Phosphorylation Selectively Increases Triiodothyronine Receptor Homodimer Binding to DNA*

Akira Sugawara‡, Paul M. Yen, James W. Apriletti, Raff C. J. Ribeiro, David B. Sacks, John D. Baxter, and William W. Chinn

From the Division of Genetics, Departments of Medicine and Pathology, Brigham and Women's Hospital, Howard Hughes Medical Institute and Harvard Medical School, Boston, Massachusetts 02115 and the Restless Research Unit, University of California, San Francisco, California 94143-0540

Thyroid hormone receptors (TRs) are ligand-regulated transcription factors that bind to thyroid hormone response elements (TREs) as monomers and homodimers, and as heterodimers with nuclear proteins such as TR auxiliary proteins and retinoid X receptors. Recently, bacterially expressed human TR-β1 (hTR-β1) was shown to be phosphorylated in vitro by HeLa cytosolic extract. However, little is known about the consequences of phosphorylation on the nature of TR complexes. Therefore, we studied the effect of phosphorylation on TR binding to TREs. Bacterially expressed hTR-β1 was phosphorylated in vitro with ATP by HeLa cytosolic extract. The ratio of phosphoserine to phosphothreonine was approximately 5:1. We then analyzed phosphorylated hTR-β1 binding to several TREs by electrophoretic mobility shift assay. Phosphorylated hTR-β1 bound better as a homodimer to the TREs than hTR-β1 incubated with preheated cytosolic extract. Alkaline phosphatase treatment of the phosphorylated hTR-β1 eliminated the enhanced homodimeric binding to DNA. In contrast, phosphorylation did not affect TR/auxiliary protein or TR/retinoid X receptor heterodimer binding to DNA. Triiodothyronine decreased both phosphorylated and unphosphorylated hTR-β1 homodimer binding to several TREs, and the addition of okadaic acid did not alter this triiodothyronine effect. These results indicate that phosphorylation, in addition to ligand binding, modulates TR dimer binding to TREs. As such, it is possible that phosphorylation may also participate in TR-mediated regulation of transcription.

**Thyroid hormone receptors (TRs)** are ligand-regulated transcription factors that belong to a large superfamily of receptors including steroid hormone, vitamin D, and retinoic acid receptors (1, 2). TRs bind to specific DNA sequences called thyroid hormone response elements (TREs) as monomers and homodimers (3–7), and bind as heterodimers with nuclear proteins such as TR auxiliary proteins (TRAPs) (7–11) and retinoid X receptors (RXRs) (12–17). Several groups have reported that TRs influences TR-DNA interactions (7, 18–21). We found that T₃ decreases TR homodimer binding, but not TR/TRAP or TR/RXR heterodimer binding, to several TREs (7, 17). Therefore, T₃-liganded TR/TRAP or TR/RXR heterodimers may play an important role in T₃-mediated transcription (7, 17). Additionally, since unliganded TRs may suppress basal transcription (18, 22), unliganded TR homodimers may be involved in basal repression.

In addition to the effect of ligand, phosphorylation may be involved in the transcriptional activity and/or DNA binding ability of members of the steroid/thyroid hormone receptor superfamily. Recently, it has been reported that phosphorylation enhances transcriptional activity of the progesterone receptor (23, 24), and both transcriptional and DNA binding activities of the glucocorticoid receptor (25). Chick TRα, v-erbA, and bacterially expressed human TR-β1 (hTR-β1) are also known to be phosphorylated (26–29). However, the physiological significance of these phosphorylation events is still unclear. The chick TRα and v-erbA are phosphorylated at serine residues that are not conserved in mammalian TRs such as human and rat TR isoforms. The hTR-β1 was phosphorylated in vitro by HeLa cytosolic extract, which resulted in an increase in hTR-β1 DNA binding using an avidin-biotin complex DNA binding assay (29). However, that study did not address the effect of phosphorylation on the nature of TR complexes. Accordingly, we have examined this question using electrophoretic mobility shift assays (EMSA) and in vitro phosphorylated hTR-β1. Surprisingly, phosphorylation of hTR-β1 increased homodimer binding to DNA, without changing TR/RXR or TR/TRA heterodimer binding to DNA.

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**MATERIALS AND METHODS**

**HeLa Cytosolic Extract Preparation**—HeLa or HeLa thymidine kinase-negative (tk−) kindly provided by Dr. M. Pfahl, La Jolla Cancer Research Foundation, La Jolla, CA) cells were grown in monolayer culture at 37 °C, 5% CO₂ in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cytosolic extracts were prepared as described previously (29).

