Thyroxine Promotes Association of Mitogen-activated Protein Kinase and Nuclear Thyroid Hormone Receptor (TR) and Causes Serine Phosphorylation of TR*

Paul J. Davis¶§, Ai Shih‡, Hung-Yun Lin‡, Leon J. Martinó, and Faith B. Davis¶§

From the ‡Samuel S. Stratton Veterans Affairs Medical Center and the §Molecular and Cellular Medicine Program, Department of Medicine and the Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York 12208

Activated nongenomically by l-thyroxine (T₄), mitogen-activated protein kinase (MAPK) complexed in 10–20 min with endogenous nuclear thyroid hormone receptor (TRβ1 or TR) in nuclear fractions of 293T cells, resulting in serine phosphorylation of TR. Treatment of cells with the MAPK kinase inhibitor, PD 98059, prevented both T₄-induced nuclear MAPK-TR co-immunoprecipitation and serine phosphorylation of TR. T₄ treatment caused dissociation of TR and SMRT (silencing mediator of retinoid and thyroid hormone receptor), an effect also inhibited by PD 98059 and presumably a result of association of nuclear MAPK with TR. Transfection into CV-1 cells of TR gene constructs in which one or both zinc fingers in the TR DNA-binding domain were replaced with those from the glucocorticoid receptor localized the site of TR phosphorylation by T₄-activated MAPK to a serine in the second zinc finger of the TR DNA-binding domain. In an in vitro cell- and hormone-free system, purified activated MAPK phosphorylated recombinant human TRβ1 (102–461). Thus, T₄ activates MAPK and causes MAPK-mediated serine phosphorylation of TRβ1 and dissociation of TR and the co-repressor SMRT.

We have demonstrated in cultured cells that l-thyroxine (T₄) can nongenomically activate signal transduction proteins such as mitogen-activated protein kinase (MAPK) (1) and, through serine phosphorylation by MAPK, can enhance the activity of several nuclear transactivator proteins. Among the latter are the signal transducer and activator of transcription (STAT) proteins that mediate growth factor (2) and cytokine (1, 3) signals. This T₄ effect is initiated by a G protein-coupled receptor in the plasma membrane (1) and has been observed in cells that contain endogenous nuclear thyroid hormone receptor (TR), such as 293T cells and human skin fibroblasts (BG-9), and in cells which are devoid of functional TR (CV-1 and HeLa (4)). In contrast, genomic actions of thyroid hormone require binding of the hormone, predominantly 3,5,3′-triiodo-l-thyronine (T₃), by specific receptors in the cell nucleus. T₃-ligated TR may bind as a monomer or a homo- or heterodimer with retinoid X receptor to thyroid hormone response elements in the regulatory upstream regions of specific hormone-responsive genes (5–7). In the absence of T₃, TR exists in the transcriptionally inactive (repressed) state. This state is imposed by the binding to unliganded TR of the co-repressor proteins, SMRT (silencing mediator of retinoid and thyroid hormone receptors) and NCoR (nuclear co-repressor) (8). SMRT binding to TR has been localized to the hinge region of the receptor (amino acids 211–240) (9). Binding of T₃ to TR results in dissociation of co-repressor proteins from TR and the recruitment of activator proteins that facilitate enhanced transcriptional activity of the receptor (10).

Serine phosphorylation of TR isoforms has been described by several laboratories (11–15). In such studies phosphorylation has been inferred from stimulation of the activity of cellular cAMP-dependent protein kinase (PKA) (11, 12), a serine/threonine kinase, or from serine kinase inhibition or phosphatase inhibition in treated cells (14, 16). There have been several results of such phosphorylation in these model systems. For example, serine phosphorylation of TRα1 has been shown to decrease TR monomer binding to DNA (11). TRβ1 is selectively stabilized against protease degradation by serine phosphorylation and transcriptional activity of the receptor is significantly increased (14, 15). Leitman et al. (12) have also shown increased transcriptional activity of TRβ1 in response to phosphorylation. Comparing phosphorylatable and nonphosphorylatable forms of TRα2, Katz et al. (17) concluded that serine phosphorylation of the receptor isoform decreased its ability to heterodimerize with retinoid X receptor at a thyroid hormone response element site. Using a serine/threonine kinase inhibitor, H7, Jones et al. (16) showed a reduction in T₃-induced transcriptional activity of both TRα1 and TRβ1. In some of these studies (12), the results were highly cell line-specific.

