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Functional Regulation of Thyroid Hormone Receptor Variant TRα2 by Phosphorylation

DEBORAH KATZ,† MAURICIO J. REGINATO, AND MITCHELL A. LAZAR*

Departments of Medicine, Genetics, and Pharmacology, Division of Endocrinology, Diabetes, and Metabolism, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

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The thyroid hormone (T3) receptor (TR) variant TRα2 is abundant in brain but does not bind T3 because of its unique C terminus. The only known function of TRα2, inhibition of TR-dependent transactivation, involves competition for T3 response elements. Paradoxically, in vitro-translated TRα2 bound poorly to these sites. We report here that dephosphorylation of TRα2 restored its DNA binding. Mutation of C-terminal serine residues to alanine (TRα2-SA) was equally effective. The C terminus of TRα2 was phosphorylated in a human cell line, whereas that of TRα2-SA was not. Conversely, TRα2-SA was a much better inhibitor of T3 action than was wild-type TRα2. The dominant negative activity of TRα2-SA was less than stoichiometric with TR concentration, possibly because it was unable to heterodimerize with retinoid X receptor, which enhances the binding of other TRs. Purified casein kinase II as well as a reticulocyte casein kinase II-like activity phosphorylated TRα2 on serines 474 and 475. Mutation of these two residues to alanine was sufficient to restore DNA binding. Thus, DNA binding by TRα2 is regulated by phosphorylation at a site distant from the DNA-binding domain. The increased dominant negative activity of a nonphosphorylatable form of TRα2 suggests that phosphorylation may provide a rapid, T3-independent mechanism for cell-specific modulation of the expression of T3-responsive genes.

Normal vertebrate development requires precise regulation of thyroid hormone (T3) action in early life (54). The effects of T3 are mediated by nuclear T3 receptors (TRs), members of the steroid/thyroid hormone receptor superfamily of transcription factors which are characterized by modular domains that regulate activity via hormone binding, DNA binding, and interaction with other transcription factors (34). A unique feature of transcription regulation by TRs is the existence of TRα2, an alternative splice product of the TRα gene whose unique C terminus does not bind T3 or activate transcription, unlike the conserved C termini of true TRs (5, 23, 38, 49, 52). Instead, TRα2 dominantly inhibits T3-induced transactivation (30, 37).

We have previously shown that inhibition of T3 action by TRα2 at least in part involves competition with other TRs for DNA binding (26). Somewhat paradoxically, TRα2 binds DNA much more weakly than TRα1 when the proteins are translated in reticulocyte lysates (24). Correspondingly, TRα2 is a weak inhibitor of TRα1 activity (60). However, truncation of the C terminus of TRα2 improves its DNA binding and dominant negative activities (24, 26). We therefore considered whether the activity of wild-type TRα2 could be regulated by posttranslational modification of its unique C terminus. Other transcription factors, including steroid hormone receptors (for a review see reference 57), are regulated by phosphorylation (for reviews, see references 22 and 47). Additionally, phosphorylation of TRβ increases its DNA binding (42, 65), and the transforming activity of the oncogenic form of TR, v-ErbA, is regulated by phosphorylation of N-terminal serines by activation of protein kinase A (PKA) and C pathways (17). TRα1 is also phosphorylated on its N terminus (shared with TRα2) by casein kinase II (CKII), but the function of this is not known (16).

In this paper, we show that TRα2 binds DNA poorly because of phosphorylation of its unique C terminus. This serine phosphorylation occurs in mammalian cells and reduces the inhibitory activity of TRα2. We further show that TRα2 is phosphorylated by CKII on two principal serine residues in the C terminus in vitro. Mutation of these two serines to alanine restores the DNA binding of TRα2. Thus, the DNA binding and dominant negative activities of TRα2 are regulated by phosphorylation.

MATERIALS AND METHODS

Proteins. Proteins were produced in vitro and labeled with [^{35}S]methionine, using the T3 polymerase TNT reticulocyte lysate transcription and translation system (Promega). Alkaline phosphatase treatment of proteins (5 to 10 μl) was done for 30 min at 30°C with 2 μl of lyophilized enzyme (20 to 100 mg/ml; Boehringer Mannheim).

Serine-to-alanine mutated proteins were created by the double-overlap fusion PCR technique (21). Two outside primers (all primers are written 5′ to 3′) were made to cover the unique 5′ *Nsi*I site (GGGGGATCCCTCGGAATGCATGT TGTTCAGGGT) and the 3′ *Sph*I site (GGGGGGATCCGGAGTAGCATG CTCCTTCTCC), each with a *Bam* site included outside of the restriction sites for possible future use. These primers were used with complementary sense and antisense sets of internal primers (Table 1). All mutations and ligation junctions were confirmed by sequencing. For expression in mammalian cells, these constructs were inserted into the *Not*I and *Hin*dIII site of the vector pCDM (3). An N-terminal deletion mutant of TRα2 (TRα2ΔN) in pCDM was derived from the full-length TRα2 cDNA by ligation, at *Hin*dIII and *Acc*I sites, of a PCR product which incorporated a *Hin*dIII site and a methionine in frame and upstream of amino acid 116. This reaction used the primers AAAAAGCTTGGATCCAT GATCGCTGTGGGCATGGCC and CCACGCGGGTGATGGCC. The serine-to-alanine mutant of TRα2ΔN was cloned into pCDM by ligating the *Hin*dIII-*Acc*I fragment of TRα2ΔN into the TRα2-SA backbone.

Proteins were expressed in *Escherichia coli* by cloning their cDNAs into a pET vector (64) modified to produce an N-terminal fusion with six histidine residues (gift of S. Stevens). The 5′ fusion was made by ligation of an *Eco*RI-*Xho* digest of the TRα1 cDNA in the pAR vector (9, 36). TRα2 was made by a partial digestion of the TRα1 construct with *Acc*I to liberate a fragment including the 5′ histidine fusion and the N terminus of TRα to the origin of replication of the pET vector. This fragment was ligated to one made by complete digestion of the

* Corresponding author. Mailing address: University of Pennsylvania School of Medicine, 611 CRB, 415 Curie Blvd., Philadelphia, PA 19104-6149. Phone: (215) 898-0198. Fax: (215) 898-5408. Electronic mail address: Lazar@mail.med.upenn.edu.

