Fluorescence Imaging Reveals the Nuclear Behavior of Peroxisome Proliferator-activated Receptor/Retinoid X Receptor Heterodimers in the Absence and Presence of Ligand*

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In a global approach combining fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS), and fluorescence resonance energy transfer (FRET), we address the behavior in living cells of the peroxisome proliferator-activated receptors (PPARs), a family of nuclear receptors involved in lipid and glucose metabolism, inflammation control, and wound healing. We first demonstrate that unlike several other nuclear receptors, PPARs do not form speckles upon ligand activation. The subnuclear structures that may be observed under some experimental conditions result from overexpression of the protein and our immunolabeling experiments suggest that these structures are subjected to degradation by the proteasome. Interestingly and in contrast to a general assumption, PPARs readily heterodimerize with retinoid X receptor (RXR) in the absence of ligand in living cells. PPAR diffusion coefficients indicate that all the receptors are engaged in complexes of very high molecular masses and/or interact with relatively immobile nuclear components. PPARs are not immobilized by ligand binding. However, they exhibit a ligand-induced reduction of mobility, probably due to enhanced interactions with cofactors and/or chromatin. Our study draws attention to the limitations and pitfalls of fluorescent chimera imaging and demonstrates the usefulness of the combination of FCS, FRAP, and FRET to assess the behavior of nuclear receptors and their mode of action in living cells.

The nucleus comprises different subdomains whose biological functions remain often very elusive, if known at all, and that differ fundamentally from cytoplasmic compartments in that they are not delineated by membranes (1, 2). Several proteins or complexes involved in processes as diverse as chromatin remodeling, transcription, and RNA processing form nuclear speckles throughout the nucleoplasm. These compartments would actually result from the temporary association of highly mobile components, which shuttle between these sites (3).

Nuclear receptors are one of the most abundant classes of transcriptional regulators in metazoans. They compose a large family of ligand-activated proteins among which are the steroid hormone receptors, the thyroid hormone receptors (TRs), the vitamin D receptor (VDR), the retinoic acid receptors (RARs), as well as the peroxisome proliferator-activated receptors (PPARs). It is generally assumed that, upon ligand binding, nuclear receptors either homodimerize or heterodimerize with the retinoid X receptor (RXR) and bind to specific responsive elements in the enhancer regions of their target genes. This promotes gene activation, after a complex array of events including both dissociation from and association with numerous cofactors (4). The three PPAR isotypes (named α, β, and γ or NR1C1, NR1C2, and NR1C3 (5)) are mainly involved in lipid and glucose homeostasis, regulation of food intake and body weight, and control of inflammation and wound healing (6, 7). They are activated by naturally occurring or metabolized fatty acids derived from the diet or from intracellular signaling pathways and induce gene transcription as heterodimers with RXRs (8). PPARs are also very important therapeutic targets for the treatment of hyperlipidemia and type 2 diabetes.

Recent developments in live cell imaging have prompted investigations on the localization and mobility of nuclear receptors to decipher the molecular mechanisms underlying transcriptional activation. Genetically encoded fluorescent proteins (FPs) allow to monitor directly in living cells the behavior of proteins in their physiological context (9). For instance, it is possible to assess the dynamics of a protein by fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) and to evaluate the interaction between two proteins by fluorescence resonance energy transfer (FRET).

Classical confocal microscopy combined with the use of FPs has first allowed to address a particularly interesting issue: the modulation of nuclear receptor localization upon ligand binding. Ligand treatment induces a redistribution of the glucocor-

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1 The abbreviations used are: TR, thyroid hormone receptor; VDR, vitamin D receptor; RAR, retinoic acid receptor; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; RXR, retinoid X receptor; FP, fluorescent protein; GFP, green FP; YFP, yellow FP; CFP, cyan FP; EYFP, enhanced YFP; ECFP, enhanced CFP; FRAP, fluorescence recovery after photobleaching; FCS, fluorescence correlation spectroscopy; FRET, fluorescence resonance energy transfer; GR, glucocorticoid receptor; AR, androgen receptor; PR, progesterone receptor; MR, mineralocorticoid receptor; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; RXR, retinoid X receptor.
ticoid receptor (GR) (10), the androgen receptor (AR) (11, 12), the progesterone receptor (PR) (13), the mineralocorticoid receptor (MR) (14), and VDR (15) from the cytoplasm to the nucleus of various cell types. In contrast, other nuclear receptors such as the estrogen receptor (ER) (16–18), TR (19–21), RAR (19), and RXR (22) are confined in the nucleus both in the presence and absence of ligand. Ligand binding promotes the formation of nuclear speckled patterns for ER, GR, AR, TR, RAR, and RXR (see Ref. 23 for a review). These induced clusters only partially overlap with sites of transcription (24), and their role in nuclear receptor function awaits further elucidation. The need for quantification for these events then lead to the use of more elaborated techniques, such as FRAP. Mobility studies have focused on the diffusion of receptors through the nucleoplasm on the one hand and at specific interaction sites on chromatin on the other (25, 26). FRAP revealed that nuclear receptors move rapidly and that, at least for ER and GR, their motion in the nucleoplasm can be modulated by ligand binding (18, 19, 27, 28). Interestingly, other receptors such as TR and RAR do not exhibit the decreased mobility observed for ER and GR in the presence of ligand (19). Although recent reports have highlighted the influence of some molecular chaperones and of the proteasome on nuclear receptor mobility (18, 28–30), it is not clear at present which factors modulate and orientate nuclear receptor trafficking in the cell. The steps required to initiate transcription upon ligand binding (i.e. interaction with cofactors that allow chromatin remodeling and recruitment of the basic transcription machinery) also possibly influence nuclear receptor mobility. With that respect, FRET has emerged as a technique to characterize the mechanisms of cofactor recruitment following ligand binding in living cells (31, 32).

