Identification of the Putative MAP Kinase Docking Site in the Thyroid Hormone Receptor-β1 DNA-Binding Domain: Functional Consequences of Mutations at the Docking Site†

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ABSTRACT: In CV-1 cells transfected with wild-type (wt) nuclear thyroid hormone receptor TRβ1 (TR), L-thyroxine (T_4) causes activation and nuclear translocation of mitogen-activated protein kinase (MAPK, ERK1/2), co-immunoprecipitation of MAPK and TR, and MAPK-dependent serine phosphorylation of TR. In the present studies, we have identified (1) the likely site of TR serine phosphorylation in the TR DNA-binding domain (DBD) by T_4-activated MAPK, (2) the site of MAPK docking on TR induced by T_4, and (3) functional consequences of TR docking site and serine phosphorylation site mutations on co-repressor and co-activator binding and on transcriptional activation by wt and mutant receptors in T_4-treated cells. Plasmids containing TR_{wt}, serine 142-substituted TR (TR_{S142A} or TR_{S142E}), TR_{K128A}, TR_{R132A}, or TR_{R133A} were transfected into CV-1 cells, and the cells were treated with 10^{-7} M T_4 for 30 min. Activated MAPK was present in nuclear fractions of all T_4-treated cells and co-immunoprecipitated prominently with TR_{wt}, TR_{S142A}, and TR_{S142E}. TR_{K128A} complexing with activated MAPK was minimally detectable, but no association of MAPK with TR_{R132A} or TR_{R133A} was seen in cells treated with T_4. Serine phosphorylation of TR_{wt}, but not of any mutants, occurred with T_4. In in vitro phosphorylation studies, constitutively activated MAPK phosphorylated only TR_{wt}. We concluded that serine 142 of the TR DBD is the likely site of phosphorylation by T_4-activated MAPK and that the docking site on TR for activated MAPK includes residues 128−133 (KGFFRR), a basic amino acid-enriched motif novel for MAPK substrates. TR mutations in the proposed MAPK docking domain and at residue 142 modulated T_4-conditioned shedding of co-repressor and recruitment of co-activator proteins by the receptor, and they altered transcriptional activity of TR in a thyroid hormone response element−luciferase reporter assay.

Thyroid hormone treatment of cells rapidly activates the mitogen-activated protein kinase (MAPK)[1] signal transduction pathway in several cell lines (*1*−*3*). In physiologic concentrations, L-thyroxine (T_4) is more effective than 3,5,3′-triiodo-L-thyronine (T_3), and T_4-agarose imitates the effect of T_4, indicating that the hormone effect is initiated at the cell membrane (*1*). T_4-activated MAPK (ERK1/2) translocates from cytoplasm to the cell nucleus in less than 15 min, with a maximal effect at 30 min, and forms transient immunoprecipitable complexes with a variety of nucleoproteins which are no longer evident in 1 h. This complex formation is associated with serine phosphorylation of MAPK substrates, including p53 (*3*), signal transducer and activator of transcription (STAT)-1α (*1*), and the nuclear thyroid hormone receptor (TRβ1) (*2*).

We have recently reported that serine phosphorylation of TRβ1 by T_4-activated MAPK results in dissociation in the cell nucleus of TR and silencing mediator of retinoid and thyroid hormone receptor (SMRT) (*2*), a co-repressor protein. The dissociation of TR and SMRT is thought to condition de-repression of transcriptional activity of TR, whereas recruitment of co-activator proteins to TR is associated with activation of transcription by TR (*4*). T_4-activated MAPK may also alter transcriptional activity of other nucleoproteins, such as p53 (*3*) and STAT1α (*1*, *5*).

In a previous study of the effect of T_4 on nuclear co-immunoprecipitation of MAPK with TR (*2*), we began to explore possible docking sites on the receptor with which activated MAPK might bind. We used TR constructs in

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[1] Abbreviations: T_4, L-thyroxine; T_3, 3,5,3′-triiodo-L-thyronine; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinases 1/2; TRβ1 or TR, thyroid hormone receptor β1; DBD, DNA-binding domain; STAT1α, signal transducer and activator of transcription 1α; SMRT, silencing mediator of retinoid and thyroid hormone receptor; NCoR, nuclear corepressor; ER, estrogen receptor; GR, glucocorticoid receptor; PR, progesterone receptor; PAGE, polyacrylamide gel electrophoresis; PTU, 6-*n*-propyl-2-thiouracil; ipodate, 3-(3-amino-2,4,6-triiodophenyl)-2-ethylpropanoic acid; DMEM, Dulbecco's modified essential medium.

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which the first or second zinc fingers of the DNA-binding domain (DBD) of the nuclear glucocorticoid receptor (GR) were substituted for the corresponding segments of TR (*6*). T_4-induced complexing of TR and MAPK and serine phosphorylation of TR did not occur in mutants containing the second portion of the DNA-binding domain of GR in place of that of TR. This finding suggested that the site of TR docking with MAPK was in the second zinc finger of the receptor. Examination of this sequence on TR reveals a basic amino acid-enriched sequence, KGFFRR (residues 128−133), which includes certain characteristics of preferred MAPK docking sites (*7*, *8*).

We also proposed that a likely site of TR serine phosphorylation was residue 142, as this serine is preceded by a proline, forming a PS sequence which has been reported as an ERK substrate (*9*). In the current study, we show evidence that serine 142 is the DBD site of MAPK phosphorylation of TR. In addition, we identify an amino acid sequence on TR for co-immunoprecipitation, or docking, with T_4-activated MAPK that is relevant to phosphorylation of serine 142, to the dissociation of the TR−SMRT complex, to association of TR with the co-activator p300, and to transcriptional activity of cellular TR in response to exposure of cells to T_4.

EXPERIMENTAL PROCEDURES

Materials. L-T_4, 3,5,3′-triiodo-L-thyronine (T_3), agarose-T_4, 6-*n*-propyl-2-thiouracil (PTU), and 3-(3-amino-2,4,6-triiodophenyl)-2-ethyl-propanoic acid (ipodate) were obtained from Sigma Chemical Co., St. Louis, MO. Stock solutions of T_4 or T_3 were prepared in 0.04 N KOH with 4% propylene glycol, and dilutions were made to final concentrations as indicated. In experiments in which T_4 was added to cultured cells in serum-free medium, the total and free T_4 concentrations were 10^{-7} and 0.7×10^{-10} M, respectively (*3*). There was no measurable T_3 in the hormone-depleted serum-supplemented medium to which T_4 was added (*2*).

Cell Culture and Transfection of TRβ1$_{wt}$ and Amino Acid-Substituted TRs for Signal Transduction Studies. CV-1 cells were maintained and grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum. The TRβ1 DNA probe is available in our laboratory (*2*). Mutations within TRβ1 receptor-encoding sequences were created using the quick-change kit (Stratagene, La Jolla, CA), and probes were verified by DNA sequencing. Mutagenic oligonucleotides were supplied by Invitrogen (Carlsbad, CA). The wt TRβ1 (TR_{wt}) and the mutants TR_{S142A}, TR_{S142E}, TR_{K128A}, TR_{R132A}, and TR_{R133A} were transformed into competent *Escherichia coli* and plated on ampicillin-treated agar plates. Single colonies were chosen and grown overnight in Circle Grow culture broth (Bio101, Carlsbad, CA) containing ampicillin (10 mg/ml). Plasmid DNA was isolated and purified by a modified alkaline lysis protocol, and for signal transduction studies, probes in pcDNA were transfected into CV-1 cells with LipofectAMINE Plus, as we have previously described (*2*). Mutations at residues 128, 132, and 133 were intended to explore the putative MAPK docking site. Mutations at residue 142 either defined the importance of a serine subject to phosphorylation (S142A), or created a charge at this site (S142E) similar to that of a phosphoserine.

