Dynamic Shuttling and Intracellular Mobility of Nuclear Hormone Receptors

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Abstract

We expressed green fluorescent proteins (GFP) chimeras of estrogen, retinoic acid, and thyroid hormone receptors (ERs, RARs, and TRs) in HeLa cells to examine nucleo-cytoplasmic shuttling and intranuclear mobility of nuclear hormone receptors (NRs) by confocal microscopy. These receptors were predominantly in the nucleus; and interestingly, underwent intranuclear reorganization after ligand treatment. Nucleo-cytoplasmic shuttling was demonstrated by hetero-karyon experiments, and energy-dependent blockade of nuclear import and leptomycin-dependent blockade of nuclear export. Ligand addition decreased shuttling by GFP-ER, whereas heterodimerization with retinoid X receptor helped maintain TR and RAR within the nucleus. Intranuclear mobility of the GFP-NRs was studied by fluorescence recovery after photo-bleaching (FRAP) +/- cognate ligands. Both GFP-TR and GFP-RAR moved rapidly in the nucleus, and ligand-binding did not significantly affect their mobility. In contrast, estrogen binding decreased the mobility of GFP-ER, and also increased the fraction of GFP-ER that was unable to diffuse. These effects were even more pronounced with tamoxifen. Co-transfection of the co-activator, SRC-1, further slowed the mobility of liganded GFP-ER. Our findings suggest E₂ and tamoxifen exert differential effects on the intranuclear mobility of GFP-ER. They also show that ligand-binding and protein-protein interactions can affect the intracellular mobility of some NRs, and thereby may contribute to their biological activity.
Introduction

Nuclear hormone receptors (NRs) comprise a large family of ligand-activated transcription factors that bind to hormone response elements (HREs) of target genes and regulate their transcription (1,2). Members of the NR superfamily include the steroid receptors (SRs), thyroid hormone, retinoic acid, and Vitamin D receptors (TRs, RARs, and VDRs), and orphan receptors whose cognate ligands are not known. NRs are structurally characterized by three distinct domains: a N-terminal domain, a central DNA-binding domain (DBD), and a C-terminal ligand binding domain (LBD).

Steroid receptors bind to their response elements as homodimers and are referred to as Type I receptors, whereas RARs, TRs, and VDRs bind to their response elements and are called Type II receptors as heterodimers with retinoid-X receptors (RXRs). Both Type I and Type II receptors can recruit co-activators in the presence of ligand, whereas Type II receptors, notably TRs and RARs, can recruit co-repressors such as silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear corepressor (NCoR), in the absence of ligand, and repress the transcription of target genes (1,3). The formation of NR/co-activator complexes is associated with histone acetylation (1,4) of the promoter region whereas NR/corepressor complexes recruit histone deacetylases. Although various protein-protein interactions and enzymatic activities seem to play important roles in mediating transcriptional activity by NRs, it is possible that other mechanisms, such as the sub-cellular distribution and intranuclear dynamics of NRs, may contribute to hormonal responses mediated by these receptors.
Recently, several groups have shown that progesterone and glucocorticoid receptors (PRs, GRs), VDRs, and TRs continuously shuttle between the cytoplasm and nucleus (5-8). We previously used green fluorescent protein (GFP) fusions of TRβ to show that entry into the nucleus is an energy-mediated process whereas export out of the nucleus is a passive one (9). Furthermore, our results showed that ablation of the DNA-binding capacity of TR did not prevent its nuclear accumulation. Using TR mutants that were defective in their interaction with various co-regulators, we showed that heterodimerization with RXR and interaction with NCoR help maintain unliganded TR within the nucleus.

