Hormone-induced Translocation of Thyroid Hormone Receptors in Living Cells Visualized Using a Receptor Green Fluorescent Protein Chimera*

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Thyroid hormone nuclear receptors (TRs) are ligand-dependent transcription factors that regulate growth, differentiation, and development. To understand the role of the hormone, 3,3′,5-triiodo-L-thyronine (T3), in the nuclear translocation and targeting of TRs to the regulatory sites in chromatin, we appended green fluorescent protein (GFP) to the human TR subtype β1 (TRβ1). The fusion of GFP to the amino terminus of TRβ1 protein did not alter T3 binding or transcriptional activities of the receptor. The subcellular localization of GFP-TRβ1 in living cells was visualized by laser-scanning confocal microscopy. In the presence of T3, the expressed GFP-TRβ1 was predominately localized in the nucleus, exhibiting a nuclear/cytoplasmic ratio of ~5.5. No GFP-TRβ1 was detected in the nucleolus. In the absence of T3, more GFP-TRβ1 was present in the cytoplasm, exhibiting a nuclear/cytoplasmic ratio of ~1.5. In these cells, cytoplasmic GFP-TRβ1 could be induced to enter the nucleus by T3. The T3-induced translocation was blocked when Lys184-Arg185 in domain D of TRβ1 was mutated to Ala184-Ala185. Furthermore, the inability of the mutant TR to translocate to the nucleus correlated with the lack of most of its transcriptional activity. These results suggest that TR functions may, in part, be regulated by T3-induced nuclear entry.

To function as transcription factors, TRs have to interact with transcriptional machinery in the nucleus. However, the process by which TRs are targeted to the nucleus is poorly understood. Before the genes encoding TRs were isolated, high affinity, low capacity T3 binding sites were detected in the nuclear fractions of tissues and cultured cells by subcellular fractionation (5–9). Subsequently, when anti-TR antibodies became available, TRs were found only in the nuclei of fixed cells by immunocytochemistry and immunohistochemistry (10–12). However, in these studies, dynamic cytoplasmic nuclear trafficking of TRs and the effect of T3 on nuclear trafficking were not addressed. In the present study, we appended the green fluorescence protein (GFP) to the human TR subtype β1 (TRβ1) allowing direct examination of the nuclear transport of TRs in living cells. TR exhibited both constitutive nuclear localization and hormone-dependent nuclear localization. Furthermore, we identified a nuclear localization signal in D domain that mediates the T3-dependent cytoplasm-nucleus translocation.

MATERIALS AND METHODS

Construction of the Plasmid Containing the cDNA Encoding GFP-TR Fusion Protein—The plasmid pCI-nGFP-C656G containing the GFP–glucocorticoid receptor fusion gene was digested with the restriction enzymes BsoI and PalI to release the glucocorticoid receptor cDNA (13). The plasmid containing the BsoI cloned from human placenta (14) was digested with NcoI and PalI or PalI and EcoRI to obtain the NcoI-PalI and PalI-EcoRI DNA fragments, respectively. The two fragments were ligated onto the plasmid pCI-nGFP BshIII-EcoRI vector using BshI-NcoI adaptor. The resultant plasmid is denoted as pGFP-TRβ1. GFP was also fused to the amino termini of two mutants to obtain GFP-TRβ2A and -TR5A. GFP-TRβ2A and -5A were constructed by replacing the sequence AAGAGG (Lys184-Arg185) of pGFP-TRβ1 with GCGCGC (Ala184-Ala185) and AAGAGCTGGCGCAAGGAG (Lys184,Arg185-Leu186-Ala187-Lys188,Arg189-Lys190) of pGFP-TRβ1 with GCGGCCGTCGGCGCAAGGAG (Ala184-Ala185-Leu186-Ala187-Ala188-Ala189-Lys189,Arg189-Lys190), respectively. The sequences of the mutants were confirmed by restriction analyses and direct DNA sequencing. The size of the mutant proteins was also confirmed by SDS–polyacrylamide gel electrophoresis analysis.