† Present address: Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030.

TABLE 1. Internal primers used for PCR

Primer	Sequence
S463-475A	
Sense	GCTGCTGAGGCAGCTGCTCTGGCTGCTGCTGCTGCTGACGAGGACACGGAGGTCTGC
Antisense	AGCAGCAGCAGCAGCCAGAGCAGCTGCCTCAGCAGCGTCGTCTTCCCCACAGACCGC
S464-469A	
Sense	GTCTGTGGGGAAGACGACGCAGCAGAGGCAGCAGCACTGAGCTCCTCTTCCTCT
Antisense	AGAGGAAGAGGAGCTCAGTGCTGCTGCCTCTGCTGCGTCGTCTTCCCCACAGAC
S471-475A	
Sense	GCTGCTGCTGCTGCTGACGAGGACACGGAGGTC
Antisense	AGCAGCAGCAGCAGCCAGGGAGCTTGCCTCACT
S472A	
Sense	GAGGCAAGCTCCCTGAGCGCTTCTTCCTCTGACGAGGAC
Antisense	GTCCTCGTCAGAGGAAGAAGCGCTCAGGGAGCTTGCCTC
S474A	
Sense	TCCCTGAGCTCCTCTGCTTCTGACGAGGACACGGAG
Antisense	CTCCGTGTCCTCGTCAGAAGCAGAGGAGCTCAGGGAGCT
S475A	
Sense	TCCCTGAGCTCCTCTTCCGCTGACGAGGACACGGAGGTC
Antisense	GACCTCCGTGTCCTCGTCAGCGGAAGAGGAGCTCAGGGA
S474-475A	
Sense	AGCTCCCTGAGCTCCTCTGCAGCAGACGAGGACACGGAGGTC
Antisense	GACCTCCGTGTCCTCGTCTGCTGCAGAGGAGCTCAGGGAGCT
S455A	
Sense	CACCGAAGCGGAATTCTCCATGCTCGAGCGGTCTGTGGGGAA
Antisense	TTCCCCACAGACCGCTCGAGCATGGAGAATTCCGCTTCG

TRα2 C terminus in the pAR vector. The N-terminally truncated (ΔN) His_6-TRα2 was expressed from a plasmid made by ligation of the *Nco*I site in the polylinker of the pET vector to the internal in-frame *Nco*I site of the TRα2 cDNA. Serine-to-alanine mutations in the ΔN construct were made by ligation of *Eco*RI fragments of the pBS constructs described above. The TRα2ΔN without the His_6 tag has been previously described (26). TRα1ΔN was made from the TRα2ΔN by replacement of the *Nco*I-*Eco*RI fragment. The expression vectors were transformed into the *E. coli* BL21 pLysS and grown in the presence of ampicillin to log phase at 30°C, when they were induced for 3 to 4 h with 100 μM isopropylthiogalactopyranoside (IPTG). His_6 fusion proteins were used either in unpurified form or after purification to near homogeneity as described previously (13).

EMSA of DNA binding. The electrophoretic mobility shift assay (EMSA) was performed as previously described (35), using *Hin*dIII-*Xba*I restriction fragments of the pBluescript polylinker containing either TREp (AGGTCATGACCT) or DR4 (AGGTCACAGGAGGTCA).

ABCD assay of DNA binding. The avidin-biotin complex with DNA (ABCD) assay was modified from that of Glass et al. (15). TRα1, TRα2, and TRα2-SA were translated in reticulocyte lysate as described above in the presence of [^{35}S]methionine. For each protein, 100,000 cpm of trichloroacetic acid-precipitable material was incubated with ABCD buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.8], 50 mM KCl, 1 mM β-mercaptoethanol, 20% glycerol, 0.1% Nonidet P-40 [NP-40]), poly(dI-dC) (10 μg), and 200 fmol of biotinylated oligonucleotide containing the DR4 sequence (GATC CCAGCTTCAGGTCACAGGAGGTCAGAGAG) in a final volume of 50 μl for 40 min at room temperature. Next 20 μl of slurried streptavidin agarose (Pierce) was added, and the mixture was gently agitated at 4°C for 10 min. Beads were washed three times with 1 ml of ice-cold ABCD buffer and vigorous shaking and then subjected to microcentrifugation for 20 s. Radioactivity of the pellets was then measured by liquid scintillation counting. Nonspecific binding was measured by preincubation with a 500-fold molar excess (100 pmol) of DR4-containing, nonbiotinylated oligonucleotide for 10 min prior to addition of biotinylated oligonucleotide. For TRα1, total binding was typically 15,000 to 20,000 cpm and nonspecific binding was typically 4,000 to 6,000 cpm. Specific binding was calculated as total minus nonspecific binding.

Kinase reactions. Proteins captured on antibody-protein A complexes were washed two times in the buffer in which they were isolated and then washed twice in the appropriate 1× kinase buffer (for CKII, the buffer contained 20 mM Tris [pH 8.0], 8 mM $MgCl_2$, 150 mM NaCl, and 0.1 mM dithiothreitol [DTT]; for PKA, the buffer contained 20 mM Tris [pH 7.4], 1 mM DTT, 100 mM NaCl, 12 mM and $MgCl_2$). Kinase (60 U of CKII [gift of Roberto Weinmann] [71] or 2 μl of 2 mM PKA [Sigma] diluted in 6 mg of DTT per ml) plus 1× buffer was added along with [γ-^{32}P]ATP or -GTP (3,000 Ci/mmol; NEN), and the mixture was incubated at 30°C for 30 min. Bovine serum albumin (0.5 μg/μl) was also added to CKII reactions. The phosphoproteins were then washed twice in cold Pansorbin C buffer and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Antibodies. A mouse monoclonal antibody recognizing both TRα1 and TRα2 has been previously described (24). A rabbit polyclonal antiserum was raised against the peptide sequence LRGPVLQHQSPKSPQQR, corresponding to amino acids 426 to 442 of rat TRα2. In addition to the characterization in this report, this antiserum was specific in immunoprecipitation and gel shift analysis of ^{35}S-labeled TRα2 (7). A rabbit polyclonal antiserum raised against a TRα1 His_6 fusion protein was also used in some experiments. This antiserum recognized both TRα1 and TRα2 in immunoprecipitation and gel shift assays (7).