We and others have shown that GFP chimeras of ER, GR, and TR form a punctate pattern in the nucleus after ligand addition, suggesting that intranuclear diffusion and reorganization of NRs may be important regulatory processes (10-12). Recently, Mancini et al. (13) have shown that unliganded ER exhibits high mobility whereas estrogen-bound ER has reduced mobility in the nucleus. Additionally, we have shown that GR rapidly exchanges with regulatory DNA sequences and is not statically bound to hormone response elements as previously thought (14). In this paper, we have used GFP chimeras of ER, RAR, and TR, as examples of Type I and II receptors, to examine and compare nucleo-cytoplasmic shuttling and intranuclear diffusion of NRs in more detail. In particular, we have used energy depletion studies, heterokaryon experiments, and fluorescence after photo-bleaching (FRAP) to examine the movement of these receptors in the absence or presence of ligand and co-regulators. Our studies suggest that ligand-binding and protein-protein interactions can significantly influence the mobility of these NRs, and thereby contribute to their biological activity.
Materials and Methods

Plasmids: Expression vectors for human ERα, RARβ and TRβ were previously described (15). GFP fusions were constructed into the multiple cloning site of pEGFP-C1 (Clontech, Palo Alto, CA) vector by PCR amplification method at Xho-I and EcoR-I site at the C-terminal end of the coding sequence of NRs. Molecular weight of the receptors was confirmed by western blotting using GFP monoclonal antibodies (Clontech, Palo Alto, CA) and the integrity of all the constructs was confirmed by DNA sequencing (Veritas, Rockville, MD). Functional characterization was done by cotransfecting 0.1 µg of the cDNA with 1.7 µg of reporter TRE (F2) and 1 µg of β-gal into HeLa cells in 35 mm dishes and luciferase activity was determined in the lysates using Berthold lumat-LB 9507 luminometer.

Cell culture, DNA transfection: HeLa (human cervical carcinoma line, ATCC, Manassas VA) cells were regularly maintained in DMEM with 10% FBS and antibiotics (100 U/ml penicillin and streptomycin, 0.5 mg/ml gentamycin; (GIBCO BRL, Gaithersburg, MD) and l-glutamine, 2 mM in 5% CO2 incubator at 37 °C. Various NRs were transiently expressed in HeLa cells grown on cover slips (1 X 10^5 cells/well) by calcium phosphate (Invitrogen, Carlsbad, CA) method (16).

Microscopic studies: The cells expressing various GFP-NRs were viewed under a Leica TCS SP laser scanning confocal microscope mounted on a DMRB inverted epifluorescent microscope equipped with a 63X 1.4 N.A. oil immersion lens (Heidelberg, Germany). The GFP fluorescence was excited with 488 nm
laser line from an air-cooled fiber-coupled argon laser (Coherent Inc., Santa Clara, CA). Typical laser output was less than 10% of its maximal power. DAPI fluorescence was excited by a 385 nm laser line from a water-cooled argon laser at 25% power (Coherent Inc., Santa Clara, CA). GFP emission was monitored between 505 and 590 nm, DAPI emission was followed between 405 and 490 nm. Both GFP and DAPI were visualized with a pinhole of 1.0 (Airy units) and detected. Quantitative analysis of receptor distribution was done as previously described (9).

ATP depletion experiments: ATP depletion experiments were performed as previously described (9). Cells were incubated for 2 hr with 10 mM sodium azide (Merck, Darmstadt, Germany) in the presence of 2-deoxyglucose 6 mM prior to imaging.

Heterokaryon assays: The shuttling nature of NRs was studied by interspecies heterokaryon assay as described previously (17). Briefly, 2 X 10^5 mouse (NIH3T3) cells were plated onto glass cover slips in 6-well multi-dishes on day one. Subsequently the cells were transfected with 0.1 µg of GFP-NR along with carrier DNA by calcium phosphate method. After 48 hrs 1 X 10^6 human (HeLa) cells were plated onto the cover slips. After 3 hr cells were washed thoroughly with DPBS and 100 µl of pre warmed PEG1500 were added and incubated at 37°C the cells for 2 min. The cells were washed again with DPBS and incubated with DMEM with 50 µg/ml of cycloheximide incubated for another 3 hr and fixed with 4% para-formaldehyde and mounted on the glass slides after counter staining with DAPI for microscopy.