Cell Treatment and Preparation of Nuclear Fractions. CV-1 cells were maintained after transfection in DMEM supplemented with 10% fetal bovine serum for 24 h, then for 2 days in medium with 0.25% serum previously depleted of thyroid hormone by the method of Samuels et al. (*10*), as modified by Weinstein et al. (*11*), and in serum-free medium for 2 h. Cells were then treated with T_4 for 30 min at 37 °C, harvested, and washed twice in ice-cold phosphate-buffered saline. For preparation of nuclear fractions, the cells were then lysed in hypotonic buffer containing 20 mM HEPES buffer (pH 7.9), 10 mM KCl, 0.1 mM Na_3VO_4, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 3 μg/mL aprotinin, 1 mg/mL pepstatin, 20 mM NaF, and 1 mM dithiothreitol with 0.2% NP-40, and placed on ice for 10 min (*1*). After centrifugation at 4 °C and 13,000 rpm for 1 min, supernatants were collected as cytoplasmic extracts. The precipitates containing crude nuclei were resuspended in high-salt buffer (hypotonic buffer with 20% glycerol and 420 mM NaCl) at 4 °C with rocking for 30 min. After subsequent centrifugation at 4 °C and 13 000 rpm for 10 min, the supernatants containing nucleoproteins were collected. In selected studies, cells were exposed to DMEM containing PTU (1 mM) or ipodate (100 μM) in medium without serum and the cells incubated for 16 h prior to treatment with T_4 or diluent, to eliminate the possibility of cellular conversion of T_4 to T_3.

Immunoprecipitation and Immunoblotting. After normalization of nuclear protein content, immunoprecipitation was performed using a monoclonal antibody to the amino-terminal half of the AB domain of TRβ1 (Santa Cruz, Santa Cruz, CA), to MAPK (ERK2, Transduction Laboratories, Lexington, KY), or with a polyclonal antibody to serine-phosphorylated proteins (Research Diagnostics, Flanders, NJ) or to activated MAPK (pERK1/2) (New England BioLabs, Beverly, MA). Aliquots of immunoprecipitates from samples in a given experiment were separated by discontinuous SDS−PAGE, and resulting proteins were electroblotted to Immobilon membranes (Millipore, Bedford, MA). Polyclonal antibodies to SMRT and p300 (Santa Cruz) or to activated ERK (pERK1/2) were used to detect those antigens in TR immunoprecipitates. Monoclonal antibody to the C-terminal amino acids 235−414 of TRβ1 (Affinity Bioreagents, Inc., Golden, CO) was used for immunoblots of TR immunoprecipitates. Immunoblots were visualized by enhanced luminescence (ECL, Amersham Life Sciences, Arlington Heights, IL) and illustrated by digital imaging (BioImage, Millipore). All results shown are representative of three or more studies.

In Vitro Phosphorylation of TRs by Activated MAPK. Equal amounts of nuclear extracts from CV-1 cells transfected with wt or mutant receptors were immunoprecipitated with anti-TRβ1 antibody. Aliquots of the immunoprecipitates were incubated for 30 min at 30 °C with 5 units of activated MAPK (New England BioLabs) in 50 mM Tris-HCl (pH 8.0) with 0.5 mM EDTA, 25 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, and 20 μM ATP, and 0.05 μCi [γ-^{32}P]-ATP (PerkinElmer Life Sciences), following the method of Kato et al. (*12*). After incubation, the proteins were solubilized, separated by PAGE, and radioautographed.

Transcriptional Activation Studies. CV-1 cells were grown in DMEM with 10% serum until 50−70% confluent. They were then diluted in 6-well plates, incubated overnight, and in the absence of serum or antibiotics transfected with the following: 0.5 μg of β-galactosidase DNA in a pSVL vector,

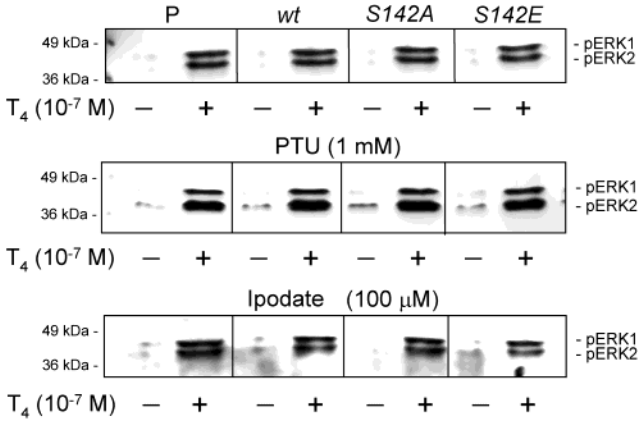

FIGURE 1: Effect of T_4 on nuclear uptake of activated MAPK in CV-1 cells transfected with TRβ1$_{wt}$ (wt) or TR serine 142 mutants (S142A, S142E). CV-1 cells were transfected with TR$_{wt}$, or S142A or S142E TR mutants, and then treated with T_4, 10^{-7} M, for 30 min. Selected experiments were performed after cell treatment with PTU (1 mM) or ipodate (100 μM), as described in Experimental Procedures. Nuclear proteins from each sample were immunoblotted with anti-phosphorylated MAPK (pERK1/2). All cells treated with T_4 (+), including cells which received no receptor (empty plasmid, P), showed increased nuclear pERK1/2 compared to similarly prepared cells without T_4 treatment (−). Neither PTU nor ipodate altered the T_4 effect.

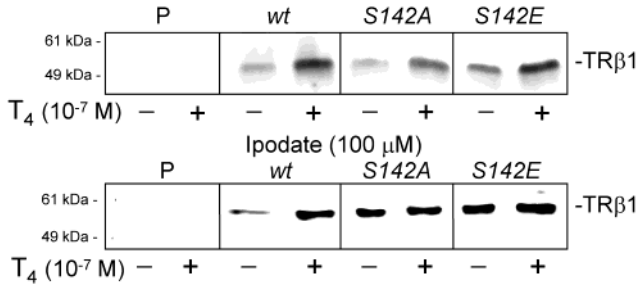

FIGURE 2: Effect of T_4 on nuclear uptake of TR in CV-1 cells transfected with TR$_{wt}$ (wt) or a serine 142-substituted mutant receptor, S142A or S142E. CV-1 cells were treated with T_4 (10^{-7} M) for 30 min as described in Figure 1. All cells transfected with wt or mutant receptor showed presence of some TR in nuclei of cells in the absence of T_4 (−). Cells which were not transfected with TR (P) showed no receptor. In TR$_{wt}$ cells treated with T_4 (+), nuclear receptor content was increased 2.3 ± 0.1-fold in three experiments. Cells transfected with S142A or S142E mutants showed a similar increase in nuclear TR content with T_4 treatment (2.1 ± 0.5- and 1.6 ± 0.1-fold increase, respectively). Pretreatment of cells with ipodate (100 μM) did not alter this T_4 effect (lower panel).

