Nuclear-Cytoplasmic Shuttling by Thyroid Hormone Receptors:  
Multiple Protein Interactions are Required for Nuclear Retention

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Summary

In this report, we have studied the intracellular dynamics and distribution of the thyroid hormone receptor beta (TRβ) in living cells, utilizing fusions to the green fluorescent protein. Wild-type TRβ was mostly nuclear in both the absence and presence of T₃; however T₃ induced a nuclear reorganization of TRβ. By mutating defined regions of TRβ, we found that both N-CoR and RXR are involved in maintaining the unliganded receptor within the nucleus. A TRβ mutant defective in DNA-binding had only a slightly altered nuclear/cytoplasmic distribution compared to wild-type TRβ; thus, site-specific DNA-binding is not essential for maintaining TRβ within the nucleus. Both ATP-depletion studies and heterokaryon analysis demonstrated that TRβ rapidly shuttles between the nuclear and the cytoplasmic compartments. Co-transfection of N-CoR and RXR markedly decreased the shuttling by maintaining unliganded TRβ within the nucleus. In summary, our findings demonstrate that TRβ rapidly shuttles between the nucleus and the cytoplasm and that protein-protein interactions of TRβ with various co-factors, rather than specific DNA interactions, play the predominant role in determining the intracellular distribution of the receptor.
Introduction

Thyroid hormone receptors (TRs) are nuclear hormone receptors, which can directly regulate transcription by binding to thyroid hormone response elements of target genes. In the absence of T3, TRs repress basal transcription of positively-regulated genes whereas in the presence of T3, TRs activate transcription (1,2). In basal repression, TRs associate with co-repressors such as N-CoR and SMRT, forming a co-repressor complex (3,4). This complex includes other components, such as histone deacetylases, which in turn, can alter the chromatin structure of target genes. During transcriptional activation, liganded TRs associate with co-activators such as SRC-1 and form co-activator complexes, including components such as the CREB-binding protein (CBP), p300 and P/CAF (5). Several of these factors have histone acetyltransferase activity (6,7); thus, chromatin remodeling is thought to be a component of both activation and repression by nuclear receptors. Although these protein-protein interactions are important for transcriptional activity, it is possible that other mechanisms, such as the subcellular distribution of TRs in the absence or presence of ligand, may contribute to regulation of TR transcriptional activity. In this connection, steroid hormone receptors, such as the glucocorticoid (8-10), progesterone (11,12) and androgen receptors (13) are at least partially cytoplasmic in the absence of hormone, and translocate into the nucleus in the presence of hormone. In their unliganded states, these receptors are believed to be associated with a complex of chaperone proteins in the cytoplasm, including heat shock proteins 70 and 90 (14-18). It is thought that these interactions are critical in maintaining the cytoplasmic distribution of the unliganded receptor.
The intracellular distribution of TRβ has been characterized both by cell fractionation and indirect immunofluorescence approaches. Using these techniques, the receptor is found completely in the nucleus, both in the presence and absence of ligand (19-21). Since the receptor will bind specific DNA recognition sites in the presence and absence of ligand (22), the prevailing model holds that the constitutive association with the nuclear compartment is due to DNA binding by TRβ. Conversely, an earlier study with a GFP-chimera of TRβ identified a significant pool of unliganded receptor in the cytoplasm (23). To examine the potential roles of various receptor functions on the intracellular distribution of TRβ, we have examined the intracellular distribution of wild-type TRβ and a battery of receptor mutants, which are defective in specific receptor functions, including ligand-binding, DNA-binding, homodimerization, and nuclear co-repressor (N-CoR) interaction. These studies have been performed in living cells using GFP-chimeras. Our findings suggest that heterodimerization with RXR, and interaction with N-CoR both play important roles in the ligand-independent nuclear retention of TRβ. In particular, TRβ mutants that cannot bind N-CoR have markedly decreased nuclear localization in the absence of hormone. Furthermore, ablation of the DNA-binding capacity of TRβ does not prevent its nuclear accumulation. While a significant fraction of wild-type TRβ is likely bound to DNA in the absence of hormone, we conclude that the formation of multi-factor complexes with heterodimer partners and co-repressors is primarily responsible for nuclear accumulation of the unliganded thyroid receptor.
Experimental procedures

Plasmids

Expression vectors for wild-type TRβ, the TRβ mutants (24), RXR (25) and N-CoR (26) were described previously. GFP fusions vectors of wild-type TRβ (pEGFP-TRβ) and the TRβ mutants (pEGFP-TRβ-mutant) were constructed as follows. The TRβ coding region was amplified by the polymerase chain reaction with primers that inserted an XhoI site at the 5'-end and an EcoRI site at the 3' end of the TRβ coding sequence. Each was then cloned in frame into XhoI/EcoRI cut pEGFP-C1 (Clontech, Palo Alto CA) resulting in a fusion protein where GFP was at the N-terminus of TRβ. pEGFP-RXR was constructed as described above for pEGFP-TRβ. The integrity of all GFP fusions were confirmed by DNA sequencing.

