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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.

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

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; Hager et al., 1998). It is now generally accepted that nuclear receptors shuttle between cytoplasm and a 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 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 (Elmogly 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 (Schlatterner 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 significant potential usage of EcR/Usp heterocomplex 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 Mupisterone 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 *Sma*I 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 *Sal*I, *Xba*I restriction sites. Sequences encoding the fusion proteins YN-Usp, YC-Usp and YC alone were cloned using *Kpn*I, *Bgl*II restriction sites and YN-EcR was cloned using *Not*I, *Bsp*119I into the MCS2 of pBudCE4.1.

2.1. Cell culture and DNA transfection

CHO-K1, chinese hamster ovarocytes (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% CO₂. 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 μ g of the appropriate pBudCE4.1 derivative in the presence of 10^{-5} M Muristerone A (Invitrogen).

2.2. 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. 18h after transfection the culture medium was replaced by DMEM/F12 buffered with 15 mM HEPES without phenol red (Sigma). For the BiFC experiments after 36h 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 60x 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.

2.3. BiFC analysis with flow cytometry

Prior to flow cytometry experiments, 36h after transfection, cells were incubated for 4h 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 488nm. 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.

2.4. FRAP assay and data analysis

Fluorescence recovery after photobleaching (FRAP) experiments and analysis were carried out using Laserssharp 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 second 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 neighbouring 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 $\pm$ SEM of the τ values from 15-30 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.

3. Results

3.1. Analysis of the interaction of EcR and Usp within living cells

The main goal of this work was to develop a methodology for examining in a direct way the interaction of EcR and Usp. Initially, we applied the technique of fluorescence recovery after photobleaching (FRAP) (Reits and Neefjes, 2001). FRAP relies on the ability of a laser scanning instrument to rapidly and irreversibly photobleach a small region of interest (ROI) within a cell, and subsequently follow the recovery of fluorescence in this region. The fluorescence recovery occurs as unbleached proteins migrate into the ROI from adjacent regions. Proteins that are stably associated with other proteins or with cellular structures will move more slowly than those that are freely mobile. For the FRAP experiments we used EcR and Usp fused with derivatives of green fluorescent protein, which had been previously characterized (Nieva et al., 2005). In particular, EcR and Usp were tagged with a yellow fluorescent protein (YFP-EcR and YFP-Usp, respectively) or with a cyan fluorescent protein (CFP-EcR and CFP-Usp, respectively). Initial measurements showed that for YFP or CFP expressed alone in CHO-K1 cells, the mean fluorescence decreased in illuminated as well as unilluminated regions. This indicated that the mobility of isolated fluorescent labels was too fast to be measured under the conditions of our experiment, and could occur if the halftime of the mean fluorescence recovery after photobleaching (τ) was shorter than the time required for a single measurement (≤ 0.5 s; data not shown). For YFP-Usp and YFP-EcR derivatives τ was greater (4.8 ± 0.4 s and 5.2 ± 0.3 s respectively, Fig. 1). Coexpression of YFP-Usp with CFP-EcR or YFP-EcR with CFP-Usp caused a further, two fold increase of τ and resulted in values of 12.7 ± 0.8 and 12.2 ± 0.8 , measured for YFP-Usp and YFP-EcR respectively (Fig. 1). Labeling EcR and Usp with different GFP derivatives allowed us to define and analyze cells expressing both fusion proteins. The mobility of YFP was very high. The fast movement of this protein strongly suggests that it was not bound to other proteins or nuclear structures. The

diminished mobility of YFP-Usp and YFP-EcR in comparison with YFP alone can be interpreted as the result of an increased molecular weight, as the consequence of interactions with other cellular components, or a combination of both of these factors. Notably, the greater increase of τ in the coexpression experiments may have been caused by the interaction between EcR and Usp.

