Association with Coregulators Is the Major Determinant Governing Peroxisome Proliferator-activated Receptor Mobility in Living Cells*

The nucleus is an extremely dynamic compartment, and protein mobility represents a key factor in transcriptional regulation. We showed in a previous study that the diffusion of peroxisome proliferator-activated receptors (PPARs), a family of nuclear receptors regulating major cellular and metabolic functions, is modulated by ligand binding. In this study, we combine fluorescence correlation spectroscopy, dual color fluorescence cross-correlation microscopy, and fluorescence resonance energy transfer to dissect the molecular mechanisms controlling PPAR mobility and transcriptional activity in living cells. First, we bring new evidence that in vivo a high percentage of PPARs and retinoid X receptors is associated even in the absence of ligand. Second, we demonstrate that coregulator recruitment (and not DNA binding) plays a crucial role in receptor mobility, suggesting that transcriptional complexes are formed prior to promoter binding. In addition, association with coactivators in the absence of a ligand in living cells, both through the N-terminal AB domain and the AF-2 function of the ligand binding domain, provides a molecular basis to explain PPAR constitutive activity.

Peroxisome proliferator-activated receptors (PPAR)\(^4\) \(\alpha\) (NR1C1), \(\beta/\delta\) (NR1C2 referred to as \(\beta\) herein), and \(\gamma\) (NR1C3) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily (1). PPARs are lipid sensors capable of adapting gene expression to integrate various lipid signals coming from intracellular signaling pathways, from inter-organ cross-talk, or even from the diet (2). Given their partially overlapping yet specific expression patterns, the three receptors cooperate to efficiently regulate metabolic functions (3, 4) as well as other cellular processes such as proliferation, differentiation, or apoptosis that are essential to the fate of the tissues and organs in which they are expressed (2, 5). PPARs are also important pharmaceutical targets as specific synthetic ligands exist for the different isotypes, and these are either currently used or hold promise for the treatment of major metabolic disorders such as hyperlipidemia, type 2 diabetes, and obesity (6). Thus, understanding the molecular mechanisms that account for target gene specificity represents a major challenge that will help to develop safer and more efficacious drugs.

At the structural level, PPARs are composed of distinct domains that constitute the hallmark of the nuclear receptor superfamily. The central DNA binding domain allows binding to specific PPAR-response elements located in the promoter of target genes. Binding to these elements requires heterodimerization with the retinoid X receptor (RXR), the PPAR/RXR complex being constitutively associated prior to ligand binding (7). The C-terminal ligand binding domain encompasses the ligand-dependent transactivating function (called AF-2). It accounts for the major effects the receptors exert on gene transcription by recruiting accessory proteins called coactivators, which can remodel chromatin and contact the basal transcriptional machinery (8). In contrast, the N-terminal AB domain is less structured but includes a weak ligand-independent transactivating function (called AF-1), which presumably acts through the basal recruitment of coactivators (9) and by modulating the structure and the activity of the entire receptor when post-translationally modified (2, 10). Interestingly, the specificity and the efficacy of target gene regulation by the different PPARs rely on an interplay between the actions of these different domains, the balance between corepressor and coactivator binding, and the promoter context (2, 11–13).

---

*This work was supported by grants from the National Research Project 50, the Swiss National Science Foundation, the Etat de Vaud (to W. W. and B. D.), the Research Council (to C. T.), Grant GOA 2006/09 (to Y. E.) from the Katholieke Universiteit Leuven, and Project G.0584.06 from the Fund for Scientific Research Flanders (to Y. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence may be addressed: Laboratory of Biomolecular Dynamics, Katholieke Universiteit Leuven, Celestijnenlaan 200G, B-3001 Leuven, Belgium. Tel.: 32-16-32-71-60; Fax: 32-16-32-79-74; E-mail: yves.engelborghs@fys.kuleuven.be.

2 To whom correspondence may be addressed: Friedrich Miescher Institute, WRO 1066.0.52, Maulbeerstrasse 66, CH-4058 Basel, Switzerland. Tel.: 41-61-69-78-590; E-mail: laurent.gelman@fmi.ch.

3 The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; AD, anomalous diffusion; AOTF, acousto-optic tunable filter; AR, androgen receptor; CFP, cyan fluorescent protein; dCFCCS, dual color fluorescence cross-correlation spectroscopy; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FCS, fluorescence correlation spectroscopy; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; EGFP, enhanced GFP; GR, glucocorticoid receptor; mRFP, monomeric red fluorescent protein; PIC, protease inhibitor cocktail; RAR, retinoic acid receptor; RID, receptor interacting domain; RXR, retinoid X receptor; WT, wild type; YFP, yellow fluorescent protein; ER, estrogen receptor; PMSF, phenylmethylsulfonyl fluoride.
PPAR Dynamics and Interactions in Living Cells

Most structural and regulatory proteins of the nucleus are extremely dynamic, and their mobility is associated with major events, such as the control of nuclear architecture or the regulation of gene expression and mRNA export (14), mobility being required for embryonic stem cell differentiation programs, for example (15). As a consequence, the association of a large number of nuclear proteins and transcription factors with chromatin is transient, suggesting that these proteins find their targets through a three-dimensional scanning of the genome (16). The interactions of nuclear receptors with chromatin are also highly dynamic because their residence time on promoters has been estimated to be in the range of 10 s using models with multimerized response elements compatible with live cell imaging (17). In addition, the global association of nuclear receptors and coregulators with endogenous promoters is cyclical (18, 19). Finally, using fluorescence recovery after photobleaching (FRAP), certain receptors such as the estrogen, glucocorticoid, and androgen receptors (ER, GR, and AR) exhibit reduced mobility and partial immobilization upon agonist treatment (20–24). On the contrary, this is not observed with other receptors such as PPARs (7) and the thyroid hormone and retinoic acid receptors (21). As reduction in mobility and enhanced immobilization upon ligand binding affect only certain receptors and correlate with their ability to accumulate in subnuclear structures termed speckles, it has been speculated that these reduced dynamics could actually be linked to the engagement of the receptor in these so far poorly defined structures (22, 25).