The microscopy studies published so far point to two major issues: first, despite some common features, each nuclear receptor displays specific distributions and dynamics, justifying a detailed analysis of each member of this family. Second, each receptor has its own spatial and temporal resolutions, which sometimes limit a comprehensive study. Our aim here was to study PPAR action in living cells by combining complementary microscopy techniques. As a first step, we characterize the function of enhanced yellow fluorescent protein (EYFP)-PPAR fluorescent chimeras. The presence and the physiological relevance of the fluorescent spots that may arise under certain experimental conditions was analyzed, and we demonstrate that these structures are non physiological and result from overexpression in individual cells. Regarding mobility, FRAP indicates that PPARs are highly mobile receptors that are not immobilized by ligand binding. A FCS study undertaken to circumvent FRAP time scale limitations gives two new major insights into PPAR behavior in living cells. First, in the absence of ligand, PPARs diffuse in the nucleus either in association with very big complexes and/or transiently interact with relatively immobile nuclear components. Second, ligand binding slows down PPARs, most probably by enhancing interactions with cofactors and chromatin. Moreover, FRET experiments demonstrate that PPARs readily form heterodimers with RXR in the absence of ligand. Finally, this study suggests guidelines for an appropriate examination of molecular behaviors in living cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rosiglitazone and 17β-estradiol were purchased from Sigma, Wy14,643 from Cayman Chemical Co. (Ann Arbor, MI), and 9-cis-retinoic acid from Biomol Laboratories (Plymouth Meeting, PA). L-165041 was synthesized in the laboratory by Marco Alves. The antibody directed against PPARγ has been previously described (33), and those directed against PPARβ, PPARγ, and GFP were purchased from Affinity Bioreagents (Golden, CO), Walk-Chemie (Steinbach, Germany), and Roche Diagnostics (Rotkreuz, Switzerland), respectively. The anti-bodies against the αβ/δ subunits of the 20 S proteasome and ubiquitylated proteins were from Affiniti Research Products (Manheim, UK).

**Plasmid Constructs**—DNAs encoding mouse PPARα, PPARγ, and PPAR-1 as well as RXRs were subcloned after PCR amplification into the pEYFP-N1 and -C1, and pECFP-N1 and -C1 plasmids (Clontech) using Xhol/BamHI, BglII/SalI, Xhol/KpnI, and BglII/SalI restriction sites, respectively. Wild-type mPPARα was expressed from a cDNA cloned in the pBK-CMV vector. The pEYFP1-C-ERα vector, the (PPRE),-luciferase (Luc) reporter construct and the ECFP-DEVD-EYFP construct were kind gifts of Drs. H. Vogel (EPFL, Lausanne, Switzerland), R. M. Evans (Salk institute, San Diego, CA), and J. M. Tavarez (Westphalian Wilhelms University, Münster, Germany), respectively.

**Cell Culture and Transient Transfection Assays**—COS-7 and MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen). Penicillin and streptomycin (Invitrogen) were added to the medium at 100 units/ml and 100 μg/ml, respectively.

Transient transfection assays were performed using Lipofectamine 2000 (Invitrogen), and luciferase activity assays were performed with the Promega dual reporter kit, according to the manufacturers’ instructions. Renilla luciferase encoded by the normalization vector pRLTK (Promega) was used as an internal control for firefly luciferase normalization. Cells were plated in 12-well plates for luciferase assays or 6-cm plastic dishes (Nunc) for live-cell images. Unless otherwise stated, transfections were performed with 100 ng of vector per cm² cell culture plate. After transfection, cells were left to recover in medium supplemented with 10% fetal calf serum for 5 h and grown in serum-free medium in the presence of PPAR ligands or vehicles for 18 h. Ligand concentrations were: 10⁻⁵ M for Wy14,643, 5.10⁻⁶ M for 1,165041, 10⁻⁷ M for rosiglitazone, 10⁻⁵ M for 9-cis-retinoic acid, and 10⁻⁷ M for 17β-estradiol.

**Western Blots**—Cells or tissues were lysed in ice-cold lysis buffer (20 mM Tris, 400 mM KCl, 2 mM diethiothreitol, 0.1 mM EDTA, glycerol 20%) supplemented with complete protease inhibitors (Roche Diagnostics) with a Dounce homogenizer. Protein extracts were resolved by SDS-PAGE as previously described (34). Primary antibodies against PPARα, PPARγ, and PPAR-γ were all used at dilutions of 1:2000 and the anti-Glc3P antibody was diluted 1:5000. Detection was performed using chemiluminescence (ECL advance, Amersham Biosciences).

**Pull-down Experiments**—For DNA pull-downs, plates were coated with oligonucleotides containing the malic enzyme PPAR response element as previously described (35). For cofactor pull-downs, the GST-p3002–516 fusion protein was purified as described previously (34). For both DNA and cofactor pull-downs, whole-cell extracts from transfected COS-7 cell were incubated 2.5 h at 4 °C in pull-down buffer in the oligonucleotide coated plates or with the GST-p3002–516 coated beads, and the appropriate PPAR ligands (Wy14,643 at 100 μM, L-165041 at 50 μM, and rosiglitazone at 10 μM). Plates or beads were washed four times with binding buffer and samples were boiled with 40 μl of 2× SDS-PAGE buffer and analyzed by Western blot.

**Wounded skin**—Wounded skin was prepared as described previously (36). Skin cryosections or transfected cells were fixed in 4% paraformaldehyde for 10 min. For the anti-PPARγ immunofluorescence, after an antigen-unmasking step (citrate buffer, pH 6, 100 °C, 10 min), the following processing was applied: wash with PBS, 0.1% Triton X-100 for 10 min; PBS for 5 min; 10% normal goat serum (Vector Laboratories) for 1 h; PBS, 2% BSA for 5 min. The primary antibody against PPARγ (Affinity Bioreagents) was applied overnight at 4 °C at 1/100th in PBS, 2% BSA, and slices were then incubated with a fluorescein isothiocyanate-coupled goat anti-rabbit IgG secondary antibody. Antibody incubations were followed by two 5-min washes in PBS, 2% BSA, and samples were mounted in DAPI containing Vectashield mounting medium (Vector Laboratories). For the anti-20 S proteasome and anti-ubiquitin immunofluorescence, a classical protocol was applied using PBS, 2% BSA as a blocking agent and antibody dilutions of 1/1000 for 1 h and 1/500 overnight at 4 °C, respectively. Labeling was then performed with a chicken anti-mouse secondary antibody coupled to Alexa594 (Molecular Probes).