To test Usp-EcR interaction in a more direct manner, we decided to apply the BiFC assay (Hu et al., 2002), which has been proven to be an invaluable tool to monitor protein-protein interactions *in vivo* and in real time (Kerppola, 2006). To the best of our knowledge the BiFC technique has never been applied to the investigation of nuclear receptors. The BiFC assay is based on the formation of a fluorescent complex from non-fluorescent fragments of a fluorescent protein, i.e., N-terminal (YN) and C-terminal (YC) fragments of YFP when they are brought together by interactions between the analyzed proteins fused to each fragment. If the two proteins interact with each other, the YC and YN are close to each other for a sufficiently long enough time to irreversibly form a fluorescent protein. However, the interaction between the YN and YC fragments does not influence the potential interactions between the investigated proteins (Hu et al., 2002). To investigate the interaction between EcR and Usp, the YN fragment was attached to the N-terminus of EcR (YN-EcR), whereas the YC fragment to the N-terminus of Usp (YC-Usp). The coexpression of both fusion proteins in COS-7 and CHO-K1 cells resulted in fluorescence confined to a nucleus (Fig. 2A). After the same transfection procedure we monitored transfected cells with flow cytometry. As shown in Fig. 3 $20.9 \pm 2.3\%$ of cells exhibited fluorescence after the coexpression of YN-EcR and YC-Usp. This value did not significantly change (to the value of $23.4 \pm 1.45\%$) when Muristerone A, the ligand which has been widely used for induction of ecdysteroid receptor-dependent transcription in mammalian cells (Lafont and Dinan, 2003; Palli et al., 2005), was added (Fig. 3). Simultaneously, confocal microscopy analysis demonstrated that Muristerone

A induced no change in the subcellular localization of the fluorescence signal, i.e., fluorescence was observed only in nuclei (data not shown).

An essential criterion for using the fusion proteins in the BiFC analysis is that the fluorescent-protein fragments should not associate with each other in the absence of interaction between the proteins which are fused to these fragments (Hu et al., 2002). To test if this criterion is fulfilled, we examined YN-EcR coexpressed with YC or YC-Usp with YN or YN with YC. In the case of YN-EcR and YC proteins we did not observe any fluorescence, for two other controls we observed only faint fluorescence in about 1.5-2.0% of cells (Fig. 3). Collectively, the data presented above indicate that the formation of the bimolecular fluorescent complex requires specific heterodimerization between EcR and Usp and that the nonspecific interactions (directed solely by YN and YC fragments) do not contribute significantly to fluorescence complementation. This interaction between EcR and Usp does not rely on the Muristerone A hormone and is restricted to the nuclei of analyzed cells. Although the presence of the hormone to some extent increases the amount of the complexes it does not change their subcellular localization.

3.2. Usp and EcR expressed alone are able to form complexes in living cells

As discussed in the Introduction, EcR and Usp appear to be key components of a dynamic network that triggers various effects in different cell types and possibly in different compartments of cells. The results presented in this study indicate that the heterodimer EcR/Usp, which is believed to be the main element of this network, is localized in the nucleus. Since there are biochemical data suggesting that isolated Usp is able to homodimerize *in vitro* (Fang et al., 2005; Rymarczyk et al., 2003) we decided to investigate, using a BiFC assay, whether the interaction of Usp molecules can take place in living cells and establish the subcellular localization of this putative complex. We also decided to carry out similar analysis for EcR. Thus, both YFP fragments were fused to Usp or EcR and the

respective constructs were used for cell transfection. As shown in Fig. 2B fluorescence in the cells in which YC-Usp and YN-Usp were expressed was visible only in the nucleus. Flow cytometry demonstrated that about $20.1 \pm 1.6\%$ of COS-7 cells exhibited fluorescence of the interacting YC-Usp and YN-Usp (Fig. 3). This value was significantly higher than the 2% value measured in control experiments for cells expressing YN-Usp and YC (Fig. 3). Under the same experimental conditions the coexpression of YN-EcR and YC-EcR resulted in nuclear fluorescence (Fig. 2C) in about $10.5 \pm 0.2\%$ of analyzed cells (Fig. 3). Again, the Muristerone A did not significantly change the number of fluorescent cells (Fig. 3) and the subcellular localization of the fluorescence was restricted to the nuclei as judged by confocal microscopy (not shown). Taken together, these results demonstrate that Usp forms complexes in the nuclei of living cells in the absence of the cognate dimerization partner. One can not rule out the possibility that a similar phenomenon exists for EcR. Importantly, formation of EcR complexes does not rely on Muristerone A.