The mechanism by which serine phosphorylation of TRβ1 is achieved is unclear, aside from the possible involvement of PKA (11, 12). In the study reported here, we describe a signal transduction mechanism by which T₄, acting at the cell surface, promotes MAPK-dependent serine phosphorylation of TRβ1 and resultant dissociation of TR and SMRT. We also identify a

*This work was supported in part by funds from the Office of Research Development, Medical Research Service, Department of Veterans Affairs (to P. J. D.) and by a grant from the Candace King Weir Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom all correspondence should be addressed: Dept. of Medicine, MC-16, Albany Medical College, Albany, NY 12208. Tel.: 518-262-6138; Fax: 518-262-5008; E-mail: pjdadavis@albany.net.

¶The abbreviations used are: T₄, l-thyroxine; T₃, 3,5,3′-triiodo-l-thyronine; tetrac, tetraiodothyroacetic acid; triac, triiodothyroacetic acid; rT₃, 3,3′,5′-triiodo-l-thyronine; TR, thyroid hormone receptor β1; DDBD, DNA-binding domain; LBD, ligand-binding domain; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PD, PD 98059; FTU, 6-α-propyl-2-thiouracil; MBP, myelin basic protein; STAT, signal transducer and activator of transcription; PKA, cyclic AMP-dependent protein kinase; 8-BrcAMP, 8-bromo-cyclic AMP; GR, glucocorticoid receptor; PAGE, polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-O-(3-thio)triphosphate.
region of TRβ1 that is required for MAPK binding to the receptor.

EXPERIMENTAL PROCEDURES

Materials—t-T4, t-T3, b-T4, b-T3, T3, T3, 3',3',5'-triiodothyronine (tT3), tetraiodothyroacetic acid (tetrac), 3,5,3'-triiodothyroacetic acid (triac), 8-bromo-cyclic-AMP (8-Br-cAMP), myelin basic protein (MBP), 6-n-propyl-2-thiouracil (PTU), T₄-agarose, and protein A-agarose were obtained from Sigma. Stock solutions of thyroid hormone and analogues were prepared in 0.04 M ROH, 4% propylene glycol, and dilutions were made to final analogue concentrations as indicated. In all experiments in which T₃ was added to cultured cells, the total and free T₃ concentrations were 10⁻⁷ and 10⁻¹⁰ M, respectively, and total and free T₄ concentrations were below the limits of detection. PD 98059 was obtained from Calbiochem (La Jolla, CA), and geldanamycin was from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD). Stock solutions of these inhibitors were prepared in 100% Me₂SO, so that a final concentration of 0.1% Me₂SO was achieved in cell cultures and had no effect on experimental results. KT5720 was obtained from Kamiya Biomedical Co. (Thousand Oaks, CA). LipofectAMINE Plus was obtained from Life Technologies, Inc.

TR nucleotides, expressed in pDNA1/Amp and including full-length hTRβ1, rTR-3N (containing DBDs and LBDs), and rTR-LBD (LBD only), were generously provided by Dr. Paul M. Yen (NIDDK, NIH, Bethesda, MD), as were a TGT hybrid construct of TRβ1 with the DBD of the guccorticoid receptor (GR) substituted for that of TR (provided with permission of Dr. Ronald Evans, Salk Institute, La Jolla, CA). Two TR hybrid mutants in which one or the other zinc finger of the TR DBD is replaced with the corresponding zinc finger of GR (T-GT-T or T-GT-T; obtained with permission of Dr. J. Larry Jameson, Northwestern University, Chicago, IL). Recombinant TRβ1 (residues 102–461) was generously provided by Dr. Brian L. West (Metabolic Research Unit, University of California-San Francisco, CA).

Cell Cultures, Treatment, and Preparation of Nuclear Fractions—TR-replete 293T cells were obtained from Dr. K. Pumiglia (Albany Medical College, Albany, NY); CV-1 cells, which lack TR, were on hand in the laboratory (1). All cell lines were maintained in Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum. Almost confluent cells were placed for 2 days in medium containing 0.25% serum that had been previously depleted of thyroid hormone by the method of Samuels et al. (18), as modified by Weinstein et al. (19), and then in serum-free medium for 2 h. Cells were then treated with T₃ or other analogues for the times indicated. In selected experiments the inhibitors PD 98059 and geldanamycin were added to cells for 5 h, and 8-Br-cAMP and T₄ were added for the last 30 min; KT5720 was added for 16 h and then treated with T₄ (10⁻⁷ M) or diluted for 30 min and harvested for preparation of nuclear fractions as described above. Nuclear samples were immunoblotted with a TRβ1 antibody that detects TRβ1 and all mutants or were immuno precipitated with the same antibody, and precipitated proteins were then separated by PAGE and immunoblotted with antibodies to MAPK, phosphoserine, or phosphothreonine.

