Thyroid Hormone Promotes Serine Phosphorylation of p53 by Mitogen-Activated Protein Kinase†

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ABSTRACT: L-Thyroxine (T_4) nongenomically promotes association of mitogen-activated protein kinase (MAPK) and thyroid hormone receptor TRβ1 (TR) in the cell nucleus, leading to serine phosphorylation of the receptor. The oncogene suppressor protein, p53, is serine phosphorylated by several kinases and is known to interact with TRβ1. We studied whether association of p53 and TR is modulated by T_4 and involves serine phosphorylation of p53 by MAPK. TR-replete 293T human kidney cells were incubated with a physiological concentration of T_4 for 10−90 min. Nuclear fractions were immunoprecipitated and the resulting proteins separated and immunoblotted for co-immunoprecipitated proteins. Activated MAPK immunoprecipitates of nuclei from T_4-treated cells accumulated p53 in a time-dependent manner; T_4 and T_4−agarose were more effective than T_3. T_4-induced nuclear complexing of p53 and MAPK was inhibited by PD 98059 (PD) and U0126, two MAPK kinase (MEK) inhibitors, and was absent in cells treated with MEK antisense oligonucleotide and in dominant negative Ras cells. T_4 also caused nuclear co-immunoprecipitation of TRβ1 and p53, an effect also inhibited by PD. Nuclear complexing of p53 and MAPK also occurred in HeLa cells, which lack functional TR. Constitutively activated MAPK caused phosphorylation of a recombinant p53-GST fusion protein in vitro; thus, p53 is a substrate for MAPK. An indicator of p53 transcriptional activity, accumulation of the immediate−early gene product, c-Jun, was inhibited by T_4. This T_4 effect was reversed by PD, indicating that the transcriptional activity of p53 was altered by T_4-directed MAPK−p53 interaction.

p53 is an oncogene suppressor protein activated in settings in which cellular DNA damage occurs (*1*, *2*). The p53 protein causes transcription of *p21*, whose gene product arrests cells in the G_1 phase of the cell cycle by binding to the G_1 cyclin−Cdk2 protein complex (*3*) that is required to move cells into S phase (*4*). Multiple sites on the p53 protein undergo serine phosphorylation (*4*). The importance of such phosphorylation to p53 stability and action is under review in several laboratories (*1*, *5*, *6*). The serine kinases implicated in phosphorylation of p53 include casein kinase I or a DNA-dependent protein kinase (*7*−*9*) for serines in the acidic, α-helical region, a cyclin-activated p34-cdc-2-like kinase for residue 316 (*10*), and casein kinase II for serine 392 (*4*). The primary nuclear localization signal for p53 is in the region of serine 316 (*11*). A role for mitogen-activated protein kinase (MAPK)[1] in the serine phosphorylation of p53 has been described (*12*), but the mechanism for regulation of this action of MAPK is not known.

We have recently shown in several cell lines that thyroid hormone nongenomically promotes phosphorylation and nuclear uptake of MAPK (*13*, *14*). Activated by a physiologic concentration of L-thyroxine (T_4), nuclear MAPK is able to complex with and serine phosphorylate *s*ignal *t*ransducer and *a*ctivator of *t*ranscription (STAT) proteins STAT1α (*13*) and STAT3 (*14*), enhancing their transcriptional activities. The effects of T_4 are blocked by MAPK pathway inhibitors, including PD 98059 (*13*, *14*) and geldanamycin (*14*), as well as by tetraiodothyroacetic acid (tetrac), an inactive thyroid hormone analogue which inhibits both T_4 binding to the cell membrane (*15*) and T_4-induced activation of MAPK (*13*) and STAT1α (*16*). We have also described serine phosphorylation by MAPK of the nuclear thyroid hormone receptor, TRβ1 (TR), in cells treated with T_4, leading to dissociation of the corepressor SMRT from TR (*17*). These effects are inhibited by tetrac and PD 98059. Yap et al. and Bhat et al. have reported 3,5,3′-triiodo-L-thyronine (T_3)-induced association of TR and p53 in the cell nucleus, an interaction that results in decreased transcriptional activity of both proteins (*18*, *19*). Mechanisms by which the association of endogenous TR and p53 is promoted have not been described. We report here the MAPK-dependent serine

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[1] Abbreviations: MAPK, mitogen-activated protein kinase; pMAPK, activated MAPK; MEK, MAPK kinase; T_4, L-thyroxine; STAT, signal transducer and activator of transcription; TRβ1 or TR, thyroid hormone receptor β1; T_3, 3,3′,5-triiodo-L-thyronine; tetrac, tetraiodothyroacetic acid; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein; GST, glutathione *S*-transferase; GST-p53, GST-p53 fusion protein; GST-p53-ip, anti-p53-immunoprecipitated GST-p53; RV, resveratrol; DMEM, Dulbecco's modified Eagle medium; IFN-γ, interferon-γ; ER, estrogen receptor; GR, glucocorticoid receptor; GPCR, G-protein-coupled receptor.