Cell culture and DNA transfection

HeLa cells (human cervical carcinoma line, ATCC, Manassas VA) were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 0.5 mg/ml gentamycin and 2 mM L-glutamine in 5% CO₂ at 37°C. 100 ng of the indicated GFP-TRβ fusion vector were transfected into HeLa cells grown on #1.5 glass cover slips (1 X 10⁶ cells/well) with calcium phosphate (Invitrogen, Carlsbad CA) for 18 hours. Cells were then washed 2X with PBS, fed with fresh media and imaged as described below.
**Microscopic studies**

Cells expressing GFP-fusion proteins were imaged on a Leica TCS SP laser scanning confocal microscope mounted on a DMIRBE inverted epifluorescence microscope equipped with a 63X 1.4 N.A. oil immersion lens. GFP fluorescence was excited by a 488 nM laser line from an air cooled fiber-coupled argon laser (Coherent Laser, 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 Laser, CA). GFP emission was monitored between 505 and 590 nM and 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 on a 8 bit PMT. All images obtained represent the average of 4 sequentially obtained images.

**Quantitative Analysis of Receptor Distributions**

The percent of GFP-TRβ within the nucleus was determined as follows using the analysis features built into the Leica TCS SP software package. First, the nucleus was encircled (using the polygon tool) and the total fluorescence intensity and total area of the nucleus was determined. This was then done for the entire cell to yield the total fluorescence intensity and total area of the entire cell. Background fluorescence was determined by measuring the total fluorescence of a random region within the field of view and dividing that value by the total area of that region. This yields the total background per unit area (BA) within the field of view. The BA was then multiplied by the total area of both the nucleus and the entire cell to give the total background within the nucleus (BN) and within the entire cell respectively (BC). The BN was then subtracted from the total fluorescence intensity of the nucleus and BC from the total fluorescence intensity.
of entire cell to yield the background-corrected intensity of both the nucleus (FN) and of the entire cell (FC). Finally, FN was divided by FC to give the percent of total fluorescence found within the nucleus. For each condition, this analysis was done on a minimum of 15 cells from at least two independent experiments.

Measurements of the coefficient of variation of the nuclear localized GFP-TRβ were done essentially as described previously (27) with the following modifications. Data analysis was performed on a Gateway E-5200 with Scion Image (Scioncorp, Frederick MD). For each cell, a minimum of 7 line profiles were generated and for each condition, a minimum of 10 cells were analyzed from two independent experiments.

**Heterokaryon analysis**

Heterokaryon analysis was performed as described previously (28). Briefly, 2X10⁵ mouse cells (NIH3T3) were plated on glass coverslips in a 6-well dish. The following day, the cells were transfected with 1 mg of pEGFP-TRβ as described above. After 48 hours, 1X10⁶ human cells (HeLa) were plated on the same coverslips. Following a 3 hour incubation, the cells were washed thoroughly with PBS and treated with 100 ml of warmed PEG1500. 2 hours later the cells were washed again with PBS and incubated with DMEM with 50 mg/ml of cycloheximide for 3 hours, fixed with 4% paraformaldehyde, stained with DAPI (to visualize chromatin) and mounted on glass slides. Cells were imaged by confocal microscopy as described above.
**ATP-depletion experiments**

ATP-depletion studies were performed as described previously (29). Cells were incubated for 2 hours in the presence of sodium azide prior to imaging.

**Cell extraction**

Cell extraction procedures were carried out according to the method of (30,31). Briefly, GFP-TRβ expressing cells were grown to 70% confluency and treated with or without 10⁻⁶ M T₃. Cells were washed once with PBS and then treated with 100 mM NaCl and 0.5% Triton X-100 for 3 minutes to remove the soluble components of the cell followed by DNAse treatment for 50 minutes at 32 °C to remove DNA. Cells were then treated with 1M ammonium sulfate to remove chromatin. Finally cells were washed with PBS, stained with DAPI (to visualize DNA) and fixed with 4 % paraformaldehyde. GFP fluorescence was visualized after each extraction step as described above.
Results

To study the intracellular distribution of TRβ in living cells, we generated a GFP fusion to the N-terminus of wild-type TRβ (GFP-TRβ; Fig. 1A). In addition, GFP fusions were made to a series of TRβ mutants (Fig. 1B). GFP-TRβ-AHT contains three point mutations in the hinge region (A223G, H224G and T227G) which abrogate its interaction with the nuclear co-repressor N-CoR (32). GFP-TRβ-127 contains a mutation within the DNA-binding domain (C127A) that destroys the integrity of the first zinc finger and prevents the receptor from binding DNA in vitro (33). GFP-TRβ-429 (R429Q) and GFP-TRβ-345 (G345R) are natural mutants from patients with resistance to thyroid hormone and are deficient in homodimerization and ligand-binding respectively (33). Western blot analysis indicated that all GFP fusion constructs generated proteins of the predicted size (data not shown). In addition, the transcriptional activity of each GFP-fusion was assayed in transient co-transfection studies, and found to be similar to that reported previously for the unfused receptors (data not shown).