**Confocal Imaging**—Live cells on LabTek chambered coverslides were washed once with phenol red free Optimem medium (Invitrogen) and the appropriate PPAR ligands or their vehicles and observed in a whole-cell configuration. For perfusion experiments, cells were grown on 18-mm round coverslides, placed in a Ludin perfusion chamber (Life Imaging Service), and perfused at 200 μl/min. All observations were performed at 37 °C on a TCS SP2 AOBS confocal microscope (Leica) equipped with a whole-microscope incubator (Life Imaging Service). Acquisitions were performed with a 63×/NA 1.2 water immersion objective for live-cell imaging and a 63×/NA 1.4 oil immersion objective for immunofluorescence.
cence observation. Observation of EYFP fusion proteins was done by exciting at 514 nm and detecting emission between 525 and 575 nm. DAPI, fluorescein isothiocyanate, and Alexa594 were measured in the following respective settings: excitation 410 nm/emission 410–465 nm, excitation 488 nm/emission 500–600 nm, and excitation 594 nm/emission 620–660 nm. For quantitative analyses, background-corrected 8-bit images were acquired with identical settings (same laser power and detector gain) and quantified using the Leica Confocal Software version 2.4.

FRET Experiments—Transfections were performed as described above, but expression levels of donor and acceptor proteins were adjusted to similar levels by Western blot. Fluorescence was recorded in three different settings: CFP*, 405 nm/CFP, 465–485 nm; YFP*, 514 nm/YFP, 525–545 nm; FRET*, 405 nm/FRET, 525–545 nm. Laser power and detector gain were adjusted in the different channels to observe equimolar concentrations of CFP and YFP at equal intensities (equimolar concentrations of CFP and YFP were obtained by expressing a fusion protein of CFP and YFP spaced by 475 residues). Settings were kept unchanged for analysis of all samples. CFP and YFP spectral bleeds-throughs (BT) in the FRET setting were determined on cells expressing CFP or YFP alone by calculating the intensity (I) ratios in the appropriate settings (I_{FRET}/I_{CFP} and I_{FRET}/I_{YFP}, respectively). FRET measured in co-expressing cells was then corrected for spectral bleed-throughs and normalized (NPFRET) for expression levels according to the following formula (37).

\[
\text{NPFRET} = \frac{I_{\text{FRET}} - I_{\text{YFP}}B_{\text{CFP}} - I_{\text{YFP}}B_{\text{YFP}}}{I_{\text{CFP}}} \quad \text{(Eq. 1)}
\]

The image of FRET was generated with the “PixFRET” plug-in for the ImageJ software. This plug-in can be downloaded from www.unil.ch/cig/papers1989.html.

FRAP Experiments—FRAP experiments were performed on Leica TCS SP2 AOBS or Zeiss LSM510 confocal microscopes. The LSM510 microscope was equipped with an argon 488-nm laser and a 505–550-nm bandpass filter, and image analysis was performed with the Zeiss LSM software version 3.2. For qualitative analysis (images only), images were acquired with a 256 × 256-pixel resolution. However, for the quantitative analysis presented in Fig. 5 (recovory curves), the resolution was switched to 128 × 128 pixels, and forward-reverse scanning was applied allowing acquisition of images every 100 ms. Photo-bleaching was performed with maximal laser power of the 488 laser line, and bleaching time was minimized by analyzing bleaching efficiency on fixed cells (eight scans at 800 Hz on the TCS SP2 and 50 iterations on the LSM510). In Fig. 6 (LSM510), the bleached region was a thin strip (100 × 5 pixels) across the nucleus not exceeding 10% of the total nuclear area. Experiments were performed with a pinhole set to 2.5 airy units. Fluorescence intensities were calculated in the bleached zone and over the entire cell nucleus with NIH ImageJ version 1.30 or Zeiss LSM softwares. Fluorescence recovery was normalized to the bleaching of the entire nucleus as described previously (38): recovery = \[
\frac{I(t) - I(0)}{I(^{\infty} - I(0))} \]

where I(0) is the average intensity in the region of interest in time point t, I(t) the average intensity in the region of interest during prebleach, T is the total nuclear intensity at time point t, and T is the average total nuclear intensity during prebleach. To extract half-recovery times and percentages of immobilization, the Origin 7.5 software (OriginLab, Northampton, MA) was used for nonlinear regression analysis. Recovery curves were fitted to the following equation.

\[
y(t) = y_0 + \frac{A_y}{1 - e^{-B_y t}} + A_y \left( 1 - e^{-B_y t} \right) \quad \text{(Eq. 2)}
\]

Maximal recovery (Mr) was the sum of \(y_0 + A_y\), and \(A_y\), and half-recovery time the value of t when \(y = Mr/2\).

FCS Experiments—FCS measurements were performed on a commercial LSM510 Confocor2 combination system (Zeiss, Jena, Germany) at room temperature. The 488-nm line of an argon-ion laser was focused through a Zeiss C-Apochromat 40×, NA 1.2 water immersion objective, and the fluorescence emission was recorded between 505 and 550 nm. The pinhole diameter was set to 90 μm, and detection was achieved using an avalanche photodiode. The 40×/40×/40× lateral radii of the detection volume were determined to be ~ 0.18 μm (488 nm excitation) from calibration measurements using standard dye (Rhodamine 6G, Molecular Probes). This value was used to convert diffusion times into diffusion coefficients. The setup was shown to be able to produce correct diffusion coefficients (39). Fluorescence intensity fluctuations were recorded at five spots in each nucleus. At each spot, measurements were performed over 25 s and repeated five times. On average, nine cells were analyzed. The autocorrelation curves were fitted to a one component model of free diffusion in three dimensions to derive the translational diffusion time through the confocal volume. Data were evaluated by Levenberg-Marquardt nonlinear least squares fitting to the appropriate model equations, using the ConfoCor2 or Origin software. The sizes of the EYFP-PPAR complexes were estimated by multiplying the molecular mass of EYFP by the factor \((D_{\text{EYFP}}/D_{\text{EYFP-PPAR}})^{2/3}\), assuming spherical symmetry of the complexes and normal diffusion in the viscous medium of the nucleus (i.e. EYFP experiences the same viscosity as the EYFP-PPAR complexes (40)).