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phosphorylation of p53 and association of nuclear TR and p53 which is induced by physiologic concentrations of T_4.

EXPERIMENTAL PROCEDURES

Materials. T_4, T_3, tetraiodothyroacetic acid (tetrac), T_4−agarose, and myelin basic protein (MBP) were obtained from Sigma Chemical Co. (St. Louis, MO). 293T cells were generously provided by Dr. Kevin Pumiglia (Albany Medical College, Albany, NY) and NIH 3T3 and stably transfected dominant negative Ras cells (N17) by Dr. Geoffrey M. Cooper (Boston University School of Medicine, Boston, MA) (*20*, *21*). These cells express an Asn-17 *ras* gene that has a significantly reduced affinity for GTP and has been shown to inhibit cellular *ras* activity in vivo without reducing cellular *ras* content (*20*). HeLa cells are on hand in the laboratory. PD 98059, U0126, apigenin, activated MAPK, and resveratrol were obtained from Calbiochem (La Jolla, CA); geldanamycin was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). Recombinant GST-p53 fusion protein was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Preparation of Nuclear Fractions. Cells were cultured and nuclear fractions prepared as we have previously described (*13*, *17*). In brief, TRβ1-replete 293T cells were grown to confluence and then exposed for 48 h to Dulbecco's modified Eagle medium (DMEM) supplemented with 0.25% fetal bovine serum which was depleted of thyroid hormone as previously described (*21*). The cells were then maintained in serum-free medium for 2 h prior to treatment. NIH3T3 and N17 cells were cultured in the same manner but with G418, 400 μg/mL, added to the culture medium. Endogenous thyroid hormone contributed by hormone-depleted serum was at or below the lower limits of detection (*22*). The preparation of stock thyroid hormone solutions was as previously reported (*13*). T_4 was then added in a final total hormone concentration of 10^{-7} M for the indicated time periods. A resulting free T_4 concentration of 0.7×10^{-10} M was measured in representative samples in the Clinical Chemistry Laboratory of the Albany Medical Center Hospital. The cells were harvested and nuclear proteins prepared as described previously (*13*). PD 98059 (90 min), U0126 (90 min), and geldanamycin (16 h) were added to cells in a final DMSO concentration of 1%, with exposure of cells to iodothyronine taking place during the final 30 min of inhibitor incubation.

Immunoprecipitation and Immunoblotting. These procedures were carried out according to our previously reported methods (*13*, *14*, *16*, *17*). The immunoprecipitation/immunoblotting protocols were as follows: (1) immunoprecipitation with polyclonal anti-activated MAPK (anti-pMAPK, New England BioLabs, Beverly, MA), followed by solubilization of immunoprecipitates and separation by SDS−PAGE, and then immunoblotting with monoclonal anti-p53 (Santa Cruz, Santa Cruz, CA); (2) immunoprecipitation with monoclonal anti-p53 and then immunoblotting with either polyclonal anti-pMAPK, anti-MAPK (anti-ERK2, Santa Cruz), or anti-phosphoserine (Research Diagnostics, Flanders, NJ); (3) immunoprecipitation with polyclonal anti-TRβ1 (to amino acids 62−82, Affinity BioReagents, Golden, CO) and immunoblotting with monoclonal anti-p53 or anti-MAPK; (4) immunoprecipitation with monoclonal anti-TRβ1 (to the C-terminal half of the A/B domain; Santa Cruz) and immunoblotting with polyclonal anti-p53 or anti-ERK2 (Santa Cruz). Analysis by immunoblot of supernatants from all such immunoprecipitations revealed no remaining specific antigen. Secondary antibodies were either rabbit anti-mouse or goat anti-rabbit IgG (DAKO Corp., Carpenteria, CA). Selected samples were immunoblotted with anti-serine-15-phosphorylated p53 (New England BioLabs), anti-pMAPK, anti-ERK2, anti-TRβ1, anti-p53, or c-Jun antibody (Santa Cruz) without prior immunoprecipitation. All immunoblots were visualized by enhanced chemiluminescence (ECL, Amersham Life Science, Arlington Heights, IL) and subjected to quantitation by digital imaging (BioImage, Millipore). Immunoblots are representative of two or more experiments. As we have previously indicated (*13*, *17*), the accumulation of nuclear MAPK in response to T_4, due to dual tyrosine−threonine phosphorylation (activation) and nuclear translocation from cytosol, is demonstrated by immunoblotting of nuclear extracts with antibody either to MAPK or to activated (phosphorylated) MAPK, since only activated MAPK translocates to the nucleus.

