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Phosphorylation stimulates the transcriptional activity of the human β1 thyroid hormone nuclear receptor

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ABSTRACT **The role of phosphorylation on the gene activation activity of the human β1 thyroid hormone nuclear receptor (h-TRβ1) was examined. h-TRβ1 was found to be a phosphoprotein when expressed in COS-1 cells, with serine, threonine, and tyrosine (85:10:5) as the phosphorylation sites. Okadaic acid (a potent inhibitor of phosphatases 1 and 2A) at 0.1, 0.25, and 0.5 μM increased the phosphorylation of h-TRβ1 by 3-, 7-, and 11-fold, respectively. The increase in phosphorylation was accompanied by a concomitant increase in receptor-mediated transcription in transient transfection assays. h-TRβ1 purified from *Escherichia coli* was phosphorylated *in vitro* by the endogenous kinase from cellular extracts. Serine, threonine, and tyrosine were phosphorylated in a similar ratio to that found in COS-1 cells. The *in vitro* phosphorylation was stimulated by okadaic acid. Phosphorylation did not affect the binding of h-TRβ1 to 3,3′,5-triiodo-L-thyronine. However, phosphorylation of h-TRβ1 resulted in an increase of its binding to DNA and conferred on it the ability to bind to nuclear accessory proteins. The results indicate that phosphorylation plays an important role in the transcriptional activity of h-TRβ1.**

The steroid/thyroid hormone receptors comprise a superfamily of ligand-dependent transcription factors that play a crucial role in regulating homeostasis, reproduction, and differentiation. Upon binding to their cognate ligands, these receptors activate or repress expression of target genes by binding to specific DNA sequences known as hormone response elements (1). The selectivity and recognition of the hormone response element for different receptors are dictated by the relative orientation and spacing of the half-site motifs (2, 3). However, one critical question to address is how the transcriptional activity is modulated once these receptors are positioned on their cognate hormone response elements.

Modulation of the transcriptional activity could be achieved by changing the level of receptors through the regulation of protein synthesis or by changing the activity of the receptors by protein modification via phosphorylation. Phosphorylation plays an important role in regulating the activity of sequence-specific transcription factors. Several mechanisms by which phosphorylation modulates the transcriptional activity of the factors have been reported. (*i*) Phosphorylation can influence the translocation of transcription factors between cytoplasm and nucleus (4, 5). (*ii*) Phosphorylation alters the DNA-binding activity of the factors (6–8). (*iii*) Phosphorylation activates the constitutively bound transcription factors (9). Several studies reported a correlation between transcriptional activity and phosphorylation without demonstrating directly an increase in binding to DNA (10). To explore the role of phosphorylation in receptor functions, we studied the effect of phosphorylation of human β1 thyroid hormone receptor (h-TRβ1) on transcriptional activity in COS-1 cells. The molecular basis for the activation was assessed by *in vitro* phosphorylation of recombinant h-TRβ1 purified from *Escherichia coli*. Phosphorylation resulted in the increase in the binding of h-TRβ1 to a thyroid hormone response element (TRE). Furthermore, phosphorylation confers on the receptor the ability to interact with the essential transcriptional factors.

MATERIALS AND METHODS

Construction of h-TRβ1 Expression Vector pCLC51. Plasmid pCLC51 was derived from rat TRβ1 expression vector pCDMerb62 (11). pCDMerb62 was treated with *Xho* I and *Not* I to remove the sequence coding for rat TRβ1. The insert containing the coding sequence for h-TRβ1 was synthesized by polymerase chain reaction using peA101 (12) as a template. The synthesized insert was purified and ligated into the vector prepared above. The nucleotide sequence at the 5′ and 3′ ends of the h-TRβ1 coding sequence (≈800 bases) was confirmed by DNA sequencing. Detailed restriction analyses further confirmed the sequence.

Phosphorylation of h-TRβ1 in Cultured Cells. Monkey COS-1 cells (7×10^5 per 100-mm dish) were plated 1 day before transfection in Dulbecco's modified Eagle's medium containing 10% thyroid hormone-depleted (Td) fetal bovine serum (Td medium) (13). Cells were transfected with 15 μg of h-TRβ1 expression vector pCLC51 by a calcium phosphate precipitation method (14). After 24 hr, medium was changed to Td medium supplemented with 100 nM 3,3′,5-triiodo-L-thyronine (T_3). After 24 hr, cells were incubated in phosphate-free medium containing 100 nM T_3 and 5% dialyzed Td serum for 90 min. The medium was then replaced by 2.5 ml of fresh medium and incubated with 2 mCi (74 MBq) of carrier-free [^{32}P]orthophosphate and 50 μM sodium vanadate in the absence or presence of various concentrations of okadaic acid (0.1–0.5 μM) for 3 hr. In some experiments, cells were treated with 8-bromo-cAMP, forskolin, or phorbol 12-myristate 13-acetate in the last 30 min of incubation with [^{32}P]orthophosphoric acid.