Electrophoresis Gel Mobility Shift Assay—Electrophoresis gel mobility shift assay was carried out as described (15, 16). Briefly, equal amounts of in vitro translated GFP-TRβ1 or GFP-TR mutants (TNT kit, Promega) were incubated with [32P]Lys- and Pal-TRE in the binding buffer (25 mM Heps, pH 7.5, 5 mM MgCl2, 4 mM EDTA, 10 mM dithiothreitol, 0.11 mM NaCl, and 0.4 µg ssDNA). In some experiments, RXRβ was added in the reaction mixture (15, 16). After incubation for 30 min at 25 °C, the reaction mixture was loaded onto a 5% polyacrylamide gel and electrophoresed at 4 °C for 2–3 h at a constant voltage of 250 V. The gel was dried and autoradiographed.

Binding of [32P]T3 to the TR Receptors—Fusion microsomes of the in vitro translated GFP-TRβ1 wild type or its mutant proteins was incubated with 0.2 nM [32P]T3 in the presence or absence of unlabeled T3 in 0.25 ml of buffer B (50 mM Tris-HCl, pH 8.0, 0.2 mM NaCl, 0.01% Lubrol, and 20% glycerol) for 90 min at 25 °C. Protein-bound [32P]T3 was separated from the unbound radioligand in a Sephadex G-25 column (fine) as
described previously (16). The binding data were analyzed similarly as described (14).

**Transient Transfection Assays Using CAT Reporters**—CV1 cells were transfected with TR1 or GFP-TR1 together with the TRE-containing reporter plasmids (each 0.2 µg) using the LipofectAMINE transfection method according to the manufacturer’s procedure. Twelve hours later, the medium was replaced with Dulbecco’s modified Eagle’s medium containing 10% T3-depleted serum for an additional 12 h. Fifteen hours before cells were harvested, T3 (100 nM) was added to the appropriate dishes. The cells were lysed, and the CAT activity was analyzed as described (16). The CAT activity was normalized for the protein concentrations of the lysates.

**Western Blotting**—Cell lysates (60 µg) from transient transfection experiments described above were loaded onto a 12% SDS-polyacrylamide electrophoresis gel. Western blotting was carried out as described (16). The antibodies used for detecting GFP-TR1 were monoclonal antibody C4 (1 µg/ml) and monoclonal anti-GFP antibodies (1 µg/ml, CLONTEC).

**Expression of GFP-TR Proteins in CV1 Cells for Evaluation of Cytoplasmic Nuclear Trafficking**—CV1 cells were plated 24 h before transfection in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum at a density of 4.5 × 10⁵ cells in a Lab-Tek chambered cover glass cuvette (Nage Nunc International, Naperville, IL). Cells were transfected with the appropriate plasmids (0.2 µg) using the LipofectAMINE transfection method described above. Fifteen hours later, the medium was replaced with Dulbecco’s modified Eagle’s medium containing 10% T3-depleted serum, and cells were further incubated for an additional 12 h. Cells were observed as described below.

**Confocal Microscopy and Image Analysis**—Microscopy was performed using a Zeiss LSM410 laser-scanning confocal microscope. All images were captured using a 100 × 1.4 numerical aperture objective lens. Image analysis and determination of nuclear/cytoplasmic ratios were carried out using standard functions of the LSM410 software. The ratios were determined by averaging the intensities of 20 random regions within the nucleoplasm and cytoplasm of 50–60 living cells. All images were scanned at a fixed gain and black level.

**RESULTS**

**Hormone and DNA Binding Activities of GFP-TR1**—It is known that the COOH-terminal region is essential for the function of TR1 (17), whereas the amino-terminal A/B domain is not critically involved in the hormone and transcriptional activity of TR1 (16, 18). We therefore fused GFP to the amino terminus of TR1 (Fig. 1A). The GFP used was a variant derived from the jellyfish Aequorea victoria, containing a serine to threonine substitution at amino acid 65, which makes this variant not only a stronger chromophore but also more resistant to photobleaching than the wild-type GFP (20).