Western blot (immunoblot) analysis. Western blotting of bacterial and transfected mammalian cell extracts was performed with a monoclonal antibody which recognizes both TRα1 and TRα2 as previously described (24, 26) except that incubation with primary antibody was performed overnight at 4°C.

Metabolic labeling. JEG-3 cells transfected with 100 μg of expression plasmid were washed with Dulbecco modified Eagle medium minus phosphate for 30 min. $^{32}P_i$ (1 mCi/ml) was added to each culture, and the cultures were incubated for 3 to 4 h. Cells were washed and lysed in 50 mM Tris (pH 8.0)–500 mM NaCl–1.0% NP-40 for 30 min on ice. Extracts were cleared of debris and immunoprecipitated as described below.

Immunoprecipitation. Samples prepared from either lysed bacterial cells or high-salt lysis of ^{32}P-labeled JEG-3 cells were precleared with 5 μl of normal whole rabbit serum (Cappel) in a 40% slurry of protein A-agarose beads (Gibco BRL) which had been washed five times in Pansorbin B solution (2 mM Tris [pH 7.4], 0.1% SDS, 0.1% NP-40). The supernatant was incubated with 5 μl of TRα2 rabbit polyclonal antiserum overnight at 4°C. Immune complexes were then precipitated with 40% protein A beads and washed five times in Pansorbin solution C (2 mM Tris [pH 7.4], 0.1% SDS, 0.1% NP-40, 0.15 M NaCl). In some experiments, low-stringency immunoprecipitation was performed on unlabeled JEG-3 cells after transfection with 100 μg of expression plasmid. After cells were resuspended in 1 ml of low-stringency (LS) buffer (50 mM HEPES [pH 7.9], 250 mM NaCl, 0.1% NP-40, phenylmethylsulfonyl fluoride) and agitated for 30 min at 4°C, the supernatant was precleared and immunoprecipitated in LS buffer without the final high-stringency washes. The resulting complexes were washed twice in LS buffer and then twice in CKII kinase buffer. Kinase reactions were performed as described above in the presence or absence of CKII inhibitors (70) or without added kinase.

Transfections. Cell transfections were performed by using calcium phosphate precipitation as previously described. For transient transcription rate assays, chloramphenicol acetyltransferase activity was normalized to expression of β-galactosidase from a cotransfected plasmid as described previously (26).

RESULTS

The source of TRα2 influences its DNA binding. Figure 1A shows that when rat TRα1 and TRα2 were translated in rabbit reticulocyte lysate, the binding of TRα2 to a T3 response element (TRE) consisting of two directly repeated AGGTCA half-sites separated by 4 bp (DR4) was much less than that of

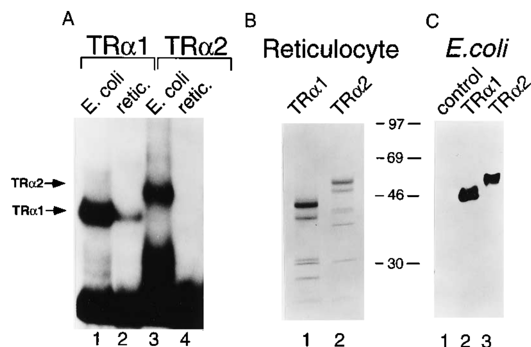

FIG. 1. DNA binding of recombinant TRα2 varies according to its source. (A) Purified TRα1 His_6 fusion protein produced in *E. coli* (lane 1), TRα1 produced in reticulocyte lysate (retic.; lane 2), purified TRα2 His_6 fusion protein produced in *E. coli* (lane 3), and TRα2 produced in reticulocyte lysate (lane 4) were used with a ^{32}P-labeled DR4 probe in EMSA. (B) SDS-PAGE analysis of ^{35}S-labeled TRα1 and TRα2 used in panel A. TRα2 contains one less methionine residue than TRα1, which was taken into account in the EMSA. (C) Western analysis of purified TRα1 and TRα2 His_6 fusion proteins using a monoclonal antibody to an epitope common to both. Sizes are indicated in kilodaltons.

TRα1 (compare lanes 2 and 4). Similar results were observed with an inverted repeat (TREp; data not shown). As previously shown, in vitro-translated TRα1 bound to DNA primarily as a monomer under these conditions (35). When the proteins were produced in *E. coli*, the DNA binding activities of TRα1 and TRα2 were much more similar (lanes 1 and 3). In the experiment shown, monomeric binding was observed although bacterially expressed TRα1 and TRα2 both bind to DR4 and TREp as homodimers as well (26). The difference in DNA binding of the reticulocyte proteins was not due to less TRα2 because the amounts of protein used were equalized by normalization to [^{35}S]methionine incorporation (Fig. 1B). Similar amounts of bacterial TRα1 and TRα2 were also used, as shown by Western analysis (Fig. 1C).

Dephosphorylation of TRα2 restores its DNA binding. We have previously shown that the C terminus of TRα2 is responsible for its reduced DNA binding in reticulocyte lysate (24). Therefore, we examined this region closely for a site of potential posttranslational modification which might affect DNA binding. Indeed, the C terminus of TRα2 is serine rich, and the truncation which improves DNA binding is within a cluster of nine serine residues (Fig. 2A). This finding suggested that DNA binding might be modulated by serine phosphorylation involving a protein kinase present in reticulocytes but not in bacteria.