Transfection of Antisense and Scrambled MEK Oligonucleotides. Phosphorothioated antisense and scrambled MEK oligonucleotides, as described by Hu et al. (*23*), were obtained from GIBCO BRL (Rockville, MD). The sequences were as follows: antisense, 5′-GCTTCTTCTTGGGCATCT-3′; scrambled, 5′-AAATTCGTGGACGTTCGC-3′. 293T cells grown in 10 mL culture dishes were transfected with 6 μg of oligonucleotide in the presence of Lipofectamine Plus reagent (GIBCO BRL) for 6 h. The medium was then replaced with fresh medium containing 0.25% hormone-depleted fetal bovine serum for 24 h. T_4 treatment was carried out for 30 min, after which time cells were harvested and nuclear and cytosolic fractions prepared as described above. Cytosols were analyzed by SDS−PAGE and immunoblotted with anti-MEK1 antibody (Santa Cruz) to confirm success of the transfections.

Induction of c-Jun Protein in Resveratrol-Treated Cells. Resveratrol induces accumulation of p53 (*24*, *25*), and p53 can induce accumulation in certain cell lines of immediate−early gene products [c-Jun (*26*); c-Fos (*27*)]. We tested 293T cells for an effect of resveratrol on c-Jun abundance as an index of transcriptional activity of p53. Confluent 293T cells grown in 100 mm Petri dishes were cultured in medium containing 0.25% hormone-depleted serum as described above. Resveratrol (RV, 100 μM) was added to cell cultures for 24 h to induce c-Jun expression, without T_4 or with 10^{-7} M T_4 for the same 24 h. PD (0.3 or 30 μM) was added to selected cell samples for 24 h. Cells were harvested and nuclear proteins extracted as described above for immunoblotting with antibodies to c-Jun.

In Vitro Phosphorylation of p53 by Activated MAPK. Recombinant GST-p53 fusion protein (amino acids 1−393; Santa Cruz; 2 μg) was immunoprecipitated with p53 antibody; 2.5 μg of the immunoprecipitate (GST-p53-ip) was incubated for 30 min at 30 °C with activated MAPK (5 units/μL) in 50 mM Tris-HCl, pH 8.0, and 20 μM ATP including 0.05 μCi of [γ-^{32}P]ATP, 0.5 mM EDTA, 25 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol, as described by Kato et al. (*28*). A control sample contained 1.0 μg of myelin basic protein (MBP) and activated MAPK, and an additional sample utilized 5 μg of immunoprecipitated p53 from 293T