1.0 μg of reporter vector containing the thyroid hormone response element DR4 linked with a mouse TSH-β-subunit gene, the herpes simplex virus thymidine kinase promotor and luciferase cDNA, and 1.0 μg of TR$_{wt}$ or mutants in a CMX derivative containing a CMV promoter, using the LipofectAMINE Plus protocol as described above. Incubation was for 5 h at 37 °C, followed by addition of DMEM with 20% serum and an overnight incubation. Cells were then placed in DMEM plus 0.25% hormone-stripped serum, penicillin, streptomycin, and 1 mM PTU, and treated with L-T_4, 10^{-7} M, for 24 h. Luciferase and β-galactosidase activities from each cell sample were measured in triplicate with the Dual Light Assay System (Tropix, Bedford, MA), and luciferase activity was normalized to the level of β-galactosidase in the same sample. Results are presented normalized to a luciferase value of 1.0 in samples without receptor.

RESULTS

Thyroxine Causes Activation and Nuclear Translocation of Activated MAPK in CV-1 Cells in the Presence or Absence of TRβ1. CV-1 cells were transfected with TR$_{wt}$, or the mutants TR$_{S142A}$ or TR$_{S142E}$. The cells were then exposed to T_4, 10^{-7} M, for 30 min. The accumulation of activated MAPK in nuclear fractions of T_4-treated cells was determined by immunoblotting with antibody to tyrosine-threonine-phosphorylated MAPK isoforms ERK1 and ERK2 (pERK1/2). In Figure 1 it is clearly seen that activated MAPK was absent from nuclei of untreated cells, but that in cells treated with T_4 for 30 min, accumulation of nuclear activated MAPK occurred. As we have previously reported (*1*, *2*), this effect was independent of whether cells contained endogenous TR, as CV-1 cells which received plasmid (P) without TR also showed MAPK activation upon T_4 treatment. Also evident is that the presence of the serine 142-substituted mutant receptors did not alter the T_4 effect on activation and nuclear translocation of activated ERK1/2. Neither PTU nor ipodate altered this effect of T_4, indicating that conversion of T_4 to T_3 by these cells is not a consideration in these experiments.

Thyroxine Causes Nuclear Accumulation of TR in Cells Containing wt TR and in S142A and S142E Mutants. In CV-1 cells transfected with TR$_{wt}$, TR$_{S142A}$, or TR$_{S142E}$, TR was evident in nuclei in the absence of T_4 treatment (Figure 2), confirming the effectiveness of the transfections. T_4, 10^{-7} M, caused increased nuclear accumulation of wt, S142A, and S142E TR in CV-1 cells. Cell samples which received plasmid alone served as negative controls and showed absence of TR in nuclei of control and T_4-treated cells.

Effect of Thyroxine Treatment of CV-1 Cells Transfected with wt TR or TR S142 Mutants on Nuclear Co-Immunoprecipitation of Receptors with MAPK. CV-1 cells transfected with wt or S142 mutants were treated with T_4 or control solvent for 30 min, and nuclear proteins were immunoprecipitated with antibody to TRβ1. The immunoprecipitated proteins were separated by PAGE and immunoblotted with antibody to ERK2 or to pERK1/2. Co-immunoprecipitation of nuclear TR and ERK2 was absent in cells which were not exposed to T_4 (Figure 3A). On the other hand, the presence of ERK2 in TR immunoprecipitates of T_4-treated cells signifies nuclear complexing, or docking, of these two proteins in response to the hormone, as we have previously described (*2*). This effect of T_4 occurred in cells transfected with wt, S142A, and S142E probes, with or without pretreatment with PTU, 1 mM.

In additional experiments, nuclear proteins of T_4-treated and control cells were immunoprecipitated with anti-TRβ1, and the precipitates were analyzed for the presence of phosphorylated ERK1/2 (Figure 3B). Again, T_4 caused nuclear localization and complexing, or docking, of TRβ1 and phosphorylated ERK1/2 isoforms. The absence of a serine at residue 142 did not diminish co-immunoprecipitation of TR and ERK1/2 in T_4-treated cells. In the study shown, cells were pretreated with ipodate, and there was no effect of this 5′-deiodinase inhibitor on the action of T_4, again indicating that the T_4 effect is not mediated by conversion of T_4 to T_3.

T_4-Activated MAPK Serine-Phosphorylates TRβ1$_{wt}$, but Not TR with Mutations at Residue 142. CV-1 cells were

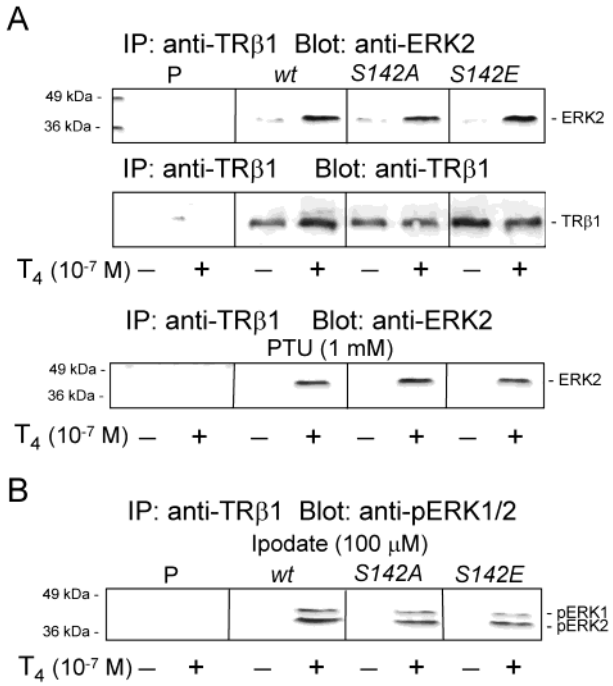

FIGURE 3: T_4 causes nuclear complexing of ERK1/2 with wt TR and with TR containing mutations at serine 142 in CV-1 cells. Cells were transfected with wt, S142A, or S142E constructs as in Figure 2, and then exposed for 30 min to T_4, 10^{-7} M, or control solvent. (A) Nuclear fractions of cells were immunoprecipitated with anti-TRβ1, and the precipitated proteins were separated by PAGE and immunoblotted with anti-ERK2. All cells transfected with wt or serine 142-substituted TR showed co-immunoprecipitation of ERK2 with the receptor in response to T_4 treatment (upper panel). As there was no TR in cells transfected with empty plasmid (P), no complexing of receptor and MAPK was seen in those cells. In the second panel, TRβ1 immunoblots of the TR immunoprecipitates are shown to indicate evidence of receptor protein in each sample. The presence of PTU, 1 mM, did not alter the results of these experiments (Figure 3A, lower panel). (B) Similar studies were carried out using anti-pERK1/2 antibody for immunoblotting to detect T_4-induced immunocomplexing of wt and serine 142 TR mutants with ERK1/2 in cells pretreated with ipodate. Again, T_4 induced co-immunoprecipitation of both wt TR and TR serine 142 mutants with ERK1/2. The T_4 effect was not altered in the presence of ipodate, another inhibitor of T_4 to T_3 conversion by 5′-deiodinase.

