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Biochemistry

Phosphorylation enhances the target gene sequence-dependent dimerization of thyroid hormone receptor with retinoid X receptor

(heterodimer/transactivation/hormone response element/DNA binding/9-*cis*-retinoic acid)

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ABSTRACT **To understand the molecular basis of the phosphorylation-enhanced transcriptional activity of human thyroid hormone nuclear receptor subtype β1 (hTRβ1), we studied the effect of phosphorylation on the interaction of hTRβ1 with the retinoid X receptor β (RXRβ). *In vitro*, the extent of hTRβ1·RXRβ heterodimer bound to various thyroid hormone response elements (TREs) was compared before and after phosphorylation of hTRβ1. Without phosphorylation, hTRβ1·RXRβ heterodimer was barely detectable under the experimental conditions. After phosphorylation of hTRβ1, heterodimer bound to (*i*) the chicken lysozyme gene TRE, (*ii*) a TRE consisting of direct repeats of half-site binding motifs separated by four gaps, and (*iii*) a palindromic TRE was enhanced by approximately 10-, 7-, and 6-fold, respectively. The effect of phosphorylation on hTRβ1·RXRβ heterodimerization was reversible. Dephosphorylation of the phosphorylated hTRβ1 by alkaline phosphatase led to loss of the ability of hTRβ1 to form a heterodimer with RXRβ in either the absence or the presence of DNA. These results indicate that the heterodimerization is enhanced by phosphorylation. To evaluate the effect of phosphorylation on the interaction of hTRβ1 with RXRβ *in vivo*, we cotransfected hTRβ1, RXRβ and TRE-chloramphenicol acetyltransferase (CAT) expression plasmids into CV-1 cells. CAT activity was assessed in the presence or absence of okadaic acid. Okadaic acid is a potent inhibitor of phosphatases 1 and 2A and increases the *in vivo* phosphorylation of hTRβ1 by ≈10-fold. Using the CAT reporter gene under control of the TRE from the malic enzyme gene, we found that RXRβ increased the okadaic acid-enhanced hTRβ1-mediated CAT activity by 2- to 3-fold in the presence of 3,3′,5-triiodo-L-thyronine. However, 9-*cis*-retinoic acid did not enhance the effect of okadaic acid. Our results indicate that phosphorylation is essential for the interaction of hTRβ1 with RXRβ. Thus, phosphorylation plays a pivotal role in the gene-regulating activity of hTRβ1.**

Thyroid hormone nuclear receptors (TRs) are members of the steroid/retinoic acid (RA) receptor superfamily. They are ligand-dependent transcription factors which regulate growth, differentiation, and development (1). Two genes for TRs have been identified, TRα and TRβ, on human chromosomes 17 and 3, respectively. Sequence analysis of the promoters of target genes shows that TRs bind to thyroid hormone response elements (TREs) with half-site binding motifs in the orientation of palindrome, direct repeat, or inverted palindrome. TRs bind to these TREs with different affinities (2) and the binding is affected differentially by 3,3′,5-triiodo-L-thyronine (T_3) (3). The transcriptional activity of TRs is also modulated by heterodimerization with other TR nuclear accessory proteins (TRAPs).

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One class of TRAPs which has been extensively characterized is retinoid X receptors (RXRα, -β, and -γ). RXRs enhance the specificity and efficiency in the interaction of TRs with TREs by heterodimerization with TRs. RXRs also modulate the transcriptional activity of TRs (4). Thus, by heterodimerization with RXRs, not only are the biological activities of TRs regulated, but also the gene regulatory activities of TRs are connected to other hormonal pathways. However, how this important heterodimeric interaction is regulated at the molecular level has not been addressed. Here we consider the possibility that heterodimerization is regulated by phosphorylation of TRs.

Phosphorylation plays an important role in regulating the activity of transcription factors, possibly by altering their ability to translocate from cytoplasm to nucleus, by changing their DNA-binding activity, and/or by affecting their interaction with the transcriptional machinery (5). Moreover, we have shown that the human TRβ1 (hTRβ1) is a phosphoprotein (6). *In vitro*, phosphorylation not only increases the binding of hTRβ1 to DNA but also enables it to bind to TRAPs. In addition, phosphorylation increases the transcriptional activity of hTRβ1 *in vivo* (6). Because RXRs are potent heterodimer partners of TRs, we have evaluated whether the interaction of hTRβ1 with RXRβ is modulated by phosphorylation. We found that phosphorylation plays a pivotal role in regulating the transcriptional activity of hTRβ1 by enhancing its heterodimerization with RXRβ.