RESULTS

Characterization of EYFP-PPAR Chimeras—To monitor PPAR action in living cells, we constructed expression vectors for the different PPAR isotypes fused to EYFP either at their N or C terminus (EYFP-PPAR and PPAR-EYFP, respectively). Proper expression of the EYFP-PPARα, β, and γ1 fusion proteins in COS-7 cells was confirmed by Western blot (Fig. 1A). Expression levels were not affected by the position of the fluorescent tag (data not shown). The main properties of nuclear receptors, i.e., ligand, DNA, and cofactor binding, were assessed for each of the fusion proteins. DNA binding was tested by analyzing by Western blot the retention of wild-type PPARα, EYFP-PPARα, and PPARα-EYFP from COS-7 cell extracts on a matrix coated with PPRE-containing oligonucleotides. In a similar assay, cofactor binding was assessed on a matrix coated with GST-p300. In these in vitro assays, PPARα (Fig. 1B), PPARδ, and PPARγ (data not shown) chimeras bound DNA and recruited p300 as well as their wild-type counterparts, and the EYFP fusion proteins displayed similar ligand-dependent binding to p300. We next assessed in COS-7 cells the transcriptional activity of these EYFP chimeras and of their wild-type counterparts on a reporter construct containing three copies of a PPRE. Importantly, because the vectors used to express the fusion proteins (pEYFP-NC1) confer high expression levels, the amounts of vector transfected were adjusted to have similar expression of the wild-type and EYFP fusion proteins. The PPARα fusion proteins could induce the transcription of the luciferase reporter gene by ~3-fold in the presence of a 10 μM concentration of the PPARα agonist Wy14,643 (Fig. 1C), whereas under conditions giving similar expression levels, the wild-type protein could activate transcription of the reporter gene ~5-fold. Transfection of higher amounts of EYFP-PPARα encoding vectors did not increase transcriptional activity significantly, suggesting that at 50 ng a plateau had almost been reached (Fig. 1C, inset). Similar activation profiles were obtained for wild-type and EYFP fusions to PPARβ and PPARγ1 (data not shown). These experiments show that the chimeras are active but induce transcription to reduced levels. Interestingly, the loss of activity caused by the EYFP tag was comparable in the N- and C-terminal chimeras. Decreased ligand affinity did not account for this impairment, since increasing the concentration of ligand up to 40 times did not further enhance transactivation, and both wild-type and chimeric receptors reached a plateau at the same ligand concentration (Fig. 1D). Moreover, ligand-dependent recruitment of p300 was not altered by the presence of the EYFP moiety in the pull-down assay (Fig. 1B). Altogether, these results demonstrate that the chimeric proteins are functional but display reduced transcriptional activities that do not result from a major defect in DNA binding (meaning that heterodimerization with RXR is not altered either), ligand binding, or recruitment of p300. These proteins can thus be used for further functional analysis.

EYFP-PPARs Are Localized in the Nucleus and Exhibit Three Distinct Expression Patterns—Insuring that expression levels of EYFP-PPARs are close to those of the endogenous proteins is a requisite for the biological relevance of the observations. We thus compared by Western blot the expression

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levels of EYFP-PPARα in transfected COS-7 cells to those of the wild-type receptor in mouse liver and kidney (Fig. 1E). Rather than using a housekeeping gene whose relative expression level in COS-7, liver, and kidney cells is not known, we adjusted total protein concentration in the lysates for the comparison of the different samples. Transfections with 200 ng of vector per cm² plate and an efficiency of 60–70% give average expression levels in protein extracts from tissues and COS-7 cells transfected with increasing amounts of an expression vector for EYFP-PPARα, EYFP-PPARβ, or EYFP-PPARγ expression in transfected COS-7 cells. Samples were resolved as described under “Experimental Procedures” with isotype-specific antibodies. As a first approach to understand the significance of the three patterns obtained for EYFP-PPARα, EYFP-PPARβ, or EYFP-PPARγ, we measured the diffuse pattern that was reshaped upon ligand treatment to a speckled pattern in all cells analyzed. Moreover, although the resolution of light imaging does not allow a precise description of the individual shape of flakes and speckles, a careful examination of the overall aspect of the nuclei allows to distinguish the two patterns. The speckles look like fuzzy patches fading in a diffuse background, whereas the structures formed by PPARs look sharper and more contrasted. These results suggest that the structures displayed by PPAR and ER reflect different nuclear organizations.

As a first approach to understand the significance of the variance of the fluorescence signal (see profiles along white lines in Fig. 2A). Cells with flakes and foci exhibited a fluorescent signal in average five times higher than cells with a diffuse pattern. Similar results were obtained when PPARα was fused to the N or C terminus of EYFP. Thus, whereas Western blot analysis showed that the average expres-

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**Fig. 1. Functional characterization of the EYFP-PPARs.** A, Western blot analysis of EYFP-PPARα, EYFP-PPARβ, and EYFP-PPARγ expression in transfected COS-7 cells. Samples were resolved as described under “Experimental Procedures” with isotype-specific antibodies. B, independent DNA and cofactor interaction assays were performed using either a biotinylated double-stranded oligonucleotide containing a PPRE (DNA pull-down) or GST-p300 (cofactor pull-down). Interactions with PPARα, EYFP-PPARα, or PPARγ-EYFP from COS-7 cell extracts were analyzed in the absence or presence of 100 μM of Wy14,643 and resolved with an anti-PPARα antibody. C, COS-7 cells were transfected with a (PPRE)₃-Luc reporter construct, increasing amounts of expression vectors for PPARα, EYFP-PPARα, or PPARγ-EYFP, and a control expression vector for Renilla luciferase. They were then grown 18 h in the presence or absence of 10⁻⁷ M Wy14,643. Each point was performed in triplicate, and firefly luciferase values were normalized to Renilla luciferase activity. D, transfections were performed as described for C with 500, 50, and 50 ng of vectors for PPARα, PPARγ-EYFP, or EYFP-PPARγ, respectively, and relative luciferase activity was analyzed after treatment with increasing concentrations of Wy14,643. E, Western blot analysis of PPARα expression levels in protein extracts from tissues and COS-7 cells transfected with increasing amounts of an expression vector for EYFP-PPARα (0, 10, 50, 100, and 200 ng/cm²). Identical extraction protocols were used, and equal amounts of total protein extracts were loaded on the gel. WT, wild type.
Expression of EYFP-PPARα in cells results in three different ligand-independent subnuclear localization patterns. COS-7 (A–C) or MCF-7 (D and E) cells were transfected with 200 ng/cm² of expression vectors for EYFP-PPARα (A–C) or EYFP-ERα (D and E) and analyzed on a Leica TCS SP2 AOBS confocal microscope. A, three typical localization patterns were observed in living cells expressing EYFP-PPARα. Fluorescence intensity fluctuation along the white line is presented under each picture. While quantification of fluorescence intensities along the white line was done on pictures acquired with identical settings (same laser power and detector gain), images with similar intensities were obtained by adjusting laser power and photomultiplier gain for each cell. Since maximal intensity values varied greatly between cell types (from 1 to 34), the fluorescence profiles had to be rescaled for presentation. B, different localization patterns are associated with different expression levels of EYFP-PPARα. Quantification of fluorescence intensities was done on pictures acquired with identical settings. C, the proportion of each localization pattern is not influenced by 10 μM Wy14,643. D, the continuous perfusion of 10 nM 17β-estradiol induces the formation of speckles whose aspect differs from the flakes and foci observed with PPAR. E, ER forms speckles in a ligand-dependent manner in all transfected cells. F, transfecting decreasing amounts of EYFP-PPARα expression vector decreases the proportion of cells exhibiting flakes and foci. All quantitative analyses were performed on at least 100 nuclei.