transfected with TR_{wt}, TR_{S142A}, or TR_{S142E} mutants and treated with 10^{-7} M T_4 for 30 min. Nuclear fractions were immunoprecipitated with antibody to serine-phosphorylated proteins, and the resulting immunoprecipitates separated and immunoblotted with anti-TRβ1. In Figure 4 it is evident that the only CV-1 cells showing serine-phosphorylated TR in response to T_4 treatment were the cells transfected with wt

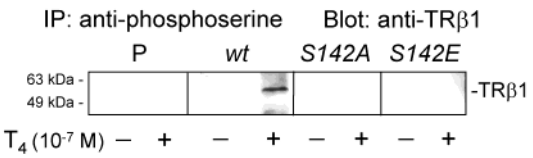

FIGURE 4: T_4 causes serine phosphorylation of TR at residue 142. CV-1 cells were transfected as described in Figures 1−3. Nuclear fractions of cells treated with T_4, 10^{-7} M, or control solvent for 30 min were immunoprecipitated with antibody to serine-phosphorylated proteins, and the immunoprecipitates examined for the presence of TR by immunoblot. Cells transfected with wt TR showed serine phosphorylation of the receptor in response to T_4, while the serine 142-substituted mutant receptors did not demonstrate serine phosphorylation.

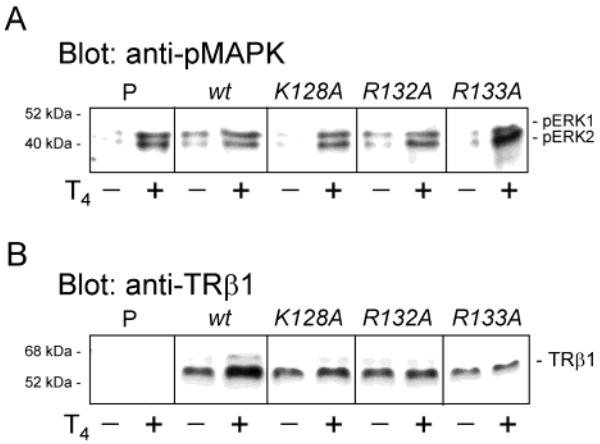

FIGURE 5: CV-1 cells transfected with TR_{wt} or TR mutants at residues 128, 132, or 133 (K128A, R132A, R133A) demonstrate nuclear accumulation of activated MAPK and TR_{wt}, but nuclear accumulation of the mutant receptors is impaired or absent in T_4-treated cells. (A) Nuclear accumulation of phosphorylated ERK1/2 was seen in all cells treated with 10^{-7} M T_4 for 30 min, regardless of the presence or absence of TR, or of the structure of TR. (B) An increase in nuclear TR was clearly seen in response to T_4 in TR_{wt}-transfected cells (1.9 ± 0.4-fold, $n = 3$ experiments), and to a lesser extent in TR_{K128A} cells (1.6 ± 0.2-fold, $n = 3$), while a similar increase was not evident in cells transfected with R132A or R133A mutant probes (1.2 ± 0.01- and 1.2 ± 0.1-fold, respectively, $n = 3$).

TRβ1. The serine 142-substituted mutant receptor proteins showed no evidence of serine phosphorylation at another site on the receptor.

Mutations at Residues 128, 132, and 133 of TRβ1 Prevent Both T_4-Induced Docking of MAPK and TRβ1 and Serine Phosphorylation of TRβ1. To define the docking site of MAPK on TRβ1, we substituted one of each of three basic amino acids in the TRβ1 DNA-binding domain (DBD) which contribute to a basic amino acid-enriched site (KGFFRR, residues 128−133) on the receptor. Translocation of activated ERK1/2 to CV-1 cell nuclei after T_4 treatment is seen in the presence of these mutant receptors as well as with wt TR and in cells without receptor (Figure 5A). An increase in nuclear TR_{wt} is evident in cells treated with T_4, but comparatively little T_4-induced increase is seen in cells with mutant receptors at residues 128, 132, or 133 (Figure 5B). This finding raised the possibility that reduced nuclear translocation of these mutant receptors in the presence of T_4 could be due to reduced docking of the receptors with T_4-activated MAPK.

A T_4-induced increase in nuclear TR_{wt} co-immunoprecipitated with ERK2 or pERK1/2 is seen in panels A (upper panel) and B of Figure 6, while in the R132A and R133A mutant TR-transfected cells, little or no increase in nuclear TRβ1, co-immunoprecipitated with ERK, is seen after T_4 treatment. In Figure 6B, the same findings are evident, even though the order of antibodies for immunoprecipitation and immunoblotting was reversed. In the TR_{K128A} cells, there is a small amount of co-immunoprecipitation of the mutant receptor with ERK proteins in Figure 6A,B. Nuclear accumulation of serine-phosphorylated TRβ1 in cells treated with T_4 is apparent only in TR_{wt}-transfected cells (Figure 6C). From these studies we conclude that the basic amino acids lysine and arginine in the sequence KGFFRR of TRβ1 must be present in the DNA-binding

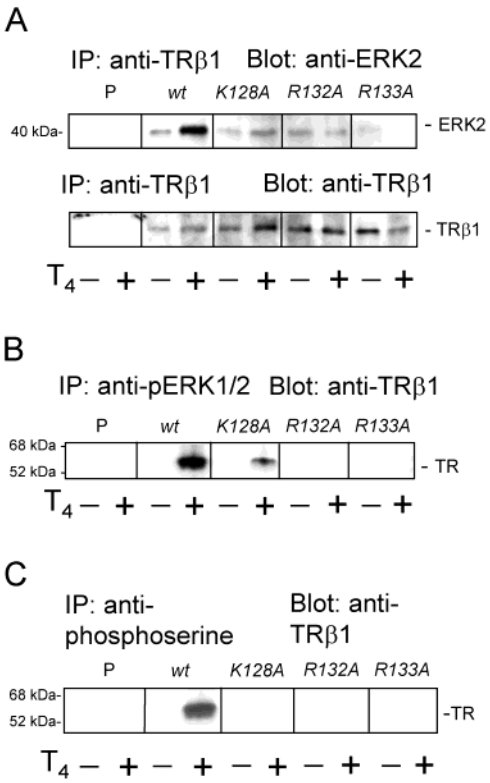

FIGURE 6: Mutation of TR at residues 128, 132, or 133 results in decreased docking of MAPK with TR in response to T_4. (A) CV-1 cells transfected with wt TR or mutants as in Figure 5 were treated with T_4, 10^{-7} M, or solvent, for 30 min. Nuclear fractions were immunoprecipitated with anti-TR, and resulting proteins separated by electrophoresis and immunoblotted with anti-ERK2. T_4 caused a clear increase in nuclear complex formation between wt TR and ERK2, shown in the upper panel. A similar increase was not evident in the cells transfected with the R132A or R133A mutants, and in the cells transfected with K128A, there was minimal nuclear complex formation with T_4 treatment. The lower panel shows a TRβ1 immunoblot of an aliquot of each immunoprecipitate to indicate the amount of immunoprecipitated TR added to each lane. (B) Cell nuclei from similar cell samples were immunoprecipitated with anti-phosphorylated MAPK, and the immunoprecipitates were separated by gel electrophoresis and immunoblotted with anti-TR. With this reversal of antibodies for immunoprecipitation and immunoblotting compared to (A), T_4-induced nuclear complex formation is again clearly seen only in the cells transfected with the wt receptor, and minimal complex formation is seen with the *K128A* mutants. (C) Using a similar set of transfected and T_4-treated cells, immunoprecipitates were made with anti-phosphoserine, and the resulting proteins were immunoblotted with anti-TR. The only transfected cells to show serine phosphorylation of TR in response to T_4 were those containing the wt receptor, even though the serine at residue 142 was present in these mutants.

domain for docking of ERK1/2 and TRβ1 to take place in cells treated with T_4. Furthermore, serine phosphorylation of TR does not occur in the absence of receptor docking with ERK1/2.