RESULTS

T₄ Induces Activation of MAPK and Nuclear Translocation of Co-immunoprecipitated MAPK and TRβ1—Human embryonic kidney (293T) cells contain endogenous TRβ1. To study co-immunoprecipitation, or complexing, of TRβ1 and MAPK, nuclear fractions were prepared from 293T cell lysates of control samples and of cells treated with T₄ (10⁻⁷ M) for 10–90 min and were immunoprecipitated with antibody to the carboxy-terminal half of the AB domain of TRβ1. The immunoprecipitated proteins were eluted, separated by SDS-polyacrylamide gel electrophoresis, and transferred to membranes for immunoblotting with antibody to MAPK. Shown in Fig. 1A is a representative immunoblot. There is an increase in nuclear MAPK complexed with TRβ1 immunoprecipitates that reaches a peak in 40 min (22-fold increase over control in the figure, decreasing to 12-fold in 90 min). In nine experiments, the fold increase in nuclear MAPK associated with TRβ1 in 30 and 40 min was 7 ± 1.5 (mean ± S.E.) and 8.6 ± 2.5, respectively, as shown in Fig. 1B. These changes parallel the timing of T₄-induced increases in tyrosine phosphorylation and nuclear translocation of MAPK that we have previously reported (1).

In studies using a selective antibody to tyrosine/threonine-phosphorylated, or activated, MAPK, for MAPK immunoprecipitates, we observed 21- and 24-fold in
In time course experiments similar to the study shown in Fig. 1A, nuclear extracts of 293T cells treated with T4 were immunoblotted with TRβ1 antibody without prior immunoprecipitation; Western blots demonstrated 2–5-fold increases in nuclear TRβ1 in 10–60 min, respectively, as shown in Fig. 2A. Nuclear samples were also immunoprecipitated with monoclonal TRβ1 antibody, and the precipitates were subjected to PAGE and immunoblotted with polyclonal anti-TRβ1 (Fig. 2B). Again, progressive nuclear accumulation of the receptor during T4 treatment is seen.

We determined in 293T cells whether T4 treatment for 30 min altered the abundance of TRβ1 per unit of nuclear fraction protein (10 μg of protein/lane). A representative blot is shown in Fig. 2C. It is assumed that the increase in nuclear TRβ1 originated from the pool of cytosolic TRβ1 also shown in this figure. We have also found cytosolic TRβ1 in another cell line (NIH3T3 cells; not shown), and others have reported the presence of TR in cytoplasm (22). Extranuclear TRβ1 concentration in our studies was 4.5 ± 2.5% of nuclear receptor concentration, where relative concentration was expressed as band integrated optical density per 10 μg of lane protein. The pool of cytosolic TRβ1 may be larger than Fig. 2C implies, however, given the 2-fold greater concentration of total protein in cytoplasm than in nuclear fractions and the relative volumes of nucleus and cytosol in intact cells.

Idothyronine Analogues and Formation of TRβ1-MAPK Complexes—We have previously reported that physiologic concentrations of T4 are more effective in nongenomic models of hormone action than physiologic concentrations of T3 (1–3) and that triac and tetrac may block the action of T3 at the plasma membrane (1, 3). We therefore studied whether T3, d-T3, rT3, triac, and tetrac caused complexing of TRβ1 and MAPK in
92T cell nuclei. In Fig. 3A, T4 is shown to enhance co-immunoprecipitation of TRβ1 and MAPK by 11.6-fold. In contrast, L-T3 in concentrations of 10^{-10} to 10^{-7} M was ineffective in promoting this complex in five studies. d-T4, d-T3, and rT3 were similarly inactive in the same studies compared with control cells. The ability of both tetrac and triac (10^{-7} M) to block the effect of T4 is seen in Fig. 3B. In this study T4 enhanced the amount of MAPK in the TRβ1 immunoprecipitate 10.4-fold. Tetrac and triac had negligible effects when present alone, but both analogues almost completely blocked T4 action.