METHODS

DNA-Binding Assay. Binding of DNA to hTRβ1 was assayed by electrophoretic gel mobility shift (3). hTRβ1 was purified from inclusion bodies of *Escherichia coli* by the method of Park *et al.* (7). Phosphorylation of hTRβ1 was carried out as described by Lin *et al.* (6). In some experiments, the phosphorylated hTRβ1 was treated with alkaline phosphatase for 1 hr at 37°C. In some experiments, 1 μg of monoclonal antibody (mAb) J51 (8) or 1 μg of anti-RXRβ mAb (1317) (9) was added to supershift hTRβ1 or RXRβ, respectively.

Evaluation of Heterodimerization by Coimmunoprecipitation. Coimmunoprecipitation was carried out as described by Bogazzi *et al.* (10), with minor modifications. After incubation of ^{35}S-labeled RXRβ (3 μl) with ^{35}S-labeled phosphory-

Abbreviations: T_3, 3,3′,5-triiodo-L-thyronine; TR, thyroid hormone nuclear receptor; hTRβ1, human TR subtype β1; TRAP, TR nuclear accessory protein; RXR, retinoid X receptor; TRE, thyroid hormone response element; Pal, palindrome TRE; DR4, TRE consisting of direct repeats of half-site binding motifs separated by four gaps; Lyz, lysozyme gene TRE; ME, malic enzyme gene TRE; RA, retinoic acid; CAT, chloramphenicol acetyltransferase; mAb, monoclonal antibody; OA, okadaic acid.

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lated or dephosphorylated hTRβ1 (3 μl) at 4°C for 20 hr, 1 μg of mAb J51 was added. *Staphylococcus aureus* cells pretreated with bovine serum albumin (1 mg/ml) and unlabeled RXRβ (50 μg/ml) were incubated with rabbit anti-mouse IgG at 4°C for 30 min. Fifty microliters of IgG-bound *S. aureus* suspension was incubated with the receptor/antibody complex at 4°C for 1 hr. After centrifugation at 500 × *g* for 5 min, the pellet was washed and the immunoprecipitates were analyzed (8).

Transcriptional Activity of hTRβ1 and RXRβ. CV-1 monkey cells (4 × 10^5 per 60-mm dish) were transfected with TRE-containing chloramphenicol acetyltransferase (CAT) expression plasmid (2 μg), hTRβ1 expression plasmid pCLC51 (0.5 μg), pCMV-RXRβ (1 μg), and β-galactosidase expression plasmid pCH110 (1 μg). pBluescript plasmid (Stratagene) was used to adjust the total DNA to 5 μg per dish and transfection was carried out by the calcium phosphate method (3). T_3 or 1 μM 9-*cis*-RA was added to the cells 12–14 hr before cells were lysed for analysis of CAT activity (11). The transfection efficiency was normalized to β-galactosidase activity.

Phosphorylation of hTRβ1 in CV-1 Cells. CV-1 cells (8.5 × 10^5 per 100-mm dish) were plated and transfected with the hTRβ1 expression plasmid pCLC51 (15 μg) as described above. Forty-eight hours after transfection, cells were metabolically labeled with carrier-free [^{32}P]orthophosphate or [^{35}S]methionine as described (6). ^{32}P- or ^{35}S-labeled hTRβ1 was immunoprecipitated by C4, a mAb raised against hTRα1 expressed in *E. coli*. It crossreacts with hTRβ1 (unpublished results).

RESULTS

Enhancement of hTRβ1 Binding to TRE by RXRβ is Phosphorylation-Dependent. Binding of hTRβ1 to TRE is enhanced by formation of a heterodimer with RXRβ (9, 12). To evaluate whether phosphorylation plays a role in heterodimerization, we compared the extent of enhancement by RXRβ in the binding of nonphosphorylated or phosphorylated hTRβ1 to TREs with different orientation of the half-site binding motifs. They are Lyz (the chicken lysozyme gene TRE; an inverted palindrome), DR4 (direct repeats separated by four gaps), and Pal (a palindromic TRE) (7). The binding was analyzed by mobility-shift assay.