4. Discussion

The response of cells to extracellular stimuli and the execution of developmental programs are mediated by networks of protein interactions. The identification and characterization of these interactions is a prerequisite to understanding biological processes on a molecular level. Protein-protein interactions have been investigated using a number of methods that enable the direct detection of interactions (Piehler, 2005). Unfortunately many experimental approaches such as co-precipitation and co-purification require the removal of proteins from their natural environment. By contrast, direct visualization of protein complexes in living cells enables the investigation of protein interactions in the cellular context. Two methods have been used to visualize protein interactions in living cells - fluorescence/förster resonance energy transfer (Yan and Marriott, 2003) and the BiFC technique, which has been recently successfully used

for validating the interaction between putative interaction partners and to identify the subcellular localization of these interactions (Kerppola, 2006). The BiFC assay has been used, for example, to study interactions among different structural classes of transcription factors. These studies demonstrated that, in many cases, the localization of a protein complex differs from the localization of the interacting partners, suggesting that complex formation can regulate the localization and hence the function of the interacting transcription factors (Grinberg et al., 2004). Taking these data into consideration we applied the BiFC assay to analyze the subcellular localization of the complex formed by EcR and Usp.

Although components of the EcR/Usp complex are active in mammalian cells and are thus potentially useful in the regulation of transgene expression in mammalian cells and transgenic animals (Lafont and Dinan, 2003; Palli et al., 2005), there is still little data concerning the molecular mechanisms governing the subcellular distribution of the complex and its components. Therefore, earlier we had analyzed the subcellular trafficking of the EcRB1 isoform and Usp in mammalian cells (CHO-K1, COS-7 and HeLa) which are routinely used for EcR/Usp heterologous studies (Gwozdz et al., 2007; Nieva et al., 2005). The experiments showed that the intracellular distribution of EcR was dependent on time and cell type. In contrast, Usp was localized exclusively in the nucleus independently of time and cell line. However, when these two proteins were coexpressed we noticed a significant change in EcR distribution; EcR was found exclusively in the nucleus. This was the first indirect experimental indication of the possible interaction of full-length EcR and Usp in living cells. Two independent methods, FRAP and BiFC, clearly indicate that Usp and EcR are components of the complexes localized exclusively within the nucleus. Taking into account current data about nuclear receptors (Laudet and Gronemeyer, 2002), including our recently published data (Gwozdz et al., 2007; Nieva et al., 2005), it is reasonable to assume that the EcR/Usp complexes are formed due to the direct interaction between the ligand binding

domains (LBDs) of both receptors. However, the interaction that brings together EcR and Usp and thus localizes them in the nucleus, may not be a direct interaction. It is known that fusion proteins that can be brought together by assembly in a macromolecular complex can produce bimolecular fluorescence complexes in the absence of direct contact between the proteins that are fused to the fragments (Kerppola, 2006). Similarly, an increase in the τ value in the FRAP experiments may be observed, even if the analyzed proteins do not contact each other directly. Previous observations have shown that Usp is able to form dimeric and oligomeric complexes *in vitro* (Fang et al., 2005; Rymarczyk et al., 2003). The BIFC data presented here show for the first time that Usp is able to form homomeric complexes in living cells as well. The exclusive nuclear localization of the Usp complex is not surprising, since Usp apparently contains only nuclear localization signal (NLS) activity within its DNA binding domain (Gwozdz et al., 2007). Regarding EcR there hasn't been any information about the possibility of interaction between full-length molecules of EcR. Here we show for the first time that EcR is also able to form homomeric complexes in living cells. However, EcR in comparison with Usp, exhibits a lesser tendency to form complexes as judged by flow cytometry measurements. Certainly, the above presented results do not prove the *in vivo* relevance of EcR and Usp homomeric complexes and the BiFC methodology cannot be directly used to study this relevance. Whether all the observed complexes of EcR and Usp (homo- and heteromeric) coexist in one cell at the same time remains, to be discovered too.