To be certain that the tagging of GFP to TR1 did not significantly alter the functional characteristics of TR1, we evaluated the hormone and DNA binding activities of GFP-TR1. GFP-TR1 was prepared by in vitro transcription/translation, and its binding to T3 was assessed. No significant differences were detected in the competitive displacement curves from the binding of T3 to TR1 or GFP-TR1 (data not shown). The dissociation constants (Kd) were determined to be 0.20 ± 0.05 and 0.14 ± 0.04 nM for TR1 and GFP-TR1, respectively, indicating that tagging the GFP to the amino terminus of TR1 did not affect the T3 binding activity of TR1.

We further evaluated the DNA binding activity of GFP-TR1 by using two different TREs. Fig. 2A shows that GFP-TR1 bound to Lys-TRE not only as a homodimer (lane 3), but also as a heterodimer with the RXRβ (lane 6). The binding, however, was weaker than the binding of the wild-type TR1 to Lys-TRE as compared with the homodimeric and heterodimeric shown in lanes 2 and 5, respectively. We also examined the binding of GFP-TR1 to Pal-TRE. Consistent with the much weaker binding of the wild-type TR1 to Pal-TRE as compared with Lys-TRE (Fig. 2B, lane 2), binding of GFP-TR1 to Pal-TRE was not detectable under the experimental conditions (Fig. 2B, lane 3). However, GFP-TR1 bound to Pal-TRE as a heterodimer with RXRβ as shown in lane 6 even though its binding was weaker than the wild-type TR1 (lane 5).

**GFP-TR1 Is Transcriptionally Active**—To further characterize the functional properties of GFP-TR1, we examined the transcriptional activity of GFP-TR1 using the CAT reporter system. CV1 cells were co-transfected with the GFP-TR1 or the wild-type TR1 expression plasmid with the TRE-containing CAT expression vectors. Fig. 3A shows that in using Lys-TRE, the transcriptional activity of GFP-TR1 was indistinguishable from that of TR1 in that the extent of T3-dependent transcriptional activity was not significantly different (bar 6 versus 4), and it also similarly repressed the basal transcriptional activity in the absence of T3 (bars 5 and 3 versus bar 1). Bars 1 and 2 are controls to indicate the basal transcriptional activity. Similar results were also obtained when Pal-TRE was used. Bars 4 and 6 of Fig. 3B indicate that no significant differences were detected in the T3-dependent transactivation activities between the wild-type TR1 and GFP-TR1. Taken together, these results indicate that GFP-TR1 was transcriptionally competent and is a valid probe to study the intracellular movement of TR1.

**T3-induced Nuclear Translocation of GFP-TR1**—Using fixed cultured cells, T3-bound TRs were shown to be localized in the nucleus (12). To understand whether T3 regulates the nuclear translocation of TRs in living cells, CV1 cells transfected with the GFP-TR1 expression vector were grown in T3-containing or T3-depleted medium, and the expressed GFP-TR1 was visualized by confocal microscopy. We determined the intracellular distribution of GFP-TR1 by measuring the intensities of 20 random regions in the cytoplasm and nucleus of 50–60 living cells. The plot of frequency of cells with nuclear/
cytoplasmic ratios is shown in Fig. 4. Fig. 4A shows that in the absence of T3, cells had nuclear/cytoplasmic ratios in the range of 1–4 with the highest frequency of cells having the ratio of 2. However, no cell with a ratio higher than 4 was detected. Including the cells exhibiting only cytoplasmic localization, the average nuclear/cytoplasmic ratio was calculated to be ~1.5. In contrast, in the presence of T3 (Fig. 4B), most of the cells had higher nuclear/cytoplasmic ratios, ranging from 2–20, with an average ratio of ~5.5. Representative cells showing localization of GFP-TRβ1 in both the nucleus and cytoplasm in the absence of T3 are shown in Fig. 5A and those in the presence of T3 showing localization in the nucleus are shown in Fig. 5B. The distribution profiles shown in Fig. 4 were independent of the expression levels of GFP-TRβ1. We had also determined the distribution in cells transfected with a 10-fold higher concentration of GFP-TRβ1 plasmid. When a higher concentration of plasmid was transfected, GFP-TRβ1 was expressed in more cells and the intensity was higher. However, no significant differences in the nuclear/cytoplasmic ratios were detected (data not shown).