To determine if phosphorylation in reticulocyte lysate was responsible for the reduced DNA binding of TRα2, TRα1 and TRα2 were treated with alkaline phosphatase prior to gel shift assays. Figure 2B shows that alkaline phosphatase treatment of TRα1 had no effect on its ability to bind to DR4 (lanes 1 and 2). Remarkably, dephosphorylation of TRα2 enhanced its DNA binding (lanes 3 and 4). Similar effects of dephosphorylation were observed with TREp (25). Furthermore, the DNA binding of human TRα2, whose C terminus is similar but not identical to that of rat TRα2, was similarly restored by dephosphorylation (25). However, the DNA binding of bacterially expressed TRα2 was not increased by treatment with alkaline phosphatase (lanes 7 and 8). To test whether the dephosphorylation of reticulocyte-translated TRα2 involved any of the nine serine residues highlighted in Fig. 2A, these were all mutated to alanines in the TRα2 protein (TRα2-SA). As shown in lanes 5 and 6, TRα2-SA bound DR4 without a requirement for dephosphorylation. In addition, alkaline phosphatase treatment did not enhance binding by the serine-to-alanine mutant, strongly suggesting that phosphorylation of one or more of these nine serines was entirely responsible for the inhibition of DNA binding of reticulocyte-translated TRα2.

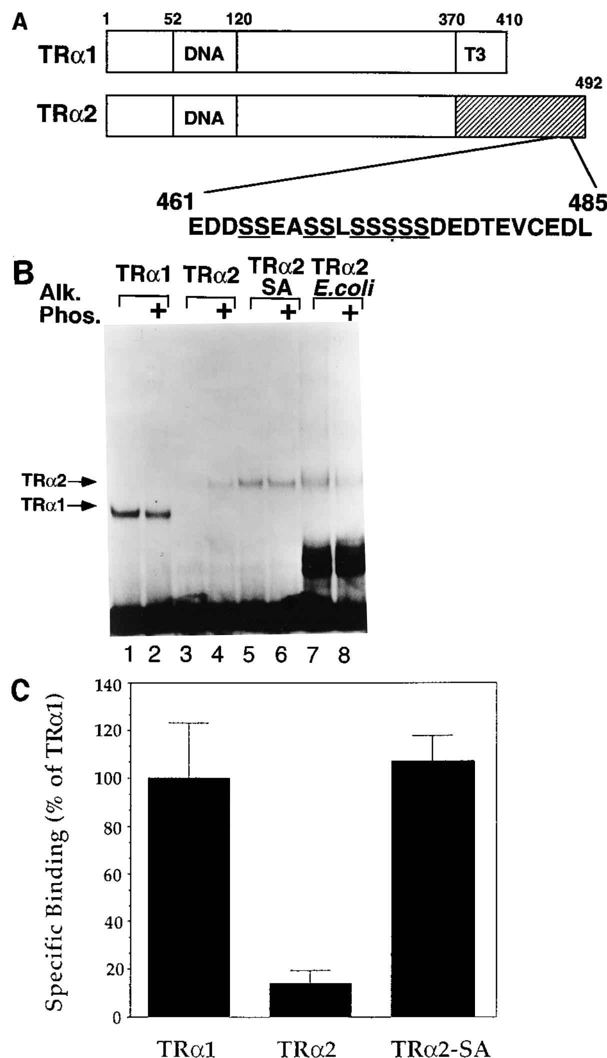

FIG. 2. Dephosphorylation or serine-to-alanine mutation of TRα2 improves its DNA binding. (A) Serine-rich domain of the TRα2 C terminus. Structures of TRα1 and TRα2 are depicted schematically. The nine serines mutated to alanine in TRα2-SA are underlined. (B) Effect of phosphorylation on DNA binding by TRα2. Reticulocyte-translated TRα1 (lanes 1 and 2), TRα2 (lanes 3 and 4), TRα2-SA (nine serines highlighted in panel A mutated to alanine; lanes 5 and 6), and bacterially expressed TRα2 (lanes 7 and 8) were treated with 20 mg of alkaline phosphatase (Alk. Phos.), native (lanes 2, 4, and 6) or inactivated by boiling (lanes 1, 3, and 5), per ml, bound to ^{32}P labeled DR4 element, and subjected to EMSA. (C) ABCD assay. Specific bindings of TRα1, TRα2, and TRα2-SA to DR4 are reported as percentages of mean TRα1 binding (± standard error, n = 3).

The enhanced ability of TRα2-SA to bind DR4 was confirmed in the ABCD assay, in which binding of equal amounts of [^{35}S]methionine-labeled TRα1, TRα2, and TRα2-SA to unlabeled biotinylated DNA was quantitated (15, 32). Figure 2C shows that the binding of reticulocyte lysate-synthesized TRα2 to DR4 was measurable in this assay, unlike in the EMSA. However, the magnitude of TRα2 binding was dramatically reduced, to less than 20% of the binding of TRα1. In contrast,

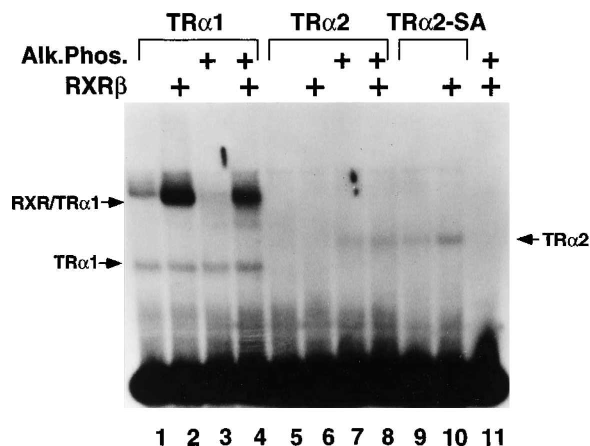

FIG. 3. Phosphorylated and unphosphorylated TRα2 do not heterodimerize with RXRβ. EMSA analysis of reticulocyte-translated TRα1 (lanes 1 to 4), TRα2 (lanes 5 to 8), or TRα2 SA (lanes 9 and 10), treated with alkaline phosphatase (Alk.Phos.; lanes 3, 4, 7, and 8) or with boiled alkaline phosphatase (lanes 1, 2, 5, 6, 9, and 10), using DR4 as the probe. RXRβ was added to the binding reaction in even-numbered lanes. Alkaline phosphatase-treated RXRβ alone is shown in lane 11.