hTRβ1 expressed in and purified from the inclusion bodies of *E. coli* is not phosphorylated (6). In the avidin–biotin complex DNA-binding assay, nonphosphorylated hTRβ1 binds only very weakly to the rat growth hormone TRE and is incapable of interacting with the nuclear factors in the extracts (6). Consistent with these findings, Fig. 1 shows typical results for the binding of phosphorylated or nonphosphorylated hTRβ1 to TRE in electrophoretic mobility-shift assays. The binding of nonphosphorylated hTRβ1 to Lyz was very weak (Fig. 1*A*, lane 1). When RXRβ was added, binding of the nonphosphorylated hTRβ1 was visible but still low (lane 2). However, a dramatic increase in the formation of hTRβ1·RXRβ heterodimer was seen after hTRβ1 was phosphorylated by the kinases in HeLa cytosolic extract (lane 5). The weak radioactive band with a more retarded mobility in lane 5 presumably represents the binding of RXRβ homodimer to Lyz (ref. 12; see below). Both the low- and high-mobility bands are specific: in the presence of 100-fold excess unlabeled Lyz, neither band was detectable (lane 6).

The enhancement seen in the hTRβ1·RXRβ band is not only due to the increase in homodimer formation as a result of phosphorylation. Binding of hTRβ1 to Lyz was increased ≈2-fold by phosphorylation (Fig. 1*A*, lane 3). However, the heterodimerization was increased ≈10-fold.

For phosphorylation to be a functionally regulatory mechanism, it has to be reversible. We therefore treated the

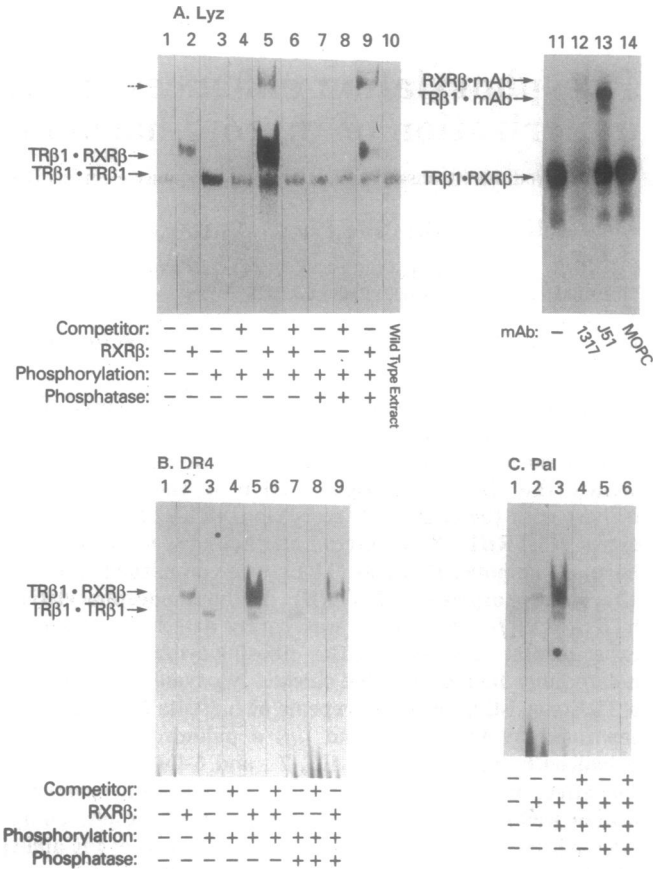

FIG. 1. Effect of phosphorylation on the binding of hTRβ1 to TREs and RXRβ. Phosphorylated or nonphosphorylated hTRβ1 (≈0.5 μg) was incubated with ^{32}P-labeled Lyz (*A*), DR4 (*B*), or Pal (*C*). Where appropriate, 50 ng of nuclear extract protein containing RXRβ was added. Dotted arrow indicates RXRβ bound to Lyz.