The use of a fluorescent chimeric protein may introduce a bias by potentially preventing the interaction of PPAR with partners dictating its localization within the cell. To confirm our results, immunolabeling experiments of the endogenous alpha and beta isotypes were performed on sections from various PPAR-expressing tissues. To assess the specificity of the labeling, we checked that: (i) absence of the primary antibody, (ii) incubation of the primary antibody with the epitope peptide, and (iii) labeling of sections from PPAR knock-out mice resulted in no or very faint background labeling. Only an antibody directed against PPARβ fully fulfilled these requirements. In line with our live cell data, we observed a diffuse nuclear localization of PPARβ in wounded skin sections (Fig. 3), a condition under which the expression of PPARβ is activated in keratinocytes (36). The absence of flakes or foci in vivo raised the question of the relevance of these substructures in transfected cells. Indeed, speckle formation is an important feature of the behavior of certain nuclear receptors, and to avoid misinterpretation, it is of importance to clarify the physiological relevance of speckle-like patterns such as flakes and foci.

One possibility was that these structures result from the aggregation of the EYFP fusion proteins. Such aggregates should favor the formation of PPAR homodimers that could be detected by FRET. Cyan and yellow fluorescent proteins (ECFP and EYFP) were used as donor and acceptor fluorophores. As controls for the absence and presence of FRET we used cells either co-transfected with separate expression vectors for EYPF and ECFP or with an expression vector encoding EYFP and ECFP in a single protein. Energy transfer occurred between PPARα-ECFP and PPARα-EYFP in cells with flakes and foci patterns and was significantly higher than in the negative control where ECFP and EYFP were co-expressed alone (Fig. 4A). The FRET obtained did not result from the weak dimerization of ECFP and EYFP (41), since FRET was neither detected in control cells expressing ECFP and EYFP alone nor in

**Fig. 2.** Expression of EYFP-PPARα in cells results in three different ligand-independent subnuclear localization patterns. COS-7 (A–C) or MCF-7 (D and E) cells were transfected with 200 ng/cm² of expression vectors for EYFP-PPARα (A–C) or EYFP-ERα (D and E) and analyzed on a Leica TCS SP2 AOBS confocal microscope. A, three typical localization patterns were observed in living cells expressing EYFP-PPARα. Fluorescence intensity fluctuation along the white line is presented under each picture. While quantification of fluorescence intensities along the white line was done on pictures acquired with identical settings (same laser power and detector gain), images with similar intensities were obtained by adjusting laser power and photomultiplier gain for each cell. Since maximal intensity values varied greatly between cell types (from 1 to 34), the fluorescence profiles had to be rescaled for presentation. B, different localization patterns are associated with different expression levels of EYFP-PPARα. Quantification of fluorescence intensities was done on pictures acquired with identical settings. C, the proportion of each localization pattern is not influenced by 10 μM Wy14,643. D, the continuous perfusion of 10 nM 17β-estradiol induces the formation of speckles whose aspect differs from the flakes and foci observed with PPAR. E, ER forms speckles in a ligand-dependent manner in all transfected cells. F, transfecting decreasing amounts of EYFP-PPARα expression vector decreases the proportion of cells exhibiting flakes and foci. All quantitative analyses were performed on at least 100 nuclei.

**Fig. 3.** PPARβ displays a diffuse expression in the nucleus of keratinocytes in wounded skin sections. Wounded skin sections were immunolabeled for PPARβ using a fluorescein isothiocyanate-coupled secondary antibody as described under the methods section. Two magnifications are shown. SC, stratum corneum; HF, hair follicle.
cells expressing PPARs tagged at their N terminus. To evaluate FRET in cells with a diffuse pattern, microscope settings required to be adjusted to the lower level of fluorescence (higher laser power and detector gain). In these cells, the normalized FRET signal between PPAR/H9251-ECFP and PPAR/H9251-EYFP was not statistically different from the negative control (Fig. 4B). These results therefore reveal that PPAR/H9251 aggregates only in cells exhibiting flakes and foci patterns. Co-expressing unlabeled RXR/H9251 affected neither flakes and foci formation nor FRET (data not shown). Since PPARs only activate transcription as heterodimers with RXR, these results support the idea that these structures correspond to a transcriptionally inactive pool of receptor, resulting from its overexpression.