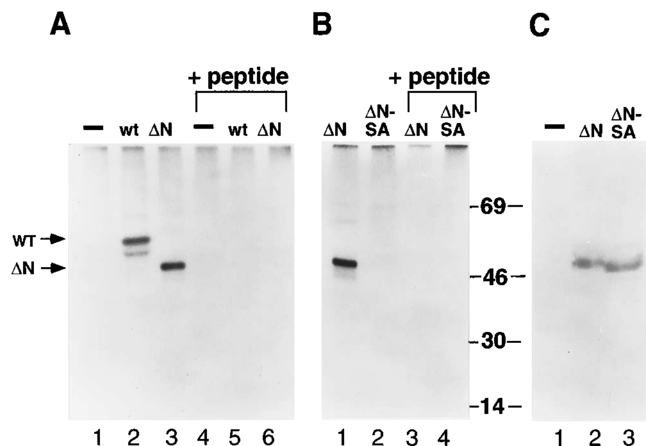

FIG. 4. The C terminus of TRα2 is phosphorylated on specific serine residues in vivo. JEG-3 cells were metabolically labeled with $^{32}P_i$ after transfection. (A) Comparison of wild-type (wt) and N-terminally deleted TRα2. Cells were transfected with control vector (lanes 1 and 4), full-length TRα2 (lanes 2 and 5), or N-terminally truncated TRα2 (TRα2ΔN; lanes 3 and 6). Immunoprecipitations were performed with a TRα2-specific polyclonal antiserum in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of 100 μg of added immunizing peptide and analyzed by SDS-PAGE. (B) Mutation of serines in the TRα2 C terminus prevents phosphorylation. Cells were transfected with TRα2ΔN, wild type (lanes 1 and 3) or with the nine serines highlighted in Fig. 2A mutated to alanines (TRα2ΔN-SA; lanes 2 and 4). Blocking peptide was added in lanes 3 and 4. (C) Western analysis of JEG-3 cells, using a TRα antibody after transfection of vector alone (lane 1), TRα2ΔN (lane 2), or TRα2ΔN-SA (lane 3). Sizes are indicated in kilodaltons.

mutation of the C-terminal serines to alanine (TRα2-SA) enhanced the binding of TRα2 to approximately the level of TRα1, consistent with the results of EMSA analysis.

We also studied the effects of phosphorylation on the ability of TRα2 to heterodimerize with retinoid X receptor (RXR), which increases the DNA binding affinity of other TRs (8, 29, 39, 46, 69, 72). Figure 3 shows that while RXR heterodimerized with TRα1 and increased its binding to a DR4 probe (lane 2), it did not enhance the ability of wild-type TRα2 to interact with DR4 (lane 6), as we have previously reported (26) but in contrast to another published report (50). We wondered whether differences in phosphorylation might explain this discrepancy. However, even after improvement of its DR4 binding by dephosphorylation or by mutation of the nine serines to alanine, DNA binding by TRα2 was not augmented by RXR (lanes 8 and 10). Most likely the failure of TRα2 to interact with RXR is due to lack of a critical leucine residue which is conserved in the ninth heptad region of TR and the retinoic acid receptor and is required for heterodimerization (4, 51). Indeed, RXR-TRα1 heterodimerization in solution was independently verified by coimmunoprecipitation of RXR with TRα1, whereas we were unable to coimmunoprecipitate RXR (or TRα1) with either TRα2 or TRα2-SA (59).

TRα2 is a phosphoprotein in vivo. We next determined whether TRα2 was phosphorylated in intact mammalian cells. JEG-3 human choriocarcinoma cells were transfected with a plasmid expressing either wild-type TRα2 or its C terminus (amino acids 122 to 492). The latter was used because of the known kinase site in the N terminus of TRα1 (16) and also because its molecular mass differed from that of full-length TRα2. Transfected cells were metabolically labeled with P_i, and cell extracts were immunoprecipitated and electrophoresed. Figure 4A shows that cells transfected with a control plasmid did not contain phosphorylated TRα2. In contrast, both wild-type TRα2 (lane 2) and an N-terminal deletion of TRα2 (TRα2ΔN; lane 3) were radiolabeled in the presence of ^{32}P. Immunoprecipitation was specifically abolished by coincubation with the TRα2 peptide against which the antiserum was raised (lanes 5 and 6). Phosphoamino acid analysis demonstrated that the proteins were phosphorylated almost entirely on serines (data not shown). The identity of the phosphoproteins as TRα2 was confirmed by their appropriate molecular masses, the dependence on the transfection of TRα2, and the complete block of immunoprecipitation by the peptide against which the TRα2-specific antiserum was raised (lanes 4 to 6).

To determine if the phosphorylation of TRα2 occurred in the region implicated by the DNA binding studies shown earlier, JEG-3 cells were also transfected with a mutated form of the TRα2ΔN, with the nine serines between amino acids 461 and 485 mutated to alanine (TRα2ΔN-SA). Figure 4B shows that this protein was almost negligibly phosphorylated compared with the wild type (lanes 1 and 2). This was not due to differences in protein expression because Western blots of transfected cells indicated that the two proteins were synthesized at similar levels (Fig. 4C).