Fluorescence recovery after photo-bleaching (FRAP): These studies were
carried out as previously described with minor modifications (14). Briefly, HeLa cells were grown on cover-slips in live cell chambers and maintained in DMEM with 10% FBS at 37°C. They were transfected with GFP-NR vectors and treated with ligands. FRAP experiments were carried out using Leica TCS SP laser scanning confocal microscope. A full power beam of 488- and 514-nm laser lines was focused on a defined region for 0.5 sec to bleach the region and then followed the recovery of fluorescence that region was monitored over time. Fluorescence intensities of regions of interest were obtained using Leica TCS NT software and analyzed by Microsoft Excel. The images were imported as TIFF files and final images were generated using Corel 9.0 software.

Immuno-fluorescence studies: Immuno fluorescence was performed on GFP-NR transfected HeLa cells on cover slips using anti CRM-1 antibodies (1:1000) (Santa Cruz bio tech, Santa Cruz CA) and visualized under Leica-Fish microscope and Images were collected using metamorph software. The images were processed using Corel 9.0 software.

Results

In order to study the intracellular distribution of NRs in living cells, we created vectors expressing GFP chimeras fused to the N-terminal ends of TRβ, ERα, and RARβ (Fig. 1A). Western blot analysis of extracts obtained from transfected cells showed that all the fusion constructs encoded full-length proteins of correct size (data not shown). The transcriptional activities of the GFP-NRs were assayed in a transient transfection system, and the levels of ligand-dependent transactivation were similar to unfused receptors (Fig. 1B and data not shown).
We examined the intracellular distribution of GFP-ER and GFP-RAR in live cells, and found that these receptors localized mostly in the nucleus, similar to previous observations for TRβ and ERα (9,18) (Figs. 2A, 3A). To quantitate the percentage of NRs present within the nucleus and cytoplasm, we measured the area-corrected intensity of GFP-NR fluorescence in both the nucleus and cytoplasm. From these analyses, we observed that only about 10-15% of unliganded GFP-ER (Fig. 2A) was present in the cytoplasm, and ER effectively translocated into the nucleus after estradiol (E₂ 10⁻⁶ M) or tamoxifen (Txn 10⁻⁸ M) treatment for 1 hr (Figs. 2B and C). Similar to GFP-ER, approximately 20% of unliganded GFP-RAR (Fig. 3B) was present in the cytoplasm, and GFP-RAR almost entirely translocated into the nucleus after retinoic acid (RA 10⁻⁶ M) treatment (Fig. 3 B). These changes in the nucleo-cytoplasmic distribution of ER and RAR after ligand addition contrast with our previous observations for TRβ, which maintained approximately 10% of receptor in the cytoplasm regardless whether ligand was present or not (9). The observed nuclear/cytoplasmic distribution was independent of the amount of expression vector transfected, as it remained constant when varying concentrations of GFP-ER and GFP-RAR vectors were transfected into HeLa cells (data not shown). In these experiments, we also observed that ligand induced intranuclear reorganization of GFP-ER and GFP-RAR (Figs. 2A, B, C, and 3A, B) similar to previous observations for GR, ER, and, TR (9-11). In the absence of ligand, GFP-ER and GFP-RAR displayed a diffuse, reticular pattern whereas in the presence of their cognate ligands, these receptors exhibited a discrete, punctate pattern. These effects were rapid with equilibrium patterns occurring within 10 min after ligand addition (data not shown).

To further delineate the nucleo-cytoplasmic shuttling of these receptors,
the intracellular distribution of these receptors was studied after treatment with sodium azide, which depletes intracellular stores of ATP and blocks the active transport of proteins across the nuclear membrane (Figs. 2 and 3). We previously showed nuclear import of GFP-TRβ is an energy-mediated process and its export is a passive one (9). We now observed similar findings for both GFP-ER and GFP-RAR. After a 2 hr treatment with sodium azide, more than 20% of GFP-ER redistributed to the cytoplasm indicating that a subpopulation of GFP-ER shuttles between the nucleus and cytoplasm (Fig. 2D). Interestingly, treatment with either E2 or tamoxifen prior to sodium azide treatment prevented cytoplasmic accumulation (Figs. 2E and F) indicating that ligand-binding to GFP-ER decreases shuttling out of the nucleus. Similarly, a significant amount of GFP-RAR was found to undergo nucleo-cytoplasmic shuttling (Fig. 3D). This shuttling out of the nucleus was prevented by either ligand treatment or co-expression of unfused RXR (Fig. 3E and F), and suggests that ligand-binding and heterodimerization help retain GFP-RAR in the nucleus.