**In Vivo Phosphorylation of TR**—Bacterially expressed full-length hTR-β1* (98% pure, 20 pmol/30 µl) was incubated with 10 µCi of [γ-³²P]ATP, 50 µM ATP, and 15 µg of HeLa cytosolic extract in the presence of 200 µM sodium orthovanadate (Na₂VO₃) and 1 µM okadaic acid in a final volume of 50 µl at 30 °C for 50 min as previously described (29). As a control, HeLa cytosolic extract preheated at 90 °C for 5 min was incubated under the same conditions. In the case of samples used for stoichiometry determination and phosphoamino acid analysis, the reaction was stopped with an equal volume of 10% (w/v) trichloroacetic acid, and 40 µl of 0.1% bovine serum albumin was added. Samples were processed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide gel) and autoradiography as previously described (30). Stoichiometry was determined by excising

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‡To whom correspondence should be addressed: G. W. Thorn Research Bldg., Rm. 905, Brigham and Women's Hospital, 20 Shattuck St., Boston, MA 02115. Tel.: 617-732-5858; Fax: 617-732-5123.

1 The abbreviations used are: TR, thyroid hormone receptor; TRE, thyroid hormone response element; RXR, retinoid X receptor; TRAP, TR auxiliary protein; T₃, triiodothyronine; h, human; m, mouse; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay.

2 J. W. Apriletti, manuscript in preparation.
the phosphorylated hTRP-1 from the gel; solubilizing in 1:2.10 (v/v/v) of water, Soluene, and Ultimagold; and performing liquid scintillation spectrometry. Background was measured in a piece of gel without specifically enhanced radioactivity.

Western Blotting—After SDS-PAGE, proteins were transferred to polyvinylidene difluoride, phosphorylated hTRP-1 was identified by autoradiography and hydrolyzed with 6 M HCl at 110°C for 2 h. Phosphoamino acids were separated on cellulose plates in 7% (v/v) formic acid at 600 V for 7 h and identified by autoradiography. The position of migration of phosphoamino acids was determined by ninhydrin staining of standards (phosphoserine, phosphothreonine, and phosphotyrosine).

Immunoprecipitation in vitro Phosphorylated TRs—Polycyclonal anti-TRβ1 antibody (32) was incubated with the same volume of immunizing peptide solution (1 µg/µl) or phosphate-buffered saline for 1 h at 4°C. As a control, preimmune serum was also incubated with the same volume of phosphate-buffered saline. After centrifugation at 10,000 x g for 5 min, 10 µl of the supernatant was incubated with 10 µl of in vitro phosphorylated hTRP-1 and 30 µl of 1 µL immunoprecipitation buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% (v/v) Triton X-100, 0.1% (w/v) SDS, 10 mM NaF, 10 mM Na2P2O7, 10 mM EDTA, 10 mM EGTA, 2 mM Na3VO4 for 2 h at 4°C. Slurry (100 µl) of 10% (v/v) preheated cytosolic extract in the presence of ATP) to several preheated cytosolic extract in the absence of ATP (2 µl) was used. In vitro transcription and translation of human RXRα (hRXRα) in pBKS (kindly provided by Dr. M. G. Rosenfeld, University of California, San Diego, CA) and mouse RXRβ (mRXRβ) in PBS (kindly provided by Dr. K. Ozato, National Institutes of Health, Bethesda, MD) were performed, and the proteins were used for EMSA as previously described (17). Nuclear extracts of 235-1 pituitary cells used for EMSA were prepared as previously described (32). The rat liver TR auxiliary factor (TAF3) was obtained from fractions eluted out the high performance liquid chromatography sizing column during large scale purification of endogenous TRs (34). They did not contain endogenous TR activity, as evidenced by their lack of T3 (data not shown) and DNA (Fig. 3B, lane 10) binding activities. The densities of the bands detected by EMSA were quantitated by scanning laser densitometry (Molecular Dynamics, Sunnyvale, CA).