Effect of Activated MAPK in Vitro on Phosphorylation of TRβ1 and Associated Mutants in a Cell-Free System. We have previously demonstrated that commercially available activated MAPK causes phosphorylation of both TRβ1 (*2*) and p53 (*3*) in vitro in a cell-free system. Similar findings have been reported with estrogen receptor-α (ERα) in which the serine residue phosphorylated by MAPK has been identified (*12*). TRβ1 immunoprecipitates of CV-1 cells transfected with TR_{wt}, TR_{S142A}, and TR_{S142E} were incubated in an in vitro phosphorylation assay with activated MAPK and [γ-^{32}P]-ATP. The wt receptor was clearly phosphorylated in vitro by MAPK (Figure 7A), while neither of the serine 142-substituted receptors were labeled. These findings further point to serine 142 of TRβ1 as the site of phosphorylation by MAPK (ERK1/2).

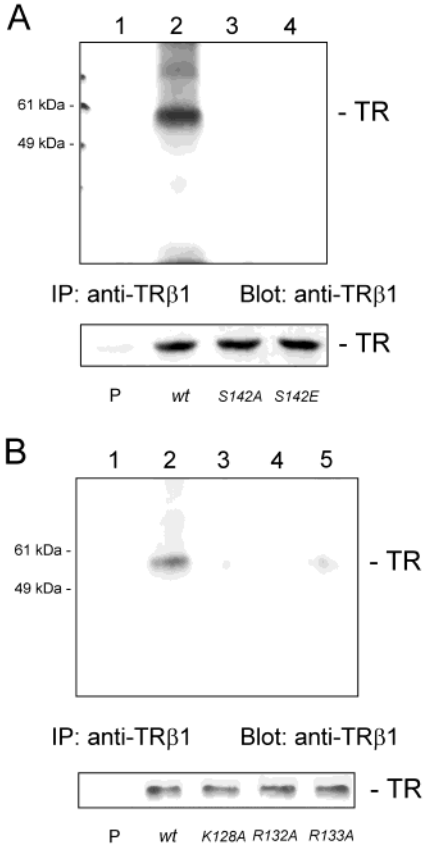

FIGURE 7: In vitro phosphorylation studies confirm that activated MAPK requires K128, R132, R133, and S142 of TRβ1 for phosphorylation of the receptor. (A) TRβ1 immunoprecipitates of CV-1 cells transfected with TR_{wt}, TR_{S142A}, and TR_{S142E} were exposed to commercially available activated MAPK in the presence of [γ-^{32}P]-ATP for 30 min, and the proteins were separated by PAGE. Activated MAPK caused in vitro phosphorylation of a TR immunoprecipitate from TR_{wt}-transfected CV-1 cells (wt, lane 2) but not of immunoprecipitates from cells transfected with S142A or S142E mutants (lanes 3 and 4), indicating that serine 142 is the likely site of phosphorylation by activated MAPK. The lower panel shows a TRβ1 immunoblot of similar aliquots from each TR immunoprecipitate. (B) In an in vitro phosphorylation study similar to that shown in panel A above, activated MAPK caused in vitro phosphorylation of a TR_{wt} immunoprecipitate (lane 2), but no phosphorylation of immunoprecipitates of TR mutants at residues 128, 132, or 133, even though serine 142 was present on these receptors. This finding provides further evidence that an intact basic amino acid sequence at residues 128−133 is required for docking of MAPK on TR. The lower panel indicates an immunoblot of similar aliquots from each immunoprecipitate.

Immunoprecipitates of nuclear TR_{wt} and TR_{K128A}, TR_{R132A}, and TR_{R133A} mutants were studied in a similar manner (Figure 7B). None of the mutant proteins were labeled by MAPK in vitro, even though serine 142 was present. This finding again supports the role of the KGFFRR sequence as a docking site for ERK1/2 on wt TRβ1.

Effect of T_4 on Binding of the Co-Activator Protein p300 and the Co-Repressor SMRT by wt and Mutant Receptors. In the presence of T_4, the co-activator p300 co-immunoprecipitated with wt TR, in contrast to the result obtained in the absence of T_4 (Figure 8, upper panel). This finding also

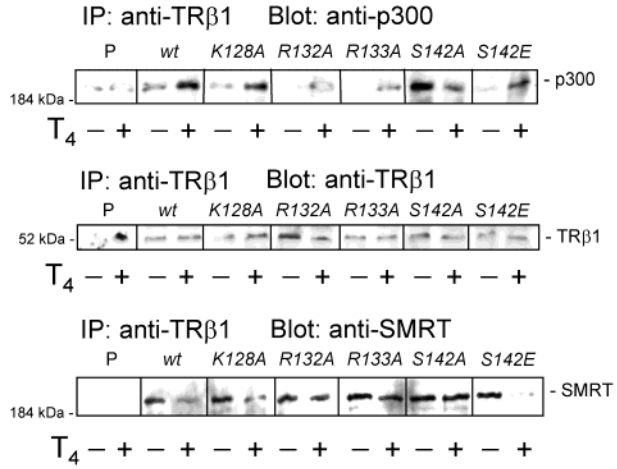

FIGURE 8: Effect of T_4 on interactions of p300 and SMRT with TR_{wt} and mutants. The co-immunoprecipitation of wt TR and mutants with the co-activator p300, or with the co-repressor SMRT, were studied after treatment with 10^{-7} M L-T_4 or solvent for 30 min. TR_{wt}, TR_{K128A}, and TR_{S142E} cells showed little p300 bound to the receptor in the absence of T_4, and a marked increase in binding of p300 to the receptor with T_4 treatment. In contrast, the R132A and R133A mutants did not bind p300 either with or without T_4 treatment, and the S142A mutant cells bound p300 more in the absence of T_4 than with hormone present. In the center panel are immunoblots of TRβ1 in aliquots of each immunoprecipitate used for the studies above and below. Shown in the third panel, SMRT was displaced from wt, K128A, and S142E receptors with T_4 treatment. The R132A and R133A mutants, however, bound SMRT in the absence or presence of T_4, as did the S142A cells.

occurred with the mutants TR_{K128A} and TR_{S142E}. In contrast, TR_{S142A} co-immunoprecipitated with p300 in the absence of T_4, and T_4 treatment of cells transfected with TR_{S142A} resulted in shedding of co-activator, rather than further recruitment. The R132A and R133A mutants did not bind p300 either in the presence or absence of T_4.