Analysis of the intracellular distribution of GFP-TRβ in living cells demonstrated that the majority of TRβ localized within the nucleus in both the absence and presence of T3 (Figs. 2A-B). To quantitate the percentage of GFP-TRβ present within the nucleus, the area-corrected intensity of GFP-TRβ fluorescence in both the nucleus and the cytoplasm was calculated (see Materials and Methods). From these analyses, we determined that 85-90% of GFP-TRβ was localized within the nucleus in both the absence and presence of T3 (Figs. 2A-B, Table 1). In addition, GFP-TRβ was excluded from the nucleoli, as has been reported for other nuclear receptors [Figs. 2A-B (8,11,13,27)]. The observed nuclear/cytoplasmic ratio was independent of the amount of
DNA transfected, indicating that protein expression levels did not impact on the observed intracellular distribution of GFP-TRβ (data not shown). We found a somewhat lower cytoplasmic concentration of TRβ in hormone-free cells than that reported by Zhu et al. 1998 (nuclear/cytoplasmic=1.5 or ~60% nuclear); in the presence of ligand, our results are in complete accord. The quantitative difference in the amount of unliganded receptor in the cytoplasm may be due to differences in experimental conditions between our studies and that of Zhu et al. 1998.

In previous studies on the intranuclear distribution of other nuclear receptors, such as the estrogen receptor, addition of ligand led to an intranuclear rearrangement of the receptor (27,34). Similarly, upon T3 addition, an intranuclear rearrangement of GFP-TRβ occurred. In the absence of T3, GFP-TRβ was organized in a diffuse, reticular pattern (Fig. 2A). Upon addition of T3, GFP-TRβ redistributed into a discrete punctate pattern (Fig. 2B). To quantitate the intranuclear distribution of GFP-TRβ in the absence and presence of T3, we adopted the method of Htun et al. 1999. Briefly, the average intensity of GFP-TRβ along a random linear line was determined. Dividing the standard deviation of the average intensity by the average intensity yielded the coefficient of variation: an intensity corrected value representing the range of GFP-TRβ intensities along a defined line. A low coefficient of variation, indicative of a more diffuse distribution, would be represented by a relatively smooth line profile, as shown in figure 2C. A high coefficient of variation would indicate a more focal distribution and is represented by uneven line profile (Fig. 2D). In the absence of ligand, the coefficient of variation for GFP-TRβ was 0.11 +/- 0.01, whereas in the presence of T3, the coefficient of variation increased to 0.25 +/- 0.07. Therefore, GFP-TRβ undergoes a quantitative intranuclear redistribution upon the addition of T3.
We then examined the intracellular distribution of the TRβ mutants described above. GFP-TRβ-345, which binds ligand poorly, exhibited a similar intracellular distribution as GFP-TRβ in the absence and presence of T3 (Figs. 3A-B, Table 1) with no change in the intranuclear distribution of this receptor (Figs. 3A-B, data not shown). Next, the intracellular distribution of GFP-TRβ-127 was studied. This receptor contains a disrupted zinc finger within the DNA-binding domain and is unable to bind DNA. In both the absence and presence of T3, approximately 80% of GFP-TRβ-127 was found within the nucleus (Figs. 3C-D, Table 1). Even though both liganded and unliganded TRβ are believed to be associated with DNA, these results suggest that site-specific DNA-binding plays only a minor role in maintaining the nuclear localization of wild-type TRβ.

Surprisingly, GFP-TRβ-AHT, which does not interact with N-CoR, was significantly more cytoplasmic in the absence of ligand than GFP-TRβ (Fig. 4A, Tables 1-2). Quantitative analyses of the intracellular distribution of this mutant demonstrated that less than 50% of the GFP-TRβ-AHT was localized to the nucleus (Tables 1-2). However, after addition of T3, a substantial portion of GFP-TRβ-AHT translocated into the nucleus, resulting in ~85% of the receptor localizing within the nucleus (Fig. 4B, Tables 1-2). These results suggest that, in the absence of T3, N-CoR interactions likely play an important role in maintaining the nuclear localization observed with wild-type TRβ. However, in the presence of ligand, N-CoR interactions are dispensable for maintaining TRβ within the nucleus.

To investigate if additional factors were required for retaining TRβ within the nucleus, the intracellular distribution of GFP-TRβ-429 was studied. GFP-TRβ-429 selectively forms heterodimers with the retinoid X receptor (RXR) and is defective in homodimerization. In the
absence of ligand, GFP-TRβ-429 was ~75% nuclear (Fig. 5A, Table 1), similar to that seen with GFP-TRβ-127 (Fig. 3C, Table 1). However, in the presence of T3, GFP-TRβ-429 translocated to the nucleus and adopted an intracellular distribution that was indistinguishable from wild-type TRβ (Fig. 5B). These results demonstrate that homodimerization has only a minor role in maintaining unliganded TRβ within the nucleus and is not required for the nuclear localization of liganded TRβ.