In contrast to some vertebrate steroid nuclear receptors which are translocated from cytoplasm into a nucleus only in the presence of the cognate hormone (Htun et al., 1996; Tyagi et al., 2000) complexes of EcR/Usp can be observed within a nucleus in the absence of Muristerone A. Since EcR in the absence of Usp can be distributed within the whole cell (Nieva et al., 2005), at least in the case of the CHO-K1 cell line, it is reasonable to assume that the nuclear localization of the EcR/Usp complex is determined by the exclusively nuclear Usp. As shown

previously, EcR contains several putative nuclear export signals (NESs) within its LBD (Gwozdz et al., 2007). We suppose that the interaction between the LBDs of EcR and Usp may lead to the inactivation/masking of the NESs. This could strengthen the ability of Usp to retain the whole complex within the nucleus. As mentioned above, EcR expressed without Usp in CHO-K1 cells could also be distributed in the cytoplasm (Nieva et al., 2005), although we observed EcR complexes only within the nucleus. We suppose that the interaction of EcR with some cytoplasmatic proteins, like for example hsp family members, could prevent the formation of homomeric EcR complexes in that compartment of a cell as well as the EcR import into the nucleus. The latter would be possible if we assume that EcR molecules interact through LBDs as in the case of the EcR/Usp complex (Gwozdz et al., 2007) causing the inactivation of NESs. In that case the NLS within the EcR molecule would be able to direct the whole EcR/EcR complex to the nucleus.

The main goal of our investigation was to obtain data that would lay the foundation for understanding how EcR and Usp function in mammalian cells. Simultaneously, owing to the usage of mammalian cell cultures we were able to dissect the properties of EcR and Usp in a neutral environment that lacks a cognate partner. This may allow better comprehension of some of the previous observations that have been made for insect cell cultures and organisms. For example, it has been previously reported that EcR is able to function as an inductor or repressor of gene transcription. Both these functions are exhibited in the presence and absence of 20E, respectively (Dobens et al., 1991). Obviously, the nuclear localization of EcR, which according to our data is determined by Usp, is essential in both cases. Therefore, EcR and Usp that are present within a cell in the absence of hormone would form obligatory nuclear complexes exhibiting a repressive function. These preformed and “prelocalized” complexes would be able to activate transcription as a result of the conformational change of EcR after binding 20E or its agonists. Accordingly, the 20E hormone emerges as a factor which would

control activity and possibly the stability of the EcR/Usp complex without affecting its subcellular distribution. The above presented data support this hypothesis, especially the fact that the EcR/Usp complex formation is limited to the nuclei independently of the presence of the hormone.

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References

- Black, B.E., Holaska, J.M., Rastinejad, F. and Paschal, B.M., 2001. DNA binding domains in diverse nuclear receptors function as nuclear export signals. *Curr. Biol.* 11, 1749-1758.
- Bunn, C.F., Neidig, J.A., Freidinger, K.E., Stankiewicz, T.A., Weaver, B.S., McGrew, J. and Allison, L.A., 2001. Nucleocytoplasmic shuttling of the thyroid hormone receptor alpha. *Mol. Endocrinol.* 15, 512-533.
- Christopherson, K.S., Mark, M.R., Bajaj, V. and Godowski, P.J., 1992. Ecdysteroid-dependent regulation of genes in mammalian cells by a *Drosophila* ecdysone receptor and chimeric transactivators. *Proc. Natl. Acad. Sci. U.S.A.* 89, 6314-6318.
- Dobens, L., Rudolph, K. and Berger, E.M., 1991. Ecdysterone regulatory elements function as both transcriptional activators and repressors. *Mol. Cell. Biol.* 11, 1846-1853.
- Elmogy, M., Iwami, M. and Sakurai, S., 2004. Presence of membrane ecdysone receptor in the anterior silk gland of the silkworm *Bombyx mori*. *Eur. J. Biochem.* 271, 3171-3179.
- Escriva, H., Bertrand, S. and Laudet, V., 2004. The evolution of the nuclear receptor superfamily. *Essays Biochem.* 40, 11-26.
- Fang, F., Xu, Y., Jones, D. and Jones, G., 2005. Interactions of ultraspiracle with ecdysone receptor in the transduction of ecdysone- and juvenile hormone-signaling. *FEBS J.* 272, 1577-1589.
- Grebe, M., Fauth, T. and Spindler-Barth, M., 2004. Dynamic of ligand binding to *Drosophila melanogaster* ecdysteroid receptor. *Insect. Biochem. Mol. Biol.* 34, 981-989.
- Grinberg, A.V., Hu, C.D. and Kerppola, T.K., 2004. Visualization of Myc/Max/Mad family dimers and the competition for dimerization in living cells. *Mol. Cell. Biol.* 24, 4294-4308.