The co-repressor SMRT was bound to TR_{wt} in untreated CV-1 cells, while in T_4-treated cells SMRT was no longer bound to the receptor (Figure 8, lower panel). The same findings were observed in the TR_{K128A} and TR_{S142E} mutants. In the TR_{R132A}, TR_{R133A}, and TR_{S142A} mutants, however, there was little loss of SMRT from the receptor in T_4-treated cells.

Transcriptional Activation by WT and Mutant TRβ1 in Response to T_4. CV-1 cells were transfected with wt and mutant receptors, then treated with T_4, 10^{-7} M, for 24 h in the presence of 1 mM PTU to prevent conversion of T_4 to T_3. Results of luciferase activity studies, corrected for transfectional efficiency, are shown in Figure 9. Cells without TR showed no transcription, while cells with TR_{wt} showed repression of transcription in the absence of T_4 (Figure 9A,B). This latter finding is mediated by the binding of co-repressors to the receptor in the absence of thyroid hormone, as seen in Figure 8 (*13*). With T_4, 10^{-7} M, there was a consistent loss of repression and a 2-fold increase in luciferase activity in TR_{wt} cells. T_4-agarose (10^{-7} M) produced the same effect (results not shown), providing evidence that a T_4-mediated effect at the plasma membrane can stimulate transcriptional activity by wt TRβ1. CV-1 cells transfected with TR_{S142A} showed enhanced luciferase activity in the absence of T_4 (Figure 9A,B) consistent with co-activator binding (Figure 8). With T_4 treatment there was no significant change in transcription, perhaps due to persistent binding of the co-repressor SMRT by TR_{S142A} (Figure 8). In contrast, cells with

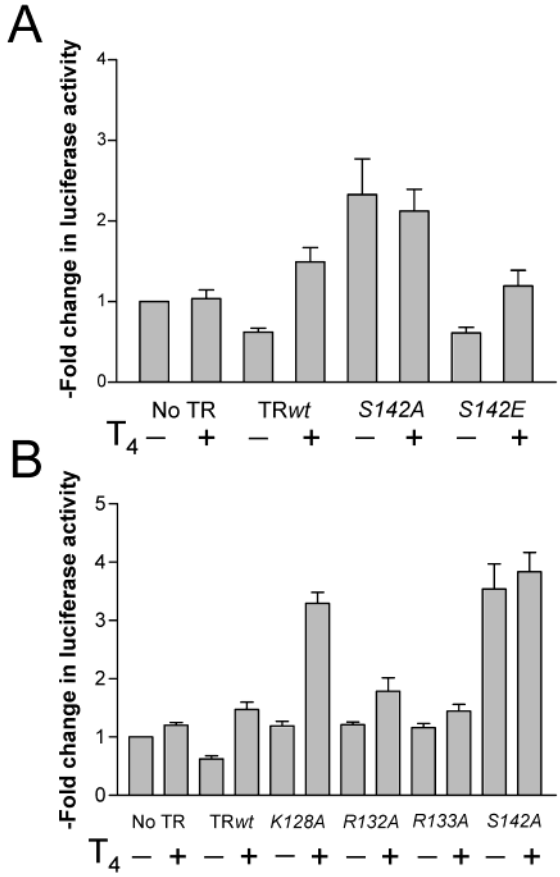

FIGURE 9: Mutations in serine 142 and the proposed MAPK docking site on TRβ1 cause changes in luciferase expression in the absence and presence of T_4. CV-1 cells were transfected with TR_{wt} or mutant receptors as described in Experimental Procedures above, and the response of these transfected cells to T_4 treatment studied in a transcriptional activation assay with measurement of luciferase activity normalized to β-galactosidase activity in the same cells. (A) In CV-1 cells with TR_{wt}, transcriptional repression was seen in the absence of T_4, and a 2-fold increase occurred with T_4, 10^{-7} M, in 24 h. Similar results were seen with TR_{S142E}, whereas with TR_{S142A}, there was increased luciferase activity in the absence of T_4 and no further change with T_4 treatment. (B) Luciferase activity levels in TR_{wt} and TR_{142A} cells with and without T_4 treatment showed a pattern similar to those seen in panel A above. The mutants K128A, R132A, and R133A showed loss of repression in the absence of T_4. With T_4 treatment, K128A cells demonstrated enhanced luciferase activity, while the other two docking site mutants showed little increase in luciferase expression.

TR_{S142E} showed results similar to those with TR_{wt}: repression of transcription in the absence of T_4, and stimulation of transcription with T_4 (Figure 9A). The patterns of p300 and SMRT binding to the S142E mutant were also similar to those seen with the wt receptor. These changes were not unexpected, since the glutamate substitution was intended to mimic, at least in part, the charge obtained with a phosphorylated serine at residue 142 in TR_{wt}.

The proposed MAPK docking site mutants, TR_{K128A}, TR_{R132A}, and TR_{R133A}, showed no repression of transcription by the receptors in the absence of T_4 (Figure 9B). With T_4 treatment of cells, there was more than 2-fold activation of transcription with the TR_{K128A} mutant (Figure 9B) which was associated with loss of SMRT and gain of p300 by the receptor (Figure 8). In contrast, TR_{R132A} and TR_{R133A} mutants showed a minimal increase in transcriptional activity with T_4, associated with minimal binding of p300, and persistent

Table 1: Summary of Studies in TRβ1 wt and Mutant Receptors Exposed to L-Thyroxine: Transcriptional Activation and Co-Repressor/Co-Activator Binding

TRβ1	treatment	transcriptional activity	MAPK binding to receptor	Co-R binding	Co-A binding
wt	control	repression	−	+	−
	T_4	activation	+	−	+
TR142A	control	activation	−	+	+++
	T_4	no additional activation	+	+	+
TR142E	control	repression	−	+	−
	T_4	activation	+	−	+
TR128A	control	no repression	−	+	−
	T_4	activation	minimal	−	+
TR132A	control	no repression	−	+	−
	T_4	minimal activation	−	+	−
TR133A	control	no repression	−	+	−
	T_4	minimal activation	−	+	−

SMRT binding (Figure 8). A summary of these co-activator and co-repressor binding and transcriptional activation results is provided in Table 1.

We note that 3,5,3′-triiodo-L-thyronine (T_3) is the natural ligand for the nuclear thyroid hormone receptor, TRβ1 (*14*), on the basis of the higher affinity of the receptor for T_3 than T_4. However, T_3 was not a focus of the current studies, since T_4 has been shown by us to be, on a molar basis, more effective than T_3 in activating the nongenomic MAPK signal transduction cascade, and such activation occurs at a physiological concentration of T_4 (*3*). The luciferase assay was tested for its responsiveness to T_3, and we found that 10^{-9} M and 10^{-7} M T_3 produced 2.8- and 4.9-fold increases, respectively, in the luciferase signal (results not shown). We have observed that T_4-agarose, 10^{-7} M, stimulates luciferase activity to the same extent as L-T_4 at the same concentration (results not shown).