**T4-induced Nuclear Complexing of TRβ1 and MAPK and Serine Phosphorylation of TRβ1 Are Blocked by a MEK Inhibitor**—To study the contribution of the T4-activated MAPK pathway to the complexing of MAPK and TRβ1, the MEK inhibitor PD 98059 was used. MEK is a dual tyrosine-threonine kinase that activates MAPK, a serine or threonine kinase (23). We have previously shown that PD 98059 (30 μM) blocks T4-induced tyrosine phosphorylation and nuclear translocation of MAPK (1) and the complexing of MAPK with both STAT1α and STAT3 (2). 293T cells were pretreated with PD 98059 (30 μM) for 5 h and with T4 (10^{-7} M) for the last 30 min. Fig. 4A demonstrates T4-induced co-immunoprecipitation of TRβ1 and tyrosine-phosphorylated MAPK, with a 2.4-fold increase in activated MAPK in T4-treated cells (comparing lanes 1 and 2). PD 98059 inhibited the T4 effect by 88% in the study shown (lane 2 versus lane 3). Additional studies examined the effect of PD 98059 on serine phosphorylation of TRβ1. Samples were immunoprecipitated with anti-TRβ1, and precipitates were immunoblotted with anti-phosphoserine. In the experiment shown in Fig. 4B, there is 2.9-fold enhancement of TRβ1 serine phosphorylation by T4 (lanes 1 and 2) and 97% inhibition of serine phosphorylation by PD 98059 (lanes 2 and 3). T4 Effect on TRβ1/MAPK Complex Formation Is PKA-mediated—Because a role for PKA in the serine phosphorylation of TRβ1 has been suggested (11, 12), we examined the effect of KT5720 and 8-Br-cAMP, respectively, an inhibitor and an agonist of PKA (24). In the study shown in Fig. 5A, T4 enhanced 293T cell nuclear uptake of TRβ1 complexed with MAPK 7.7-fold (lane 2). KT5720 inhibited T4-induced complexing of TRβ1 and MAPK (lanes 3 and 4, 33 and 61% inhibition, respectively). 8-Br-cAMP added to the effect of T4 (lane 8) and was itself agonistic (lane 5). The effect of 8-Br-cAMP was also partially blocked by KT5720 (lanes 6 and 7).

The PKA-mediated T4 Effect on Nuclear TRβ1/MAPK Complex Formation Depends on MEK Activity—To further study whether the effect of 8-Br-cAMP on nuclear complexing of TRβ1 and MAPK was direct via PKA or mediated through the MAPK pathway, we examined the effects of T4 and 8-Br-cAMP in 293T cells in the absence and presence of PD 98059. In the experiment shown in Fig. 5B, T4 enhanced complexing of TRβ1 and MAPK (lane 2), as did 8-Br-cAMP (lane 4). PD 98059 inhibited the effect of T4 by 65% (lane 3) and the effect of 8-Br-cAMP by 62% (lane 5), indicating that the action of PKA on nuclear TRβ1/MAPK association takes place at, or upstream of, MEK in the MAPK signal transduction pathway.

We then examined the effect of T4, 8-Br-cAMP, and PD 98059 on the appearance of TRβ1 in anti-phosphoserine immunoprecipitates. Fig. 5C demonstrates 15-fold nuclear accumulation of serine-phosphorylated TRβ1 in nuclei of T4-treated 293T cells (lane 2) and 6-fold accumulation with 8-Br-cAMP (lane 4). Inhibition of both T4 and 8-Br-cAMP effects by PD 98059 was evident (lanes 3 and 5, respectively). These studies suggest that PKA, whether activated by T4 or by 8-Br-cAMP, requires MEK action on MAPK to bring about serine phosphorylation of TRβ1.

T4-induced Nuclear Complexing of MAPK with Transfected TRβ1 and Mutants of TRβ1 in CV-1 Cells—Having established that T4 induces TRβ1/MAPK complexing and serine phosphorylation of the endogenous receptor in 293T cells, we studied CV-1 cells transfected with TRβ1 and found that T4 produced the same effects. Preliminary studies with two truncated mutants of TRβ1, rTR-ΔN (amino acids 94–461, containing both DBD and LBD) and rTR-ΔBD (amino acids 174–461, without DBD) showed that the DBD must be present for T4-induced complexing of MAPK and receptor to occur (results not shown).