The GFP-TRβ1, which was localized in the cytoplasm, could be induced to translocate into the nucleus by T3 in a time-dependent manner. Fig. 5B shows that the GFP-TRβ1, which was in the cytoplasm shown in Fig. 5A, was translocated into the nucleus after 30 min at 37 °C. Fig. 5C shows another representative cell in which GFP-TRβ1 was detected in both the nucleus and the cytoplasm in the absence of T3. However, as shown in Fig. 5D, l-thyronine, which is a biologically inactive analog of T3, did not induce the translocation of GFP-TRβ1. The distribution of GFP-TRβ1 in the cell shown in Fig. 5D was the same as that in Fig. 5C. Thus, although a major fraction of GFP-TRβ1 is “constitutively” localized in the nucleus, GFP-TRβ1 can also respond to hormone to undergo nuclear translocation.

The Nuclear Localization Signal of TRβ1 Is Located in Domain D—Based on the studies of many nuclear proteins including steroid receptors, the consensus sequence mediating the nuclear translocation has been determined to consist of two clusters of 2–3 basic amino acids separated by two nonbasic amino acids ((K/R)(K/R)(K/R)) (21). Analysis of the amino acid sequence of TRβ1 identified a possible nuclear localization signal in domain D (Lys184-Arg185-Leu186-Ala187-Lys188-Arg189-Lys190). To verify that this indeed mediated the nuclear translocation for TRβ1, we mutated the Lys184-Arg185 to AA. The mutated TRβ1 was fused to GFP to yield GFP-TR2A whose expression was under the same cytomegalovirus promoter as in GFP-TRβ1 (Fig. 1B). In living CV1 cells, which had been cultured in the presence of T3, two different distribution patterns of GFP-TR2A were observed. Approximately 20–30% of the cell population had GFP-TR2A equally distributed in the nucleus and cytoplasm. However, about 70–80% of the cell population had most of the GFP-TR2A localized in the cytoplasm, indicating the inability of GFP-TR2A to translocate despite the presence of T3. Representative cells are shown in Fig. 6B. This is in contrast to GFP-TRβ1 in that all GFP-TRβ1 was located in the nucleus in the presence of T3 (Figs. 6A and 5B). Panels C and D of Fig. 6 are the corresponding phase contrast micrographs for the cells shown in panels A and B, respectively. We also mutated Lys184-Arg185-Lys190 to AAA to obtain GFP-TR5A (Ala184-Ala185-Leu186-Ala187-Ala188-Ala189). These additional mutations only slightly increased the cell population having the receptors localized in the cytoplasm (80–90%). T3 Fifteen hours later, the medium was replaced with Dulbecco’s modified Eagle’s medium containing T3-depleted serum with or without 100 nM T3. The intensities and intracellular distribution of GFP-TRβ1 were determined after cells were incubated for an additional 12 h. The resulting nuclear/cytoplasmic ratios were measured as described under “Materials and Methods.”
Bar 8 of Fig. 3 shows that ~75 and ~90% of T₃-dependent transactivation activity of GFP-TR2A was lost as compared with that of GFP-TRβ1 (Fig. 3, A and B, bar 4) on Lys- and Pal-TRE, respectively.