Fluorescence correlation spectroscopy (FCS) is an alternative to FRAP for the analysis of protein mobility in living cells with high spatial and temporal resolution. By using this technique, we were able to demonstrate that although PPARs do not form ligand-induced speckles and are not immobilized upon ligand binding in FRAP experiments, they nevertheless have a smaller diffusion coefficient than expected for a PPAR/RXR heterodimer and do exhibit a ligand-dependent reduction in mobility (7). In this study, we dissected the molecular mechanisms governing PPAR mobility. PPAR diffusion is governed by the association with coregulators, both in the absence and presence of ligand, and the size of the complexes is enlarged upon agonist binding. In contrast, DNA binding has no global impact on the diffusion of the entire population, and PPARs most probably do not perform a three-dimensional scanning of chromatin to reach their targets. Interestingly, our data also demonstrate that the N-terminal AB domain plays an important role in coregulator recruitment in living cells.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The cloning of the cDNAs encoding mouse PPARα, PPARβ, and PPARγ1 as well as RXRα into the pEYFP-N1 and -C1 and pECFP-N1 and C1 plasmids (Clontech) has been described previously (7). The ECFP-DEVD-EYFP construct was a kind gift of Dr. J. M. Tavare (Westphalian Wilhelms University, Münster, Germany). The cDNA for the monomeric red fluorescent protein (mRFP) was kindly donated by Dr. R. Tsien (26). The expression vector for the mRFP-PPARα fusion protein was generated by flanking the mRFP cDNA with the Nhel and Xhol sites by PCR amplification and cloning of the PCR product into pEYFP-C1-mPPARα in place of EYFP. The EYFP-mPPARα13 construct was generated by PCR amplification of the truncated cDNA followed by cloning into pEYFP-C1 using the Xhol and BamHI restriction sites. The EYFP-C1-mPPARαC122S construct was generated from the corresponding WT vector by site-directed mutagenesis of codon 122 (TGT to AGT) and the EYFP-mPPARαΔAB by deletion of the bases corresponding to residues 21–99. The plasmid encoding EYFP-p300N-term was constructed by cloning bases 1–756 from pEGFP-p300 into pEYFP-C1 using HindIII and BamHI as restriction sites. The EYFP-Med1 receptor interacting domain (RID) construct was generated by cloning a PCR amplification product corresponding to residues 550–716 of Med1 into pEYFP-C1, using BglII and Sall.

Cell Culture and Transient Transfection Assays—Cells were grown and transfected as described before (7). The generation of the HeLa H2B-EYFP cell line has been reported previously (27). Wy14,643 (Cayman Chemical Co.), L-165041 (sized at custom in the CIG Laboratory), rosiglitazone (Sigma), GW6471 (synthesized at custom by Zydis, India), and 9-cis-retinoic acid (Biomol) were used at respective final concentrations of 10−5, 5 × 10−6, 10−6, 10−5, and 10−6 M.

FCS Experiments—Transfections were performed as described previously in 8-well chambered cover glasses with 100 ng of vector per cm2 of cell culture plate (7). Measurements in living cells were also performed as reported previously on a commercial LSM510 Confocor2 combination system (Zeiss) at room temperature (7). Fluorescence intensity fluctuations were recorded using an avalanche photodiode at five spots in each nucleus. At each spot, measurements were performed over 25 s and repeated five times. On average, 10 cells per condition were analyzed. For the nuclear extracts a laser beam was focused 200 μm above the bottom of a cover slide where 10 μl of extract was deposited.

dcFCCS Experiments—Cellular dcFCCS measurements were performed on the LSM510/Confocor2 system with a water immersion objective (C-Apochromat, ×40, 1.2NA; Zeiss). The 488-nm line of the argon laser (AOTF 0.5%, ~25 microwatts) was used to excite EGFP, and the 543-nm line of the HeNe laser (AOTF 7%, ~70 microwatts) was used to excite mRFP. The excitation light was reflected by a dichroic mirror (HFT 488/543) and focused through a type C-Apochromat ×40/1.2W objective lens. The fluorescence emission light was split by a second dichroic mirror (NFT 570) into two separate beam paths and passed through a 505–530-nm bandpass filter for EGFP fluorescence and a 600–650-nm bandpass filter for mRFP fluorescence. Each confocal pinhole diameter was set to 70 μm. After preparation of the cells in 8-well chambered cover glasses (NUNC A/S), laser scanning microscopy was used to search for cells suitable for dcFCCS. The laser beam was focused at a selected spot, and a dcFCCS measurement (10 times for 20 s) was performed. After the fitting of the curves (see below), the relative cross-correlation (degree of binding) was calculated from the following formula: 

\[ CC_{rel} = \frac{G_{cross}(0) + G_{cross}(0)}{G_{cross}(0) + G_{cross}(0)} \]

with \( G_{cross}(0) \) and \( G_{green}(0) \) being the amplitudes of the cross- and green autocorrelation curves, respectively. The
cross-talk between channels was also taken into account for the calculation of interactions (28).