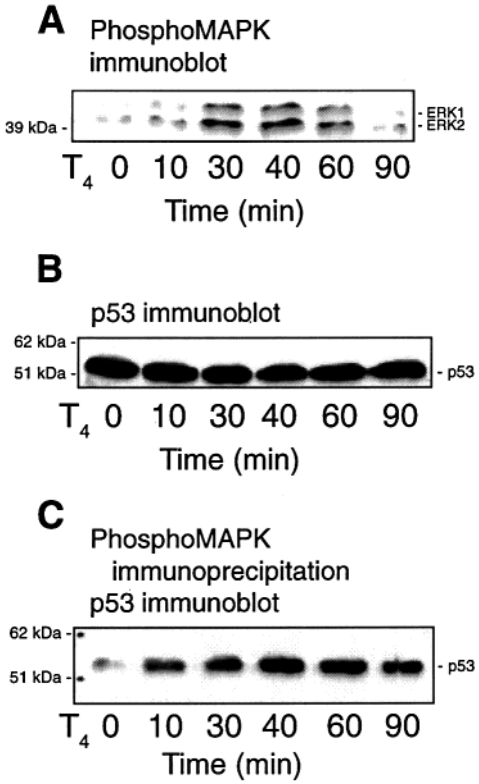

FIGURE 1: T_4 activates MAPK and stimulates nuclear co-immunoprecipitation of phosphoMAPK and p53 in 293T cells. Cells were treated with T_4 (10^{-7} M) for 10−90 min, after which time nuclear fractions were isolated. (A) A representative immunoblot with antibody to tyrosine−threonine-phosphorylated (activated) MAPK (phosphoMAPK) shows a progressive increase in activated MAPK up to 40 min: 13.4- and 18.6-fold increases in ERK1 (44 kDa) and ERK2 (42 kDa) isoforms, respectively, at 40 min in the study shown and 11.4- and 17.6-fold increases in four similar experiments. (B) An immunoblot of similar samples shows no appreciable change in total nuclear p53 content over the same time period. (C) Comparable nuclear samples were immunoprecipitated with antibody to phosphoMAPK, and the precipitated proteins were separated by PAGE and immunoblotted with antibody to p53. Shown in this figure is progressive nuclear complexing of phosphoMAPK and p53, with a 21.8-fold increase seen at 40 min.

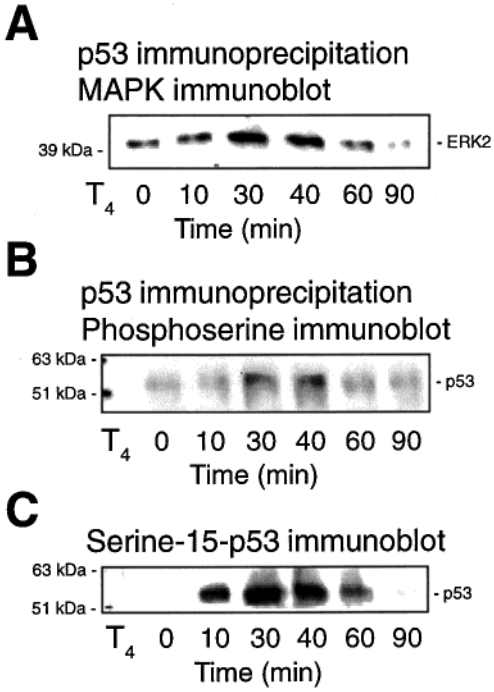

FIGURE 2: T_4 induces serine phosphorylation of p53 in 293T cells. (A) T_4-treated nuclear samples from a study similar to those in Figure 1 were prepared and immunoprecipitated with antibody to p53. The immunoprecipitates were separated by PAGE and immunoblotted with anti-MAPK. Findings in this study were similar to those in Figure 1C, indicating that reversal of antibody order does not significantly alter the results obtained. (B) p53 immunoprecipitates were immunoblotted with antiphosphoserine. T_4 caused an increase in serine phosphorylation of p53 in 30 and 40 min. (C) Nuclear samples immunoblotted with antibody to serine-15-phosphorylated p53, without prior immunoprecipitation, also demonstrated increases in serine-15 phosphorylation of p53 in 30 and 40 min.

cells not exposed to T_4. After incubation solubilized samples were separated by SDS−PAGE, and radioautography was performed.

RESULTS

Thyroid Hormone Causes Nuclear Accumulation of Activated MAPK, Association of Nuclear MAPK and p53, and Serine Phosphorylation of p53 in 293T Cells. Consistent with results which we have previously reported (*13*), T_4 (in 10^{-7} M total and 0.7×10^{-10} M free hormone concentrations) caused nuclear accumulation in 293T cells of tyrosine-phosphorylated (activated) MAPK in 10−60 min, with a maximal effect seen in 30−40 min and loss of the effect by 90 min (Figure 1A). Immunoblots of nuclear fractions revealed a relatively constant nuclear content of p53 over the 10−90 min period of incubation with T_4 (Figure 1B). Immunoprecipitates of activated MAPK contained p53 after exposure of cells for 10−90 min to T_4 (Figure 1C), with maximal co-immunoprecipitation of the two proteins seen at 40 min.

In Figure 2A evidence of T_4-induced nuclear co-immunoprecipitation of p53 and MAPK is again presented, but with the order of antibodies in immunoprecipitation and immunoblotting steps reversed compared with the experiment shown in Figure 1C. The same p53 immunoprecipitate used in Figure 2A was immunoblotted with antibody to phosphoserine, and results showed a time-dependent increase in serine phosphorylation of the protein (Figure 2B), with a maximal effect seen at 30−40 min. Additional immunoblots of nuclear protein using antibody to serine-15-phosphorylated p53 revealed similar results (Figure 2C). Control cells incubated for 30 min with diluent for thyroid hormone showed no activation of MAPK, complexing of MAPK and p53, or serine phosphorylation of p53.