The mobility of nuclear proteins is an important parameter to understand the temporal organization of nuclear processes and their adaptation to physiological challenges. Thus, we first investigated by FRAP the mobility of PPARs in cells exhibiting different fluorescence profiles. In cells with a diffuse pattern, EYFP-PPAR (Fig. 4C) as well as EYFP-PPARβ and EYFP-PPARγ (data not shown) were highly mobile, and no immobilization of the proteins was observed as total re-colonization of the bleached zone could be obtained. When cells displaying flakes or foci patterns were photobleached, only 40% of the initial fluorescence signal could be recovered, both in the presence and absence of ligand, therefore revealing a high degree of immobilization of the receptor in those structures (Fig. 4C). It is noteworthy that cells in which a heterogeneous labeling is hardly distinguishable from the diffuse background already display altered dynamics (Fig. 4C). Not only the degree but also the rate of recovery was extremely low, indicating that very limited shuttling occurs between foci. Together with the previous demonstration by FRET of aggregate formation, the FRAP data further indicate that flakes and foci constitute an inactive cellular pool.

These results demonstrating the nonphysiological formation of flakes and foci suggested that the aggregated receptors might undergo an active degradation process. Cells expressing EYFP-PPARα were therefore immunolabeled with antibodies directed against the 20 S subunits of the proteasome or against ubiquitylated proteins. A clear co-localization between the 20 S proteasome and the flakes and foci could be observed (Fig. 5A), whereas the patterns observed with the antibody directed against ubiquitylated proteins did not overlap with these structures (Fig. 5B).

**FRAP and FCS Study of PPAR Mobility in Diffuse Cells**—The effect of ligand treatment on PPAR mobility was then investigated for the three PPAR isotypes as well as for RXR in the relevant model of a diffuse expression pattern (Fig. 6). As outlined previously, the diffusion of EYFP-PPARs was very fast and no immobilization of the receptors was observed. Ligand
treatment did not influence the recovery rates (Fig. 6, A, C, and D) and the half recovery times of both liganded and unliganded receptors were estimated in the 100-millisecond time range (Table I). As PPARs are active as heterodimers with RXR, we analyzed the influence of RXR on PPAR mobility. Co-expressing unlabeled RXR with EYFP-PPAR did not affect the recovery of EYFP-PPAR.
PPARs and ER differ not only in their subnuclear localization ligand treatment. This study also highlights that liganded are highly mobile receptors which are not immobilized upon (Table I), in agreement with previous reports (18, 19, 27).

of the receptors and a 3.3-fold increase of the half-recovery time whereas estrogen treatment induced an immobilization of 32% in the absence of ligand, 10% of the receptors were immobilized

B

and MCF-7 cells (data not shown). In the

D

FRET experiments strongly suggest that, even in the absence that heterodimer formation is not restricted to a subcompart-

ment. Similar distributions were observed in the absence (Fig. 6B) or presence (data not shown) of ligand. Altogether, these

FRAP studies reveal that PPARs and RXR are highly mobile receptors which are not immobilized upon ligand treatment. This study also highlights that liganded PPARs and ER differ not only in their subnuclear localization but also in their mobility.

Fluorescence correlation spectroscopy is a method based on the analysis of fluctuations of fluorescence intensity due to the diffusion of a labeled protein through a small volume (in the range of 1 μm³) (42, 43). It allows us to study diffusion of mobile molecules at a higher spatial and temporal (microsecond range) resolution than FRAP.

FCS measurements were done on COS-7 cells expressing EYFP, EYFP-PPARα, EYFP-PPARβ, or EYFP-PPARγ. Although we observed a pronounced heterogeneity of diffusion times for each individual measurement (Fig. 7A), data were fitted with a one-component model of free diffusion (Fig. 7B; Table II) with no necessity to imply a second slower component, a reduced dimensionality factor, or anomalous diffusion (44). The respective concentrations of all the fluorescent proteins were estimated to range between 3 and 100 nM, after initial photobleaching. Assuming that the volume of the cell nucleus is 2000 μm³, we calculated that 0.8 μm² s⁻¹ nm² 0.10 ± 0.06, 0.8 ± 1.7

EYFP-PPARγ + L-165041

0.16 ± 0.05, 3.0 ± 2.7

EYFP-PPARγ + 9-cis-retinoic acid

0.25 ± 0.08, 6.4 ± 2.8°

EYFP-PPARα + RXRα

0.15 ± 0.07, 4.7 ± 4.1

EYFP-PPARα + RXRα + Wy14,643

0.17 ± 0.08, 2.1 ± 1.4

EYFP-ERα

0.24 ± 0.09°, 9.9 ± 6.9°

EYFP-ERα + 17β-estradiol

0.80 ± 0.37°, 31.7 ± 9.4°

% immobilization

s

Table 1

| Fusion Protein | 1/2 t-recovery | % Immobilization |
|---------------|---------------|-----------------|
| EYFP-PPARα    | 0.13 ± 0.05   | 1.7 ± 1.9       |
| EYFP-PPARα + Wy14,643 | 0.15 ± 0.06 | 2.4 ± 2.5       |
| EYFP-PPARβ    | 0.10 ± 0.06   | 1.4 ± 1.7       |
| EYFP-PPARβ + L-165041 | 0.16 ± 0.05 | 3.0 ± 2.7       |
| EYFP-PPARγ    | 0.10 ± 0.07   | 5.0 ± 5.7       |
| EYFP-PPARγ + rosiglitazone | 0.12 ± 0.08 | 3.3 ± 2.5       |
| EYFP-RXRα     | 0.20 ± 0.08   | 1.9 ± 2.9°      |
| EYFP-RXRα + 9-cis-retinoic acid | 0.25 ± 0.08 | 6.4 ± 2.8°      |
| EYFP-PPARα + RXRα | 0.15 ± 0.07 | 4.7 ± 4.1       |
| EYFP-PPARα + RXRα + Wy14,643 | 0.17 ± 0.08 | 2.1 ± 1.4       |
| EYFP-ERα      | 0.24 ± 0.09°  | 9.9 ± 6.9°      |
| EYFP-ERα + 17β-estradiol | 0.80 ± 0.37° | 31.7 ± 9.4°     |

a Statistically significant difference according to a t test with p value < 0.01.

PPARα both in the absence and presence of a PPARα ligand (Fig. 6E). Moreover, EYFP-RXRα displayed a diffusion similar to PPARα in the absence of ligand (Fig. 6E and Table I). As a control of ligand effect on mobility, the diffusion of EYFP-ERα was analyzed in the absence and presence of 17β-estradiol both in COS-7 (Fig. 6B) and MCF-7 cells (data not shown). In the absence of ligand, 10% of the receptors were immobilized whereas estrogen treatment induced an immobilization of 32% of the receptors and a 3.3-fold increase of the half-recovery time (Table I), in agreement with previous reports (18, 19, 27).