DISCUSSION

Activation of the MAPK signal transduction pathway results in the nuclear translocation of tyrosine−threonine-phosphorylated (activated) MAPK and serine phosphorylation of a variety of nucleoproteins (*15*, *16*). We have shown elsewhere that T_4 nongenomically activates the MAPK (ERK1/2) cascade with a maximal effect occurring in 30 min (*1*−*3*). These effects of T_4 have been documented in HeLa and CV-1 cells, which do not have a functional nuclear TR (*1*−*3*), and in the present study, activation and nuclear translocation of ERK1/2 in response to T_4 treatment are shown in CV-1 cells which were transfected with plasmid but no thyroid hormone receptor. Consequences of MAPK activation by T_4 are serine phosphorylation of the oncogene suppressor protein, p53 (*3*), STAT proteins (*1*, *5*, *17*), and TRβ1 (*2*), the nuclear receptor for L-T_3 (*14*). This action of T_4 is reproduced by agarose-T_4 and is blocked by tetraiodothyroacetic acid (tetrac) which inhibits T_4 binding to plasma membranes of cells (*2*).

In an earlier report, we described in vitro phosphorylation of recombinant TRβ1 by constitutively activated MAPK, specifically ERK2 (*2*). TR is therefore a substrate for MAPK but lacks the consensus MAPK phosphorylation sequence (PX_{1-2}[S/T]P) (*12*), as well as the minimal sequences SP or TP (*15*). We did not identify in the earlier study the specific serine phosphorylated by T_4-directed MAPK. However, a PS sequence exists at residues 141−142 in human TRβ1 and was postulated by us to be a possible site of MAPK-dependent serine phosphorylation (*2*). The PS sequence has been shown to be a MAPK phosphorylation site in the HIV Vif protein (*9*) and other nonconsensus motifs have been reported for MAPK substrates (*18*, *19*).

To establish that serine 142 in the TR DBD is a MAPK phosphorylation site, we transfected TRβ1 DNA constructs containing alanine or glutamate substitutions at residue 142 into CV-1 cells and treated the cells with T_4 to activate MAPK. Neither serine 142 mutant construct was serine-phosphorylated in these cells in response to T_4, although transfected $TR\beta1_{wt}$ was serine-phosphorylated in hormone-treated cells in parallel samples. The possibility existed that mutation of residue 142 from serine to alanine or glutamate might produce changes in the configuration of the receptor protein that prevented docking of MAPK with TR. However, T_4-activated MAPK co-immunoprecipitated with both serine 142 mutant receptors as well as with wt receptor in nuclear fractions of T_4-treated cells. Thus, T_4 treatment of CV-1 cells transfected with $TR\beta1_{wt}$ promotes MAPK activation, formation of an immunoprecipitable complex of MAPK and TRβ1, and serine phosphorylation of TRβ1 at residue 142. In separate in vitro studies carried out in the absence of T_4, constitutively activated MAPK phosphorylated $TR\beta1_{wt}$ expressed in CV-1 cells but did not phosphorylate either TR in which a serine 142 mutation had been made.

We have previously reported that in CV-1 cells transfected with a TRβ1 construct containing the second zinc finger of the glucorticoid receptor (446−488) instead of residues 132−176 of TR ($TR\beta1_{T-TG-T}$) (*6*), T_4 treatment brought about neither co-immunoprecipitation of the receptor with MAPK in cell nuclei, nor serine phosphorylation of the receptor (*2*). This finding suggested that a potential docking site for MAPK on TRβ1 might reside in the second zinc finger of TRβ1. We therefore examined complexing of MAPK with constructs of TR carrying mutations in the DNA-binding domain, residues 128−133 (KGFFRR). This portion of the receptor is basic amino acid-enriched and similar to the D domain described by Yang et al. (*7*, *8*) and Jacobs (*20*). This sequence in ERK substrates is usually, but not always, N-terminal to the site of serine phosphorylation (*20*).

Our results with mutants of TR with substitutions at residues 128, 132, and 133 demonstrate that in the absence of any one of these three basic amino acids, there is no co-immunoprecipitation, or docking, of ERK and TRβ1, and no serine phosphorylation of the receptor in response to T_4. In addition, none of these three mutants was phosphorylated by constitutively activated MAPK in vitro. Thus, neither activated MAPK in vitro, nor nuclear activated MAPK in T_4-treated cells, formed a complex with TR mutants in which there was disruption of the KGFFRR sequence at residues 128, 132, or 133.

TRE-luciferase assays of wild type and mutated TRs were used to define the relationships of co-repressors, co-activators, and TR to transcriptional activity of the receptor in T_4-treated cells. In these studies, TR_{wt} was transcriptionally derepressed in the TRE-luciferase assay when the receptor was subjected to serine phosphorylation by T_4-directed MAPK (Figure 9A,B). This is to be expected from our studies of

co-repressor shedding in the setting of serine phosphorylation of TR (*2*) and from the present studies of SMRT binding to wt TR (Figure 8). Transfection of the glutamate-substituted TR mutant (TR_{S142E}) also resulted in repressed transcriptional activity and SMRT binding to the receptor in cells without T_4 treatment. Hormone treatment of cells transfected with this mutant caused decreased binding of SMRT and increased p300 binding and was associated with increased transcriptional activity, similar to findings with TR_{wt}. As indicated above, the glutamate substitution was expected to simulate the charge of a phosphorylated serine at residue 142 in TR_{wt}.

The alanine-substituted mutant TR_{S142A}, on the other hand, was constitutively active in the transcriptional assay, and there was no further increase in activity with T_4 treatment of cells transfected with this mutant. In the absence of T_4, TR_{S142A} bound substantial quantities of co-activator (p300) and some co-repressor (SMRT). Thus, this substitution produces a conformational change in the receptor that is favorable to co-activator binding. The lack of a serine at residue 142 that is subject to phosphorylation also resulted in persistent co-repressor binding. The fact that activation, rather than repression, was obtained with this interesting TR mutant suggests dominance of co-activator molecules over co-repressors and/or determination of the activation state of the receptor by the ratio of co-repressor and co-activator molecules associated with the receptor pool, as suggested by Shibata et al. (*13*).

Cells transfected with the TR_{K128A} mutant showed no repression of transcriptional activity in the absence of T_4 treatment, although SMRT binding was evident. This suggests that binding of SMRT by TR_{K128A} is insufficient to induce repression of transcriptional activity by this mutant, but that binding of SMRT is certainly sufficient and necessary in the wt and $_{S142E}$ forms of TR. With T_4 treatment of cells containing TR_{K128A}, luciferase activity increased, along with increased p300 binding and decreased SMRT binding. These changes, although similar to those seen with TR_{wt}, were associated with minimal, but detectable, hormone-induced TR/MAPK docking, and no serine phosphorylation of the receptor.

From the above observations, we conclude that phosphorylation of serine 142 by T_4-directed MAPK relieves repression of transcription that is conferred on TR_{wt} by SMRT-binding. That induction of repression may be more complicated than simply the binding of co-repressor by the receptor is suggested by inability of SMRT to cause repression in TR_{K128A} cells, despite formation of a SMRT−TR complex. Further, when the binding of SMRT by this TR construct was insufficient to cause repression of transcription, then a small amount of MAPK binding by the mutated receptor did not result in shedding by the TR of SMRT. This indicates that the binding of SMRT by the K128A mutant does not mimic the association of co-repressor and receptor in the TR_{wt}−SMRT complex.