Nonphosphorylatable TRα2 is a better dominant negative inhibitor than the wild type. We have previously related the weak DNA binding of TRα2 to its weak ability to inhibit T3 action. Therefore, we hypothesized that TRα2-SA, which is not phosphorylated, would be more competitive than wild-type TRα2 as an inhibitor of TRα1 function in JEG-3 cells. Indeed, as shown in Fig. 5A, TRα2-SA repressed T3 activation of transcription by TRα1 more than did wild-type TRα2. In contrast, mutation of a serine outside of the implicated region (serine 455) had no effect on dominant negative function. Western analysis revealed that comparable levels of mutant and wild-type proteins were produced in the transfected cells (Fig. 5B), and this was confirmed by metabolic labeling with [^{35}S]methionine followed by immunoprecipitation of transfected cells (not shown). The correlation between the increased dominant negative and DNA binding activity of the nonphosphorylatable TRα2 supports our earlier work suggesting that TRα2 inhibits TRα1 action by competing for TREs. Interestingly, TRα2-SA migrated slightly faster than wild-type TRα2 in the SDS-polyacrylamide gel system, consistent with phosphorylation of the majority of the wild-type protein after

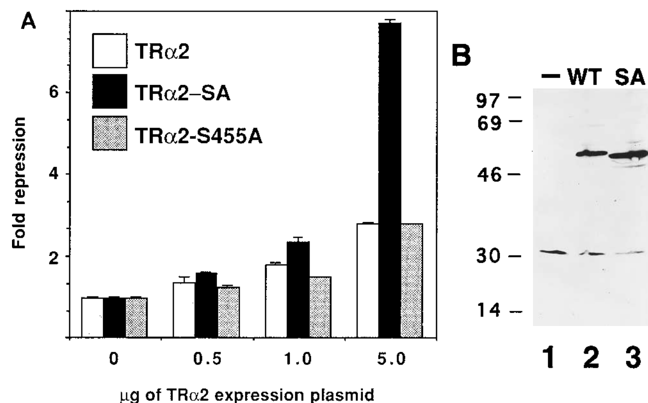

FIG. 5. TRα2-SA is a better inhibitor than wild-type TRα2. (A) Repression of TRα1-stimulated transcription by TRα2. JEG-3 cells were transfected with a chloramphenicol acetyltransferase reporter containing a modified rat growth hormone TRE (TK35BA [10]). Increasing amounts of either wild-type TRα2 (white bars), TRα2-SA (solid black bars), or TRα2 with a different serine mutated to alanine (TRα2-S455A) (grey bars), all in pCDM, were transfected along with TRα1 (in pECE; 2.5 μg). Results were normalized to the activity of a cotransfected β-galactosidase expression plasmid and are reported as the fold repression (± standard error of the mean) of TRα1 activity. (B) Western analysis of extracts of cells transfected with 100 μg of vector alone (lane 1) or vector expressing wild-type (WT) TRα2 (lane 2) or TRα2-SA (lane 3), using a TRα monoclonal antibody. Sizes are indicated in kilodaltons.

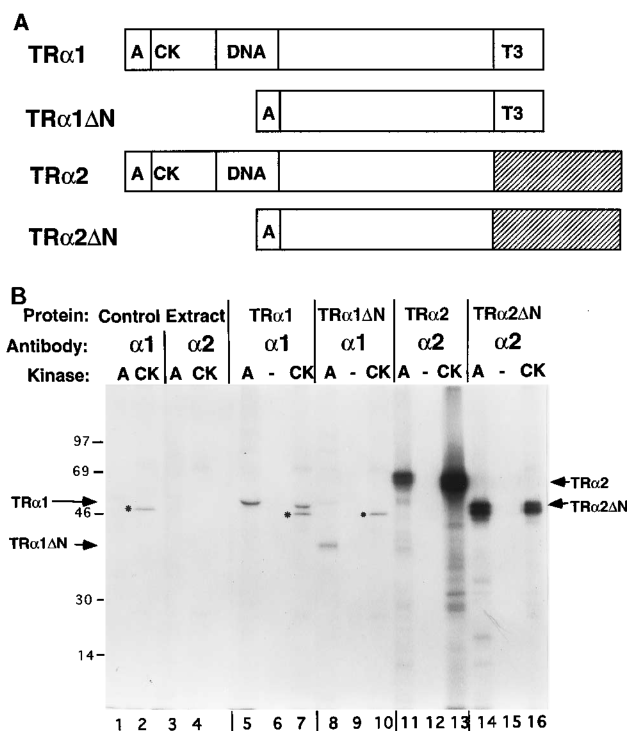

FIG. 6. Phosphorylation of the C terminus of TRα2 by CKII in vitro. (A) Protein substrates. Full-length or N-terminally deleted (ΔN) TRα1 and TRα2 were expressed in *E. coli* fused to an N-terminal PKA site (labeled A). The CKII site in the N terminus of TRα1 and TRα2 is marked by CK. Note that this is deleted in the ΔN proteins. (B) Phosphorylation by CKII. No TR, lanes 1 to 4; full-length TRα1, lanes 5 to 7; TRα1ΔN, lanes 8 to 10; full-length TRα2, lanes 11 to 13; TRα2ΔN, lanes 14 to 16. Proteins were immunoprecipitated with polyclonal antisera recognizing TRα1 (lanes 1, 2, and 5 to 10) or TRα2 (lanes 3, 4, and 11 to 16). Kinase reactions were performed with PKA (lanes 1, 3, 5, 8, 11, and 14), CKII (lanes 2, 4, 7, 10, 13, and 16), or no added kinase (lanes 6, 9, 12, and 15) and then subjected to SDS-PAGE. A protein which is phosphorylated by CKII and nonspecifically precipitated by the TRα1 antiserum is marked with an asterisk in lanes 2, 7, and 10. This protein is unlikely to be CKII itself, whose phosphorylated subunit is 24 kDa (19), but could be a bacterial extract protein. It was not observed with the TRα2 antibody. Sizes are indicated in kilodaltons.

expression in JEG-3 cells (a similar difference in mobility was observed between TRα2ΔN and TRα2ΔN-SA in Fig. 4C). Of note, the TRα1 expression vector yields considerably less protein than do the TRα2 vectors (26). Therefore, the dominant negative activity of the TRα2-SA, while greater than that of TRα2, was not stoichiometric with TRα1 protein concentration. This is most likely because JEG-3 cells contain abundant RXRα (12), which greatly enhances the DNA binding by TRα1 but not TRα2.