To further investigate whether heterodimerization played a role in the nuclear localization of the unliganded TRβ we expressed unfused RXR in the presence of GFP-TRβ-AHT, which was mostly cytoplasmic in the absence of ligand. If RXR was involved in the nuclear retention of the receptor, we predicted that co-expression of RXR should increase the nuclear localization of the predominately cytoplasmic GFP-TRβ-AHT. Figure 4C demonstrates that this was indeed the case as GFP-TRβ-AHT localized to the nucleus when cotransfected with RXR. In addition, as the amount of RXR expression vector transfected into the cell was increased, the amount of GFP-TRβ-AHT retained within the nucleus also increased (Table 2). As expected, GFP-RXR was found almost completely in the nucleus in both the absence and presence of its ligand, 9-cis-retinoic acid (9C-RA; Figs. 5C-D). Co-expression of N-CoR with GFP-TRβ-AHT had no effect on the intracellular distribution of this mutant (Fig. 4D, Table 2) as would be predicted by the inability of GFP-TRβ-AHT to interact with N-CoR. In addition, co-expressing unfused TRβ had no effect on the intracellular distribution of GFP-TRβ-AHT (Fig. 4E, Table 2), consistent with the results seen with the heterodimer-specific GFP-TRβ-429. Taken together, these findings further underscore the role of heterodimerization on nuclear localization of the unliganded TRβ.
Previous studies by Milgrom and associates have shown that both the estrogen and progesterone receptors are actively transported into the nucleus and return to the cytoplasm by passive diffusion (29). Our studies with GFP-TRβ-AHT and RXR suggest that, at least a portion of the intracellular TRβ may also shuttle between the nucleus and the cytoplasm. To confirm this hypothesis, the intracellular distribution of GFP-TRβ was studied after treatment with sodium azide, which depletes intracellular stores of ATP and previously has been shown to block the ATP-dependent uptake of both the estrogen and progesterone receptors into the nucleus (29). After a two hour treatment with sodium azide, approximately 10% of the nuclear localized GFP-TRβ redistributed to the cytoplasm (Figs. 6A-B, Table 3), demonstrating that a sub-population of GFP-TRβ shuttles between the nucleus and cytoplasm. In addition, treatment of cells with T3, either before or after the addition of sodium azide had no effect on the intracellular redistribution of GFP-TRβ (Figs. 6C-D, Table 3) demonstrating that the population of GFP-TRβ that shuttles was unaffected by ligand binding. This observation was confirmed by heterokaryon analysis (Fig. 6G). Here, GFP-TRβ expressing NIH3T3 cells were fused to nonexpressing HeLa cells and GFP-TRβ was found to translocate from the NIH3T3 nuclei to the HeLa nuclei, again confirming that GFP-TRβ can rapidly shuttle between the nuclear and the cytoplasmic compartments.

These findings suggested that nuclear import of both the unliganded and liganded TRβ was an energy-dependent process. In further support of this point, the effect of sodium azide on GFP-TRβ-AHT was studied. In the presence of sodium azide, a significant redistribution of this receptor was observed, with ~ 50% of the nuclear-localized GFP-TRβ-AHT redistributing to the cytoplasm (Table 3). As shown above, addition of ligand to GFP-TRβ-AHT led to a
redistribution of the receptor from the cytoplasm to the nucleus (Figs. 4A-B, Table 2). However in the presence of sodium azide, this nuclear translocation was blocked (Table 3), again demonstrating that nuclear import of TRβ is an ATP-dependent process.

As described above (Figs. 4-5, Table 2), both N-CoR and RXR interactions played important roles in maintaining the correct intracellular distribution of TRβ. To further confirm these observations, cells were co-transfected with GFP-TRβ and either RXR or N-CoR, and then treated with sodium azide. Co-expression of either N-CoR (Fig. 6E, Table 3) or RXR (Fig. 6F, Table 3) blocked the sodium azide-induced redistribution of GFP-TRβ, and demonstrated again the importance of both RXR and N-CoR in maintaining TRβ within the nucleus.

Previous experiments with the estrogen receptor found that the ligand-bound ER is in a tighter association with the nucleus than the unliganded receptor (27,34). We have found very similar results with TRβ. In both the absence (Fig. 7A) and presence (Fig. 7B) of ligand, high salt and detergent extractions of GFP-TRβ expressing cells failed to remove all of the receptor from the nuclei, indicating that the receptor was tightly associated with one or more factors within the nucleus. However, after DNase and ammonium sulfate treatment to remove the chromatin, the unliganded GFP-TRβ was completely lost from the nucleus, whereas a portion of the liganded GFP-TRβ remained associated with an extraction-resistant component of the nucleus (Fig. 7B bottom panel, data not shown). Therefore, ligand binding not only induces a change in the intranuclear distribution of TRβ but also increases the association of the receptor with non-chromatin components of the nucleus.
Discussion

Nuclear receptors can be divided into three broad categories, based on their unliganded distributions: those that are primarily nuclear [estrogen receptor (27,35,36) and TRβ (23,our results)], those that are primarily cytoplasmic [glucocorticoid receptor (8,10,37) and androgen receptor (13)] and those with a mixed distribution [mineralocorticoid receptor (38) and progesterone receptor (11,12)]. However, in all cases, addition of ligand leads to a near complete nuclear localization of the receptors [reviewed in (39)]. Thus it is possible there may be a continuum of cellular/nuclear distributions among the unliganded nuclear hormone receptors that plays a role in modulating their activity in both the absence and presence of ligand.