We then used heterokaryon system to further study the nucleo-cytoplasmic shuttling by GFP-ER. In these studies, NIH3T3 cells expressing GFP-ER were fused with nonexpressing HeLa cells, both in the presence and absence of ligands. We observed that GFP-ER translocated from the NIH3T3 nucleus to the HeLa nucleus, indicating that GFP-ER rapidly shuttles between the nucleus and cytoplasm (Fig. 4A and B). However in the presence of either E2 or tamoxifen, translocation of GFP-ER into the HeLa nucleus was reduced (Figs. 4C-F) indicating decreased nucleo-cytoplasmic shuttling.

In order to characterize the mechanism of shuttling, we studied ER diffusion in the heterokaryon system, in the presence of leptomycin B, an
inhibitor of CRM-1 (involved in nucleocytoplasmic export) (Figure 5). GFP-ER was retained in the HeLa cell nuclei in the absence of ligand, E2, and tamoxifen suggesting that ER export occurs by CRM-1 regardless of its ligand-binding state. Similar results also were observed for GFP-TRβ and GFP-RAR (data not shown).

These foregoing findings suggest that GFP-ER and GFP-RAR shuttle between the nuclear and cytoplasmic compartments. Additionally, our findings in Figs. 2 and 3 suggest that these receptors reorganize within the nucleus upon ligand-binding. To better understand this latter phenomenon, we used fluorescence recovery after photo-bleaching (FRAP) to study the intranuclear mobility of GFP-ER, GFP-RAR, and GFP-TR in transfected cells in the presence and absence of ligand. In these studies, a full power laser beam was focused for 0.5 sec on the nuclei of GFP-NR expressing cells, causing fluorescence loss in a defined nuclear zone. The mobility of GFP-NR molecules was then measured as a function of fluorescence recovery over time in the bleached region.

Under these conditions, we determined the recovery rate of a mobile fraction of GFP-NRs in which fluorescence recovery could be measured (t1/2), and observed an immobile fraction in which no recovery occurred even after 3 min (original fluorescence (100%)-%recovery after 3 min). The calculated data of ten individual cells from 6 different experiments are shown in Table 1. The effect of ligands on the mobility of GFP-ER was studied using FRAP technique (Figs. 6, 7A). In cells transfected with GFP-ER vector, bleaching for 0.5 sec caused only a 55% reduction in fluorescence within the nuclear zone, indicating that unliganded GFP-ER was extremely mobile and rapidly moved back to the bleached zone, even during the time between the bleaching process and first
measurement (Figs. 6A and 7A). Rapid recovery was observed ($t_{1/2} = 1.6$ sec) as more than 90% of fluorescence recovery occurred within 3 sec, and maximal recovery occurred in less than 12 sec. The immobile fraction was 14%. In order to observe the effect of ligands on the mobility of GFP-ER, we incubated transfected cells with either $E_2$ or tamoxifen for 1 hr, and then subjected the cells to FRAP (Figs. 6B, C and Fig. 7A). In these studies, we found that cells treated with $E_2$ had more than 70% loss in fluorescence initially, and $t_{1/2} = 5.8$ sec, with an immobile fraction of 44% (Fig. 7A, Table 1). These findings demonstrated that $E_2$ reduced the rate at which GFP-ER returns to the bleached zone, and increased the amount of ER that was unable to diffuse readily. Interestingly, treatment with the ER antagonist, tamoxifen, resulted in almost total loss in fluorescence, with little fluorescence recovery even after 3 min due to a large immobile fraction (Figs. 6C, Fig. 7A, Table 1, and data not shown). In all these experiments, the recovered fluorescence was normalized to unbleached control cells in the same experiments. All the experiments were repeated more than six times with similar findings.