For the labeling of h-TRβ1 with L-[^{35}S]methionine, 24 hr after transfection, cells were incubated with 100 μCi of [^{35}S]methionine in the absence or presence of various concentrations of okadaic acid in 4 ml of methionine-free Td medium containing 100 nM T_3. After incubation with [^{32}P]orthophosphoric acid or [^{35}S]methionine for 3 hr, cells were rinsed in ice-cold phosphate-buffered saline and lysed at 4°C for 10 min with 0.5 ml of EBC buffer (50 mM Tris, pH 8.0/120 mM NaCl/0.5% Nonidet P-40) containing 100 mM NaF, 0.2 mM Na_3VO_4, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and 200 μM phenylmethylsulfonyl fluoride. The cells

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Abbreviations: T_3, 3,3′,5-triiodo-L-thyronine; h-TRβ1, human thyroid hormone nuclear receptor β1; CAT, chloramphenicol acetyltransferase; mAb, monoclonal antibody; TRE, thyroid hormone response element; r-TRE, rat TRE.

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were scraped with a rubber policeman and the lysates were then centrifuged (16,000 × *g*) at 4°C for 30 min. Half of the supernatant was mixed with 0.5 ml of NET-N buffer (20 mM Tris, pH 8.0/100 mM NaCl/1 mM EDTA/0.5% Nonidet P-40) containing 5 μg of monoclonal antibody (mAb) J52 (15) or 5 μg of the control antibody MOPC21. The mixture was incubated for 2 hr on ice and 55 μl of washed formalin-fixed *Staphylococcus aureus* was added. After incubation for 30 min, the mixture was centrifuged (1100 × *g*) for 10 min and the pellet was washed essentially as described (15). The immunoprecipitates were analyzed by gel electrophoresis. The ^{32}P-labeled or ^{35}S-labeled h-TRβ1 was quantitated by densitometric scanning of autoradiograms or by excision of the radioactive bands followed by scintillation counting.

Determination of h-TRβ1-Mediated Transcription. COS-1 cells (5 × 10^5 per 60-mm dish) were plated and cultured for transfection as described above. The cells were cotransfected with the h-TRβ1 expression vector (pCLC51; 2 μg), a reporter gene that contained two copies of palindromic thyroid hormone response elements in tandem upstream of the chloramphenicol acetyltransferase (CAT) gene (pTK28m; 2.5 μg), and a human growth hormone expression plasmid (pXGH5; 1 μg) as an internal control for transfection efficiency. Transfection was carried out by calcium phosphate precipitation. Twenty-four hours after transfection, the cells were washed and fresh medium was added with 100 nM T_3. Cells were cultured for another 24 hr. In the last 3 hr, various concentrations of okadaic acid were present. After incubation, the cells were harvested and lysed, and the CAT activity was determined (16).

Phosphorylation of h-TRβ1 *in Vitro*. Purified h-TRβ1 (0.3 μg/30 μl), prepared as described (17), was incubated with 10 μCi of [γ-^{32}P]ATP (specific activity, ≈6000 Ci/mmol) and cytosolic extract of HeLa cells (20 μg/10 μl) in a final volume of 50 μl containing 30 mM Hepes (pH 7.5), 6 mM $MgCl_2$, and 50 μM ATP. In some experiments, 200 μM Na_3VO_4 or 1 μM okadaic acid was added to the phosphorylation mixture. Cytosolic extracts of HeLa cells were prepared as described (18). Phosphorylation was carried out at 30°C for various times, depending on the purpose of the experiments. After phosphorylation, the phosphorylated h-TRβ1 was immunoprecipitated with 1 μg of mAb J52. The immunoprecipitates were analyzed by gel electrophoresis and the radioactivity was determined as described above.