Altogether, our FRAP studies reveal that PPARs and RXR are highly mobile receptors which are not immobilized upon ligand treatment. This study also highlights that liganded PPARs and ER differ not only in their subnuclear localization but also in their mobility.

PPAR Dynamics and Interactions in Living Cells

DISCUSSION

Understanding protein behavior, localization, trafficking, and participation to complexes in living cells has remained so far difficult. With that respect, our analysis provides important clues to establish the appropriate and biologically relevant settings to further study fluorescent fusion proteins in living cells. Moreover, a detailed approach allowed a reliable examination of the behavior of the three PPAR isotypes and thereby unveiled important new insights on PPAR mode of action.

Criteria for a Relevant Model to Study PPAR Action in Living Cells—First, the different properties of the fluorescent receptors were evaluated. Ligand, DNA, and pS300 binding were not affected by addition of the EYFP moiety, and the various constructions were able to activate transcription, albeit to a lower extent than their wild-type counterparts. Proper DNA binding also attested of their normal capabilities to heterodimerize with RXR. However, these experiments are not sensitive enough to detect subtle changes and the decreased transcriptional activity of the fusion proteins could result from the addition of small impairments. Also, we cannot rule out that

μm² s⁻¹, and 2.3 ± 1.1 μm² s⁻¹ for the α, β, and γ isotypes, respectively (Fig. 7, C–E; Table II). This shift in mobility indicates that the conformational change induced by ligand results in the docking of new factors to the receptor and/or strengthens the interaction with slow nuclear components.

PPAR and RXR Form Heterodimers in Living Cells in the Absence of Ligand—Our FCS measurements suggested that PPARs contact numerous nuclear components, as suggested by the broad distribution of the diffusion coefficients, even in the absence of ligand. One question that has never been clearly answered so far is whether PPAR and RXR form heterodimers prior to ligand binding. A first clue was that PPAR and RXR exhibit similar diffusion coefficients (Fig. 7F; Table II), but FRET experiments were necessary to prove a direct interaction in living cells. Cells were transfected with combinations of expression vectors for ECFP/PPARα, ECFP/PPARβ, ECFP/ PPARγ, and EYFP/RXRα giving equivalent expression levels of donor and acceptor proteins (data not shown). Energy transfer between any PPAR-ECFP and RXRα-EYFP was significantly higher than in the negative control where ECFP and EYFP were co-expressed alone (Fig. 8A). This positive FRET signal yet occurred at lower levels than in the positive control were CFP and YFP are fused, possibly because of less favorable fluorophore positioning in the PPAR-ECFP/RXRα-EYFP dimer. While adding ligand did not change FRET values for PPARβ and PPARγ, addition of a PPARα ligand induced a small but reproducible increase of FRET. This suggests that ligand binding either increases the affinity of PPARα for RXR or orientates the ECFP moiety in a more favorable manner for FRET. Note that FRET did not occur between the N-terminal fusion proteins ECFP-PPARs and EYFP-RXRα, suggesting that, in such heterodimers, the N-terminal domains of the receptors are too far from each other to allow energy transfer. To localize heterodimer formation within the nucleus, a pixel-by-pixel analysis was performed on cells transfected with PPARα-ECFP and RXRα-EYFP (Fig. 8B). When the FRET channel was corrected for the spectral ECFP and EYFP bleedthroughs and for fluorophore expression levels, an NRET signal could be observed throughout the entire nucleus, showing that heterodimer formation is not restricted to a subcompartment. Similar distributions were observed in the absence (Fig. 8B) or presence (data not shown) of ligand. Altogether, these FRET experiments strongly suggest that, even in the absence of ligand, PPAR and RXR readily form heterodimers in the nucleus of living cells.
the binding of other cofactors than p300 in the cell is affected, accounting also for the reduced transactivation observed.

The second important issue concerns the localization of PPARs and the discrimination between relevant speckles and nonfunctional aggregates. We observed that the three PPAR isotypes and RXRα were localized in the nucleus of living COS-7 cells and excluded from nucleoli, in accordance to previous reports (22, 45, 46). Immunolabeling of PPARα in mouse skin sections corroborated the results obtained with EYFP-PPARs. PPARs did not form the physiological ligand-induced speckles observed for ER. Neither did RXRα, in contrast to previously reported data (22). The fluorescent patterns observed after transfection, which we deliberately termed flakes and foci and not speckles, appeared to be artifacts due to overexpression and aggregation of the fusion proteins in individual cells. Importantly, we also observed such flakes and foci for RXRα and ERα in cells expressing high levels of the receptors. Abnormal localization of the estrogen receptor due to overexpression has been also reported before and was shown to bias studies on nuclear matrix association (16, 47, 48). Problems linked to the overexpression of RXR have also been pointed to recently (49). This immobilization in nonfunctional structures is therefore likely to be of general occurrence for nuclear receptors and possibly for other nuclear proteins upon transfection. Mislocalization and abnormal mobilities of proteins due to overexpression or mutations have been linked to nuclear matrix association and/or proteasome function in several studies (see Ref. 48 for a review). We observed a co-localization between the flakes and foci formed by EYFP-PPARα and the subunits of the 20 S proteasome, which constitute the catalytic core of the complex, but these structures were not enriched in ubiquitin. Interestingly, some unfolded proteins can be degraded by the proteasome in the absence of ubiquitylation (50). It is therefore tempting to speculate that the flakes and foci correspond to an

**Fig. 7.** Ligands reduce the diffusion rates of EYFP-PPARs. Diffusion of EYFP (A and B), EYFP-PPARα (C), EYFP-PPARβ (D), EYFP-PPARγ (E), and EYFP-RXRα (F) was analyzed by FCS in transfected COS-7 cells in the absence or presence of the respective ligands. B, the autocorrelation curves, shown here for EYFP, were fitted with a one-component model of free diffusion. A and C–F, frequency histograms of the diffusion coefficients representing at least 200 measurements.
PPAR Dynamics and Interactions in Living Cells

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PPAR Are Associated with RXR Prior to Ligand Binding—Our FRET data demonstrate for the first time that in living cells PPARs readily associate with RXR in the absence of ligand. This result challenges the current general view of PPAR action where heterodimerization with RXR is often presented as a consequence of ligand binding. Moreover, our data also suggest that PPAR/RXR dimerization occurs prior to promoter binding because: (i) the receptors are in large excess with respect to the number of PPAR target genes, (ii) the FRET signal is present all over the nucleus with no particular sign of enrichment in any region, and (iii) we recently published chromatin immunoprecipitation experiments showing that stable promoter binding occurs in vivo only after ligand treatment on the PDK1 promoter (35).