phosphorylated hTRβ1 with alkaline phosphatase and evaluated the effect of dephosphorylation on heterodimerization. When the phosphate was removed by phosphatase, hTRβ1 lost most of its ability to bind to Lyz (Fig. 1*A*, lane 7), and the formation of heterodimer was reduced by ≈90% (lane 9). To be certain that the markedly enhanced band seen in lane 5 was due to the interaction of RXRβ with hTRβ1, we carried out the following control experiments. When nuclear extract obtained from wild-type Sf9 cells was used, no heterodimer was detected (lane 10). In addition, to be certain that the enhanced band in lane 5 was the result of hTRβ1 complexing with RXRβ, we carried out a binding experiment in which the anti-hTRβ1 mAb J51 or anti-RXRβ mAb 1317 (9) was present. RXRβ was supershifted by mAb 1317 (lane 12). Binding of mAb 1317 caused the dissociation of hTRβ1·RXRβ as evidenced by the weakening of the intensity of the hTRβ1·RXRβ heterodimer. TRβ1 was also supershifted by mAb J51 (lane 13). The weakening of the heterodimer band induced by binding of mAb J51 to hTRβ1 was less than that caused by mAb 1317. However, when a control antibody, MOPC, was used, no supershifted band was detected, nor did it decrease the intensity of the hTRβ1·RXRβ band (lane 14). These results indicate that the markedly enhanced band seen in lane 5 was indeed hTRβ1·RXRβ heterodimer.

To evaluate whether the phosphorylation-dependent enhancement in the binding of hTRβ1 to TRE via heterodimer formation was TRE-dependent, we evaluated hTRβ1·RXRβ binding to two other TREs. We found that the effect of phosphorylation on the enhanced binding of hTRβ1 to DR4 was similar to that with Lyz. Phosphorylation enhanced homodimer formation ≈2-fold (Fig. 1*B*, lane 2). The

hTRβ1·RXRβ heterodimer was increased ≈7-fold. Treating the phosphorylated hTRβ1 with alkaline phosphatase decreased its ability to bind to DR4 (lane 7) and weakened its interaction with RXRβ (lane 9).

Complex formation by hTRβ1 with RXRβ and Pal increased ≈6-fold after phosphorylation (Fig. 1*C*). Because TRβ1 homodimer binding to Pal was not detectable, it was not possible to obtain the fold increase in the homodimer. Removal of phosphate from hTRβ1 resulted in the loss of its ability to bind to RXRβ (lane 5).

The enhanced heterodimer bands shown in Fig. 1 *A–C* were obtained by using the same amounts of hTRβ1, RXRβ, and TRE. Comparison of the intensity of the heterodimer bands indicates that phosphorylation-dependent enhancement varies with TRE in the order Lyz > DR4 > Pal.

Enhancement in the Binding of *in Vitro* Translated hTRβ1 to TRE by RXRβ Is Phosphorylation-Dependent. Binding of hTRβ1 prepared by *in vitro* transcription/translation to TRE is enhanced by RXRβ (4, 10). If phosphorylation is important for binding of hTRβ1 to RXRβ, the *in vitro* translated hTRβ1 must already be phosphorylated. We tested this possibility first by evaluating whether the *in vitro* translated hTRβ1 could be phosphorylated with [γ-^{32}P]ATP by the cytosolic kinases from HeLa cells. No incorporation of $^{32}P_i$ was detected (data not shown). We then treated the *in vitro* translated hTRβ1 with potato acid phosphatase. The ability of hTRβ1 to form heterodimer was much weakened when it was bound to Lyz or completely lost when it was bound to DR4 after dephosphorylation (Fig. 2, lanes 5). Removal of phosphate from the *in vitro* translated hTRβ1 also led to the loss of its ability to form heterodimer on Pal (data not shown). The heterodimers bound to the TREs were complexes of hTRβ1 and RXRβ as shown by supershifting the receptors with the corresponding mAb J51 or 1317 (data not shown). These results indicate that the *in vitro* translated hTRβ1 is a phosphoprotein.

Dephosphorylation of hTRβ1 Blocks the Heterodimerization of hTRβ1 and RXRβ in the Absence of DNA. The phosphorylation-enhanced heterodimers (Fig. 1 *A* and *B*, lane 5; Fig. 1*C*, lane 3; Fig. 2, lanes 4) were bound to TREs. This enhancement could result not only from the increase in the binding of TRβ1 to TRE but also from increased interaction directly between TRβ1 and RXRβ. To assess whether the latter possibility plays a role in phosphorylation-induced enhancement, we evaluated the association of hTRβ1 and RXRβ in the absence of DNA. ^{35}S-labeled TRβ1 and RXRβ were prepared by *in vitro* transcription/translation. After incubation of the two proteins in the absence of TRE, mAb