Fitting of FCS Correlation Curves—Auto- and cross-correlation curves were fitted with the Origin software (OriginLab) by a Levenberg-Marquardt nonlinear least square method to a free (nuclear extracts or living cells) or an anomalous (living cells only) diffusion model to derive the translational diffusion time through the confocal volume. The apparent diffusion coefficients obtained for each curve were then averaged or represented as a frequency histogram.

Nuclear Extracts—COS-7 cells were transfected in 6-well plates. At 36 h post-transfection, cells were washed with phosphate-buffered saline, harvested, and centrifuged. The pellet was lysed in 400 μl of hypo-osmotic buffer (10 mM Tris, 10 mM NaCl, 0.5 mM dithioretiol, 1× protease inhibitor mixture (PIC), 0.2 mM PMSF) with a Dounce homogenizer. Nuclei were then isolated by centrifugation at 12,000 rpm during 5 min and lysed in 50 μl of hyper-osmotic buffer (50 mM Tris, 400 mM KCl, 1 mM EDTA, 0.1% Triton, 10% glycerol, 1× PIC, 0.2 mM PMSF). After centrifugation, the supernatant was diluted with 3 volumes of 50 mM Tris, 1 mM EDTA, 10% glycerol, 1× PIC, 0.2 mM PMSF to bring the salt concentration to 100 mM. Nuclear extracts were stored at −20 °C and incubated with ligands for 2 h at 4 °C before FCS analysis.

Pulldown Experiments—DNA and coregulator pulldowns were performed as described previously (9, 29). Ligands were both used at 100 μM.

Western Blots—Cells were lysed and protein extracts were resolved by SDS-PAGE as described previously (9). The primary antibody against GFP (Roche Applied Science) was diluted at 1:5000 and detection was performed using enhanced chemiluminescence (ECL advance; Amersham Biosciences).

FRET Experiments—Donor and acceptor proteins were adjusted to similar levels by Western blot, and sensitized emission FRET was performed as described previously (30). Briefly, fluorescence was recorded in three different settings as follows: CFPex, 405 nm/CFPem, 465–485 nm; YFPex, 514 nm/YFPem, 525–545 nm; and FRETex, 405 nm/FRETem, 525–545 nm. Laser power and detector gain were adjusted in the different channels in order to observe equimolar concentrations of CFP and YFP at equal intensities, and settings were kept unchanged for analysis of all samples. FRET was calculated using the expNFRET formula that corrects spectral bleed throughs by using an exponential fit of the spectral bleed through ratio and normalizes for differences in donor and acceptor expression (30).

Statistical Analyses—Data are presented as means ± S.E., and p values for statistical significance were evaluated with a Student’s t test.

RESULTS

An Anomalous Diffusion Model Is a Better Model for Fitting PPAR FCS Autocorrelation Curves—FCS is one of the most sensitive techniques currently available in single molecule analysis. FCS is based on an analysis of fluctuations of fluorescence intensity because of the diffusion of fluorescent molecules through a very small volume (in the range of 1 μm3). Among a multitude of physical parameters that are accessible by FCS, it most conveniently enables the direct measurement of diffusion coefficients and local concentrations (31). The fluorescence signal recorded at microsecond resolution (Fig. 1A) is used to extract a so-called “autocorrelation curve” (Fig. 1B, upper panels). The experimental points along this curve are then fitted with an autocorrelation function G(t) that mathematically reflects the diffusion properties of the fluorescent molecules.

In a previous study analyzing the localization and diffusion of EYFP-tagged PPARs in living cells (7), we used a one-component model of free diffusion to fit autocorrelation curves and thereby obtained satisfactory statistical (χ2) values. However, with the nucleus being extremely crowded and presenting numerous potential obstacles and binding sites for a transcription factor such as PPAR, we realized that anomalous diffusion (AD) might actually better describe PPAR mobility in living cells (32). Although the mean square displacement of a particle following normal diffusion is proportional to time (⟨r2(t)⟩ ∼ t), anomalous diffusion is defined by (⟨r2(t)⟩ ∼ tα), where the anomalous coefficient α reflects the interaction of the diffusing particle with its environment. This coefficient varies between 0.1 and 1, a value of 1 actually indicating free diffusion in a homogeneous environment. When autocorrelation curves of EYFP-PPAR diffusion in living cells were fitted with a one-component model, the residual curves were noisy, indicating some systematic deviations, whereas these deviations were much smaller when the AD model was used (Fig. 1B, lower panels). AD particularly improved the fit in the 0.1–10-ms range, a zone in which the accuracy of diffusion time determination is particularly sensitive. It is noteworthy that the diffusion coefficients obtained with anomalous diffusion are close to those originally obtained with a one-component model (Table 1) and confirm our former conclusions as follows: (i) PPAR diffusion in the absence of ligand is slower than expected if the receptor was roaming the nucleus as a monomer or even as a heterodimer with RXR, and (ii) ligand binding further slows down the diffu-
sion of the receptor. Because the AD model is more precise and reflects better the physiological nuclear environment experienced by the receptor, we applied this model to all our measurements in living cells. In contrast, when experiments were performed in vitro in nuclear extracts where the nucleoplasm is highly diluted and structures impairing free diffusion have been removed, AD modeling gave an anomalous parameter $\alpha = 1$, indicating free diffusion. FCS measurements in these conditions were therefore fitted with a single component model.