TRα2 is phosphorylated by CKII. We hypothesized that CKII or a similar kinase might be involved in the phosphorylation of TRα2, which inhibits its DNA binding because CKII is abundant in rabbit reticulocyte lysate, and its consensus target sequence, SXXE, often in the context of multiple serines and acidic residues (18), is found in the C terminus of TRα2, particularly serines 474 and 475 (Fig. 2A). To test the ability of TRα2 to serve as a substrate for CKII, full-length and C-terminal (ΔN) fragments of both TRα1 and TRα2 were synthesized in bacteria as fusion proteins containing a PKA phosphorylation site at the N terminus (9) to serve as a phosphorylation control (Fig. 6A). These proteins were then phosphorylated with either purified CKII or PKA. Figure 6B shows that all proteins were phosphorylated by PKA, as expected (lanes 5, 8, 11, and 14). Full-length TRα1 was also a substrate for CKII (lane 7), but TRα1ΔN was not (lane 10), confirming the presence of a CKII site in the N terminus of TRα proteins and indicating that the remainder of TRα1 is not a substrate for CKII. In contrast, TRα2ΔN (lane 16) as well as full-length TRα2 (lane 13) were phosphorylated by CKII, indicating that the unique portion of the TRα2 C terminus was a substrate for CKII.

Phosphorylation of serines 474 and 475 is responsible for the reduced DNA binding of TRα2. We next sought to identify the phosphorylated serine residues in TRα2. The nine serine residues shown in Fig. 2A were systematically mutated to alanine in the context of the TRα2ΔN. All proteins were synthesized in bacteria and used at equal concentrations (determined by Western analysis; not shown). Figure 7A shows that when serines 464 to 469 were mutated to alanine, TRα2 remained a substrate for CKII (lane 3). In contrast, mutation of serines 471 to 475 to alanine virtually eliminated phosphorylation of TRα2 (lane 4). The phosphorylation site was further sublocalized. Mutation of serine 472 to alanine had no effect (lane 5), whereas mutation of both serines 474 and 475 virtually eliminated phosphorylation of the protein (lane 8). Individually, mutation of serine 475 had a greater effect than mutation of serine 474 on overall phosphorylation (lanes 6 and 7). The same pattern of phosphorylation of the serine-to-alanine mutants was observed when the reticulocyte lysate was used as a source of kinase (Fig. 7B). This experiment was performed with [γ-^{32}P]GTP as the phosphate donor, confirming the likelihood that the major kinase phosphorylating TRα2 in reticulocyte lysate was CKII, since most other serine kinases use only [γ-^{32}P]ATP as a substrate (18). Similar results were obtained with [γ-^{32}P]ATP as the phosphate donor (25).

To confirm that phosphorylation of TRα2 on serines 474 and 475 was related to its DNA binding affinity, TRα2 mutants were produced in reticulocyte lysate, and their DNA binding before and after dephosphorylation was studied. Figure 8 shows that the DNA binding of the serine-to-alanine mutants paralleled their ability to be phosphorylated by the reticulocyte

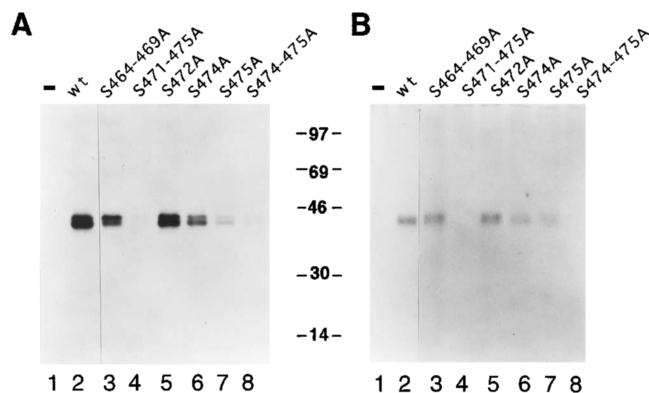

FIG. 7. CKII phosphorylates TRα2 on serines 474 and 475. (A) Phosphorylation in vitro by CKII. Bacterial extracts expressed either no insert (lane 1), TRα2ΔN (wild type [wt]; lane 2), or TRα2ΔN with specific serines mutated to alanine: 464 to 469 (lane 3), 471 to 475 (lane 4), 472 (lane 5), 474 (lane 6), 475 (lane 7), or 474 and 475 (lane 8). Proteins were immunoprecipitated and treated with added CKII and [γ-^{32}P]GTP as described in Materials and Methods. (B) Phosphorylation in vitro, using reticulocyte lysate as the source of kinase. The same TRα2 proteins as in panel A were immunoprecipitated and kinase treated as described above, but 10 μl of reticulocyte lysate was used as a source of kinase instead of CKII. Sizes are indicated in kilodaltons.

kinase. Thus, mutation of both serines 474 and 475 to alanine allowed binding in the absence of treatment with alkaline phosphatase (lanes 13 and 14). Single serine-to-alanine substitution at 474 or 475 resulted in improved but submaximal binding which increased further after dephosphorylation (lanes 9 to 12).

DISCUSSION

We have shown that phosphorylation of the unique C terminus of TRα2 greatly reduces its DNA binding. TRα2 is formally an orphan receptor, since no ligand is known to bind or regulate its activity (53). The observation that posttranslational modification of the C terminus, which is the ligand-binding domain in other members of the steroid/thyroid hormone receptor superfamily, indicates that this region of the protein can function in ways other than mediating ligand binding or protein-protein interaction. Furthermore, the ability of dephosphorylation to increase DNA binding is shared by human and rat TRα2 (25), which have highly similar C termini. Thus, interspecies conservation of C-terminal sequences may occur for reason other than the existence of a regulating ligand.