DNA-Binding Assay. Binding of DNA to phosphorylated h-TRβ1 was carried out with the avidin–biotin complex DNA-binding assay (ABCD assay) (19). The DNA used was the rat growth hormone TRE (r-TRE; nucleotides −186 to −158 relative to the transcription start site) with random sequences added at the 5′ and 3′ ends for the incorporation of biotin. In a typical binding assay, h-TRβ1 was phosphorylated as described above except that only unlabeled ATP (2 mM) was used. After phosphorylation, the mixture was chilled to 4°C. An aliquot (40 μl) of the phosphorylation mixture was incubated with 3 nM ^{125}I-T_3 at 4°C for 30 min in the absence or presence of 20–60 μl of HeLa cell nuclear extract (1 mg of protein per ml) in a final volume of 100 μl in buffer H (20 mM Hepes, pH 7.8/50 mM NaCl/1 mM 2-mercaptoethanol/1 mM EDTA/0.1% Nonidet P-40/20% glycerol). Nuclear extract was prepared as described (20). r-TRE (5 ng/μl; 10 μl) was subsequently added to the above mixture and was incubated for an additional 40 min. At the end of the incubation, 30 μl of 75% of streptavidin-agarose slurry was added. After incubation for 30 min, the mixture was centrifuged (11,000 × *g*) at 4°C for 30 sec. The radioactivity associated with streptavidin-agarose was determined by a γ-counter after the agarose was washed three times with buffer H.

Identification of Phospho Amino Acids. The phosphorylated h-TRβ1 bands were localized by autoradiography and excised from the acrylamide gel. Elution and hydrolysis of ^{32}P-labeled h-TRβ1 was carried out as described (21). Electrophoresis of the phospho amino acids was carried out in pyridine/acetic acid/water (1:10:189, vol/vol) at 1000 V for 90 min at 12–14°C. The unlabeled standards were visualized by ninhydrin (200 mg/100 ml in pyridine/acetone, 1.5:98.5, vol/vol); the radioactive phospho amino acids were localized by autoradiography.

RESULTS

h-TRβ1 Is a Phosphoprotein When Expressed in COS-1 Cells. Since it is unknown whether h-TRβ1 is phosphorylated *in vivo*, we first demonstrated that h-TRβ1 is a phosphoprotein. COS-1 cells, which are functionally deficient in T_3 receptor, were transfected with an h-TRβ1 expression vector. Nuclear extract of the transfectants was prepared. The apparent association constant for binding of T_3 to the expressed h-TRβ1 was 6 × 10^9 M^{-1}. This binding constant is similar to that of the endogenous T_3 receptors from tissues and cultured cells (22). Cells were metabolically labeled with [^{32}P]orthophosphoric acid and lysates were immunoprecipitated with mAb J52, which was raised against h-TRβ1 (15). Preliminary experiments indicated that phosphorylation of h-TRβ1 reached equilibrium after 3–4 hr. The h-TRβ1 expressed in COS-1 cells was a phosphoprotein (Fig. 1, lane 1). Cells were further treated with okadaic acid under equilibrium conditions to evaluate its effect on the phosphorylation of h-TRβ1. In the presence of 0.1, 0.25 and 0.5 μM okadaic acid, phosphorylation of h-TRβ1 was increased by 3-, 7-, and 11-fold, respectively (lanes 2–4). To examine whether the increase in phosphorylation was due to an increase in the cellular level of h-TRβ1 or a result of inhibition of dephosphorylation or both, we incubated the cells with [^{35}S]methionine in the presence of okadaic acid and then carried out immunoprecipitation with mAb J52. The rate of h-TRβ1 synthesis was virtually unchanged at all concentrations of okadaic acid (lanes 6–9), indicating that okadaic acid has no effect on the synthesis of h-TRβ1 during the period in which the level of phosphorylation was increasing. Neither ^{32}P-labeled nor ^{35}S-labeled h-TRβ1 was immunoprecipitated when a control antibody (MOPC21) was used (lanes 5 and 10). These results indicate that the increase in phosphorylation of h-TRβ1 was due to the inhibition of cellular phosphatases by okadaic acid.