The lower T₃-dependent transactivation activity of GFP-TR2A could be due to its loss of T₃ binding because of mutations, a lower protein expression level in the transfected cells, an inability to bind to TREs, and/or an impairment in nuclear translocation. Therefore, we evaluated these possibilities. We determined the T₃ binding activity of GFP-TR2A. The competitive displacement curve for the binding of GFP-TR2A to T₃ was indistinguishable from those of TRβ1 and GFP-TRβ1 (data not shown). The Kᵅ was determined to be 0.16 ± 0.06 nM, which was not significantly different from that of TRβ1 and GFP-TRβ1 (0.20 ± 0.05 and 0.14 ± 0.04 nM, respectively). These data indicate that the lower transactivation activity of GFP-TR2A was not because of its inability to bind T₃. We then evaluated protein expression levels of GFP-TRβ1 and GFP-TR2A by Western blotting. As shown in Fig. 7, lanes 3 and 4, the expression levels of the two proteins as detected by monoclonal anti-TRβ1 antibody, C4, were quite similar. The identical protein expression levels were further confirmed by using anti-GFP antibodies (Fig. 7, lanes 6 and 7). Therefore, the lower transactivation activity of GFP-TR2A was not because of a lower expression level for the chimeric receptor.

The lower transactivation activity of GFP-TR2A was also not because of its inability to bind to TREs. Lanes 6 and 7 of Fig. 2A show that GFP-TR2A bound to Lys-TRE as a heterodimer with RXRβ stronger than GFP-TRβ1. On Pal-TRE, GFP-TR2A heterodimerized with RXRβ equally well as GFP-TRβ1 (Fig. 2B, lane 7 versus 6). Consistent with much weaker homodimeric binding of TRβ1 to Pal-TRE (15), binding of GFP-TRβ1 and GFP-TR2A to Pal-TRE was not detectable under the experimental conditions (Fig. 2B, lanes 3 and 4). Taken together, these results strongly suggest that the reduction of the transactivation activity of GFP-TR2A was most likely because of failure of cytoplasm to nucleus translocation of GFP-TR2A.

DISCUSSION

The present study demonstrated that the GFP-TRβ1 localized in the cytoplasm was induced to translocate into the nucleus by T₃. We identified a nuclear localization signal that mediated the T₃-induced nuclear translocation. The mechanism by which this signal responded to T₃ for nuclear import is not clear. This signal with the sequence of Lys₁₈⁴-Arg₁₈⁵-Leu₁₈⁶-Ala₁₈⁷-Lys₁₈₈-Arg₁₈₉-His₁₉⁰ is located in the “A-box,” which is part of a long a-helix (A-helix) in domain D (22). This structure was determined from the RXRα/TRβ DNA binding domain heterodimer complexed with a direct repeat TRE. At present, it is unknown whether the structure of this A-helix in the context of the intact TRβ1 undergoes changes upon the binding of T₃ to domain E because no crystallographic studies of the entire TRβ1 are yet available. However, it is reasonable to assume that this A-helix in domain D could undergo T₃-induced conformational changes as it is clearly demonstrated that dramatic structural changes occur in the ligand binding domain E of TRβ1 when bound to T₃ (23, 24). The T₃-induced changes of A-helix in the context of the intact TRβ1 could expose the nuclear localization signal to become more accessible to the receptors that bind the nuclear localization signal (25, 26), thereby providing additional regulation of the transcriptional activity of TRβ1. Conformational changes and protein folding have been implicated in the translocation of several steroid receptors and are thought to involve molecular chaper-
Hsp90 and a number of factors associate with the glucocorticoid receptor shortly after translation (27) and are displaced from the receptor during ligand activation and nuclear translocation (28). Stable complexes between the heat shock proteins and members of the thyroid receptor and retinoic acid receptor families have not been described (27), although Yamamoto and colleagues (29) reported that Hsp90 expression is required for retinoic acid receptors and RXR function in yeast. Thus it appears that the role of the heat shock chaperones in signal transduction by nuclear receptors may be more complex than simple association with the receptors in the cytoplasm. Moreover, whether Hsp90 plays a direct or indirect role in the translocation of TRs is totally unknown.