Effect of Thyroid Hormone Analogues and T_4−Agarose on Association of p53 and MAPK in 293T Cell Nuclei. Cells were incubated with T_3 (10^{-10} or 10^{-7} M), T_4 (10^{-7} M), tetrac (10^{-7} M), or T_4−agarose (T_4 10^{-7} M) for 30 min, and nuclear p53 immunoprecipitates were prepared and immunoblotted with anti-pMAPK. The effect of T_3 on nuclear complexing was minimal at 10^{-10} M but clearly evident at a supraphysiologic concentration of 10^{-7} M (Figure 3, lane 3). In contrast, T_4 produced an appreciable effect at a physiologic concentration of 10^{-7} M (lane 4). In prior studies we have demonstrated that the effect of T_4 on signal transduction is not altered by the presence of 6-*n*-propyl-2-thiouracil (*17*), evidence that the conversion of T_4 to T_3 does not play a role in these studies.

The T_4 effect was partially blocked by tetrac, a deaminated analogue of T_4 (Figure 3, lane 6), although tetrac had no

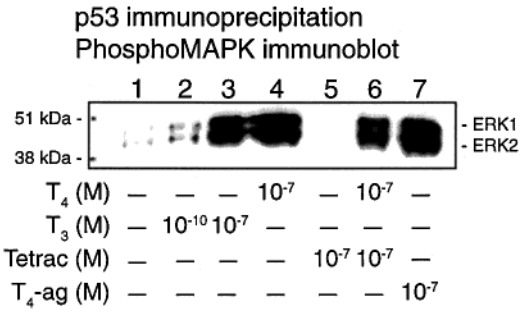

FIGURE 3: Nuclear co-immunoprecipitation of p53 and activated MAPK in 293T cells is induced by T_4 and T_4−agarose and to a lesser extent by T_3, and the T_4 effect is blocked by tetrac. 293T cells were treated with T_4, T_3, or T_4−agarose for 30 min, and nuclear samples were prepared as described. T_4, 10^{-7} M, caused substantial increases in phosphorylated ERK1 and ERK2 content in nuclear p53 immunoprecipitates (lane 4). A supraphysiologic concentration of T_3 (10^{-7} M) caused similar increases in the same experiment (lane 3) while a more physiologic concentration of T_3 (10^{-10} M) was less effective (lane 2). Tetrac alone (10^{-7} M) had no effect (lane 5) but did reduce T_4 action by 71% (ERK1) and 60% (ERK2) (lane 6 compared to lane 4). The effect of T_4−agarose (T_4−ag, lane 7) was comparable to that seen with T_4.

effect alone (lane 5). We have previously shown that tetrac blocks T_4 stimulation of MAPK activation and tyrosine phosphorylation of the *s*ignal *t*ransducer and *a*ctivator of *t*ranscription STAT1α (*13*, *16*) and blocks T_4-induced nuclear complexing of TR and MAPK (*17*). We have also shown that tetrac inhibits the binding of T_4 to human erythrocyte membranes (*15*). Further support for a plasma membrane effect of T_4 in promoting complexing of p53 and MAPK is provided by the similar action of T_4−agarose (Figure 3), a conjugate that does not gain access to the cell interior. We have previously reported the activation of MAPK, STAT1α, and STAT3 (*13*, *14*, *16*) and co-immunoprecipitation of activated MAPK and nuclear TRβ1 (*17*) to be induced by T_4−agarose containing a physiologic concentration of T_4.

Action of MAPK Pathway Inhibitors on T_4-Induced Association of p53 and MAPK in Nuclear Fractions. As T_4-induced signal transduction is dependent on activation of MAPK (*13*, *17*), the effects on T_4 action of geldanamycin (GEL), which depletes cells of Raf-1 (*29*), and PD 98059 (PD) and U0126, inhibitors of MAPK kinase (MEK) (*13*, *30*), were examined. We have previously demonstrated that both GEL and PD block T_4-induced activation of MAPK leading to T_4 potentiation of the antiviral action of interferon-γ (*13*). T_4-induced nuclear co-immunoprecipitation of TRβ1 and MAPK and serine phosphorylation of TRβ1 in 293T cells are also blocked by PD (*17*). In the present studies, 293T cells were pretreated with PD, U0126, GEL, or control solvent, with T_4, 10^{-7} M, added for the last 30 min of inhibitor incubation. Nuclear fractions were immunoprecipitated with monoclonal anti-p53, and the immunoprecipitates were immunoblotted with polyclonal anti-MAPK or -pMAPK. PD alone (30 μM) had no effect on p53−MAPK complex formation (Figure 4A), while T_4 alone stimulated formation of the complex. PD inhibited the T_4 effect in a dose-dependent manner, with an IC_{50} of approximately 0.3 μM.