Previous studies have shown that p160 co-activators such as SRC-1 interact with ER in a ligand-dependent manner and enhance its transcriptional activity (1). In order to study the effect of SRC-1 on the intranuclear mobility of GFP-ER, we performed FRAP on cells co-expressing both GFP-ER and unfused SRC-1, both in the absence and presence of $E_2$ (Fig. 6B). Interestingly, there was delayed recovery of fluorescence of unliganded GFP-ER in the presence of SRC-1 ($t_{1/2} = 3.6$ sec vs. 1.6 sec, respectively) with a maximal recovery occurring by approximately 20 sec (Fig. 7B). These findings suggest a possible association between SRC-1 and unliganded GFP-ER that may decrease the latter’s mobility. In the presence of $E_2$, more than 80% of fluorescence was bleached in cells
transfected with GFP-ER and SRC-1, compared to 70% bleaching in cells transfected with only GFP-ER. Additionally, recovery time was slower in cells transfected with GFP-ER and SRC-1 than GFP-ER alone ($t_{1/2} = 6.9$ sec vs. 5.8 sec, respectively). These findings suggest that SRC-1 may reduce the intranuclear mobility of $E_2$-bound ER.

To further investigate the effect of ligands on the mobility of nuclear hormone receptors, we performed FRAP on cells expressing GFP-RAR in the presence and absence of RA (Fig. 8A). GFP-RAR was highly mobile in the absence of ligand as there was only a 40% loss in fluorescence after photo bleaching and maximal recovery occurred by 10 sec (Fig. 8A). In the presence of RA, there was little change in the intranuclear mobility of GFP-RAR ($t_{1/2} = 2.3$ sec vs 2.0 sec). FRAP studies on unliganded GFP-TRβ showed very similar recovery kinetics as GFP-RAR as there was only a 40% loss in fluorescence after photo bleaching and almost total fluorescence recovery after 10 sec (Fig. 8B). In the presence of T$_3$, there was little change in mobility of GFP-TR as maximal fluorescence recovery occurred by 5 sec, and $t_{1/2}$ remained unchanged (1.8 sec). In contrast to SRC-1’s effect on ER mobility, co-transfection of SRC-1, as well as NCoR and HDAC-1, did not affect the intranuclear mobility of GFP-TR (data not shown). We previously observed that nuclear export of ER, RAR, and TR was mediated by CRM-1; thus, we examined whether CRM-1 may be associated with GFP-ER and modulate its intranuclear mobility (Fig. 9). However, we did not observe co-localization of CRM-1 and GFP-ER in the absence or presence of ligand, suggesting that exportin does not modulate intranuclear mobility of ER.

Discussion
We have used GFP chimeras of ER, RAR, and TR to examine the cellular localization of several prototypical Type I and Type II NRs in living cells. In the absence of ligand, all the NRs studied displayed predominantly nuclear distribution. In contrast, previous studies of some steroid hormone receptors, particularly GR and PR, showed mostly cytoplasmic distribution in the absence of ligand (12,19). This distribution is likely maintained by the formation of cytoplasmic complexes that include chaperone proteins such as hsp 70 and hsp 90 (20). The location of unliganded ER is still controversial as earlier biochemical studies suggested ER was mostly cytoplasmic, but later cell fractionation and immuno histochemical studies suggested it was predominantly nuclear (21-24). Recent studies with GFP-ER have not addressed directly the issue of unliganded ER localization (10,11). Here, we show that a small pool of unliganded ER exists in the cytoplasm, and it constantly shuttles between the cytoplasm and nucleus in live cells. Moreover, the cellular distribution and shuttling of ER is markedly affected by estrogen treatment. Interestingly, the estrogen antagonist, tamoxifen, also has similar effects as estrogen on receptor cycling and retention, suggesting it does not interfere with these particular receptor roles. We also showed that the nuclear import of ER is an energy-dependent process similar to our present findings for RAR, and previous studies for TR and PR (5,9). We also demonstrated that nuclear export of ER, RAR, and TR are mediated via the exportin, CRM-1 (25). Previous studies suggested that VDR, but not PR and GR, were blocked by leptomycin B (26-28). Thus, it is possible there may be more than one mechanism for nuclear export of nuclear hormone receptors.