- Gwozdz, T., Dutko-Gwozdz, J., Nieva, C., Betanska, K., Orlowski, M., Kowalska, A., Dobrucki, J., Spindler-Barth, M., Spindler, K.D. and Ozyhar, A., 2007. EcR and Usp, components of the ecdysteroid nuclear receptor complex, exhibit differential distribution of molecular determinants directing subcellular trafficking. *Cell. Signal.* 19, 490-503.
- Hager, G.L., Lim, C.S., Elbi, C. and Baumann, C.T., 2000. Trafficking of nuclear receptors in living cells. *J. Steroid Biochem. Mol. Biol.* 74, 249-254.
- Hager, G.L., Smith, C.L., Fragoso, G., Wolford, R., Walker, D., Barsony, J. and Htun, H., 1998. Intranuclear trafficking and gene targeting by members of the steroid/nuclear receptor superfamily. *J. Steroid. Biochem. Mol. Biol.* 65, 125-132.
- Htun, H., Barsony, J., Renyi, I., Gould, D.L. and Hager, G.L., 1996. Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera. *Proc. Natl. Acad. Sci. U.S.A.* 93, 4845-4850.
- Hu, C.D., Chinenov, Y. and Kerppola, T.K., 2002. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell* 9, 789-798.
- Hu, X., Cherbas, L. and Cherbas, P., 2003. Transcription activation by the ecdysone receptor (EcR/USP): identification of activation functions. *Mol. Endocrinol.* 17, 716-731.
- Karns, L.R., Kisielewski, A., Gulding, K.M., Seraj, J.M. and Theodorescu, D., 2001. Manipulation of gene expression by an ecdysone-inducible gene switch in tumor xenografts. *BMC Biotechnol.* 1, 11.
- Katagiri, Y., Takeda, K., Yu, Z.X., Ferrans, V.J., Ozato, K. and Guroff, G., 2000. Modulation of retinoid signalling through NGF-induced nuclear export of NGFI-B. *Nat. Cell Biol.* 2, 435-40.

- Kerppola, T.K., 2006. Visualization of molecular interactions by fluorescence complementation. *Nat. Rev. Mol. Cell. Biol.* 7, 449-456.
- King-Jones, K. and Thummel, C.S., 2005. Nuclear receptors--a perspective from *Drosophila*. *Nat. Rev. Genet.* 6, 311-323.
- Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P. and Hogness, D.S., 1991. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* 67, 59-77.
- Lafont, R. and Dinan, L., 2003. Practical uses for ecdysteroids in mammals including humans: an update. *J. Insect. Sci.* 3, 7.
- Laudet, V. and Gronemeyer, H., 2002 *The nuclear receptor: factbook*, Academic Press, San Diego.
- Nieva, C., Gwozdz, T., Dutko-Gwozdz, J., Wiedenmann, J., Spindler-Barth, M., Wieczorek, E., Dobrucki, J., Dus, D., Henrich, V., Ozyhar, A. and Spindler, K.D., 2005. Ultraspiracle promotes the nuclear localization of ecdysteroid receptor in mammalian cells. *Biol. Chem.* 386, 463-470.
- No, D., Yao, T.P. and Evans, R.M., 1996. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3346-3351.
- Oro, A.E., McKeown, M. and Evans, R.M., 1990. Relationship between the product of the *Drosophila* ultraspiracle locus and the vertebrate retinoid X receptor. *Nature* 347, 298-301.
- Palli, S.R., Hormann, R.E., Schlattner, U. and Lezzi, M., 2005. Ecdysteroid receptors and their applications in agriculture and medicine. *Vitam. Horm.* 73, 59-100.
- Piehl, J., 2005. New methodologies for measuring protein interactions in vivo and in vitro. *Curr. Opin. Struct. Biol.* 15, 4-14.