To identify the amino acids that were phosphorylated, the protein bands were eluted from the gel and acid hydrolyzed. For a positive control, we also treated human epidermal carcinoma A431 cells with [^{32}P]orthophosphoric acid and

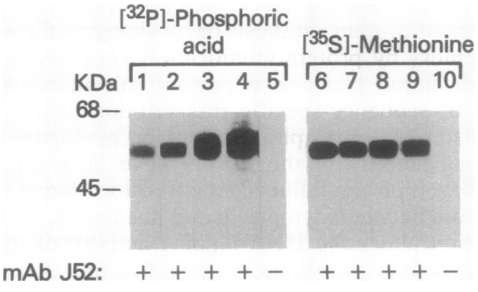

FIG. 1. Effect of okadaic acid on the phosphorylation of h-TRβ1 in COS-1 cells. COS-1 cells (7 × 10^5 per 100-mm dish) were transfected with h-TRβ1 expression vector. Cells were labeled with 2 mCi of [^{32}P]orthophosphoric acid (lanes 1–5) or 100 μCi of [^{35}S]methionine (lanes 6–10) in the absence of okadaic acid (lanes 1 and 6) or in the presence of 100 nM (lanes 2 and 7), 250 nM (lanes 3 and 8), or 500 nM (lanes 4, 5, 9, and 10) okadaic acid. After cell lysis, immunoprecipitation was carried out with monoclonal antibody J52 (lanes 1–4 and 6–9) or a control antibody, MOPC21 (lanes 5 and 10).

immunoprecipitated with anti-epidermal growth factor receptor antibody. In epidermal growth factor receptor, phosphorylated serine, threonine, and tyrosine in a ratio of 6.3:2.2:1.5 were detected (Fig. 2, lane 1). These results are similar to those described previously (23). In h-TRβ1 from cells treated with okadaic acid and vanadate, phosphorylated serine, threonine, and tyrosine in a ratio of 8.5:1:0.5 were found (lane 2). However, without vanadate, phosphotyrosine was not detectable (lane 3).

To explore the possibility that protein kinase C or cAMP-dependent protein kinase might be involved in the phosphorylation of h-TRβ1, we treated cells with various activators (8-bromo-cAMP, forskolin, or phorbol 12-myristate 13-acetate). However, no increase in the phosphorylation of h-TRβ1 was detected (data not shown).

Phosphorylation Stimulates Transcriptional Activity of h-TRβ1. The effect of phosphorylation on the regulation of h-TRβ1-mediated transcription was assessed by cotransfection of h-TRβ1 expression plasmid and a reporter gene that contained multiple copies of T_3 response element in tandem upstream of the CAT gene (pTK28m; ref. 24). When cells were transfected with a control plasmid that did not contain the h-TRβ1 DNA insert, only low CAT activity was seen (Fig. 3*B*). However, in the presence of transfected h-TRβ1, a 10- to 12-fold increase in CAT activity was detected, indicating that transcriptional activity was mediated by the receptor (Fig. 3). Because phosphorylation of h-TRβ1 was increased by treating cells with okadaic acid, we also examined the effect of okadaic acid. Indeed, concomitant with the increase in the phosphorylation of h-TRβ1, the h-TRβ1-mediated transcription as assessed by CAT was also increased in a concentration-dependent manner. At 0.5 μM okadaic acid, an $\approx$2.2-fold increase in the receptor-mediated transcription was detected (Fig. 3).

However, this increase was not a result of a general increase in the transcriptional activity of cells. We examined the effects of okadaic acid on the CAT activity of several other reporter genes. No significant increase in CAT activity was detected for CAT reporter genes using the herpes simplex virus thymidine kinase promoter (pU9KAT3; ref. 25) or a simian virus 40 early promoter (pSV2cat; ref. 26) (data not shown). These results, together with the findings that the synthesis of h-TRβ1 was not affected by okadaic acid (see Fig. 1), indicated to us that the transcriptional activity of h-TRβ1 was regulated by phosphorylation.