Our findings are in agreement with those of Alessi et al., who have documented IC_{50} values of 2−7 μM for MEK1 and 50 μM for MEK2 (*31*). In a HeLa cell antiviral assay measuring the activity of interferon-γ and potentiation of interferon action by T_4, we found an IC_{50} for PD of 3 μM (*13*). Our findings support the premise that promotion by T_4 of p53 complex formation with activated MAPK in the cell nucleus requires activation of MAPK via a pathway utilizing MEK. In Figure 4B the results of immunoblotting of nuclear fractions with anti-serine-15-phosphorylated p53 demonstrate a pattern of phosphorylation which parallels the findings in Figure 4A and indicates inhibition by PD of T_4-stimulated serine-15 phosphorylation.

While PD is thought to inhibit MEK activity by binding to nonactivated MEK (*31*), the inhibitor U0126 has a higher affinity than PD for MEK−MAPK and MEK−ATP complexes and a lower IC_{50} than PD for MEK activity, approximately 60 nM (*30*). In our studies of T_4-induced p53−MAPK co-immunoprecipitation, the IC_{50} of U0126 was less than 7 nM, as shown in Figure 4C. Even though the mechanisms of action of PD and U0126 may not be identical, the two inhibitors apparently share a binding site on MEK, and PD has been shown to displace U0126 from MEK (*30*).

In contrast, GEL depletes cells of Raf-1 by displacing heat shock protein 90 from Raf-1 (*29*), thus inhibiting activation of MEK by Raf-1. This inhibitor also blocks T_4-induced STAT3 (*14*), STAT1α, and MAPK activation (*13*) with an IC_{50} of approximately 10 μM and in the current studies inhibited T_4-induced nuclear co-immunoprecipitation of p53 and activated MAPK by 70% at a concentration of 10 μM (Figure 4D).

Effect of Antisense MEK1 Oligonucleotide Transfection on T_4-Induced Nuclear Complexing of p53 and MAPK. Transient transfection of antisense and scrambled MEK oligonucleotides was carried out in 293T cells. Cells were then treated with T_4, 10^{-7} M, or control solvent for 30 min, and cytosols and nuclear extracts were prepared. Cytosols were immunoblotted with anti-MEK1 and showed an 82% reduction in MEK content in the cells transfected with antisense oligonucleotide (Figure 5A, lane 3 vs lane 1). T_4 caused a 72% reduction in cytosol MEK content in control cells (lane 4 vs lane 1) and a 43% reduction in cells treated with scrambled oligonucleotide (lane 5 vs lane 2). This finding is consistent with transient translocation of MEK1 to the nucleus upon activation of the MAPK pathway by T_4. In antisense-treated cells T_4 caused a reduction in the small amount of remaining MEK in cytosolic fractions (comparing lanes 3 and 6).

Nuclear extracts were immunoprecipitated with anti-p53, and the precipitates were immunoblotted with anti-MAPK. In cells treated with scrambled MEK1 oligonucleotide, T_4 caused nuclear complexing of p53 and MAPK [Figure 5B, lane 5 (with T_4) compared to lane 2 (without hormone)]. These latter findings are similar to results in cells not transfected with oligonucleotide [shown in lanes 4 (with T_4) and lane 1 (without hormone)]. Cells which received antisense MEK oligonucleotide (lanes 3 and 6) showed no nuclear complexing of p53 and MAPK, with or without T_4 treatment.

Action of Thyroxine in a Dominant Negative ras Model. Upstream of MEK and Raf-1 in the MAPK (ERK1, ERK2) pathway is Ras. N17 cells stably transfected with dominant negative *ras* (*20*, *21*) showed no activation of MAPK by T_4 (Figure 6A, lane 4), whereas MAPK was activated by T_4 in parent NIH 3T3 cells (lane 2). Thyroxine also caused nuclear