Alkaline Phosphatase Treatment—hTRβ1 phosphorylated with unlabeled ATP was incubated with or without 36 units of alkaline phosphatase (Boehringer Mannheim) in a final volume of 10 µl at 37°C for 1 h. Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed by thin-layer chromatography as described (31). Briefly, after transfer to polyvinylidene difluoride, phosphorylated hTR-1 was identified by autoradiography and hydrolyzed with 6 M HCl at 110°C for 2 h. Phosphoamino acids were separated on cellulose plates in 7% (v/v) formic acid at 600 V for 7 h and identified by autoradiography. The position of migration of phosphoamino acids was determined by ninhydrin staining of standards (phosphoserine, phosphothreonine, and phosphotyrosine).

RESULTS AND DISCUSSION

Silver staining of the unmodified bacterially expressed hTRB-1 revealed two TR forms (55 and 49 kDa), with the latter probably due to translation from an internal methionine (Fig. 1A, lane 1). No bands corresponding to hTRB-1 were observed in HeLa cytosolic extract (Fig. 1A, lane 2) with or without ATP. We previously reported that co-incubation of HeLa nuclear extract with hTRB-1 formed a TR/TRAP heterodimer band that migrated more slowly than the TR homodimer band on EMSA (35). However, co-incubation with HeLa cytosolic extract did not form additional bands on EMSA, suggesting that the cytosolic extract did not contain any TRAP activity (data not shown). When hTR-B1 was phosphorylated with [γ-32P]ATP by HeLa cytosolic extract and analyzed by SDS-PAGE, three major 32P-labeled bands (95, 50, and 32 kDa) were observed (Fig. 1B). Anti-TRβ1 antibody immunoprecipitated a doublet of 32P-labeled bands (~50 kDa; Fig. 1C, lane 8), which migrated with mobilities identical to those of the 35S-labeled, in vitro translated hTRβ1 forms seen in Fig. 1C (lane 1).

These bands were not observed when anti-TRβ1 antibody was preincubated with immunizing peptide (Fig. 1C, lane 9) or when preimmune serum was used (Fig. 1C, lane 10), indicating that these 32P-labeled doublet bands represent phosphorylated hTRβ1. When cytosolic extract was incubated without hTRβ1, hTRβ1 was incubated with preheated cytosolic extract (Fig. 1C, lanes 5–7) in the presence of [γ-32P]ATP, these bands were not observed. These data confirm that hTRβ1 phosphorylation is mediated by a kinase(s) in the HeLa cytosolic extract (as previously reported; Ref. 29) whose activity was inactivated by heating. 32P-Labeled doublet bands with apparent molecular sizes of approximately 95 kDa were also immunoprecipitated (Fig. 1C, lane 8). The identity of these 95-kDa bands is unknown. It is possible that they are due to weak antibody cross-reactivity with low abundance, but highly phosphorylated, non-TR protein that was present in the bacterial and/or cytosolic extract (Fig. 1B and C).

We next performed phosphoamino acid analyses of the bands corresponding to TR and the 95-kDa protein. As shown in Fig. 2A, phosphorylation of serine, tyrosine, and threonine residues in TR was in a ratio of 90:3:7 (lane 1) and 80:4:16 (lane 2) in the absence and presence of okadaic acid and Na3VO4, respectively. The ratio in the presence of okadaic acid and Na3VO4 is similar to the ratio observed by Lin et al. (29). In contrast, the relative ratios of phosphoserine, phosphothreonine, and phosphotyrosine in the 95-kDa protein(s) were 61:2:37 (Fig. 2B, lane 1) and 55:3:42 (Fig. 2B, lane 2) in the absence and presence of okadaic acid and Na3VO4, respectively. These data suggest that the 95-kDa protein is unrelated to TR. Determination of stoichiometry showed that ~0.1 mol of phosphate/mol of hTRβ1 was incorporated. However, since cytosol is reported to contain large amounts of ATP (0.2–1.9 mM) (36, 37), the actual stoichiometry is probably higher than 0.1.

We next performed EMSA to study the binding of phosphorylated hTRβ1 (incubated with cytosolic extract in the presence of ATP) and unphosphorylated hTRβ1 (incubated with preheated cytosolic extract in the presence of ATP) to several TREs containing half-sites of different orientations. As shown in Fig. 3, panel A (lanes 2 and 3) and panel B (lanes 2, 4, 12, 14, 16, and 18), phosphorylation of hTRβ1 increased homodimer binding to F2 (inverted palindromic), rMHCα (direct repeat), DR4 (direct repeat), and TREPα (palindromic). In the latter case, unphosphorylated hTRβ1 bound mainly as monomer (Fig. 3B, lane 2), and the monomer binding was not increased by phosphorylation of hTRβ1 (Fig. 3B, lane 4). Cytosolic extract alone showed no binding to F2 (Fig. 3A, lane 1).