Based on this finding, we developed a strategy to localize the site in the TR DBD of MAPK-receptor interaction. CV-1 cells were transfected with three hybrid constructs, substituting human GR sequences for corresponding segments of human TRβ1: T-TG-T, containing the second zinc finger of GR instead of that of TR; T-GT-T, with the first zinc finger of GR replacing that of TR; and TGT, which contains the entire DBD of GR in
and immunoblotting was reversed. Similarly, 8-Br-cAMP (lane 4, 1–3 of Fig. 4) enhanced serine phosphorylation (8–5) of TR,

the immunoprecipitates then immunoblotted with anti-TR

PD 98059 (11% inhibition compared with lane 1–3). The findings in lanes 1–3 were also inhibited by PD 98059 (15% inhibition, lane 2).

enhanced serine phosphorylation of TR (lane 2, 14% inhibition compared with lane 4, n = 3). C, in 293T cell samples immunoprecipitated with anti-phosphoserine and the immunoprecipitates then immunoblotted with anti-TRβ1, T4 enhanced serine phosphorylation of TRβ1 (lane 2, 14 ± 3-fold increase, n = 3), an effect inhibited by PD 98059 (lane 3, 62 ± 4% inhibition, n = 3). The findings in lanes 1–3 of this study were similar to those in lanes 1–3 of Fig. 4B, even though the antibody order for immunoprecipitation and immunoblotting was reversed. Similarly, 8-Br-cAMP (lane 4) stimulated receptor serine phosphorylation (8–4-fold, n = 3), which was also inhibited by PD 98059 (lane 5, 65 ± 15% inhibition, n = 3). Shown at right is a sample of 293T cell nuclear extract immunoblotted with anti-TRβ1, without immunoprecipitation.

FIG. 5. Contribution of PKA activity to T4-induced formation of nuclear TRβ1-MAPK complexes in 293T cells. A, T4 caused association of TRβ1 and MAPK (lane 2, 8.8 ± 2.5-fold in three experiments) in 293T cells, an effect inhibited by KT5720 (lanes 3 and 4; KT5720 (KT) 1 or 100 mM, 61 ± 16% and 78 ± 12% inhibition, respectively, n = 3). 8-Bromo-cAMP (8-Br, 1 mM) also enhanced TRβ1-MAPK association (lane 5; 9.3-fold in the figure, 7.5 ± 3.9-fold, n = 3), an effect reduced by KT5720 (lanes 6 and 7; 32 and 37% inhibition, respectively, n = 3). The effects of T4 and 8-Br-cAMP are additive (lane 8).

Effect of Activated MAPK on Serine Phosphorylation of Recombinant TRβ1—Kato et al. (21) have demonstrated that activated MAPK can cause serine phosphorylation of the estrogen receptor in vitro. To determine whether MAPK can directly serine phosphorylate TRβ1, in vitro studies of thyroid hormone receptor phosphorylation were performed with activated MAPK and TRβ1. Recombinant TRβ1 (amino acids 102–461, 5 μg), was further purified by immunoprecipitation with the C3 antibody and then incubated for 30 min at 30 °C with activated MAPK (5 units/μl), and 20 μM ATP including 0.05 μCi of [γ-32P]ATP in the buffer medium described by Kato et al. (21). A control sample contained 1.0 μg of MBP and activated MAPK. Phosphorylation of proteins was evaluated by PAGE and radiography, and results are shown in Fig. 7. Phosphorylation of MBP by activated MAPK is seen in lane 1. In the lane 2 sample MAPK was absent, and no phosphorylation of recombinant TRβ1 is seen. The lane 3 sample contained activated MAPK and immunoprecipitated recombinant TRβ1, and a 43-kDa labeled band is seen, consistent with the size of the TRβ1 fragment. A sample of immunoprecipitated endogenous TRβ1 from 293T cells was also exposed to activated MAPK and MAPK substrates (25). Anti-phosphothreonine immunoblots of TR immunoprecipitates revealed no receptor threonine phosphorylation (not shown). These results support an essential role for the second zinc finger of TRβ1 in T4-induced receptor complexing with activated MAPK and suggest that serine 142 is the phosphorylation site.
radiolabeled ATP, and in lane 4 a band is seen at a molecular mass of 56 kDa, consistent with that of intact TRβ1.

Effect of T₄-induced Nuclear Complexing of Activated MAPK and TRβ1 on Binding of SMRT to TRβ1—293T cells were treated with T₄ and/or tetrac (10⁻⁷ m) for 30 min, and selected samples were pretreated for 1 h with 30 μM PD 98059 or 10 μM geldanamycin. The latter causes depletion of cellular Raf-1, a serine/threonine kinase responsible for activation of MEK (1). Shown in Fig. 8A is the presence of SMRT in the TRβ1 immunoprecipitate of control cells (lane 1) and a marked reduction in SMRT binding to TRβ1 when cells were treated with T₄ (lane 2). Geldanamycin and PD 98059 both inhibited the T₄ effect on SMRT binding to TRβ1 (lanes 3 and 4, respectively). Tetrac alone had no effect on SMRT binding to TRβ1 (lane 5) but completely blocked T₄-induced displacement of SMRT from the receptor (lane 6). From prior studies we know that tetrac inhibits T₄ binding to plasma membranes (26) and blocks T₄-induced activation of MAPK (1), STAT1α (3), and STAT3.² In additional studies, T₄-agarose (10⁻⁷ m T₄) inhibited SMRT binding to TRβ1 by 50% (not shown). These findings support a role for T₄ in the release of SMRT from TRβ1 in a manner that is dependent on 1) an intact MAPK pathway and 2) tetrac-inhibitable binding of T₄ to a plasma membrane receptor.