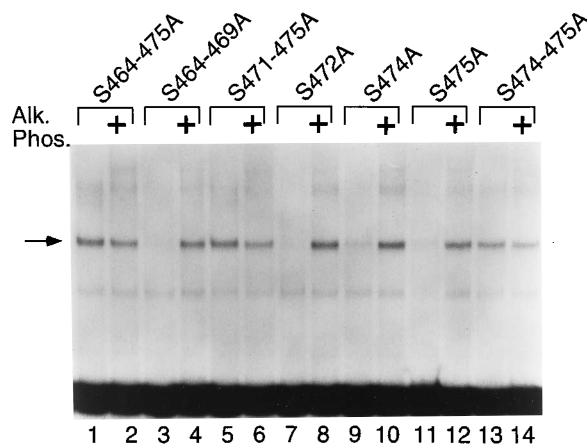

FIG. 8. Mutation of serines 474 and 475 in TRα2 restores DNA binding. TRα2 with serines 464 to 475 (lanes 1 and 2), 464 to 469 (lanes 3 and 4), 471 to 475 (lanes 5 and 6), 472 (lanes 7 and 8), 474 (lanes 9 and 10), 475 (lanes 11 and 12), or 474 and 475 (lanes 13 and 14) mutated to alanine was expressed in reticulocyte lysate and treated with alkaline phosphatase (Alk. Phos.; even-numbered lanes) or boiled alkaline phosphatase (odd-numbered lanes). Proteins were then bound to ^{32}P-labeled TREp and assayed by EMSA.

The N-terminal DNA-binding domain, consisting of two zinc fingers and adjacent A and T boxes, is necessary and sufficient for DNA binding by nuclear hormone receptors, including TRα2 (14). The ability of phosphorylation to prevent DNA binding by TRα2 is reminiscent of another orphan receptor, NGFI-B, whose DNA binding is directly and negatively regulated by phosphorylation. However, phosphorylation of NGFI-B occurs in the A box, which actively participates in DNA binding, and the reduction in DNA binding is therefore likely to be a local effect (20). In contrast, phosphorylation of the C terminus of TRα2 acts at a distance to reduce the availability or affinity of the DNA-binding domain, probably by changing the overall conformation of the protein. Phosphorylation of the C terminus of TRα2 may thus be analogous to hormone binding by other TR isoforms, whose conformation is greatly altered by the interaction between their C termini and T3 (40, 66). Ligand binding (2, 61, 68) as well as phosphorylation (42, 65) may also regulate the DNA binding affinity of bona fide TRs, but unlike the case of TRα2, these effects are likely to be secondary to changes in homodimerization or heterodimerization.

Although DNA binding by reticulocyte lysate-synthesized, phosphorylated TRα2 was below the limits of detection by EMSA, the ABCD assay revealed weak binding, suggesting that the gel shift assay underestimates the amount of phosphorylated TRα2 bound at equilibrium. The low-affinity specific binding of phosphorylated TRα2 may explain its correspondingly weak dominant negative function in vivo, where there may also be a minority of unphosphorylated TRα2 molecules in equilibrium with the phosphorylated form. Either explanation or both would be consistent with our previous observation that a TRα2-VP16 fusion protein activated transcription from TREs in JEG-3 cells, further indicating that TRα2 binds TREs weakly in vivo (26). The correlation between the DNA binding and dominant negative activities of nonphosphorylatable TRα2 strengthens the argument that the inhibition of T3 action by TRα2 is in large part due to competition for binding to TREs. However, it remains formally possible that a physical interaction between TRα2 and TRα1, although undetectable by gel shift or coimmunoprecipitation, contributes to the dominant negative effect of TRα2.

Although increased relative to that of wild-type TRα2, the dominant negative activity of nonphosphorylatable TRα2 was not stoichiometric with TR concentration. We hypothesize that this is due to a competitive advantage of true TRs due to their ability to heterodimerize with RXR, which increases their affinity for TREs containing inverted or direct repeats of AGG TCA half-sites (33, 56). It is possible that TRα2 will compete more effectively for TREs which may respond to TR monomers, such as octameric half-sites (27, 62), or other TREs which may function with TR homodimers, such as direct repeats of octameric half-sites (28) and everted repeats (33).

The findings that TRα2 is phosphorylated in vivo on the same serines as phosphorylated by CKII in vitro strongly suggest that endogenous CKII phosphorylates TRα2. Indeed, in preliminary experiments, we have noted that transfected TRα2 could be phosphorylated by a kinase which coimmunoprecipitated at low stringency. This kinase used [γ-^{32}P]GTP as a substrate and was sensitive to inhibitors of CKII (25), thus adding to the evidence that regulation of TRα2 activity in vivo involves a kinase identical or highly similar to CKII.

The present work describes the first example of CKII-dependent phosphorylation regulating the activity of a member of the steroid/thyroid receptor superfamily. CKII is present in the nuclei of most mammalian cells (31) and has been shown to regulate the DNA binding and transcriptional activities of other transcription factors, including serum response factor (44, 45), Myb (43), Max (6, 58), and Jun (41) (reviewed in reference 47). Although CKII activity appears to be constitutive in many systems, it may be regulated by growth factors (1, 11) and appears to be important during the cell cycle (55). It is also possible that the phosphorylation state of TRα2 is physiologically regulated by a phosphoprotein phosphatase.

Steady-state phosphorylation of TRα2 may also differ in different cell types as a result of the presence or absence of kinase or phosphatase activities with specificity for TRα2. In particular, the ability of phosphorylation to regulate the function of TRα2 is likely to be important in tissues such as the brain, where high levels of expression (38, 48, 63, 67) may compensate for the relatively weak dominant negative activity of TRα2. Posttranslational regulation of TRα2 activity would provide a novel, rapid, T3-independent mechanism for cell-specific modulation of the expression of T3-responsive genes.

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