We have used fluorescence microscopy in conjunction with GFP fusion proteins to examine the contribution of various receptor parameters to the intracellular distribution of TRβ in living cells. In the absence of T3, approximately 10 to 15 % of the intracellular GFP-TRβ was found within the cytoplasm. The cytoplasmic distribution seen for several members of the steroid receptor family, including the glucocorticoid and progesterone receptors, has been postulated to be stabilized by interactions with molecular chaperones, primarily hsp70 and hsp90 (40). A previous study did not demonstrate TRβ association with heat shock proteins (22). However, this study was performed in vitro, and a potential association between TRβ and the growing family of chaperones has not been exhaustively investigated. Currently it is not known whether the cytoplasmic pool of TRβ exists as monomers or homodimers (our data shows RXR is exclusively nuclear, thus eliminating the possibility of cytoplasmic TRβ-RXR heterodimers). Nongenomic effects by thyroid hormone and other nuclear hormone receptor ligands have
previously been described (41). It is possible that a population of TRβ located within the cytoplasm may be responsible for some of the observed nongenomic effects of thyroid hormones.

To understand the properties of TRβ responsible for its observed intracellular distribution, several TRβ mutants were studied. Mutations that disrupted site-specific DNA-binding (GFP-TRβ-127), ligand binding (GFP-TRβ-345) and homodimerization (GFP-TRβ-429) have minimal effects on the intracellular distribution of TRβ, demonstrating that these receptor properties are not major contributors to the intracellular localization of the receptor.

We have demonstrated several lines of evidence which suggest that interactions with N-CoR play an important role in maintaining the correct intracellular distribution of TRβ. First, GFP-TRβ-AHT was primarily cytoplasmic in the absence of T₃; upon addition of T₃, this mutant translocated to the nucleus. Additionally, in the presence of sodium azide, ~50% of the nuclear-localized GFP-TRβ-AHT redistributed to the cytoplasm as compared to ~10% of wild-type TRβ. This is likely due to the fact that a larger percentage of GFP-TRβ-AHT shuttles between the cytoplasm and the nucleus as compared to GFP-TRβ (due to the inability to interact with N-CoR). Furthermore, co-expression of N-CoR with GFP-TRβ in the presence of sodium azide blocked the redistribution of the receptor observed without N-CoR. Although the x-ray crystal structure suggests that the amino acid residues mutated in TRβ-AHT may not be on the surface of the receptor (42), this mutant has been shown, by several groups, to be deficient in N-CoR interactions (32,43,44). Taken together, our data strongly implicate N-CoR in maintaining the correct intracellular distribution of the unliganded TRβ. Although the major function of N-CoR interaction with TRβ may be to mediate basal repression of transcription in positively-regulated
target genes, our data show that nuclear retention of TRβ may be another role. Previous co-transfection studies have shown that TRβ-AHT is defective in mediating basal repression (26,45). It is possible that the inability to interact with N-CoR may result in an inability of the AHT mutant to be retained within the nucleus, and hence contribute to its inability to repress basal transcription. Thus, compartmentalization of TRs within the cell may be a mechanism to modulate transcriptional activity.

In addition to N-CoR interactions, our findings demonstrate that RXR interactions contribute to the retention of TRβ within the nucleus. Co-expression of RXR enhances the nuclear localization of unliganded GFP-TRβ-AHT, suggesting that heterodimerization is important for maintaining the nuclear localization of the thyroid hormone receptor. Again, as found in the experiments with N-CoR, co-expression of RXR with GFP-TRβ blocked the azide-dependent redistribution of the wild-type receptor to the cytoplasm. Of note, GFP-RXR was exclusively localized to the nucleus even when co-expressed with TRβ or TRβ-AHT (data not shown). In addition, sodium azide treatment had no effect on the distribution of GFP-RXR under conditions that led to cytoplasmic retention of GFP-TRβ, indicating that RXR itself does not readily shuttle between the nucleus and cytoplasm. Lazar and associates have observed that RXR heterodimerization with TRβ-AHT restores basal repression by binding to N-CoR (43). Thus, TRβ/RXR/N-CoR may form a complex that maintains TRβ in the nucleus and increases basal repression (Fig. 8).