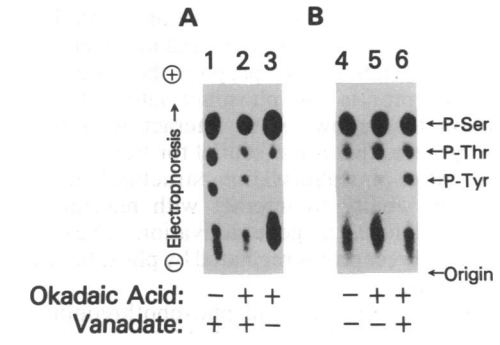

FIG. 2. Identification of phospho amino acids. Epidermal growth factor receptors (lane 1) or h-TRβ1 (lanes 2 and 3) phosphorylated in COS-1 cells (*A*) or h-TRβ1 (lanes 4–6) phosphorylated *in vitro* (*B*) were eluted from polyacrylamide gel after immunoprecipitation and autoradiography of the dry gel. The eluted receptors were hydrolyzed in 0.15 ml of 6 M HCl at 110°C for 2 hr. Phospho amino acids were analyzed by high-voltage electrophoresis followed by autoradiography. Phosphorylation in COS-1 cells was carried out in the absence or presence of okadaic acid (500 nM) or sodium vanadate (50 μM) as indicated. The okadaic acid and sodium vanadate concentrations used during *in vitro* phosphorylation were 1 μM and 200 μM, respectively.

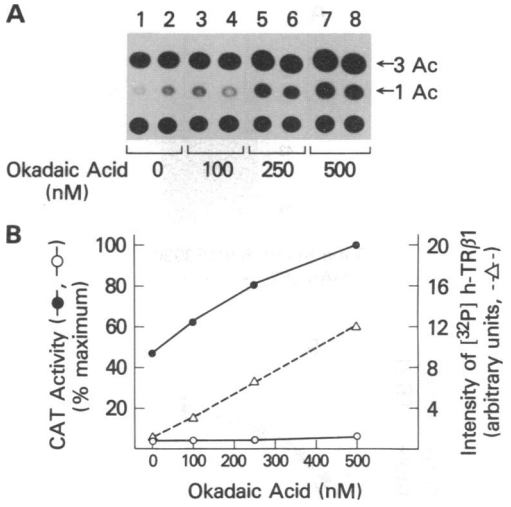

FIG. 3. Regulation of h-TRβ1-mediated transcription by okadaic acid. COS-1 cells (5×10^5 per 60-mm dish) were transfected with pCLC51 (2 μg), pTK28m (2.5 μg), and a human growth factor hormone expression plasmid (1 μg). Twenty-four hours later, cells were treated with 100 nM T_3 for 24 hr. In the last 3 hr, various concentrations of okadaic acid as indicated were added to the medium. Cells were lysed and the CAT activity was determined. (*A*) Representative positions of 3-acetylated (3Ac) and 1-acetylated (1Ac) chloramphenicol are indicated. (*B*) A summary of six experiments with duplicates in each experiment is presented with SD < 5%. CAT activity was measured from cells transfected with h-TRβ1 expression vector (●) or a control vector without h-TRβ1 (○). The increase in the level of phosphorylated h-TRβ1 is also shown (△).

h-TRβ1 Is Phosphorylated *in Vitro* by the Endogenous Kinase of Cultured Cells. We analyzed the effect of phosphorylation on the binding of h-TRβ1 to T_3 and DNA *in vitro*. h-TRβ1 purified from *E. coli* was incubated with cytosolic extract of HeLa cells in the presence of [γ-^{32}P]ATP for various lengths of time. Analysis of J52 immunoprecipitates showed that h-TRβ1 was phosphorylated in a time-dependent manner (Fig. 4*A*). Analysis of the data from several similar kinetic experiments indicated that in the absence of inhibitors, phosphorylation reached a maximum at $\approx$30 min with a stoichiometry of $\approx$0.8 mol of phosphate incorporated per mol of h-TRβ1 (Fig. 4*B*). Phosphorylation of h-TRβ1 was reduced upon longer incubation (Fig. 4*B*). In the presence of okadaic acid or vanadate, an inhibitor of tyrosine phosphatases, phosphorylation of h-TRβ1 was increased by $\approx$2-fold (Fig. 4*B*). These results demonstrate that serine/threonine kinase(s) and tyrosine kinase(s) are involved in the phosphorylation of h-TRβ1. This conclusion is further supported by phospho amino acid analysis. In the absence of okadaic acid and vanadate, phosphorylated serine, threonine, and tyrosine in a ratio of 90:6:4 were detected (Fig. 2*B*, lane 4). When okadaic acid was added during phosphorylation, a relative 2-fold increase in phosphothreonine was detected (Fig. 2*B*, lane 5). In the presence of vanadate, a relative 2- to 3-fold increase in phosphotyrosine was seen (Fig. 2*B*, lane 6). The proportions of phospho amino acids produced *in vitro* are similar to those found in h-TRβ1 expressed in COS-1 cells (Fig. 2, lanes 2 and 6).