We next treated phosphorylated hTRβ1 with alkaline phosphatase. As shown in Fig. 3C, phosphatase treatment decreased TR homodimer binding (lanes 1 and 2) to F2, confirming that hTRβ1 phosphorylation enhances homodimer binding to DNA. Similar results were also observed with another TRE, DR4 (data not shown). Comparison of silver-stained hTRβ1 samples treated with or without alkaline phosphatase showed no difference in the protein patterns, indicating that the phosphatase did not contain any protease activity (data not shown).

We have shown previously that T3 decreases in vitro translated TR homodimer binding to several TREs (7). T3 decreased both phosphorylated and unphosphorylated bacterially expressed hTRβ1 homodimer binding to F2 at similar concentrations (Fig. 4, panel A, lanes 1–4, and panel B). Furthermore, addition of the phosphatase inhibitors, Na3VO4 and okadaic acid, had no effect on the T3-mediated decrease in TR homodimer binding to F2 (Fig. 4A, lanes 5 and 6). T3 also de-
LANE and HeLa cytosolic extracts were analyzed by SDS-PAGE, and Rad) according to the manufacturer’s instructions. Through a Sephadex G-50 column, and the eluate was analyzed by extract containing hTRP-1 (10(1 pg).

HeLa cytosolic extract in the presence of [γ-32P]ATP immunoprecipitation. Bacterially expressed hTRP-1 incubated with HeLa cytosolic extract (lanes 2-4) described under “Materials and Methods.” Immunoprecipitation was performed using anti-TRP-1 antibody alone (lanes 2,5, and 8), anti-TRP-1 antibody preincubated with immunizing peptide (lanes 3, 6, and 9), and preimmune serum (lanes 4, 7, and 10). hTRP-1 bands are indicated by arrows. Abbreviations are as follows: pre, preimmune serum; cyt, cytosolic extract.

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Phosphorylation Increases TR Homodimer Binding to DNA increased phosphorylated hTRβ-1 homodimer binding to rMHCα and DR4 (Fig. 3B, lanes 15 and 19). Additionally, as previously reported for unphosphorylated hTRβ-1 (19), T3 increased both monomer and homodimer binding of phosphorylated hTRβ-1 to TREpal (Fig. 3B, lanes 4 and 5). These results also imply that the T3 influence on TR-DNA binding is not exerted by inducing dephosphorylation of the TR sites phosphorylated by the HeLa cytosolic extract.

We also performed EMSA to examine the ability of phosphorylated and unphosphorylated hTRβ-1 to form heterodimers with endogenous TRAP or in vitro translated RXR. In order to allow only heterodimer formation, 10−6 M T3 was added to each sample. In contrast to their ability to form homodimers, there was no difference between phosphorylated and unphosphorylated hTRβ-1 heterodimerization with in vitro translated RXRα, RXRβ, or endogenous TRAP in 235–1 nuclear extract using F2 as the TRE (Fig. 3A, lanes 4–12). Similar results were also observed with rat liver TR auxiliary factor/ protein(s) using TREpal as a probe (Fig. 3B, lanes 6–9). In addition, alkaline phosphatase treatment of phosphorylated hTRβ-1 incubated with cytosolic extract had no effect on heterodimerization with endogenous TRAP in 235–1 nuclear extract using F2 (Fig. 3C, lanes 3 and 4). Similar results were also observed with another TRE, DR4 (data not shown). These data suggest that phosphorylation of hTRβ-1 affects TR homodimer binding to TREs, but not TR/TRAP or TR/RXR heterodimer binding to TREs. These results are similar to those of Lin et al. (29), who observed an increase in phosphorylated TR binding to DNA. However, their work did not distinguish whether the increase was due to increased TR homo- or heterodimer binding.

Phosphorylation can alter the DNA binding activity of several other transcription factors in addition to hTRβ-1 (38). For example, glucocorticoid receptor (25), SRF (39), and CREB (40) binding to DNA is increased by phosphorylation. In contrast, c-Myb (41), c-Jun (42), and Max homodimer (43) binding to DNA is decreased by phosphorylation. Interestingly, Max phosphorylation does not affect Myc/Max heterodimer binding to DNA (43).