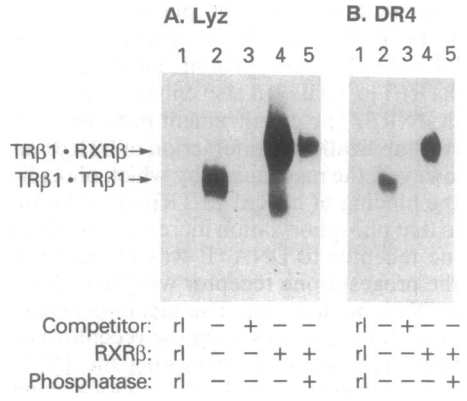

FIG. 2. Binding of *in vitro* translated hTRβ1 to TREs before or after phosphorylation. hTRβ1 (1 μl) prepared by *in vitro* transcription/translation was incubated with ^{32}P-labeled TRE (10,000–12,000 cpm). Lane 1 (rl) is the unprogrammed reticulocyte lysate control. The hTRβ1 used in lane 5 was treated with acid phosphatase at 37°C for 1 hr.

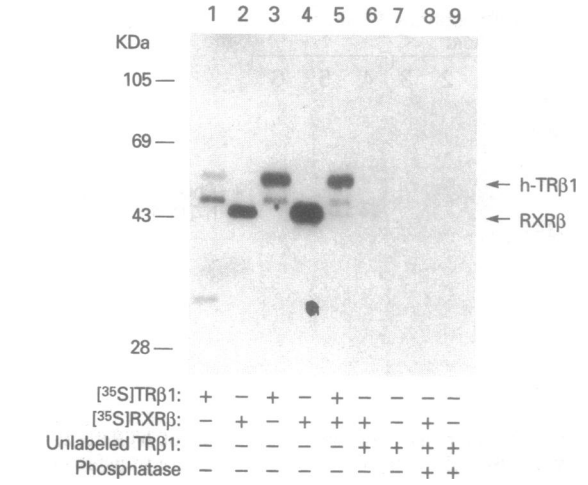

FIG. 3. Heterodimer formation of RXRβ with the phosphorylated or dephosphorylated hTRβ1. Three microliters of ^{35}S-labeled RXRβ was incubated with 3 μl of ^{35}S-labeled phosphorylated or dephosphorylated h-TRβ1. One microgram of mAb J51 was used for lanes 3 and 5–9. One microliter of mAb 1317 was used for lane 4. Lanes 1 and 2 show ^{35}S-labeled hTRβ1 and RXRβ, respectively, as standards.

J51 was used to immunoprecipitate the complex. Lanes 1 and 2 of Fig. 3 show the translated ^{35}S-labeled hTRβ1 and RXRβ, respectively. The typical three translation products are seen for hTRβ1 (8). After immunoprecipitation, the intact hTRβ1 (55-kDa protein) is enriched because J51 has a higher affinity for the intact hTRβ1 than for the truncated hTRβ1 (53-kDa protein) (8). To be certain the band shown in lane 2 is RXRβ, we have immunoprecipitated the translated ^{35}S-labeled RXRβ with mAb antibody 1317 (lane 4). In the presence of ^{35}S-labeled RXRβ, an additional band with the same electrophoretic mobility as RXRβ is seen (lane 5 vs. lanes 2 and 4), indicating the coimmunoprecipitation of RXRβ with hTRβ1. RXRβ with the same intensity can be seen more clearly in lane 6, in which unlabeled hTRβ1 was present. The RXRβ bands seen in lanes 5 and 6 are specific. When unprogrammed reticulocyte lysate containing [^{35}S]methionine was used, no RXRβ was detected (lane 7 vs. lane 6). These results indicate that in the absence of DNA, phosphorylated hTRβ1 forms a heterodimer with RXRβ in solution. Therefore, in the absence of DNA, RXRβ can form a heterodimer not only with TRα1 (13) but also with hTRβ1. Upon dephosphorylation of hTRβ1 with acid phosphatase, the RXRβ band was not detectable (lane 8). Lane 9 shows again the control in which only the unprogrammed reticulocyte lysate (without RXRβ) was treated similarly. No RXRβ is visible. These results indicate that dephosphorylation weakens the interaction between hTRβ1 and RXRβ.