To further characterize the endogenous kinases that phosphorylate h-TRβ1, the effects of several activators and inhibitors that are specific for cAMP-dependent protein kinase (protein kinase A), protein kinase C, or casein kinase II were evaluated. cAMP, 8-bromo-cAMP, protein kinase A inhibitor peptide, lipids composed of phosphatidylserine and 1,2-dioleoyl L-α-phosphatidyl-DL-glycerol, phorbol 12-myristate 13-acetate, protein kinase C inhibitor peptide, and spermine/spermidine had no significant effect on the phosphorylation

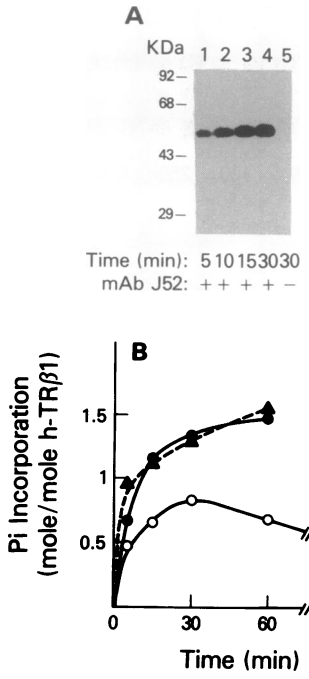

FIG. 4. *In vitro* phosphorylation of h-TRβ1 by the endogenous kinase of HeLa cell extract. Purified h-TRβ1 (0.3 μg/30 μl) was incubated with 10 μCi of [γ-^{32}P]ATP and cytosolic extract of HeLa cells (20 μg/10 μl) at 30°C for the time indicated. (*A*) Autoradiogram of the immunoprecipitates after gel electrophoresis. No inhibitor was used during phosphorylation. (*B*) Phosphorylation was carried out in the absence (○) or presence of sodium orthovanadate (200 μM) (●) or okadaic acid (1 μM) (▲). After immunoprecipitation, gel electrophoresis, and autoradiography the radioactive bands were excised from the dry gel and the radioactivity was determined.

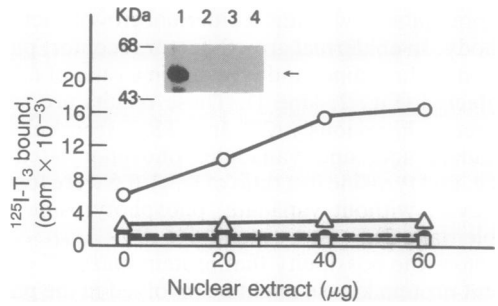

FIG. 5. Binding of h-TRβ1 to r-TRE as shown by avidin–biotin complex DNA-binding assay. Purified h-TRβ1 (0.3 μg) was phosphorylated with cytosolic extract of HeLa cells as described in the legend to Fig. 4. After phosphorylation, 3 nM ^{125}I-T$_3$ was added and subsequent binding to r-TRE (○) or adenovirus DNA (□) was carried out. Binding of r-TRE to nonphosphorylated h-TRβ1 (△) or phosphorylated h-TRβ1 that had been treated with 0.3 unit of potato acid phosphatase (●) was carried out similarly. (*Inset*) Autoradiogram of the immunoprecipitate of ^{32}P-labeled h-TRβ1 with mAb J52 (lane 1), with the control antibody MOPC21 (lane 2), or with J52 treatment of the phosphorylated h-TRβ1 with 0.3 unit of potato acid phosphatase (lane 3) or 30 units of alkaline phosphatase (lane 4). Arrow indicates position of phosphorylated h-TRβ1.

of h-TRβ1. These results suggest that h-TRβ1 was not phosphorylated by a kinase with specificity similar to that of cAMP-dependent protein kinase, protein kinase C, or casein kinase II.

Phosphorylation Stimulates Binding of h-TRβ1 to TRE and Confers its Binding to Nuclear Accessory Proteins. Purified h-TRβ1 was previously shown to bind to T_3 with affinity and analog specificity similar to those of the T_3 receptors isolated from tissues or cultured cells (17, 27). To evaluate the effect of phosphorylation on T_3 binding, the binding of h-TRβ1 to T_3 was compared before and after phosphorylation. No effect was detected (data not shown). We further examined the effect of *in vitro* phosphorylation on the binding of h-TRβ1 to TRE and nuclear accessory proteins. We used the 29 bp of the rat growth hormone gene extending from −186 to −158 from the transcription start site as the TRE (r-TRE) (21). Without phosphorylation, h-TRβ1 bound to the r-TRE (Fig. 5). The binding was low but was specific, because a similarly biotinylated oligomer with adenovirus 5 element sequence (21) did not bind. After phosphorylation, binding of h-TRβ1 to r-TRE was increased by ≈3-fold.