That MAPK-binding by the receptor may be relevant to co-activator recruitment is suggested by studies of TR_{R132A} and TR_{R133A}. These mutants did not bind MAPK and did not bind co-activator. Both mutants did bind SMRT and failed to shed the co-repressor when cells containing either of the mutant receptors were treated with T_4. While the TR transcriptional complex is traditionally viewed as an association of receptor, receptor ligand ($_L$-T_3), co-activators, and de-acetylases (*14*), we have elsewhere suggested that the T_4-stimulated complex is an enhanceosome that contains activated MAPK and other signal transducing proteins (*3*).

The basic amino acid-enriched sequence KGFFRR on TRβ1, the likely site of MAPK docking, is not accompanied by a nearby C-terminal LXL and a more distant C-terminal hydrophobic FXFP motif. These may be found in ERK substrates, but are not required (*21*). The basic amino acid sequences in activated ERK substrate dimers are thought to bind to acidic amino acid sequences on ERKs, enriched in aspartic (D) and glutamic (E) acids, called the common docking (CD) domain (*22*, *23*). The LXL motif is thought to permit docking of both Jun N-terminal kinase (JNK) and ERK with substrates (*8*, *24*). The absence of LXL in TRβ1 infers additional specificity of this substrate for the action of ERK1/2. It has been postulated that FXFP may be a docking site that interacts with a binding pocket on ERK which is different from the active site on ERK associated with phosphorylation of substrate (*20*).

The incorporation of both basic amino acid- and phenylalanine-enrichment into the MAPK docking site we have defined appears to be novel for MAPK substrates, but in fact is found on other members of the superfamily of nuclear receptors subject to serine phosphorylation by MAPK. For example, in both estrogen receptors α (ERα) and ERβ, the sequence KAFFKR, similar to KGFFRR in TRβ1, is present in the DNA-binding domains (ERα, residues 206−211 and ERβ, residues 170−175; Gene Bank Accession numbers P03372 and Q92731, respectively). These sites are located, similar to that of TRβ1, just C-terminal to the end of the first zinc finger, and C-terminal to respective sites of serine phosphorylation on serines 118 of ERα (*12*) and 105 of ERβ (*25*). Nuclear hERα is subject to serine phosphorylation by MAPK, whether the latter is activated by estrogen (*12*) or by thyroid hormone.[2] Kato has also recently described phosphorylation of hERβ by MAPK (*25*), and a report of Tremblay et al. supports these findings (*26*). Human glucocorticoid receptor (GR) and progesterone receptor (PR) contain similar amino acid sequences in their DNA-binding domains: KVFFKR at residues 442−447 of GR (Gene Bank Accession number P04150) and KVFFKR at residues 588−593 of PR (Gene Bank Accession number P06401). Transcriptional enhancement of GR (*27*) and PR (*28*) function by MAPK have been reported. The present studies conducted in TR thus appear to be relevant to other members of the hormone receptor superfamily.

In the present studies, we have employed concentrations of T_4 regarded to be physiologic (*2*). We (*2*) and others (*29*) have shown that T_3 will also nongenomically activate MAPK, but at concentrations that exceed physiologic levels. It is unlikely that T_3 contributed to results of experiments involving T_4 which we describe, since two pharmacologic inhibitors of 5′-thyronine monodeiodinases that convert T_4 to T_3 did not alter the outcome of the studies.

REFERENCES

1. Lin, H.-Y., Davis, F. B., Gordinier, J. K., Martino, L. J., and Davis, P. J. (1999) *Am. J. Physiol. 276*, C1014−C1024.

[2] Lin, H.-Y., Davis, P. J., and Davis, F. B. unpublished observations, 2002.

2. Davis, P. J., Shih, A., Lin, H.-Y., Martino, L. J., and Davis, F. B. (2000) *J. Biol. Chem. 275*, 38032−38039.
3. Shih, A., Lin, H.-Y., Davis, F. B., and Davis, P. J. (2001) *Biochemistry 40*, 2870−2878.
4. Torchia, J., Glass, C., and Rosenfeld, M. G. (1998) *Curr. Opin. Cell Biol. 10*, 373−383.
5. Lin, H.-Y., Martino, L. J., Wilcox, B. D., Davis, F. B., Gordinier, J. K., and Davis, P. J. (1998) *J. Immunol. 161*, 843−849.
6. Nagaya, T., Kopp, P., Kitajima, K., Jameson, J. L., and Seo, H. (1996) *Biochem. Biophys. Res. Commun. 222*, 524−530.
7. Yang, S.-H., Yates, P. R., Whitmarsh, A. J., Davis, R. J., and Sharrocks, A. D. (1998) *Mol. Cell. Biol. 18*, 710−720.
8. Yang, S.-H., Whitmarsh, A. J., Davis, R. J., and Sharrocks, A. D. (1998) *EMBO J. 17*, 1740−1749.
9. Yang, X., and Gabuzda, D. (1998) *J. Biol. Chem. 273*, 29879−29887.
10. Samuels, H. H., Stanley F., and Casanova, J. (1979) *Endocrinology 105*, 80−85.
11. Weinstein, S. P., Watts, J., Graves, P. N., and Haber, R. S. (1990) *Endocrinology 126*, 1421−1429.
12. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., et al. (1995) *Science 270*, 1491−1494.
13. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) *Rec. Prog. Horm. Res. 52*, 141−164.
14. Yen, P. M. (2001) *Physiol. Rev. 81*, 1097−1142.
15. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) *Adv. Cancer Res. 74*, 49−139.
16. Kolch, W. (2000) *Biochem. J. 351*, 289−305.
17. Lin, H.-Y., Shih, A., Davis, F. B., and Davis, P. J. (1999) *Biochem. J. 338*, 427−432.
18. She, Q. B., Chen, N., and Dong, Z. (2000) *J. Biol. Chem. 275*, 20444−20449.
19. Persons, D. L., Yazlovitskaya, E. M., and Pelling, J. C. (2000) *275*, 35778−35785.
20. Jacobs, D., Glossip, D., Xing, H., Muslin, A. J., and Kornfeld, K. (1999) *Genes Dev. 13*, 163−175.
21. Yang, S.-H., Galanis, A., and Sharrocks, A. D. (1999) *Mol. Cell. Biol. 19*, 4028−4038.
22. Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) *Nat. Cell Biol. 2*, 110−116.
23. Tanoue, T., Maeda, R., Adachi, M., and Nishida, E. (2001) *EMBO J. 20*, 466−479.
24. Mosselman, S., Polman, J., and Dijkema, R. (1996) *FEBS Lett. 392*, 49−53.
25. Kato, S. (2001) *Breast Cancer 8*, 3−9.
26. Tremblay, A., Tremblay, G. B., Labrie, F., and Giguere, V. (1999) *Mol. Cell 3*, 513−519.
27. Krstic, M. D., Rogatsky, I., Yamamoto, K. R., and Garabedian, M. J. (1997) *Mol. Cell. Biol. 17*, 3947−3954.
28. Shen, T., Horwitz, K. B., and Lange, C. A. (2001) *Mol. Cell. Biol. 21*, 6122−6131.
29. Kozawa, O., Hatakeyama, D., Yoshida, M., Kamiya, Y., Kondo, C., Matsuno, H., and Uematsu, T. (2001) *BBRC 286*, 1140−1143.

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