The effect of T₄ on SMRT binding to TRβ1 was compared with that of T₃. In four experiments, levels of SMRT complexed with TRβ1 were reduced by 38 ± 9 and 80 ± 6% of control levels in cells treated with 10⁻⁸ and 10⁻⁷ m T₄, respectively, and the SMRT levels were reduced by 49 ± 18 and 70 ± 9% of control in cells treated with T₃ (10⁻¹⁰ and 10⁻⁷ m, respectively). A representative blot is shown in Fig. 8B. Similar findings were obtained in studies with NCoR antibody (not shown). Thus, a physiologic concentration of T₄ (10⁻⁷ m) is more effective than a physiologic concentration of T₃ (10⁻¹⁰ m) in displacing SMRT from endogenous TRβ1 in 293T cells.

**DISCUSSION**

A number of hormones have recently been reported to influence activity of kinase cascades, including the MAPK pathway. Activation of steps in this pathway by gonadotropin-releasing hormone (27), norepinephrine (28), insulin (29), estradiol (21), ²H.-Y. Lin, unpublished observations.
To activate MAPK and then identified TRβ1 in that precipitate.

The structure-activity relationships of the iodothyronine analogues studied in this MAPK-TR model were identical to those we have previously reported in the nongenomic activation by thyroid hormone of MAPK and its association with STAT proteins (1, 2). T-T4 was more active at physiologic concentrations than 1-T3, and T4-agarose was as active as T4. The fact that deamminated analogues (tetrac and triac) were not thyroid hormone agonists but were capable of blocking the action of T4 is consistent with the existence of a cell surface receptor for thyroid hormone that we have previously described (26, 35, 36), which is pertussis toxin- and GTP·S-sensitive (37). We have previously demonstrated that tetrac and triac block T3 potentiation of the antiviral (38) and immunomodulatory (3) actions of interferon-γ, even though these analogues themselves have no effect on interferon-γ action.

We have detected TRβ1 in cytosol of 293T cells (Fig. 2B). TRβ1 is generally regarded to be restricted to the nucleus but has been reported recently by Zhu et al. (22) to be present in cytosol. In our studies of 293T cells, we found TRβ1 in cytosol in much lower concentrations than those in corresponding nuclear fractions. The role of cytosolic TRβ1 is not clear; it may be nascent in an inactivated state or may be undergoing degradation. Although nuclear estrogen receptor and plasma membrane estrogen receptor apparently represent transcripts of the same gene (39), the TRβ1 detected in cytosol in our studies is not currently thought by us to represent trafficking of TRβ1 to the cell surface. We base this conclusion on the structure-activity relationships of iodothyronine analogues in their actions at the cell surface (1, 3, 26), compared with those at the nuclear TR (40, 41).

In the present experiments, we showed that MEK activity was required to cause nuclear translocation of phosphorylated MAPK and association of MAPK with TRβ1 in cell nuclei. PD 98059, an inhibitor of MEK, prevented activation of MAPK and the nuclear association of phosphorylated MAPK and TRβ1 and inhibited T4-induced serine phosphorylation of TRβ1. Thus, the MEK-MAPK cascade that has been implicated in serine phosphorylation of estrogen receptor (21, 30) is also operative in T4-treated cells in which TRβ1 is serine-phosphorylated. In studies not presented here, we have found that T4 can also promote the nuclear association of estrogen receptor and MAPK.

The fact that TRβ1 and MAPK were found to be associated in the nucleus did not prove that the receptor was a substrate for MAPK. However, using a phosphoserine antibody we documented that endogenous nuclear TRβ1 complexed with MAPK was serine-phosphorylated in T4-treated cells and that this serine phosphorylation was inhibited by PD 98059. In contrast, we found no evidence for threonine phosphorylation of TR.