**A High Proportion of PPAR and RXR Receptors Readily Form Heterodimers in Living Cells in the Absence of Ligand**—By using FRET, we previously demonstrated that, contrary to a general assumption, PPARs and their heterodimeric partner RXR are readily associated before ligand activation (7). Only PPARα exhibited a slightly enhanced interaction with RXR upon ligand binding. However, we could not determine by FRET the proportion of receptors that were heterodimerized. This question is of importance not only with respect to PPAR mechanism of action but also to that of RXR, which can also homodimerize and heterodimerize with other partners. We therefore performed dual color fluorescence cross-correlation spectroscopy (dcFCCS) to address this issue. Briefly, dcFCCS enables the diffusion of two molecules labeled with spectrally different fluorophores to be measured and gives the concentration of free and interacting species as well as their respective diffusion coefficients (Fig. 2A and B). The concentrations of the fluorescent PPAR and RXR chimeras were derived from the autocorrelation curves, and the analysis was performed only on cells where the ratios between PPAR and RXR concentrations were between 1 and 4. The average percentage of RXR molecules interacting with PPARα was 60 and 68% in the absence and presence of the PPAR ligand Wy14,643, respectively (Fig. 2C). FCS experiments require analysis of cells where EYFP fusion proteins are expressed at concentrations in the low nanomolar range. In our previous study, we estimated EYFP-PPARα concentrations and found them to be lower than those of the endogenous protein in mouse liver cells (7), therefore suggesting that these values can be extrapolated to endogenous PPAR and RXR.

The activity of PPAR/RXR heterodimers may also be regulated by an RXR agonist (2). In Fig. 3, we represent the diffusion coefficients that can be determined from the autocorrelation curves, the measurements being represented as a frequency histogram. Consistently, addition of 9-cis-retinoic acid to living cells induced a reduction of EYFP-PPAR mobility, but on average to a lower extent than a PPAR agonist (Fig. 3 and Table 2). This result is consistent with the fact that not all PPARs interact with RXR molecules and provides further evidence for the permissive nature of the PPAR/RXR heterodimer and the role of RXR in the modulation of PPAR activity and transcriptional activation. In line with the transactivation studies we performed previously (7), this result also indicates that enough endogenous RXR is present for the action of EYFP- or mRFP-PPARs, whose expression levels are kept as low as possible (hardly detected by laser scanning microscopy) to allow proper auto-correlation function derivation and to avoid overexpression artifacts.

The combination of a PPAR and an RXR ligand does not reduce PPAR mobility further than what is observed with a PPAR ligand alone (Table 2). Additional studies will be necessary in the future to determine

---

**TABLE 1**

The diffusion coefficients of EYFP-PPARs in living cells obtained with an anomalous diffusion model are close to those obtained with a one-component model of free diffusion

|                  | One component free diffusion | Anomalous diffusion |
|------------------|------------------------------|--------------------|
|                  | Diffusion coefficient ($\mu m^2/s$) |                     |
| EYFP (nucleus)   | 19.7 ± 0.56                  | 22.6 ± 2.5         |
| EYFP-PPARα       | 4.8 ± 0.21                   | 6.4 ± 0.86         |
| EYFP-PPARα + Wy14,643 | 3.0 ± 0.19               | 3.3 ± 0.35 ($p < 0.01$) |
| EYFP-PPARβ       | 5.5 ± 0.23                   | 6.7 ± 0.58         |
| EYFP-PPARβ + L-165041 | 3.5 ± 0.17               | 4.6 ± 0.40 ($p < 0.05$) |
| EYFP-PPARγ       | 5.0 ± 0.24                   | 6.5 ± 0.53         |
| EYFP-PPARγ + rosiglitazone | 2.3 ± 0.11           | 4.2 ± 0.68 ($p < 0.01$) |

* Statistical difference with sample without ligand is shown.

---

**FIGURE 2**

The in vivo constitutive interaction of PPARα and RXRα is slightly increased by a PPAR ligand. COS-7 cells were transfected with expression vectors for EYFP-PPARα, EYFP-PPARβ, or EYFP-PPARγ in the presence or absence of their respective ligands, and diffusion was measured by FCS.

A, intensity fluctuations for mRFP-PPAR and EGFP-RXR. B, fit of the data with the anomalous diffusion model. C, percentage of RXR interacting with PPAR. D, diffusion coefficients of the mRFP-PPARα, EGFP-RXR, and mRFP-PPARα/EGFP-RXR-containing complexes. Wy, Wy14,643.
whether the nature of the complexes recruited in vivo when both receptors are liganded is the same as when the PPAR ligand only is present, as well as to determine the respective roles of the RXR AF2 and of RXR conformational changes in the recruitment of coregulators by PPAR itself.

Finally, we determined the diffusion coefficients of the complexes containing both PPAR and RXR by dcFCCS (Fig. 2D). These complexes display even smaller diffusion coefficients than those predicted for PPAR-containing complexes (which include the PPAR/RXR-containing complexes but also the complexes containing PPAR only). This result is interesting because it suggests either that RXR favors the recruitment of coregulators by PPAR or that both partners of the heterodimer recruit coregulators, leading to the formation of even bigger complexes.

**Impact of Chromatin Binding on PPAR Mobility**—In a previous study (7), we showed that EYFP-PPARs diffusion coefficients were unexpectedly smaller than those predicted for EYFP-PPAR complexes (or reciprocally their theoretical diffusion coefficients). These complexes display even smaller diffusion coefficients to this calculation, the fact that the diffusion coefficients obtained for PPAR in the absence of ligand are much smaller than those predicted from these calculations lead us to consider that these differences are significant.