Phosphorylation Stimulates RXRβ-Modulated TRβ1 Transcriptional Activity. Previously we have demonstrated that hTRβ1 expressed in COS-1 cells is a phosphoprotein. Serine and threonine in a ratio of 9:1 are the phosphorylation sites. Treatment of cells with okadaic acid (OA) not only increases the *in vivo* phosphorylation of hTRβ1 but also stimulates the hTRβ1-mediated transcriptional activity (6). To evaluate the effect of phosphorylation on the RXRβ-modulated TRβ1-mediated transactivation, we used OA as a probe. We chose CV-1 cells in the present studies because the CV-1 line has been used by many investigators to demonstrate the modulating effect of RXRs on TRs (4). To be certain that the hTRβ1 expressed in CV-1 cells is also a phosphoprotein, we incubated the cells with [^{32}P]orthophosphate in the absence or presence of OA. hTRβ1 expressed in CV-1 cells was phosphorylated (Fig. 4, lane 1), and treating the cells with OA led to an increase of ≈10-fold in phosphorylated hTRβ1 (lane 2).

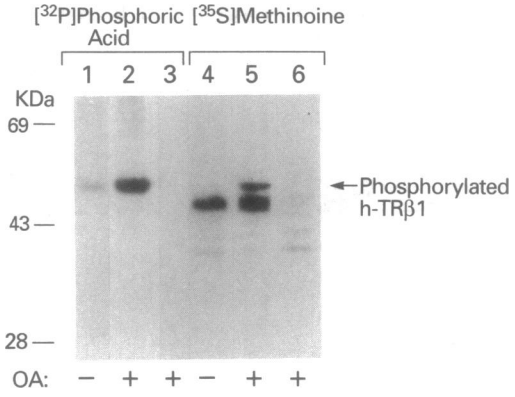

FIG. 4. OA increases the phosphorylation of hTRβ1 in CV-1 cells. CV-1 cells (8.5×10^5 per 100-mm dish) were transfected with hTRβ1 expression vector (pCLC51, 15 μg) by the calcium phosphate method. Cells were labeled with 3 mCi (111 MBq) of [^{32}P]orthophosphoric acid (lanes 1–3) or 100 μCi of [^{35}S]methionine (lanes 4–6) in the absence of OA (lanes 1 and 4) or in the presence of 250 nM OA (lanes 2, 3, 5, and 6). After cell lysis, immunoprecipitation was carried out with mAb C4 (5 μg) (lanes 1, 2, 4, and 5) or a control mAb, MOPC (5 μg) (lanes 3 and 6).

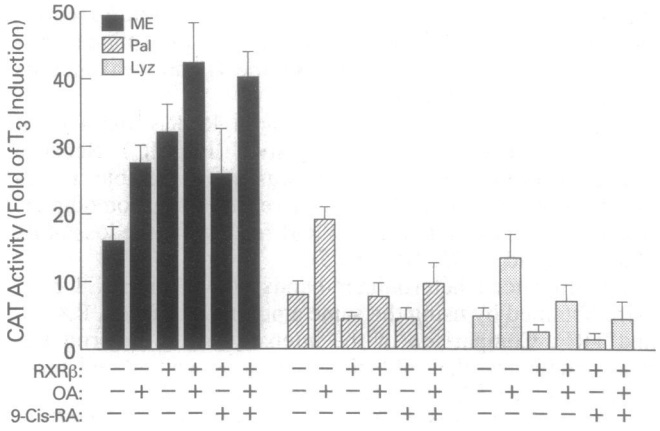

FIG. 5. Phosphorylation stimulates the RXRβ-modulated TRβ1-mediated transactivation. CV-1 cells (4×10^5 per 60-mm dish) were transfected with 2 μg of reporter plasmid in which the CAT gene was under control of ME, Pal, or Lyz and the herpes simplex virus thymidine kinase promoter (ME-TK-CAT, TREpal-TK-CAT, TRE-lys-TK-CAT); 0.5 μg of hTRβ1 expression vector (pCLC51); 1 μg of RXRβ expression vector (pCMV-RXRβ); and 1 μg of β-galactosidase expression vector (pCH110). Transfection efficiency was corrected by determining β-galactosidase activity. The results are expressed as mean and SD of four independent experiments done in duplicate. The significance of the differences in the transactivation of TRβ1 with various TREs due to RXRβ, OA (250 nM), or 9-*cis*-RA (1 μM) was analyzed by Student's *t* test and was found to be significantly different between the pairs, with >99.5% confidence.