Studies with two truncated mutants of TRβ1 suggested that to demonstrate T4-induced co-immunoprecipitation of MAPK and the receptor, the DBD must be present. With the use of hybrid constructs of TRβ1 and GR in which the zinc fingers of the DBDs were exchanged, we identified the second zinc finger of the TR DBD as a necessary participant in MAPK action. In cells with either this portion of TR or the entire TR DBD replaced with that of GR, T4 treatment did not result in co-immunoprecipitation of TR and MAPK or serine phosphorylation of the TR/GR hybrid. The second zinc finger of the DBD has also been found to be important for homodimerization of TR and transcriptional activation (42).

We also demonstrated that constitutively activated MAPK phosphorylates purified recombinant human TRβ1 in vitro. The receptor fragment we used (residues 102–461) contains 2 serines in the DBD and 14 in the LBD. From our studies described above, we concluded that MAPK binds to the DBD, and serine phosphorylates the DBD. None of the serines in TR are in environments that qualify as optimum consensus phosphorylation sites, namely, PX(S/T/P), where X is a neutral or basic amino acid and n = 1 or 2, or as the minimum sequence site (S/T/P); Ref. 21). However, MAPK substrates may lack consensus sequences, and the enzyme may phosphorylate a PS sequence (25) similar to that which occurs in the TR DBD at residues 141–142. Another PS sequence occurs at amino acids 98–99 of TR, but this segment was not present in the recombinant TRβ1 used in our studies.

It is the hinge region of TR (amino acids 211–240), located in the amino-terminal portion of the LBD, that binds the corepressor proteins SMRT and NCoR (9), which contribute to basal repression of transcriptional activation. Our studies demonstrate that T4, alone, in the absence of T3, is effective in displacing SMRT and NCoR from the receptor. This action of T4 has characteristics similar to T3-induced activation of MAPK and TRβ1/MAPK complexing, including dependence on MEK activity, effectiveness of T4-agarose (1), and inhibition by tetrac and triac (1). We therefore postulate that T4 treatment causes serine phosphorylation in the DBD of TRβ1, leading to allosteric changes in the proximal region of the LBD resulting in dissociation of SMRT from TRβ1. Such a change in the state of the receptor would result, even in the absence of T3 and binding of T4 by TRβ1, in derepression of the receptor without ligand-induced transcriptional activation. That this may be the case has recently been shown in a preliminary study, where T4, in the absence of T3 caused return of TRβ1 from a state of transcriptional repression to the basal state but did not cause transcriptional activation of the receptor. We have previously shown that thyroid hormone can potentiate the action of interferon-γ by a signal transduction pathway that involves both protein kinase C and PKA (24). We therefore studied the possibility that T4 might also promote the serine phosphorylation of TRβ1 by a PKA-dependent mechanism and found that the MEK-MAPK pathway by which T4 acts to phosphorylate TRβ1 is indeed subject to activation by 8-Br-cAMP and inhibition by KT5720. Our observations suggest that the involvement of PKA in TRβ1 phosphorylation reported by others depends upon MAPK activation and that T4-stimulated PKA contributes to activation and nuclear location of MAPK.

