding domain; NES, nuclear export signal; NLS, nuclear localization signal; Usp, product of *ultraspiracle* gene; YC, C-terminal fragment of YFP; YFP, yellow fluorescence protein; YN, N-terminal fragment of YFP.

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superfamily and the *D. melanogaster* ortholog of the mammalian retinoid X receptors (Oro et al., 1990). It has also been observed that ligand binding increases the affinity of the EcR/Usp heterodimer to 20E-response elements (Koelle et al., 1991; Oro et al., 1990; Yao et al., 1993). Consequently, a transcriptionally active EcR/Usp heterocomplex is believed to be the only functional form of the 20E receptor. This paradigm is not consistent with gene repression and activation by apo- and holo-EcR (Hu et al., 2003), dimerization of EcR and Usp with alternative partners, and very recent data suggesting that membrane-associated EcR may be involved in the transmission of non-genomic signals elicited by ecdysteroids (Elmogy et al., 2004). Thus, in contrast to the traditional static view, EcR and Usp appear to be key components of a dynamic network that triggers genomic and non-genomic action in different compartments of a cell (Schlattner et al., 2006).

Since a variety of studies have demonstrated that EcR and Usp can function not only in insects but also in mammalian cell lines, the constituents of the EcR/Usp complex, and especially EcR, are of great interest as a source for creating artificial molecular switches (Lafont and Dinan, 2003; Palli et al., 2005). The main advantage of 20E receptor-based molecular switches in mammalian systems is that ecdysteroids, which are used to induce a switch, are structurally different from mammalian steroids, and they do not bind to vertebrate steroid receptors (Lafont and Dinan, 2003). The overexpressed wild type complex EcR/Usp, as well as a variety of chimeric receptors based on EcR, have been shown to be capable of activating a reporter gene in response to several ecdysteroids and ecdysteroid agonists in mammalian cells without other discernible changes in the phenotype (Karns et al., 2001; No et al., 1996). There is a significant potential usage of EcR/Usp herocomplex elements for heterologous applications, and therefore, it is necessary to understand the functioning mechanism of the complex in mammalian cells. Determining the molecular mechanisms responsible for the intracellular distribution of the EcR/Usp complex and/or its constituents is of great importance. However, there are few data concerning this phenomenon (Gwozdz et al., 2007; Nieva et al., 2005), possibly due to the fact that an experimental approach enabling direct investigation of EcR–Usp interactions in living cells has not been described.

Here, we directly detailed for the first time interactions of EcR and Usp in living cells. Our experimental approach relies on the new technique of bimolecular fluorescence complementation (BiFC) (Hu et al., 2002). This technique which allows monitoring protein–protein interactions *in vivo* and in real time has been recently used for the successful visualization of interactions among a wide range of proteins in many cell types and organisms (Kerppola, 2006). Our data demonstrate that EcR–Usp interaction is nuclei-restricted. Moreover, BiFC analysis complemented with flow cytometry experiments allowed us to observe for the first time that Usp and EcR are able to form nuclear complexes in the absence of the cognate dimerization partner. Interestingly, the presence of Muristerone A, the ligand which has been widely used for the induction of ecdysteroid-dependent transcription in mammalian cells (Palli et al., 2005), does not significantly change the amount of EcR/Usp and EcR/EcR complexes and it does not influence their subcellular localization.

2. Materials and methods

2.1. Plasmid construction

Constructs containing the full-length EcRB1 isoform and USP from *Drosophila melanogaster* tagged with fluorescent proteins YFP and CFP have been previously described (Nieva et al., 2005).

In order to construct plasmids for BiFC experiments the sequences encoding the N-terminal fragment of YFP containing amino acids 1–154 (YN) and the C-terminal fragment of YFP containing amino acids 155–238 (YC) were amplified by PCR from pEYFP-C1 (Clontech). cDNA encoding full-length EcR and Usp were amplified by PCR and fused to YN or YC using the SmaI site. Next, the sequences encoding the fusion proteins YN-EcR, YC-EcR, YN-Usp and YN alone were cloned into the multiple cloning site 1 (MCS1) of pBudCE4.1 (Invitrogen) using SalI, XbaI restriction sites. Sequences encoding the fusion proteins YN-Usp, YC-Usp and YC alone were cloned using KpnI, BglII restriction sites and YN-EcR was cloned using NotI, Bsp119I into the MCS2 of pBudCE4.1.