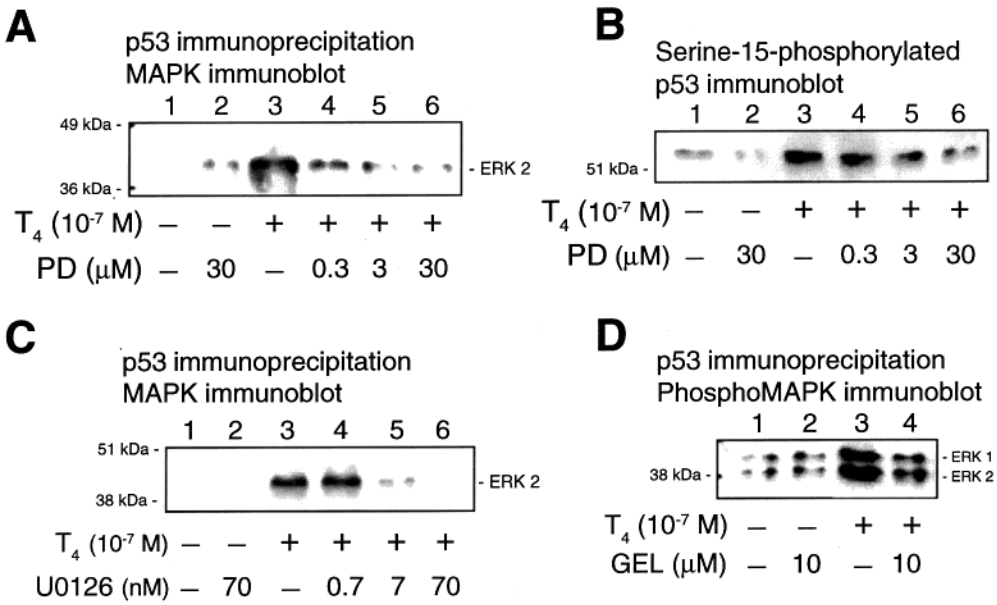

FIGURE 4: T_4-induced co-immunoprecipitation of p53 and MAPK in 293T cell nuclei is inhibited by PD 98059, U0126, and geldanamycin. Cells were treated with T_4 (10^{-7} M) for 30 min, with or without pretreatment with PD 98059 (PD, 0.3, 3.0, or 30 μM, 90 min), U0126 (0.7, 7.0, or 70 nM, 90 min), or geldanamycin (GEL, 10 μM, 16 h). In panels A, C, and D proteins from nuclear p53 immunoprecipitates were immunoblotted with antibody to MAPK (ERK2) or phosphoMAPK; both antibodies indicate activated MAPK in these nuclear samples. In panel B, nuclear samples were immunoblotted with anti-serine-15-phosphorylated p53 without immunoprecipitation. (A) PD inhibited T_4-induced nuclear complexing of p53 and MAPK by 60% and 78%, respectively, at concentrations of 0.3 and 3.0 μM. (B) Cells treated with T_4 showed serine-15-phosphorylated p53 in 30 min. This effect was inhibited by PD. (C) U0126 also inhibited T_4 action on p53−MAPK co-immunoprecipitation, with an IC_{50} between 0.7 and 7.0 nM. (D) GEL inhibited the T_4 effect by 70% at a concentration of 10 μM.

co-immunoprecipitation of p53 and MAPK in NIH 3T3 cells but not in N17 cells (Figure 6B), thus supporting a role for Ras in T_4-induced stimulation of the MAPK signal transduction pathway and nuclear complexing of p53 and activated MAPK. In Figure 6C the nuclear p53 content is shown to be the same in all samples. In the original report of N17 cells, which were provided to us by the authors, Ras was demonstrable by immunoblot, although introduction of dominant negative *ras* prevented function of the wild-type protein (*20*).

T_4-Induced Complexing of p53 and TRβ1 in 293T Cell Nuclear Fractions. 293T cells were treated with PD in several concentrations for 90 min and with T_4, 10^{-7} M, for the last 30 min of those incubations. Immunoblots of nuclear fractions with monoclonal antibodies to TRβ1 or p53 (Figure 7A) revealed a T_4-induced increase in nuclear TRβ1 (lane 3) which was inhibited by PD (lanes 4−6), while levels of nuclear p53 were not affected by T_4 or PD. Nuclear fractions were also immunoprecipitated with polyclonal anti-TRβ1 (to amino acids 62−82) and the precipitates immunoblotted with monoclonal anti-p53 or anti-MAPK. Without T_4 there was little association of TRβ1 and p53 or of TRβ1 and MAPK in 293T cell nuclei (Figure 7B), whereas with T_4 treatment, both p53 and MAPK were seen in nuclear TRβ1 immunoprecipitates in 30 min. Pretreatment of cells with PD inhibited this association of TRβ1 with p53 and MAPK. The T_4 effects were inhibited >50% by 3 μM PD and >80% by 30 μM PD, consistent with our previous findings on the effect of PD on T_4 activation of MAPK (*13*) and STAT3 (*14*).