REFERENCES
1. Lin, H.-Y., Davis, F. B., Gordiner, J. K., Martino, L. J., and Davis, P. J. (1999) Am. J. Physiol. 276, C1014–C1024
2. Lin, H.-Y., Shih, A., Davis, F. B., and Davis, P. J. (1999) Biochem. J. 338, 427–432
3. Lin, H.-Y., Martino, L. J., Wilcox, B. D., Davis, F. B., Gordiner, J. K., and Davis, P. J. (1998) J. Immunol. 161, 843–849
4. Selmi, S., and Samuels, H. H. (1993) J. Biol. Chem. 268, 11589–11593
5. Yen, P., Sugawara, A., and Chin, W. (1992) J. Biol. Chem. 267, 23244–23252
6. Lazar, M. A. (1993) Endocr. Rev. 14, 184–193
7. Chin, W. W., and Yen, P. M. (1997) in Contemporary Endocrinology: Diseases of the Thyroid (Braverman, L. E., ed) pp. 17–34, Humana Press, Totowa, NJ
8. Tagami, T., Gu, W. X., Pears, P. T., West, B. L., and Jameson, J. L. (1998) Mol. Endocrinol. 12, 1888–1902
9. Safer, D. J., Cohen, R. N., Hollenberg, A. N., and Wondisford, F. E. (1998) J. Biol. Chem. 273, 30175–30182
10. Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (1997)Curr. Opin. Cell Biol. 9, 222–232
11. Tzagarakis-Foster, C., and Privalsky, M. L. (1998) J. Biol. Chem. 273, 10926–10932
12. Leitman, D. C., Costa, C. H. R. M., Graf, H., Baxter, J. D., and Ribiero, R. C. J. (1998) J. Biol. Chem. 273, 21950–21955
13. Sugawara, A., Yen, P. M., Apriellti, J. W., Ribiero, R. C. J., Sacks, D. B., Baxter, J. D., and Chin, W. W. (1994) J. Biol. Chem. 269, 433–437
14. Ting, Y.-T., Bhat, M. K., Wong, R., and Cheng, S.-y. (1997) J. Biol. Chem. 272, 4129–4134
15. Ting, Y.-T., and Cheng, S.-y. (1997) Thyroid 7, 463–469
16. Jones, K. E., Brubaker, J. H., and Chin, W. W. (1994) Endocrinology 134, 543–548
17. Katz, D., Reginato, M. J., and Lazar, M. A. (1995) Mol. Cell. Biol. 15, 2341–2348
18. Samuels, H. H., Stanley, F., and Casanova, J. E. (1979) Endocrinology 105, 2195–2200
80–85
19. Weinstein, S. P., Watts, J., Graves, P. N., and Haber, R. S. (1990) *Endocrinology* **126**, 1421–1429
20. Wen, Z., Zhong, Z., and Darnell, J. E., Jr. (1995) *Cell* **82**, 241–250
21. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995) *Science* **270**, 1491–1494
22. Zhu, X.-G., Hanover, J. A., Hager, G. L., and Cheng, S.-y. (1998) *J. Biol. Chem.* **273**, 27058–27063
23. Cobb, M. H., Goldsmith, E. J. (1995) *J. Biol. Chem.* **270**, 14843–14846
24. Lin, H.-Y., Yen, P. M., Davis, F. B., and Davis, P. J. (1997) *Am. J. Physiol.* **273**, C1225–C1232
25. Yang, X., and Gabunza, D. (1998) *J. Biol. Chem.* **273**, 29879–29887
26. Davis, P. J., Davis, F. B., and Blas, S. D. (1982) *Life Sci.* **30**, 675–682
27. Han, X.-B., and Conn, P. M. (1999) *Endocrinology* **140**, 2241–2251
28. Yamazaki, T., Kimura, I., Zou, Y., Kudoh, S., Shiojima, I., Hirai, Y., Mizuno, T., Aikawa, R., Takano, H., and Yaraki, Y. (1997) *Circulation* **95**, 1260–1268
29. Ceresa, B. P., Horvath, C. M., and Pessin, J. E. (1997) *Endocrinology* **138**, 4131–4137
30. castoria, G., Barone, M. V., Di Domenico, M., Bilancio, A., Ametrano, D., Migliaccio, A., and Auricchio, F. (1999) *EMBO J.* **18**, 2500–2510
31. Krstic, M. D., Rogatsky, I., Yamamoto, K. R., and Garubedian, M. J. (1997) *Mol. Cell Biol.* **17**, 3947–3954
32. Denner, L. A., Weigel, N. L., Maxwell, B. L., Schrader, W. T., and O’Malley, B. W. (1990) *Science* **250**, 1740–1743
33. Lin, K.-H., Ashizawa, K., and Cheng, S.-y. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 7737–7741
34. Bhat, M. K., Ashizawa, K., and Cheng, S.-y. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 7927–7931
35. Smith, T. J., Davis, F. B., and Davis, P. J. (1992) *Biochem. J.* **284**, 583–587
36. Davis, F. B., Moffett, M. J., Davis, P. J., Al Ogaily, M. S., and Blas, S. D. (1993) *J. Clin. Endocrinol. Metab.* **77**, 1427–1430
37. Davis, F. B., Davis, P. J., Blas, S. D., and Gombas, D. Z. (1995) *Metabolism* **44**, 865–868
38. Lin, H.-Y., Thacore, H. R., Davis, F. B., and Davis, P. J. (1996) *J. Cell. Physiol.* **167**, 269–276
39. Razandi, M., Pedram, A., Greene, G. L., and Levin, E. R. (1999) *Mol. Endocrinol.* **13**, 307–319
40. Schueler, P. A., Schwartz, H. L., Strait, K. A., Mariash, C. N., and Oppenheimer, J. H. (1990) *Mol. Endocrinol.* **4**, 227–234
41. Oppenheimer, J. H., Schwartz, H. L., Dimna, W., and Surks, M. I. (1973) *Biochem. Biophys. Res. Commun.* **55**, 544–550
42. Nagaya, T., Kopp, P., Kitajima, K., Jameson, J. L., and Seo, H. (1996) *Biochem. Biophys. Res. Commun.* **222**, 524–539