- Prufer, K. and Barsony, J., 2002. Retinoid X receptor dominates the nuclear import and export of the unliganded vitamin D receptor. *Mol. Endocrinol.* 16, 1738-1751.
- Reits, E.A. and Neefjes, J.J., 2001. From fixed to FRAP: measuring protein mobility and activity in living cells. *Nat. Cell. Biol.* 3, E145-147.
- Riddiford, L.M., Cherbas, P. and Truman, J.W., 2000. Ecdysone receptors and their biological actions. *Vitam. Horm.* 60, 1-73.
- Rymarczyk, G., Grad, I., Rusek, A., Oswiecimska-Rusin, K., Niedziela-Majka, A., Kochman, M. and Ozyhar, A., 2003. Purification of *Drosophila melanogaster* ultraspiracle protein and analysis of its A/B region-dependent dimerization behavior in vitro. *Biol. Chem.* 384, 59-69.
- Schlattner, U., Vafopoulou, X., Steel, C.G., Hormann, R.E. and Lezzi, M., 2006. Non-genomic ecdysone effects and the invertebrate nuclear steroid hormone receptor EcR--new role for an "old" receptor? *Mol. Cell. Endocrinol.* 247, 64-72.
- Shank, L.C. and Paschal, B.M., 2005. Nuclear transport of steroid hormone receptors. *Crit. Rev. Eukaryot. Gene Expr.* 15, 49-73.
- Sutherland, J.D., Kozlova, T., Tzertzinis, G. and Kafatos, F.C., 1995. *Drosophila* hormone receptor 38: a second partner for *Drosophila* USP suggests an unexpected role for nuclear receptors of the nerve growth factor-induced protein B type. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7966-7970.
- Tyagi, R.K., Lavrovsky, Y., Ahn, S.C., Song, C.S., Chatterjee, B. and Roy, A.K., 2000. Dynamics of intracellular movement and nucleocytoplasmic recycling of the ligand-activated androgen receptor in living cells. *Mol. Endocrinol.* 14, 1162-1174.
- Yan, Y. and Marriott, G., 2003. Fluorescence resonance energy transfer imaging microscopy and fluorescence polarization imaging microscopy. *Methods Enzymol.* 360, 561-580.

Yao, T.P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., McKeown, M., Cherbas, P. and Evans, R.M., 1993. Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. *Nature* 366, 476-479.

Figure legends

Fig. 1. Analysis of the intracellular mobility of Usp and EcR in CHO-K1 cells.

(A) Fluorescent recovery curves recorded in a representative YFP-Usp FRAP experiment. Normalized fluorescence intensities in the ROI for YFP-Usp (filled circles) and YFP-Usp coexpressed with CFP-EcR (+CFP-EcR; open circles) were plotted, (B) Representative images from a FRAP experiment which were used to prepare the graph shown in panel A. The region of interest (ROI), indicated by a rectangle, was photobleached, and images were then recorded at the time points indicated in each image. (C) Fluorescent recovery curves for the representative YFP-EcR FRAP experiment. Normalized fluorescent intensities in the ROI for YFP-EcR (filled circles) and YFP-EcR coexpressed with CFP-Usp (+CFP-Usp; open circles) were plotted. (D) Representative images of the FRAP experiments which were used to prepare the graph shown in panel C. The region of interest (ROI), indicated by a rectangle, was photobleached, and images were then recorded at the time points indicated in each image. (E) The half-recovery time, τ , for YFP-Usp in the absence and presence of CFP-EcR and for YFP-EcR in the absence and presence of CFP-Usp was calculated as described in the Materials and Methods section. The reported τ values are the means $\pm$ SEM of the τ values from 15-30 individual experiments. Bar, 10 μ m.

Fig. 2. The BiFC analysis of the interaction between EcR and Usp in CHO-K1 and COS-7 cells. The representative images of cells coexpressing the indicated and schematically depicted fusion proteins: YN-EcR and YC-Usp (A), YN-Usp and YC-Usp (B) or YN-EcR and YC-EcR (C).

Fig. 3. Flow cytometry analysis. The graph presents the percentage of COS-7 cells with visible fluorescence resulting from BiFC. For more details see Materials and Methods. Each histogram represents the average values of three independent experiments $\pm$ SD.

Figure 3