PPARs Have Unexpected Small Diffusion Coefficients in Living Cells—When studied by FRAP, PPARs appear as highly mobile nuclear receptors which are not immobilized upon ligand binding, as similarly reported for RAR and TR (19, 51) and in contrast to ER and GR (18, 19, 27, 28). These results therefore suggest that ligand-induced immobilization of nuclear receptors could be a hallmark of homodimerization. However, the limited temporal resolution of this technique does not allow to observe very fast events such as transient interactions for example.

FCS, a technique providing quantitative information on diffusion of fluorescent molecules in the microsecond time range, was used here for the first time to assess the mobility of a nuclear receptor in living cells. A very interesting result is that unliganded PPARs have diffusion coefficients (~5 μm²s⁻¹), which are much smaller than those expected if the receptors were freely diffusing in the nucleus as monomers (~14 μm²s⁻¹) or even as heterodimers with RXR (~11.5 μm²s⁻¹). These data actually reflect the association of PPARs with other proteins and/or their transient interactions with very slow nuclear components. Assuming that PPARs are associated with soluble factors only, a diffusion coefficient of 5 μm²s⁻¹ indicates the engagement in a complex of 1 to 2 MDa (Table II). Such factors may be corepressors (52, 53) or alternatively chaperones that are thought to be essential at least for the diffusion properties of GR and PR (30). However, it is highly probable that PPARs also interact transiently with rather immobile components such as the nuclear matrix or chromatin, as suggested recently for other nuclear factors (54, 55).

Interestingly, the mobility of the three PPAR isotypes was reduced upon ligand binding. This may reflect the recruitment of cofactors, leading to the formation of complexes of higher molecular masses. However, the estimated sizes of these complexes are very important (Table II) and it is likely that the decrease in diffusion coefficients due to ligand binding also reflects enhanced interactions with very slow nuclear components such as chromatin. These increased interactions could potentially result from either an increase in affinity or from a stabilization of the interactions leading to longer binding events. Since the number of PPAR target genes in a cell is low (probably tens to hundreds) compared with the number of EYFP-PPAR molecules, some of the reported effects may result from interactions with bona fide PPREs, but most of them probably result from transient and unspecific interactions at sites resembling PPREs but not located within active promoters, as described previously for NF-κB (51). Interestingly, a

Table II

Diffusion coefficients of EYFP-PPAR complexes

Masses were estimated from the average diffusion coefficients as described under "Experimental Procedures." They are hypothetical and calculated under the assumption that the diffusion time of EYFP and EYFP-PPAR is determined by normal diffusion in a viscous (nuclear) medium.

| Complex | Diffusion coefficient | Average complex mass |
|---------|----------------------|----------------------|
| EYFP (nucleus) | 19.7 ± 7.8 | |
| EYFP-PPARα | 4.8 ± 1.9*a | 1913 |
| EYFP-PPARα + Wy14,643 | 3.0 ± 1.8*a | 7767 |
| EYFP-PPARβ | 5.5 ± 2.3*a | 1227 |
| EYFP-PPARβ + L-165041 | 3.5 ± 2.0*a | 4852 |
| EYFP-PPARαy | 5.0 ± 2.5*a | 1657 |
| EYFP-PPARαy + rosiglitazone | 2.3 ± 1.1*a | 17,182 |
| EYFP-RXα | 4.6 ± 1.7 | 2142 |

*a Statistically significant difference between vehicle and ligand treatment according to a t test with p value < 0.01.

A

B

**FIG. 8.** PPARs and RXRs form heterodimers in living cells in the absence of ligand. Live COS-7 cells transfected with 100 and 50 ng/cm² PPARs-EYFP and RXRs-EYFP, respectively, were subjected to FRET analysis. A, NFRET in the nuclei of cells with a diffuse pattern compared with NFRET in cells transfected with expression vectors for free or linked EYFP and ECFP proteins. At least 50 cells were analyzed. * and **, statistically significant difference with the negative control ECFP + EYFP (*) or with the corresponding vehicle treatment (**) according to a t test with p value < 0.01. B, pixel-by-pixel analysis of FRET on a cell expressing PPARα-EYFP and RXRα-EYFP, performed with the FijiFRET plug-in for the ImageJ software.

accumulation of misfolded receptors that the cell tries to remove using the proteasome pathway.

Thus, our results point out several technical pitfalls linked to live cell imaging; (i) when assessing the activity of a GFP-tagged transcription factor, it is of importance to compare activity at similar expression levels of the chimera and the wild-type proteins; (ii) it is also important to precisely characterize the nature and the conditions of occurrence of subnuclear structures to rule out potential artifacts. The occurrence of ligand-induced speckles should also be demonstrated by following a single cell before and after ligand addition and by statistically describing the phenomenon reported at the cell population level; (iii) since over the whole population important differences in the level of expression of transfected proteins are observed, assessing mean expression levels by Western blot is not sufficient to define relevant experimental conditions, hence, appropriate selection of the individual cells analyzed is necessary; (iv) when possible, immunolabeling of the endogenous protein, like it was done here on skin sections, should be performed to support live cell studies.

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recent publication proposes a model where transcription factors permanently perform a three-dimensional scanning of the genome (55). Extrapolating this model to PPARs is a possibility we are currently assessing, among others, by studying the mobility of PPAR mutants deficient for DNA binding and co-factor recruitment.

Altogether, this study demonstrates that both in the presence and absence of ligand, all PPARs are engaged in complexes of very high molecular masses and/or in transient interactions with very slow nuclear components. Moreover, PPAR and RXR are readily associated in the cell prior to ligand binding. Importantly, we also demonstrate here the usefulness of FCS when studying the mobility of nuclear receptors in living cells.

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