The mechanism for the nuclear retention of ER is not known; however, it is possible ligand-binding may facilitate interactions with nuclear proteins or
components. For instance, it is known that liganded ERs can form multimeric transcription complexes containing co-activators (1). Recent studies also have shown that E$_2$-bound ER and T$_3$-bound TR can bind to the nuclear matrix component (9,11), which in turn may affect intranuclear mobility and nuclear retention of these receptors.

Our studies also show that unliganded GFP-RAR and GFP-TR shuttle between the cytoplasmic and nuclear compartments, suggesting that such shuttling might be a general phenomenon among NRs. In contrast to ER, ligand-binding did not promote major changes in RAR or TR cellular distribution. Instead, GFP-RAR exhibited reduced shuttling in the presence of RXR suggesting that heterodimerization plays a major role in maintaining the normal cellular distribution of RAR. We previously used GFP chimeras of TR mutants that were defective in homodimerization or nuclear localization to demonstrate that heterodimerization with RXR helped maintain TR in the nucleus. Similar results also have been observed for TR$\alpha$ in an oocyte system and GFP-VDR (7,8). Our previous studies also showed that unliganded TR$\beta$ interaction with the co-repressor, NCoR, helped maintain nuclear localization (9). Furthermore, we previously showed that DNA-binding by TR did not significantly contribute to nuclear retention. Taken together, these findings with the other GFP-NRs, suggest that ligand-binding or protein-protein interactions are key processes that may be differentially employed by NRs for nuclear localization.

Previous studies have indicated that some NRs undergo intranuclear reorganization in response to ligand treatment (7,9-11,18). Similarly, all the NRs studied here underwent intranuclear reorganization after hormone treatment.
To better understand this phenomenon, we used FRAP to study the intranuclear mobility of the NRs, and the effect of various co-regulators on their mobility. Our studies demonstrate that NRs rapidly move within the nucleus. Additionally, the mobility of NRs can be modulated by ligand-binding as both E$_2$ and tamoxifen reduce the mobility of GFP-ER. However, E$_2$ treatment results in partial fluorescence recovery whereas tamoxifen treatment causes minimal or no fluorescence recovery as it generated a large immobile fraction of ER. These findings suggest that anti-estrogens can have profound effects on the intranuclear mobility of ER, and this process may be involved in antagonist activity. Of note, our data differ from a recently published study in which ICI decreased ER intranuclear mobility whereas tamoxifen had minimal effect (11). Additionally, these studies are consistent with recent findings that showed steroid hormone receptors are dynamic within the nucleus, and rapidly exchange between various nuclear components including chromatin (14).

In contrast to ER, ligand did not significantly affect the mobility of Type II receptors such as GFP-RAR and GFP-TR. Thus, ligands can exert differential effects on the intranuclear mobility of NRs. We also studied the effects of various transcriptional co-factors on the intranuclear mobility of NRs. GFP-ER had decreased mobility when co-expressed with SRC-1 perhaps by forming of an NR/co-activator complex or by stabilizing NR within larger multi-protein complexes. In this connection, nuclear hormone receptors have been shown to co-localize with co-activators and interact with proteins in the proteasome complex (29-31). This may potentially be a mechanism for down-regulating transcriptional activity by NRs. It also is interesting to note that SRC-1 decreased the mobility of both unliganded and E$_2$-bound ER, suggesting that SRC-1 itself may be in a dynamic equilibrium with ER, even when the latter is in
In summary, we have shown that several representative NRs undergo nucleocytoplasmic shuttling in an energy-dependent manner. These NRs also have rapid intranuclear mobilities, which in the case of ER, can be modulated by ligand-binding. Estrogen and tamoxifen have differential effects on ER mobility and tamoxifen’s marked effect on ER mobility may contribute to its anti-estrogenic properties. When our results of GFP-ER, GFP-RAR, and GFP-TR are taken together, they suggest that ligand-binding and interaction of NRs with transcriptional co-factors may exert differential effects on the nucleocytoplasmic shuttling and intranuclear mobility of NRs. These, in turn, may represent novel regulatory processes, which affect the biological activity of NRs.