In conjunction with our preceding data, we propose a model of TRβ intracellular distribution in which a subpopulation of the receptor is continually shuttling between the cytoplasm and nucleus. This continual shuttling results in a dynamic equilibrium between the
rate of ATP-dependent nuclear import and passive nuclear export (Fig. 8). The observed nuclear localization of GFP-TRβ results when the rate of nuclear import exceeds that of nuclear export. Nuclear proteins, such as RXR and N-CoR interact with the nuclear localized TRβ, decrease the rate of nuclear export and alter the equilibrium of the receptor. Since GFP-TRβ-AHT cannot interact with N-CoR, the rate of nuclear export increases, and the observed intracellular distribution of GFP-TRβ-AHT is more cytoplasmic. Co-expression of RXR with GFP-TRβ-AHT increases the localization of this mutant within the nucleus, presumably by forming GFP-TRβ-AHT/RXR heterodimers [which, based on the results of Lazar and colleagues (43), can interact with N-CoR] and thereby decrease the rate in which GFP-TRβ-AHT is exported from the nucleus. In the presence of sodium azide, nuclear import is blocked and the subpopulation of shuttling GFP-TRβ accumulates in the cytoplasm (nuclear export is unaffected by sodium azide). However, in the presence of either RXR or N-CoR, GFP-TRβ remains in the nucleus (i.e. the rate of nuclear export is decreased). In the cell, it is possible that limiting amounts of these and other co-factors may help determine which compartment unliganded TRβ will reside. Additionally, it is possible that other cytoplasmic factor(s) may help compartmentalize a subset of unliganded TRβs in the cytoplasm (i.e. decrease the rate of nuclear import). Post-translational modification of TRβ is yet another potential mechanism for regulating the intracellular distribution of TRβ. TRβ can be phosphorylated in vitro and in vivo but the effect(s) of phosphorylation on the cellular distribution of the receptor currently are not known (46,47). The factor(s) that maintain the liganded TRβ within the nucleus are less clear since all of the TRβ mutants described here are mostly nuclear in the presence of ligand. Studies are now underway to address this important issue.
The current model views unliganded TR$\beta$ as statically bound to TREs and actively involved in basal repression of positively-regulated genes through corepressor interactions (19,48). Upon addition of T$_3$, the corepressors are lost and exchanged for coactivators complexes. As a consequence, DNA-binding is thought to be critical for maintaining the unliganded receptor within the nucleus (22). Therefore, our data showing the predominantly nuclear distribution of GFP-TR$\beta$-127 was initially surprising. However, a recent study on the intranuclear dynamics of the glucocorticoid receptor has found that nuclear receptors may not be statically bound to chromatin (49). Through direct photobleaching studies, it was observed that the glucocorticoid receptor is not statically bound to chromatin but instead is rapidly exchanging between chromatin and the nucleoplasm. Based on these results, it is reasonable to speculate that the unliganded TR$\beta$ may also be rapidly exchanging between chromatin and the nucleoplasm. If so, then DNA-binding would not be a critical factor in maintaining TR$\beta$ within the nucleus.

A central tenet of the current TR$\beta$ models is that the receptor remains bound to the same chromatin response element when ligand is added and the differential activity of the receptors with and without ligand results from the different macromolecular complexes that interact with the ligand-free and ligand-bound receptors. However, we have observed an intranuclear reorganization of TR$\beta$ in the presence of T$_3$, suggesting that a significant pool of the receptor is moving from one site to another in response to ligand. Similar ligand-dependent intranuclear distributions have been observed for other nuclear hormone receptors, including the estrogen receptor, glucocorticoid receptor, mineralocorticoid receptor and androgen receptor (8,34,50,51). Therefore, ligand-bound nuclear receptors may adopt similar intranuclear distributions, though
the exact nature of these nuclear substructures is still a matter of debate. Our studies with high salt, detergent-extracted and DNase-treated, nuclei suggest that a significant portion of liganded TRβ can interact with nuclear components other than chromatin, whereas unliganded TRβ cannot. Similar results have been reported for other nuclear receptors including ER and GR (27,52). Therefore, the intranuclear redistribution of these receptors appears to recruit the receptors to an insoluble component of the nuclear substructure. Why then would the liganded nuclear receptors be resistant to DNase treatment? One possibility is that in addition to being bound to DNA, the liganded nuclear receptors interact with other nuclear substructures and it is these interactions that instill the DNase insensitivity to their distribution. A second possibility is that the DNase insensitive receptors represent a subpopulation of liganded receptors that are, for one reason or another, inactive. Previous observations have found that the ligand-bound forms of several nuclear receptors, including the estrogen, progesterone, and retinoic acid receptors are rapidly degraded by the 26S proteasome (53-57). Therefore, it is intriguing to speculate that the DNase resistant forms of TRβ and of the other nuclear receptors may represent a population of receptors that are currently being degraded or are targeted for degradation by the proteasome.

In summary, we have observed nuclear/cytoplasmic shuttling of TRβ in living cells using GFP technology. We also have observed that T3 promotes nuclear redistribution of TRβ. Additionally, we have shown that TRβ can localize in the nucleus even in the absence of ligand- or DNA-binding. Furthermore, RXR and N-CoR may promote this nuclear localization in the absence of ligand. We speculate that nuclear/cytoplasmic shuttling may be a novel mechanism for modulating transcription by TRβ and other nuclear hormone receptors. Understanding how
various factors regulate this shuttling should provide new insight into nuclear hormone receptor action.
References


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Figure Legends

Figure 1. Schematic of GFP-TRβ constructs used in this study.  A) Schematic of the pEGFP-TRβ. Locations of the CMV promoter, EGFP coding sequence, TRβ coding region, termination codon and SV40 polyA signal are all indicated.  B) Domain structure of wild-type TRβ and each of the four mutants used in this study. Location of the DNA-binding domain (DBD), hinge region, ligand binding domain (LBD) and activation function 2 (AF2) are all shown. * represents the location of the indicated mutations. The ability of each mutant to perform various receptor functions is indicated by a + or -.  