These results are similar to those found previously for COS-1 cells (6). To rule out the possibility that the hyperphosphorylation of hTRβ1 is a result of increase in the synthesis of hTRβ1, we also incubated the cells with [^{35}S]methionine. Comparison of lanes 4 and 5 shows that OA did not significantly increase the level of hTRβ1, indicating that OA has little or no effect on the synthesis of hTRβ1. The upper band in lane 5 has the same electrophoretic mobility as those in lanes 1 and 2, indicating it is the phosphorylated hTRβ1. Phosphorylation led to a slower migration of hTRβ1 in the gel, a feature commonly observed for phosphorylated proteins such as the progesterone receptor (14) and the vitamin D receptor (15). Under the experimental conditions, only ≈40% of the hTRβ1 pool was phosphorylated. In the absence of OA, the phosphorylated pool was too small to be detected by [^{35}S]methionine labeling.

Whether phosphorylation increases the RXRβ-modulated TRβ1-mediated transactivation was evaluated by cotransfection of RXRβ and hTRβ1 plasmids with CAT reporters containing the malic enzyme gene TRE (ME, a direct repeat), Pal, or Lyz in the presence or absence of OA. In the presence of T_3, OA increased hTRβ1-mediated transcriptional activity 1.7-, 2.3-, and 3-fold for ME, Pal, and Lyz, respectively (Fig. 5). When RXRβ was cotransfected with hTRβ1 in the absence of OA, a 2-fold synergistic effect was seen with ME. This RXRβ-modulated increase was further enhanced by OA.

Previously we had demonstrated that the OA-enhanced transcriptional activity of hTRβ1 was not a result of a general increase in the transcriptional activity of cells. No significant effect of OA on CAT activity was observed for CAT reporter genes without TREs (6). Therefore, the OA-induced enhancement was mediated by hTRβ1. Further, no transactivation was seen by RXRβ alone for these TREs with or without OA (data not shown). Therefore, these results indicate that concomitant with the increase in the phosphorylation of hTRβ1, not only is the ME-driven transactivation increased, but also RXRβ-modulated synergism is enhanced.

The twofold synergistic effect of RXRβ on the ME-driven hTRβ1-mediated transactivation is similar to that reported by Hallenbeck *et al.* (13). In contrast, a 30–40% repression by RXRβ was invariably seen for the Pal- and Lyz-driven hTRβ1-mediated transactivation. This repression, however, was reversed by OA in the Pal-driven transactivation. In the Lyz-driven transactivation, not only was the inhibitory effect of RXRβ overcome by the increase in phosphorylation of hTRβ1, but also an additional RXRβ-induced increase (≈20%) was seen.

To understand whether the ligand of RXRβ plays a role in the phosphorylation-dependent interaction of hTRβ1 and RXRβ, we evaluated the effect of 9-*cis*-RA. We found that 9-*cis*-RA did not increase either the RXRβ-modulated effects or the OA-mediated enhancement. These results are similar to the findings of Hallenbeck *et al.* (13) in which 9-*cis*-RA either alone or in combination with T_3 was shown not to enhance the transactivation driven by myelin basic protein TRE (an inverted Pal) or ME (16). Rosen *et al.* (17) also reported that 9-*cis*-RA did not further enhance the synergistic effect of RXRβ on hTRβ1-mediated transactivation when the TRE was a direct-repeat hexamer.

DISCUSSION

These studies demonstrate that phosphorylation plays a pivotal role in modulating the interaction of hTRβ1 with TREs and RXRβ. Phosphorylation not only increases the binding of hTRβ1 to TREs but also enhances its heterodimerization with RXRβ. The enhancement in heterodimerization is also due to stabilization of interaction of hTRβ1 and RXRβ protein. However, the mechanism by which phosphorylation increases the binding of hTRβ1 to TREs is unknown. It has been shown that phosphorylation increases the binding of the progesterone receptor to DNA. It was proposed that association of the progesterone receptor with heat shock protein masked the DNA binding site. The ligand-dependent dissociation of heat shock proteins from the receptor is mediated by phosphorylation, thereby unmasking the DNA binding site (18). This mechanism most likely cannot account for the increase in DNA binding observed in the present studies. TRs are synthesized in the DNA-binding state and are not associated with heat shock proteins (19). Further, there is no evidence that the hTRβ1 used in the binding studies was copurified with a contaminant. Therefore, the possibility that the increase in DNA binding is the result of dissociation of an