2.2. Cell culture and DNA transfection

CHO-K1, chinese hamster ovaricytes (ATCC CCL-61) were maintained in Ham's F12 medium, COS-7, african green monkey kidney fibroblasts (ATCC CRL-1651) and HeLa, human cervix adenocarcinoma (ATCC CCL-2) cells in DMEM supplemented with 1% non-essential amino acids (Gibco/Invitrogen), 1 mM sodium pyruvate and 2% glutamine (Gibco/Invitrogen). Both media were supplemented with 10% fetal calf serum (Gibco/Invitrogen). Cells were grown at 37 °C and in an atmosphere of 95% air and 5% CO_2. Cells were transfected with 4 μg of pBudCE4.1 containing YN- and YC-chimeras for BiFC or cotransfected with 4 μg of DNA (total amount) of YFP- and CFP-chimeras for FRAP assays using jetPEI (Polyplus). However, for the BiFC analysis that was done to determine the influence of Muristerone A, cells were transfected with 4 μg of the appropriate pBudCE4.1. derivative in the presence of 10^{-5} M Muristerone A (Invitrogen).

2.3. Confocal microscopy

Prior to the imaging experiments cells were grown on round, glass 0.17 mm thick coverslips (Mentzel) submerged in a culture medium, in 4 cm diameter Petri dishes. 18 h after transfection the culture medium was replaced by DMEM/F12 buffered with 15 mM HEPES without phenol red (Sigma). For the BiFC experiments after 36 h the culture medium was replaced by DMEM/F12 buffered with 15 mM HEPES and phenol red (Sigma) and maintained at 30 °C in a normal atmosphere for 2–6 h to promote YFP fluorophore maturation. Coverslips with cell cultures were transferred into a steel holder and mounted in a microscope stage microincubator (Life Science Resources). During microscopy studies the temperature of cell cultures was maintained at 37 °C. Images of fluorescently labelled proteins were acquired using a Bio-Rad MRC1024 confocal system (Bio-Rad), built on a Nikon Diaphot 300 inverted microscope (Nikon), and equipped with a 100 mW argon ion laser (ILT). A 60× PlanApo oil-immersion NA 1.4 objective lens was used. CFP and YFP fluorescence was excited by a 457 nm and 514 nm wavelength light, respectively. A z458/514rpc dual primary dichroic (Chroma) was used. To separate fluorescence emissions of CFP and YFP 510DCLP dichroic (VHS filter block) and HQ485/30 and HQ540/30 (Chroma) emission filters were used.

2.4. BiFC analysis with flow cytometry

Prior to flow cytometry experiments, 36 h after transfection, cells were incubated for 4 h at 30 °C, gently trypsinized, washed with PBS and resuspended in 0.5 ml of PBS. The cells were analyzed in a flow cytometer (FACScan, Becton Dickinson) using an excitation wavelength of 488 nm. Only cells exhibiting the forward and side scatter that is typical of live cells were included in the analysis. A threshold fluorescence intensity was selected so that none of control cells (untransfected cells) were above the threshold.

2.5. FRAP assay and data analysis

Fluorescence recovery after photobleaching (FRAP) experiments and analysis were carried out using Lasersharp 2000 software (Bio-Rad). A region of interest (ROI) of the nucleus was photobleached by a 514 nm laser line (8 mW intensity). After the bleaching scan images were collected at 1 s intervals to monitor the recovery of fluorescence.

In order to account for the photobleaching arising from serial scanning, the mean intensity values of the ROI (I_{ROI}) were corrected using the following equation: $I_{ROI} = I_{ROI,t} * (I_{Ref,0}/I_{Ref,t})$, where $I_{ROI,t}$ is the fluorescence intensity of the ROI at time t, $I_{Ref,0}$ is the intensity of the reference cell (i.e., a neighboring cell which was not exposed to a bleaching scan) before photobleaching and $I_{Ref,t}$ is the intensity of the reference cell at time t. The corrected intensities were plotted against time to construct the recovery curve. To establish the half-recovery time (τ), a regression analysis was performed using the standard biexponential decay, $I_{ROI} = I_\infty + ae^{-bt} + ce^{-dt}$ where I_∞ is the plateau of the curve, a and c are amplitude constants, b is the bleaching decay constant with reciprocal time units and d is the second bleaching rate. The reported τ values are the means ± S.E.M. of the τ values from 15 to 30 individual experiments.

To generate the graphs presented in Fig. 1A and C fluorescence intensity values were additionally normalized using the following equation, $I_{ROI} = (I_{ROI,t} - Y)/(Z - Y)$ where Y is the intensity immediately after the photobleaching (where t is equal to zero), and Z is the intensity at the final time point. This results in an initial post-bleach intensity (at $t = 0$ s) of zero and the final intensity of 1 using arbitrary units and enables a comparison of the shapes of the recovery curves.

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