At least two nonexclusive explanations for these relatively low diffusion coefficients may be presented as follows: (i) the receptor makes transient interactions with chromatin through its DNA binding domain, and thereby performs a three-dimensional scanning of the genome, as suggested for several other transcription factors (16); (ii) in the absence of ligand, the PPAR/RXR heterodimer interacts with soluble factors of high molecular mass. To test these hypotheses, we generated two EYFP-PPARα mutants, defective for DNA (PPARαC122S (33)) or for coregulator binding (PPARαΔ13 (34)). First, the DNA and coregulator binding properties of these EYFP-PPARα mutants were assessed by in vitro pulldown assays. As expected, the ability of the C122S mutant to bind to a oligonucleotide containing a PPAR-response element is almost totally abolished, the binding being hardly detectable. Note that the C122S mutant still retains the ability to interact with p300, albeit to a lesser extent than its WT counterpart. Oppositely, the interaction with the coactivator p300 is totally abolished in the C122S mutant (Fig. 4A). Upon transfection in COS-7 cells, both PPAR mutants display the same nuclear diffuse and homogeneous pattern as the WT receptor (data not shown). EYFP-PPARαC122S exhibits significantly higher diffusion coefficients than the WT receptor in the absence of ligand (Fig. 4B), indicating that transient DNA interactions may impair receptor mobility. However, the receptor is still much slower than expected if it were roaming freely as a mono- or heterodimer, indicating that if chromatin binding has an effect, it contributes only to a minor extent to receptor mobility at the scale of the entire population of receptors. In the presence of ligand, a strong reduction of mobility was observed, the C122S mutant diffusing at a speed comparable with that of the liganded WT receptor. Because EYFP-PPARαC122S cannot bind DNA, this

\[
M_{\text{EYFP-PPAR}} = M_{\text{EYFP}} \times \left( \frac{D_{\text{EYFP}}}{D_{\text{EYFP-PPAR}}} \right)^3
\]

assumes spherical symmetry of the complexes and free and normal diffusion in the nucleus (7). For example, if the diffusion coefficient of EYFP, a 27-kDa protein, is 19.7 \(\mu m^2 s^{-1}\), then the diffusion coefficient for an 80-kDa protein such as EYFP-PPARα is theoretically 13.7 and 11.7 \(\mu m^2 s^{-1}\) for a heterodimer such as EYFP-PPARα/RXR. Although some imprecision may be linked to this calculation, the fact that the diffusion coefficients obtained for PPAR in the absence of ligand are much smaller than those predicted from these calculations lead us to consider that these differences are significant.

**TABLE 2**

**Binding of 9-cis-RA by RXR decreases PPAR mobility in living cells**

The data represent the average diffusion coefficient quantified from the experiment in Fig. 3.

|                 | Diffusion coefficient (\(\mu m^2 s^{-1}\)) | Statistical analysis |
|-----------------|-------------------------------------------|----------------------|
| EYFP-PPARα      | 6.7 ± 0.50                                | \(p < 0.01^a\)       |
| EYFP-PPARα + Wy14,643 | 3.3 ± 0.28                             | \(p < 0.01^a\)       |
| EYFP-PPARα + 9-cis-RA | 4.7 ± 0.42                             | \(p < 0.01^a\)       |
| EYFP-PPARα + Wy14,643 + 9-cis-RA | 3.5 ± 0.14                           | \(p < 0.01^a\)       |
| EYFP-PPARβ      | 6.7 ± 0.50                                | \(p < 0.01^a\)       |
| EYFP-PPARβ + L-165041 | 3.5 ± 0.53                             | \(p < 0.01^a\)       |
| EYFP-PPARβ + 9-cis-RA | 5.0 ± 0.53                             | \(p = 0.029^a\)      |
| EYFP-PPARγ      | 7.5 ± 0.24                                | \(p < 0.01^a\)       |
| EYFP-PPARγ + rosiglitazone | 2.9 ± 0.50                           | \(p < 0.01^a\)       |
| EYFP-PPARγ + 9-cis-RA | 4.8 ± 0.25                             | \(p < 0.01^a\)       |

\(^a\) Statistical difference with sample without ligand is shown.

\(^b\) Statistical difference with sample with PPAR-specific ligand only is shown.

\(^c\) Statistical difference with sample with 9-cis-RA only is shown.
reduction in mobility cannot be interpreted as a more avid binding of the activated receptor to chromatin, but must rather reflect the recruitment of soluble coregulators (8, 35). In agreement with this hypothesis, the mobility of the EYFP-PPARα/H9251/H900413 mutant, which is deficient for coactivator recruitment but not for ligand and DNA binding (34), is not affected by ligand addition (Fig. 4B). Thus, the mobility shift observed upon ligand binding for the WT and C122S receptors most likely reflects their association with high molecular mass complexes of coactivators. However, it is still possible that the slightly higher mobility of the C122S mutant, compared with the WT in the absence of ligand, reflects an effect of chromatin on WT receptor diffusion.