The role of TR phosphorylation in TR action is unknown. The
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**Fig. 3.** EMSA of phosphorylated and unphosphorylated hTRP-1. A, homo- and heterodimerization of phosphorylated and unphosphorylated hTRP-1 on F2 TRE. Homodimer formation was examined using 1 μl of phosphorylated (lane 2) and unphosphorylated (lane 3) hTRP-1. Lane 1, cytosolic extract alone (2 μg). For heterodimer formation, in vitro translated hRXRa (0.5 μl, lanes 4–6), mRXRa (0.5 μl, lanes 7–9), or 235–1 cell nuclear extract (5 μg, lanes 10–12) was incubated with 0.3 μl of either phosphorylated (lanes 4, 7, and 10) or unphosphorylated (lanes 5, 8, and 11) hTRP-1 in the presence of 10^{-6} M T3. B, homo- and heterodimerization of phosphorylated and unphosphorylated hTRP-1 on different TREs. Homodimer formation was examined using 1 μl of phosphorylated (lanes 4, 5, 14, 15, 18, and 19) or unphosphorylated (lanes 2, 3, 12, 13, 16, and 17) hTRP-1 on TREpal (lanes 1–5), rMHCα (lanes 11–15), and DRA (lanes 16–19). In lanes 3, 5, 13, 15, 17, and 19, T3 (10^{-8} M) was added. Lanes 1 and 11, probe alone. Heterodimer formation was examined using 1 μl of phosphorylated (lanes 8 and 9) or unphosphorylated (lanes 6 and 7) hTRP-1 with rat liver TRAP (Liver) (0.7 μg) on TREpal in the absence (lanes 6 and 8) or presence (lanes 7 and 9) of T3 (10^{-6} M). Lane 10, rat liver TR auxiliary factor protein(s) (0.7 μg) alone. C, effect of alkaline phosphatase treatment of phosphorylated hTRP-1 on homo- and heterodimerization on F2 TRE. Phosphorylated hTRP-1 was treated with (lanes 1 and 3) or without (lanes 2 and 4) alkaline phosphatase. Then 0.8 μl of each sample was analyzed by EMSA in the absence (lanes 1 and 2) or presence of 2 μg of 235–1 nuclear extract and 10^{-6} M T3 (lanes 3 and 4). Additional abbreviations: P, phosphorylated hTRP-1; U, unphosphorylated hTRP-1; M, hTRP-1 monomer; βD, hTRP-1 homodimer; TR/Ra, hTRP-1/hRXRa heterodimer; TR/Rβ; hTRP-1/mRXRa heterodimer; HD, heterodimers of hTRP-1 formed with two endogenous TRAPs in 235–1 cells (35). Note that the two hTRP-1/hRXRa heterodimer bands are probably due to the two in vitro translated products of hRXRa. * represents nonspecific binding.

**Fig. 4.** T3 effect on TR homodimer binding to F2 TRE. A, effect of phosphatase inhibitors on the T3-induced decrease in homodimer binding to DNA. Phosphorylated (lanes 1, 3, and 5) or unphosphorylated (lanes 2, 4, and 6) hTRP-1 (0.7 μl) was analyzed by EMSA in the absence (lanes 1 and 2) or presence of 10^{-6} M T3 (lanes 3 and 4). In lanes 5 and 6, phosphatase inhibitors (200 μM Na3VO4 and 1 μM okadaic acid) were also incubated with 10^{-6} M T3. B, T3 dose-dependent effect on hTRP-1 homodimer binding to F2 TRE. Phosphorylated or unphosphorylated hTRP-1 (1 μl) was incubated with increasing concentrations of T3 and analyzed by EMSA. The intensities of the homodimer bands on the fluorograph were quantitated by laser densitometry. The density of the homodimer band in the absence of T3 is set as maximal homodimer bound (100%).

Influence of phosphorylation on DNA binding appears to be selective for homodimers and, therefore, could affect TR function when homodimers are active. This might be more prominent in the absence of T3, since the ligand tends to disrupt homodimers, and unliganded TRs may repress basal transcription. However, since okadaic acid enhances T3-mediated transcriptional activation, it is possible that phosphorylated TR may activate transcription in its liganded state. In any case, our results provide evidence for another mechanism, in addition to ligand binding, by which homodimer binding to TREs is modulated. As such, it is possible that phosphorylation of TRs may participate in TR-mediated regulation of transcription.

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