Consistent with the previous studies using fixed cells (12) and subcellular fractionation (5–9), we found that TRs are localized in the nuclei in the presence of T3. However, in the absence of T3, we found that the intracellular localization of TRs was heterogeneous with respect to nuclear/cytoplasmic ratios because of the presence of GFP-TR1 in the cytoplasm of some cells. This observation is in contrast to the previous reports in which high affinity and low capacity T3 binding sites (presumably TRs) were not detected in the cytosolic fractions of tissues and GH3 cells by radiolabeled hormone binding studies (5–9). There are several possible explanations to account for the different observations. First, subcellular fractionation of tissues and cells could lead to the loss of low amounts of the high affinity T3 binding sites present in the cytosol. Second, the tissues might still contain a sufficiently high level of endogenous T3 so that all high affinity binding sites are already located in the nucleus. Third, the radiodetection method may not be sensitive enough to detect the low level of TRs in the cytosol.

The use of GFP-labeled TRs to study the trafficking of TRs in living cells clearly offers significant advantages over the subcellular fractionation approach. However, as with all overexpression studies with GFP labeling (e.g. studies of intracellular trafficking of GFP-labeled steroid hormone receptors), other interpretations exist. First, GFP may alter the activity of TRs or its compartmentalization. Second, transport factors may be limiting or insufficient for the overexpressed TRs. This would
T₃-induced Nuclear Translocation of TRs

be studied by immunocytochemistry in fixed cells (19). In
contain some level of thyroid hormones, which might explain
TR₁ binding, DNA binding, and transactivation activities of GFP-
reflects the behavior of the endogenous receptor. This interpre-
cycles. In the absence of T₃, cells may preferentially “arrest” at
ulate cell growth (30). In a normal cultured cell population,
the transfected rat TR₁ in the cytoplasm because of the mutation of the nuclear
localization signals (GFP-TR2A). The cell cycle-dependent sub-
localization signals (E2F-4), a transcription factor involved in cell cycle progression, has been
shown to regulate its activity by changing the nuclear/cytoplasmic
distribution ratios during cell cycles (31). Furthermore, it has been shown that the retention of glucocorticoid receptors in
the nuclei is not only affected by the hormone but also by the
cell cycles. Glucocorticoid receptors are efficiently retained
within nuclei following hormone-dependent nuclear transloca-
tion in the G₀ and S phase of the synchronized cells, but not in
cells synchronized in the G₂ phase (32).

The intracellular localization of the transfected rat TRα1 has been studied by immunocytochemistry in fixed cells (19). In this study, similar to our findings, Lys₁⁸⁵-Arg₁⁸⁶-Lys₁⁸⁷ (corresponding to Lys₁⁸⁸-Arg₁⁸⁹-Lys₁⁹₀ in TRβ₁) was identified to be one of the nuclear localization signals. However, in contrast to our findings, ligand was not found to affect the localization of the transfected rat TRα1. There are several possible explanations for this discrepancy. The fixation procedures required for immunolocalization may cause major disruption of cellular architecture, causing a perturbation of the true in vivo receptor distribution. Also, the epitopes necessary for antibody recognition in a specific subcellular compartment may not be accessible in fixed cells to the large antibody complexes. Finally, the hormone-free conditions used for observing the localization of the rat TRα1 in the studies were not clearly defined. It is entirely possible that the hormone-free conditions could still contain some level of thyroid hormones, which might explain

The heterogeneous distribution of TRs with respect to nucle-
ar/cytoplasmic ratios in living cells could be the result of addi-
tional levels of control such as the cell cycle. We have observed
that synchronization of cells by serum starvation resulted in a
more uniform distribution of GFP-TRβ₁. T₃ is known to stim-
the lack of detection of the cytoplasmic localization.

The findings that TRβ₁ could be induced by T₃ to translocate
to the nucleus in a subpopulation of cells added another dimen-
sion in the regulation of TR actions. Thus, in addition to the
types of TRs and the co-regulatory proteins, which are known
to regulate transcriptional activity of TRs, the present study
demonstrates that the hormone-dependent nuclear trafficking
could play an important regulatory role in the functions of TRs.

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