Steric Hindrance Rather than DNA Binding Affects PPAR Diffusion in Heterochromatic Regions—Non-specific and transient interactions with chromatin are the basis of the very interesting model of three-dimensional genome scanning proposed for several transcription factors (16). If PPARs scan chromatin by transiently interacting with non-specific binding sites through their DNA binding domain, PPAR mobility should be affected mainly in regions where chromatin is dense and hence where the concentration of potential binding sites is high. To visualize chromatin density in living cells, we used an engineered HeLa cell line stably expressing the chimeric histone EYFP-H2B (27). EYFP intensity is directly linked to chromatin concentration and enables a discrimination to be made between heterochromatin and euchromatin. Both in the absence and presence of ligand, mRFP-PPARα/H9251 diffuses on average slower in regions where chromatin is dense (Fig. 5A). When the mobility of mRFP-PPARα was measured not only in very high and very low density regions but over a continuum of chromatin densities, a correlation appeared between the two factors (Fig. 5B). Also, the anomalous parameter reflected well the change in environment, decreasing from 0.81 in euchromatin to 0.69 in heterochromatin (data not shown).

The slower diffusion of the receptor in regions of dense chromatin could reflect transient binding to DNA, steric hindrance in a very crowded environment, or both. If DNA binding plays a role, the diffusion of the mRFP-PPARαC122S mutant as well as that of the globular protein mRFP alone should be less affected by chromatin density. Actually, both proteins exhibit slower diffusion coefficients in heterochromatin, and the difference between euchromatin and heterochromatin is comparable with that of WT PPARα (Fig. 5, C and D). Altogether, these results indicate that interactions with chromatin are not a major determinant of PPAR mobility or that they affect only a very small subpopulation of receptors in the vicinity of the target promoters.
In that respect, the slower mobility of PPARαC122S in the absence of ligand remains puzzling. To totally exclude a chromatin effect, we measured the mobility of PPARα WT and mutant receptors in nuclear extracts, where only soluble factors are present. Again, PPARαC122S was faster than the WT receptor in the absence of ligand, but slowed down to the same values in the presence of ligand (Fig. 6). These results are consistent with a weaker recruitment of coregulators by PPARαC122S, as suggested by the pulldown assay (see Fig. 4A).

**PPARα Is Associated with Coactivators in Vivo in the Absence of Ligand**—Although it clearly appears from the previous results that PPARs are associated with soluble factors in the absence of ligand, the nature of these factors remains unknown. Unliganded nuclear receptors are commonly assumed to interact with corepressors, but PPARs have also been reported to have some basal transcriptional activity and to interact with coactivators in the absence of exogenous ligand (9, 36–39).

Antagonist treatment that is expected to induce coactivator release therefore represents an interesting tool for assessing the constitutive association with coactivators. In a pulldown experiment, PPARα interacts in the absence of ligand with the p300 coactivator (Fig. 7A), and as expected, this interaction is enhanced by the Wy14,643 agonist but abolished by the GW6471 antagonist. In the absence of ligand, PPARα can also interact with the corepressor NCOR. This interaction is relieved by an agonist but is preserved and even slightly increased by an antagonist. In living cells, addition of the GW6471 antagonist increases PPARα mobility, suggesting that PPARα binds to coactivators in the absence of ligand and that these are released upon antagonist binding (Fig. 7B). Moreover, the anomalously parameter α in the presence of GW6471 is closer to 1, indicating a diffusion mode closer to free diffusion that is consistent with a complex of reduced size (Fig. 7B). The hypothesis of a ligand-independent association with coactivators is also strengthened by the observation that the antagonist had a similar effect in nuclear extracts (Fig. 7C), which demonstrates that soluble factors only are sufficient to replicate this effect. Finally, the PPARαΔ13 mutant, which cannot bind coactivators but can still bind corepressors (34), also diffuses faster than its wild-type counterpart both in living cells (Fig. 4B) and in nuclear extracts (Fig. 6). Fluorescence resonance energy transfer (FRET) was then used to determine the nature of the coactivators that are constitutively associated to PPARα. PPARα-ECFP was coexpressed either with the N terminus of p300 or the central domain of Med1 (also called TRAP220/DRIP205/PBP), both fused to EYFP (Fig. 8A). Cells coexpressing ECFP and EYFP or PPARα-ECFP and EYFP were used as negative FRET controls. In the absence of ligand, PPARα readily interacts with p300 and Med1, and the interaction is enhanced upon the addition of Wy14,643, thereby confirming that ligand-independent coactivator binding occurs in vivo. The interaction with fusions to full-length coregulators or to domains of SRC-3, PGC-1α, and CARM-1 was also tested but could not be resolved by FRET, probably because of fluorophore spacing and orientation incompatible with energy transfer.

To confirm these results, we decided to assess the ligand-independent interaction of p300 with PPARα in living cells with a complementary technique. COS-7 cells were cotransfected with expression vectors for mRFP-PPARα and either EGFP-p300RBD or EGFP alone, in the presence or absence of a PPARα agonist Wy14,643 (Wy), and the percentage of interacting receptors was determined by dcFCCS. Although EGFP does not interact with mRFP-PPARα, 24% of EGFP-p300RBD forms complexes with the receptor in the absence of ligand, and the percentage of interacting GFP-p300RBD raises to 40% upon Wy14,643 treatment (Fig. 8B).
result confirms that PPARα can readily contact coregulators in the absence of any exogenous ligands \textit{in vivo} and suggests that the low FRET values obtained are because of the distance or misorientation of the fluorophores coupled to the PPAR and p300.