inhibitor due to phosphorylation can be eliminated. That the extent of enhancement in the binding to TREs depends on the orientation of the half-site binding motifs suggests that the increase is mediated by a phosphorylation-induced conformational change. Without phosphorylation, the binding of hTRβ1 to all three TREs was weak and was in the order DR4 > Lyz > Pal. After phosphorylation, the order of binding activity changed to Lyz > DR4 > Pal. The change in the recognition pattern in the receptor would certainly necessitate the adjustment of some regional structures in the receptor molecule. This notion was further supported by the enhancement in the heterodimerization observed after phosphorylation of hTRβ1. It has been reported that the hTRβ1·RXRβ heterodimer not only depends on the dimerization domain near the C-terminal region of hTRβ1 and the DNA-binding domain, but also on the "A" and "T" boxes in the D domain (20, 21). It is conceivable that phosphorylation could change the regional structure in any one of the three regions or a combination of the three, thereby resulting in a structure which facilitates its dimerization.

Consistent with the *in vitro* binding studies, the *in vivo* results further emphasized the important role of phosphorylation in the transcriptional activity of hTRβ1. Phosphorylation of hTRβ1 was increased in the presence of OA. Concomitant with this increase, T_3-induced TRβ1-mediated transcriptional activity driven by any of the three TREs was also increased. RXRβ induced a synergistic effect in the transactivation of hTRβ1 driven by ME. An increase in the phosphorylation of hTRβ1 further enhanced this synergy. When hTRβ1 phosphorylation was at the basal level, RXRβ partially repressed the hTRβ1-mediated transactivation driven by Pal or Lyz. However, this repression was overcome when hTRβ1 became hyperphosphorylated. The TRE-dependent modulation of hTRβ1 activity by RXRβ observed in the present studies was also reported by Hallenbeck *et al.* (16). Using TRα1, those authors found that RXRβ could either enhance, not change, or inhibit the transcriptional activity of TRα1 depending on the type of TRE (16). It is clear that the molecular mechanism by which RXRs modulate the activity of TRs is more complex than previously envisioned. Even though the mechanism of the TRE-dependent modulator effect of RXRβ is unclear at the present time, the present data indicate that in all three TREs, phosphorylation enhances the interaction of hTRβ1 and RXRβ. This could be achieved either by strengthening the interaction of hTRβ1 with RXRβ or by increasing the efficiency of hTRβ1 to recruit RXRβ.

OA increases the level of phosphorylation of hTRβ1 by ≈10-fold, whereas the increase in transactivation is only 1.7- to 3-fold. This suggests that not all phosphorylation sites participate in the transactivation function. *In vivo*, hTRβ1 is phosphorylated at multiple sites (6). Serine is the major phosphorylated site and threonine and tyrosine are the minor sites (6). Therefore, phosphorylation of hTRβ1 not only modulates the transactivation activity but also is involved in other, unidentified functions.

It is evident from our results that phosphorylation regulates the transcriptional activity of hTRβ1 at several levels. If cells are devoid of RXR or other TRAPs, it is likely that hTRβ1 regulates its target genes as a homodimer. Phosphorylation enhances its activity by increasing its binding to DNA. In target cells where RXRβ is present, an additional regulation of hTRβ1 transcriptional activity by phosphorylation is achieved via modulation of heterodimerization. The selectivity and strength of this modulation lie in the orientation of the half-site binding motifs. Thus, this multifaceted regulation could be one of the mechanisms by which TR achieves its diverse biological functions.

Note. While this manuscript was in review, Sugawara *et al.* (22) reported on the effect of phosphorylation on the binding of hTRβ1 to various TREs in electrophoretic mobility-shift assays. They found that phosphorylation increased the binding of hTRβ1 homodimer to TRE, which is in agreement with our present results. However, in contrast to our studies, they found that phosphorylation did not affect TR·RXR heterodimerization. In the present studies, the effect of phosphorylation on the heterodimerization has been analyzed not only by mobility shift but also by coimmunoprecipitation studies. Further, the results of transactivation studies further support our *in vitro* binding studies. It is difficult for us to reconcile our findings with their data of the effect of phosphorylation on the *in vitro* TR·RXR heterodimerization.

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