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**Figure Legends**

**Figure 1.** Characterization of GFP-NR constructs. A) Schematic of the pGFP-NR constructs. Locations of the CMV promoter, EGFP coding sequence, NR coding region, termination codon and SV40 poly A signals are indicated. B)
Ligand-dependent transactivation by GFP-NRs with cognate response element reporters. 0.1 µg of the pGFP-NR expression vector with 1.7 µg of TRE (F2), or RARE or ERE reporter, and 1 µg of β-gal control vector were co-transfected into HeLa cells in the absence or presence of respective ligands (T3, RA, or E2). Cells were harvested after 48 hours, and luciferase activity of lysates was measured as described in Materials and Methods.

**Figure 2.** Effect of ligands on the intranuclear distribution and nucleo-cytoplasmic shuttling of GFP-ER. HeLa cells were transfected with 0.1µg of pEGFP-ER and subsequently depleted of intracellular ATP levels by treating with sodium azide (10 mM) in the presence of deoxyglucose (6 mM) and imaged after 2 hrs. Numbers at the bottom of each image represent the average nuclear localization (determined from percent nuclear to total cellular fluorescence) of at least 30 cells as described under Materials and Methods. GFP-ER expressing cells with no treatment (A), with E2 (10^{-6} M) (B), or Tamoxifen (10^{-8} M) (C) for 1 hr. GFP-ER expressing cells treated with sodium azide (10 mM) alone to deplete ATP levels (D), or cells treated with E2 (10^{-6} M) (E), or Tamoxifen (10^{-8} M) (F) for 1 hr before sodium azide treatment.

**Figure 3.** Effect of ligand-binding and heterodimerization on the intranuclear distribution and nucleo-cytoplasmic shuttling of GFP-RAR. HeLa cells were transfected with 0.1µg of pEGFP-RAR +/- 0.3 µg of pcDNA-RXR and were subsequently depleted of intracellular ATP levels as described in Fig 2. Numbers at the bottom of each image represent the average nuclear localization (determined from percent nuclear to total cellular fluorescence) of at least 30
cells as described under Materials and Methods. GFP-RAR expressing cells
with no treatment (A) or RA (10^{-6} M) for 1 hr (B), and GFP-RAR co-expressed
with pcDNA-RXR (C). GFP-RAR expressing cells treated with sodium azide as
mentioned above with no treatment (D) or treated with RA (10^{-6} M) for 1 hr prior
to sodium azide (E), or GFP-RAR co-expressed with pcDNA-RXR and then
treated with sodium azide (F).

**Figure 4.** Heterokaryon experiments to study GFP-ER nucleo-cytoplasmic
shuttling. GFP-ER expressing NIH3T3 cells were fused with HeLa cells using
PEG 1500 for 2 min as described in Materials and Methods. The cells were
treated with E_2 (10^{-6} M) or Tamoxifen (10^{-8} M). The identity of each nucleus
was determined by DAPI staining of the nuclei (right panel). NIH3T3 nucleus can
be differentiated from the HeLa nucleus by the relatively smaller size and/or the
presence of heterochromatin. A) GFP-ER distribution after fusion, B) DAPI
staining of GFP-ER expressing nuclei, C) GFP-ER distribution after fusion and E_2
(10^{-6} M) treatment, D) DAPI staining of GFP-ER expressing nuclei upon E_2
treatment, E), GFP-ER distribution after fusion and Tamoxifen treatment (10^{-8}
M), F) DAPI staining of GFP-ER expressing nuclei upon Tamoxifen treatment.
Cell membrane borders of transfected fused cells are highlighted.
Figure 5. Effect of Leptomycin B on GFP-ER shuttling in the heterokaryon system: GFP-ER expressing NIH3T3 cells were fused with HeLa cells. Cells were treated with Leptomycin B $10^{-9}$ M for 3 hrs either in the absence or presence ($E_2$ $10^{-6}$ M or Txn $10^{-8}$ M). GFP-ER distribution between heterokaryons treated with no ligand (A) presence of $E_2$ (B) or Txn. (C). DNA counter-staining with DAPI of these treatments is shown in panels D, E, and F. GFP-ER distribution between heterokaryons in the presence of Leptomyin B: GFP-ER treated with no ligand (G) $E_2$ (H) or Txn (I). DNA counter-staining with DAPI of these treatments is shown in panels J, K, and L.