\textbf{The AB Domain of PPARα Stabilizes the Interaction with Coactivators \textit{in vivo}}—The contribution of the AF-1 to transcriptional activation varies greatly between receptors, and its relevance for PPAR activity remains to be proven. However, the AB domains of nuclear receptors can also dock coactivators, either to autonomously induce transcription or to synergize or regulate the AF-2 function. In particular, the p300 coactivator was shown to interact directly with the AB domain of PPARα (9). We therefore measured the diffusion of a PPARα mutant devoid of AB domain (EYFP-PPARαΔAB) (Fig. 9). EYFP-PPARαΔAB diffuses faster than its WT counterpart, both in the absence and presence of ligand, but a decreased mobility upon ligand binding is still observed. These data suggest that the recruitment of coactivators is still effective but also more labile in the absence of the N-terminal domain. This further supports the idea that the AB domain plays an important role in the composition of ligand-dependent and -independent PPAR complexes by modulating coregulator recruitment.

\textbf{DISCUSSION}

Gene transcription is a dynamic process that requires tight regulation both in space and in time to control precise cellular programs. We applied FCS, a technique with microsecond resolution, which proved highly satisfactory to precisely measure the diffusion of PPARs in living cells (7). The nucleus consists of a crowded environment with structural meshworks such as chromatin or the putative nuclear matrix and elevated protein concentrations (40). Anomalous diffusion models were thus developed and correction factors introduced to compensate for steric hindrance or binding events that potentially alter free diffusion (32). By applying such a model to the diffusion of PPAR in living cells, we were able to improve the accuracy of the autocorrelation fits. The validity of this approach was confirmed by the observation that the anomaly parameter was always significantly smaller than 1.

Our previous study on PPAR localization and mobility in living cells had revealed that PPARs are highly mobile in the nucleus, but even in the absence of ligand, their diffusion coefficients are lower than expected for the mass of PPAR/RXR heterodimers (7), thus raising the possibility of interactions with several cellular components such as chromatin or coregulators. Quantitative FRAP studies have suggested that DNA
binding considerably affects the diffusion of transcription factors, including GR and AR, which transiently interact with specific and nonspecific sites on chromatin (16, 24, 41, 42). It does indeed seem that up to 80% of a population of transcription factor interacts transiently with DNA and does not freely diffuse, thereby performing a “three-dimensional genome scanning” until these factors reach genuine enhancer sites (16). Using a different approach, we suggest that PPAR does not perform any genome scanning through its DNA binding domain, the impact of chromatin density on receptor mobility being the same for WT PPARα, the C122S DNA-binding deficient mutant, and the mRFP protein. The slight increase in mobility observed for PPARαC122S actually results from an impairment in coregulator recruitment. However, prolonged residence times on genuine promoters may concern only a tiny and therefore undetectable fraction of the PPAR population. It may also necessitate the cooperation of additional DNA-binding molecules as demonstrated for the glucocorticoid receptor and HMGB1 (43) and for ER and FoxA1 (44). Finally, our experiments do not exclude other possible modes of scanning, such as coregulator-mediated probing of histone modifications, although there is so far no evidence for such a mechanism.

The slower mobility of PPAR in regions rich in chromatin is consistent with the recent demonstration that interphase chromatin forms meshwork-like regions (45). Complexes with molecular masses up to ~1 MDa have no limit in access to chromatin, whereas bigger complexes, such as the SWI/SNF or polymerase II complexes, may experience some local exclusion until histones are acetylated and the chromatin structure expanded. PPARs present a diffuse and regular localization pattern within nuclei and do not seem to be excluded from any region (7). As the composition of the complexes may differ locally, it will be interesting in the future to use dcFCCS with labeled PPARs and coregulators to assess the impact of chromatin density on the accessibility of specific PPAR-containing complexes.

Our data indicate that coregulator binding is actually the major determinant of PPAR mobility. The addition of a ligand considerably reduces PPAR diffusion coefficient, whereas helix 12 deletion or addition of an antagonist, which both prevent coactivator binding, increases receptor speed. Further supporting this conclusion is the fact that the mobility shift induced by ligand binding also occurs when diffusion is measured in nuclear extracts where only soluble factors are present. Consistent with our observations, AR, GR, and ER mobility is decreased upon ligand binding (20–22, 46) and deletion of GR and ER AF-2 functions increases their mobility. Moreover, overexpression of the coactivator SRC-1 reduces ER mobility (21), and reciprocally, treatment of ER-expressing cells with an ER agonist reduces SRC-1 mobility (22, 47). Finally, GR mobility is correlated with ligand affinity (25), and the distribution of ER diffusion times measured by FCS is a signature of the nature and efficacy of the agonist (47). In that regard, FCS could constitute an interesting technique for identifying and classifying selective PPAR modulators, which regulate only specific subsets of genes due a selective recruitment of coregulators.

Despite some parallels, the behavior of PPAR and of AR, ER, and GR may follow somewhat different rules. First, in contrast to AR, ER, and GR, addition of an antagonist neither promotes the immobilization nor decreases PPAR mobility, but it does enhance diffusion coefficients. Moreover, the reduction of AR, ER, and GR mobility upon ligand binding may actually also reflect the property of these receptors to accumulate into speckles, which are not observed for PPAR (7, 22, 25, 46).

PPAR is also shown here to readily interact with coactivators in the absence of exogenous ligand in vivo. Although we cannot completely rule out the possibility that some endogenous ligands are present, this observation most likely reflects a constitutive activity as the ligand binding domain of PPARγ has been shown to spontaneously adopt an active conformation in the absence of ligand (36), and this also holds true for PPARα (5). Our data therefore bring further evidence for a constitutive activity of the receptor in vivo, which should vary in each cell type depending on the ratio between coactivator and corepressor expression (48). The nature of the complexes associated with PPARs in the absence of ligand are therefore expected to be very diverse and may also include chaperones (49, 50), which have been shown to influence steroid receptor mobility (51).