Fig. 5 also shows the effect of phosphorylation on the interaction of the r-TRE–receptor complex with nuclear accessory proteins. Without phosphorylation, h-TRβ1 could not interact with nuclear accessory proteins, as evidenced by no change in the binding of h-TRβ1 to r-TRE. However, phosphorylation conferred on h-TRβ1 the ability to interact with nuclear accessory proteins. A 3- and 4-fold enhancement in the binding of h-TRβ1 to r-TRE was detected by the addition of 20 μg and 40–60 μg of nuclear extract, respectively. The enhancement in the binding of h-TRβ1 to the r-TRE by the nuclear accessory proteins due to phosphorylation was reversible. When the phosphorylated h-TRβ1 was treated with potato acid phosphatase or alkaline phosphatase, the DNA-binding activity was lost (Fig. 5). [Fig. 5 *Inset* shows that phosphorylated h-TRβ1 (lane 1) was indeed dephosphorylated upon treatment with either phosphatase (lanes 3 and 4).] Similar results were found by gel shift assay. Nuclear accessory proteins not only enhanced the binding of r-TRE to the phosphorylated h-TRβ1 but also upshifted r-TRE-bound h-TRβ1 in the gel, indicating the formation of a higher molecular weight complex (data not shown). These results indicate that phosphorylation is essential for the *in vitro* binding of receptor to r-TRE and nuclear accessory proteins.

DISCUSSION

The present study provides evidence that an increase in h-TRβ1 phosphorylation in COS-1 cells led to the stimulation of transcriptional activity. *In vitro*, phosphorylation not only resulted in an increase in the DNA-binding activity of h-TRβ1 but also conferred its ability to interact with nuclear accessory proteins. The validity of the *in vitro* results was substantiated by the similar characteristics (e.g., phospho amino acid patterns and sensitivity to activators and inhibitors) of phosphorylation of h-TRβ1 *in vitro* and in the cultured cells. Based on these findings, we propose the following model. When the receptor is not phosphorylated, it is not in a conformation that allows it to interact with the nuclear accessory proteins that are essential for transcriptional activation (28). After phosphorylation, structural changes occur that confer its ability to interact with nuclear accessory proteins, which leads to gene activation. Thus, transcriptional activity of receptor is regulated by phosphorylation and dephosphorylation.

Not only phosphoserine and phosphothreonine but also phosphotyrosine were detected in h-TRβ1 phosphorylated *in vitro* and in COS-1 cells. In contrast, only phosphoserine or only phosphotyrosine was detected in other members of the steroid/thyroid hormone receptor family (29–32). Thus, similar to the receptors for growth factors (e.g., epidermal growth factor), h-TRβ1 is phosphorylated at multiple sites. The functional role of each phosphorylation site of h-TRβ1 is unknown. However, based on the observation that phosphorylation of h-TRβ1 leads to an increase in the interaction with both DNA and transcriptional machinery, it is possible that each function requires the independent structural modification induced by phosphorylation at a unique site. It is

also conceivable that the multiple sites work in concert; phosphorylation of one site may render h-TRβ1 recognizable by a second kinase and subsequently by a third kinase. This sequential phosphorylation would generate a series of receptor forms with different properties, each to carry out a specific function. Alternatively, sequential phosphorylation may lead to a final structural form that performs both functions.

Attempts were made to identify the serine/threonine kinase(s). However, our studies suggested that the endogenous serine/threonine kinase(s) involved in the phosphorylation of h-TRβ1 did not have specificities similar to those of cAMP-dependent protein kinase, protein kinase C, or casein kinase II. In contrast, phosphorylation of chicken TRα1 was reported to be stimulated by the above three kinases (29, 33). h-TRβ1 and c-TRα1 might not be recognized by the same kinase(s). However, no information is currently available regarding the regulation of transcriptional activity of c-TRα1 by phosphorylation. In view of the finding of phosphotyrosine in h-TRβ1 but not in c-TRα1, the possibility arises that α and β forms of T_3 receptor, in spite of extensive sequence homology, might be regulated differently by phosphorylation.

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