Figure 6. Fluorescence recovery after photo-bleaching (FRAP) of nuclear GFP-ER. A small region in the nuclei of GFP-ER expressing cells was bleached with a full power laser beam for 0.5 sec and imaged continuously for the recovery of fluorescence to measure intranuclear mobility of GFP-ER (highlighted circle). A) FRAP of GFP-ER expressing cells, B) FRAP of GFP-ER expressing cells in the presence of $E_2$ ($10^{-6}$ M), C) FRAP of GFP-ER expressing cells in the presence of Tamoxifen ($10^{-8}$ M).

Figure 7. Time course of FRAP of nuclear GFP-ER in the presence of ligands and SRC-1 co-activator. A small region in the nuclei of GFP-ER expressing
cells was bleached with a full power laser beam for 0.5 sec and imaged continuously for the recovery of fluorescence as a function of movement of GFP-ER. A) FRAP of GFP-ER expressing cells in the presence or absence of ligand. FRAP of GFP-ER expressing cells (circles), FRAP of GFP-ER expressing cells in the presence of E2 (10^{-6} M) (triangles), FRAP of GFP-ER expressing cells in the presence of Tnx (10^{-8} M) (squares). B) FRAP of GFP-ER cells co-expressed with SRC-1. FRAP of GFP-ER expressing cells (circles), FRAP of GFP-ER expressing cells co-expressed with SRC-1 (open circles), FRAP of GFP-ER expressing cells in the presence of E2 (triangles), FRAP of GFP-ER expressing cells co-expressed with SRC-1 in the presence of E2 (10^{-6} M) (open triangles).

**Figure 8.** Time course of FRAP of nuclear GFP-RAR and GFP-TR in the presence of their respective ligands. The nuclei of GFP-RAR expressing cells were bleached with full power laser beam and imaged continuously as in Figure 6. A) FRAP of GFP-RAR. FRAP of GFP-RAR expressing cells (circles), FRAP of GFP-RAR expressing cells in the presence of RA (10^{-6} M) (triangles). B) FRAP of GFP-TR. FRAP of GFP-TR expressing cells (circles), FRAP of GFP-TR expressing cells in the presence of T3 (10^{-6} M) (triangles).
**Figure 9.** Co-localization of CRM-1 with GFP-ER: Immuno fluorescence was performed on GFP-ER expressing cells treated with ligands, using anti CRM-1 antibody (1:1000) and visualized by fluorescence microscopy. GFP-ER expression after treatment with no ligand (A) E\textsubscript{2} (B) or Txn (C). CRM-1 immuno fluorescence after treatment with no ligand (D) E\textsubscript{2} (E) or Txn(F). G, H, and I show overlay images for each treatment. J, K, and L show DNA counter-staining by DAPI for the same treatments.
Fig. 1
|       | t½ in s | % immobile fraction |
|-------|---------|---------------------|
| ER    | 1.6 ± 0.3 | 14                  |
| ER + E₂ | 5.8 ± 0.4 | 44                  |
| ER + SRC | 3.6 ± 0.5 | 14                  |
| ER + SRC + E₂ | 6.9 ± 0.7 | 42                  |
| ER + Ttxn | 8.3 ± 0.4 | 68                  |
| RAR   | 1.9 ± 0.2 | 18                  |
| RAR + RA | 2.3 ± 0.4 | 18                  |
| TR    | 1.8 ± 0.3 | 12                  |
| TR + T₃ | 1.8 ± 0.2 | 14                  |

n=10 for each condition