Finally, although p300 was shown to bind to the AB domain of PPARγ in vitro and in a mammalian two-hybrid system (9), no evidence for a role of the N terminus of PPARs in coregulator docking in vivo has been brought to bear so far. We show here that deletion of the AB domain translates into faster diffusion coefficients, suggesting that the AB domain stabilizes the interaction with coregulators, most probably by offering additional docking surfaces. This may be one mechanism through which the AB domain, and not the DNA-binding domain, confers target gene specificity to the receptor (13). Here again, nuclear receptor localization and action seem to be governed by receptor-specific rules, because deletion of the AB domain did not influence PPAR localization, although it is a major determinant for AR, not only regarding coregulator recruitment but also receptor localization (see Refs. 52 and 53 and references therein) and is required for the formation of ligand-induced speckles by ER (54). Moreover, deletion of the AB domain increases PPAR mobility but reduces that of GR (20).

In conclusion, a combination of different fluorescence microscopy techniques has made it possible during the past few years to dissect the molecular mechanisms underlying nuclear receptor mobility in living cells. It has revealed unexpected and receptor-specific behaviors and mechanisms of action, sometimes very different from those inferred from in vitro studies. In this study, the assessment of PPAR action in living cells by FRET and FCS unveiled the crucial role of coregulator recruitment, both by the N and C termini, in governing the mobility and the activity of the receptor in the presence and absence of ligand. We demonstrated also the usefulness of dcFCCS, a very promising technique for living cell studies enabling quantitative in vivo biochemistry studies to be carried out.

---

5 Michalik, L., Zoete, V., Krey, G., Grosdidier, A., Gelman, L., Chodanowski, P., Feige, J. N., Desvergne, B., Wahli, W., and Michielin, O. (January 2, 2007) J. Biol. Chem. 10.1074/jbcM610523200.
Acknowledgments—We thank Dr. Tsien for the gift of the mRFP cDNA, Dr. Tavare for the ECFP-EYFP construct, and Dr. Langowski for the HeLa H2B-YFP cell line.

REFERENCES

1. Nuclear Receptors Nomenclature Committee (1999) Cell 97, 161–163
2. Feige, J. N., Gelman, L., Michalik, L., Desvergne, B., and Wahli, W. (2006) J. Biol. Chem. 281, 17880–17890
3. Lonard, D. M., and O’Malley, B. W. (2006) Mol. Endocrinol. 20, 1251–1263
4. Chu, P. C., Guan, H. P., Ishizuka, T., Chui, P. C., Lehrke, M., and Lazar, M. A. (2005) J. Clin. Invest. 115, 7681–7688
5. Metivier, R., Penot, G., Hubner, M. R., Reid, G., Brand, H., Kos, M., and Misteli, T. (2006) Cell 125, 141–143
6. Brooks, B. L., Pego, R. L., Stavreva, D. A., and McNally, J. I. (2004) Biophys. J. 86, 3473–3495
7. Schaaf, M. J., Lewis-Tuffin, L. J., and Ciddorowski, J. A. (2003) Mol. Cell. Biol. 23, 1992–1994
8. Maruvada, P., Baumann, C. T., Hager, G. L., and Yen, P. M. (2003) Prog. Lipid Res. 42, 1–5
9. Desvergne, B., Michalik, L., and Wahli, W. (2006) J. Biol. Chem. 281, 17880–17890
10. Gelman, L., Michalik, L., and Wahli, W. (2006) Trends Pharmacol. Sci. 26, 244–251
11. Guan, H. P., Ishizuka, T., Chui, P. C., Lehrke, M., and Lazar, M. A. (2005) J. Clin. Invest. 115, 2216–2222
12. Guan, H. P., Ishizuka, T., Chui, P. C., Lehrke, M., and Lazar, M. A. (2005) J. Cell. Biochem. 95, 119–121
13. Nolte, V. J., Dyck, J. A., Ochs, R. L., and Evans, R. M. (1998) J. Biol. Chem. 273, 28766–28771
14. Cohen, R., and Nawata, H. (2002) Mol. Endocrinol. 16, 2244–2256
15. Juge-Aubry, C. E., Gorla-Bajszczak, A., Pernin, A., Lemberger, T., Wahli, W., Burger, A. G., and Meier, C. A. (1997) J. Biol. Chem. 272, 18171–18172
16. tandem, T., Wachsmuth, M., Waldeck, W., and Langowski, J. (2000) Mol. Endocrinol. 14, 329–347
17. Gelman, L., Zhou, G., Fajas, L., Raspe, E., Fruchart, J. C., and Auwerx, J. (2003) J. Biol. Chem. 278, 3473–3495
18. Gelman, L., Zhou, G., Fajas, L., Raspe, E., Fruchart, J. C., and Auwerx, J. (1999) J. Biol. Chem. 274, 7681–7688
19. Gelman, L., Zhou, G., Fajas, L., Raspe, E., Fruchart, J. C., and Auwerx, J. (1999) J. Biol. Chem. 274, 7681–7688
20. Schaaf, M. J., and Cidlowski, J. A. (2003) J. Biol. Chem. 278, 8493–8497
21. Schaefer, I., Kim, S. A., and Schwille, P. (2006) Nat. Meth. 3, 83–89