Figure 2. Intracellular distribution of wild-type GFP-TRβ in HeLa cells. pEGFP-TRβ (100 ng) was transfected into HeLa cells and the intracellular distribution of GFP-TRβ was determined by laser scanning confocal microscopy. Representative image of EGFP-TRβ in HeLa cells in the A) absence and B) presence of 10^-6 M T₃. Arrows indicate nucleoli. Numbers at bottom of each image represents the average nuclear localization (in percent total) as described in the materials and methods.  C) Representative line graphs displaying the variation of pixel intensities across a nuclei in the absence (left) and presence (right) of 10^-6 M T₃. Values represent the mean pixel intensity across the graph. s.d. is the standard deviation.
**Figure 3.** Intracellular distribution of GFP-TRβ-345 and GFP-TRβ-127 in HeLa cells.

pEGFP-TRβ-345 (A and B) or pEGFP-TRβ-127 (C and D) (100 ng each) was transfected into HeLa cells and the intracellular distribution of the each GFP-TRβ mutant was determined by laser scanning confocal microscopy. Representative image in the (A and C) absence and (B and D) presence of $10^{-6}$ M T₃. Numbers at bottom of each image represents the average nuclear localization (as percent total) as described in the materials and methods.

**Figure 4.** Intracellular distribution of GFP-TRβ-AHT in HeLa cells. pEGFP-TRβ-AHT (100 ng) was transfected into HeLa cells and the intracellular distribution of the receptor was followed by laser scanning confocal microscopy. A) Representative example of GFP-TRβ-AHT in the absence of $10^{-6}$ M T₃. B) Representative example of GFP-TRβ-AHT in the presence of T₃. C) Effect of RXR on the intracellular distribution of GFP-TRβ-AHT. RXR and GFP-TRβ-AHT were coexpressed in HeLa cells and the intracellular distribution of GFP-TRβ-AHT was followed as described above. D) Effect of N-CoR on the intracellular distribution of GFP-TRβ-AHT. N-CoR and GFP-TRβ-AHT were coexpressed in HeLa cells and the intracellular distribution of GFP-TRβ-AHT was followed as described above. E) Effect of wild-type TRβ on the intracellular distribution of GFP-TRβ-AHT. Wild-type TRβ and GFP-TRβ-AHT were coexpressed in HeLa cells and the intracellular distribution of GFP-TRβ-AHT was followed as described above. Values represent the average nuclear intensity (as percent total) of GFP-TRβ-AHT as described in the materials and methods.
**Figure 5.** Intracellular distribution of GFP-TRβ-429 and GFP-RXR in HeLa cells.

pEGFP-TRβ-429 (A and B) or pEGFP-RXR (C and D) (100 ng each) were transfected into HeLa cells and the intracellular distribution of the each GFP-fusion was determined by laser scanning confocal microscopy. Representative image in the (A) absence and (B) presence of 10^{-6} M T_3 or the (C) absence and (D) presence of 10^{-6} M 9C-RA. Numbers at bottom of each image represents the average nuclear localization (as percent total) as described in the materials and methods.

**Figure 6.** Nuclear-cytoplasmic shuttling of GFP-TRβ in HeLa cells. HeLa cells were transfected with 100 ng pEGFP-TRβ and subsequently depleted of ATP by sodium azide treatment as described in the materials and methods. Numbers at bottom of each image represents the average nuclear localization (as percent total) as described in the materials and methods. A) GFP-TRβ prior to treatment with sodium azide. B) GFP-TRβ expressing cells treated with sodium azide for two hours as described in materials and methods. C) GFP-TRβ expressing cells treated with sodium azide for one hour prior to the addition of 10^{-6} M T_3. D) GFP-TRβ expressing cells treated with sodium azide for two hours after the addition of 10^{-6} M T_3 for one hour. Co-expression of either N-CoR (E) or RXR (F) blocks the observed redistribution of GFP-TRβ after treatment with sodium azide.
Figure 7. Ligand bound GFP-TRβ but not unliganded GFP-TRβ can associate with an insoluble nuclear component. HeLa cells were transfected with 100 ng pEGFP-TRβ and sequentially extracted as described in the materials and methods both before (A) and after (B) treatment with 10⁻⁶ M T₃. GFP fluorescence was followed by laser scanning confocal microscopy. DAPI stained nuclei (right) are included in each image to indicated the presence or absence of DNA following each treatment.

Figure 8. Model for intracellular distribution of the unliganded TRβ. A) Wild-type TRβ (yellow) exists in an equilibrium with multiple protein factors within the cell, including RXR (green) and N-CoR (orange) and acts to repress transcription from target genes (red “x”). These interactions are proposed to maintain TRβ within the nucleus by maintaining the rate of nuclear import (large arrow) greater than that of nuclear export (small arrow). For simplicity, only N-CoR and RXR interactions are shown. B) When unable to interact with N-CoR, TRβ-AHT accumulates within the cytoplasm due to an increase in the rate of nuclear export as compared to nuclear import (right side of panel B). However, in the presence of co-expressed RXR (left half of panel B) TRβ-AHT is retained within the nucleus, either as a direct consequence of RXR interactions or through the recruitment of N-CoR by RXR to the AHT/RXR heterodimer (43). C) DNA-binding is not essential for the correct nuclear distribution of TRβ. TRβ-127, which cannot bind DNA, is still retained within the nucleus through interactions with RXR and N-CoR.
Table 1. Intracellular distribution of GFP-TRβ and GFP-TRβ mutants.

<table>
<thead>
<tr>
<th>Expressed receptor</th>
<th>T&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Average&lt;sup&gt;1&lt;/sup&gt;</th>
<th>SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-TRβ</td>
<td>-</td>
<td>88.9</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>88.7</td>
<td>4.5</td>
</tr>
<tr>
<td>GFP-TRβ-345</td>
<td>-</td>
<td>83.5</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>89.6</td>
<td>4.2</td>
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<tr>
<td>GFP-TRβ-127</td>
<td>-</td>
<td>76.2</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>80.7</td>
<td>2.9</td>
</tr>
<tr>
<td>GFP-TRβ-AHT</td>
<td>-</td>
<td>45</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>84.3</td>
<td>8.4</td>
</tr>
<tr>
<td>GFP-TRβ-429</td>
<td>-</td>
<td>74.6</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>85.3</td>
<td>3.3</td>
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</tbody>
</table>

<sup>1</sup> All values represent the percentage of expressed protein localized within the nucleus.
Table 2. Effect of associated proteins on the intracellular distribution of GFP-TRβ-AHT.

<table>
<thead>
<tr>
<th>Expressed receptor</th>
<th>RXR (ug)</th>
<th>Average</th>
<th>SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-TRβ-AHT - T₃</td>
<td>-</td>
<td>45</td>
<td>10</td>
</tr>
<tr>
<td>GFP-TRβ-AHT + T₃</td>
<td>-</td>
<td>84.3</td>
<td>8.4</td>
</tr>
<tr>
<td>GFP-TRβ-AHT + N-CoR</td>
<td>-</td>
<td>42.4</td>
<td>6</td>
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<tr>
<td>GFP-TRβ-AHT + TRβ</td>
<td>-</td>
<td>48.5</td>
<td>7</td>
</tr>
<tr>
<td>GFP-TRβ-AHT - T₃ (0.01)</td>
<td>0.01</td>
<td>55.8</td>
<td>3.3</td>
</tr>
<tr>
<td>GFP-TRβ-AHT - T₃ (0.1)</td>
<td>0.1</td>
<td>62</td>
<td>3.8</td>
</tr>
<tr>
<td>GFP-TRβ-AHT - T₃ (1.0)</td>
<td>1.0</td>
<td>67.2</td>
<td>4.3</td>
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</table>

¹All values represent there percentage of expressed protein localized within the nucleus.
Table 3. Effect of sodium azide on the intracellular distribution of GFP-TRβ and GFP-TRβ-AHT.

<table>
<thead>
<tr>
<th>Expressed receptor</th>
<th>Average</th>
<th>SD (%)</th>
<th>Cytoplasmic</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-TRβ - T₃</td>
<td>88.9</td>
<td>2.3</td>
<td>11.1</td>
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<td>GFP-TRβ induced</td>
<td>88.7</td>
<td>4.5</td>
<td>11.3</td>
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<tr>
<td>GFP-TRβ + azide</td>
<td>80.6</td>
<td>3.8</td>
<td>19.4</td>
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<tr>
<td>GFP-TRβ + azide + T₃</td>
<td>78.2</td>
<td>4.2</td>
<td>21.8</td>
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<tr>
<td>GFP-TRβ + T₃ + Azide</td>
<td>79.8</td>
<td>2.1</td>
<td>20.2</td>
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<tr>
<td>GFP-TRβ + azide + N-CoR</td>
<td>90.6</td>
<td>5.1</td>
<td>9.4</td>
</tr>
<tr>
<td>GFP-TRβ + azide + RXR</td>
<td>90.3</td>
<td>5.9</td>
<td>9.7</td>
</tr>
<tr>
<td>GFP-TRβ-AHT - T₃</td>
<td>45</td>
<td>10</td>
<td>55</td>
</tr>
<tr>
<td>GFP-TRβ-AHT + T₃</td>
<td>84.3</td>
<td>8.4</td>
<td>15.7</td>
</tr>
<tr>
<td>GFP-TRβ-AHT + azide</td>
<td>24.2</td>
<td>8.1</td>
<td>75.8</td>
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<td>GFP-TRβ-AHT + azide + T₃</td>
<td>30.3</td>
<td>10.6</td>
<td>69.7</td>
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</tbody>
</table>

¹Values represent there percentage of expressed protein localized within the nucleus.

²Cytoplasmic = 100-average nuclear intensity
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Fig. 1
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Fig. 2
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Fig 8