T_4-Stimulated Nuclear Complexing of p53 with TRβ1 or MAPK in Two Cell Lines. 293T cells were treated with T_4, 10^{-7} M, for 30 min, and nuclear extracts were immunoprecipitated with a monoclonal antibody to TRβ1, a different antibody than that used for the immunoprecipitates shown in Figure 7B. Immunoprecipitates were then immunoblotted with anti-p53, and nuclear complexing of TRβ1 and p53 was again demonstrated in T_4-treated 293T cells (Figure 8A). Complexing was inhibited by PD. These findings are comparable to those illustrated in the upper panel of Figure 7B, using two different TRβ1 and p53 antibodies for immunoprecipitation and immunoblotting, respectively.

HeLa cells do not contain a functional TR (*32*) and were used to disclose whether TR was required for p53 complexing to occur with other proteins in T_4-treated cells. In Figure 8B we demonstrate that T_4 can bring about nuclear co-immunoprecipitation of p53 with MAPK in HeLa cells, again an effect blocked by PD. These results are similar to those presented in Figure 4A from experiments in TR-replete 293T cells and indicate that TR is not required for the thyroxine effect on MAPK and p53 to be seen. These findings are consistent with our prior documentation of T_4-induced STAT1α−MAPK co-immunoprecipitation in HeLa cell nuclei (*13*).

In Vitro Phosphorylation of p53 by Activated MAPK. Serine phosphorylation of the estrogen receptor by activated MAPK has been demonstrated in vitro (*28*). We have demonstrated phosphorylation of a 102−461 amino acid fragment of TRβ1 by activated MAPK in a similar assay system (*17*). Following the method described by Kato et al. (*28*), purified activated MAPK was incubated in the presence of [γ-^{32}P]ATP with either MBP, anti-p53-immunoprecipitated recombinant GST-p53 fusion protein (GST-p53-ip), or a p53 immunoprecipitate from 293T cells not exposed to T_4. In Figure 9 the first lane shows phosphorylation of MBP by the activated MAPK. When GST-p53-ip was incubated with activated MAPK, an 80 kDa band appeared, consistent with phosphorylation of the fusion protein (lane 3), and this effect was blocked by the addition of apigenin (lane 4), a MAPK

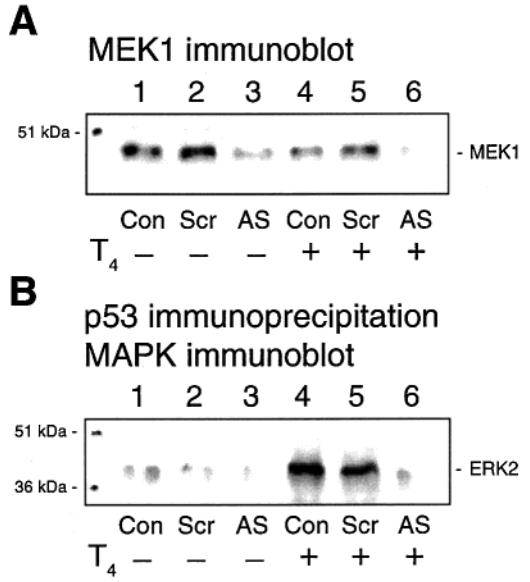

FIGURE 5: Action of thyroxine on nuclear complexing of p53 and MAPK is blocked by MEK antisense oligonucleotide transfection in 293T cells. Cells transiently transfected with antisense (AS) or scrambled (Scr) MEK oligonucleotide, or not transfected with oligonucleotide (Con), were treated with T_4, 10^{-7} M, for 30 min, and nuclear fractions were immunoprecipitated with anti-p53. Precipitates were immunoblotted with anti-ERK2. Corresponding cytosol samples were immunoblotted with anti-MEK1. (A) T_4 caused a reduction in cytosol MEK1 content in Con cells (lane 1 vs lane 4) and Scr cells (lane 2 vs lane 5). MEK1 content was reduced 78% in cells transfected with antisense MEK oligonucleotide (AS, lane 3), and after T_4 treatment minimal MEK1 was evident in cell cytosol (lane 6). (B) Lanes 1−3 show no nuclear complexing of p53 and MAPK in the absence of T_4. The hormone induced co-immunoprecipitation of p53 and MAPK in untransfected cells (Con, lane 4) and in cells transfected with scrambled MEK oligonucleotide (Scr, lane 5). Cells treated with MEK antisense oligonucleotide (AS) showed no significant nuclear complexing of p53 and MAPK after T_4 treatment (lane 6).

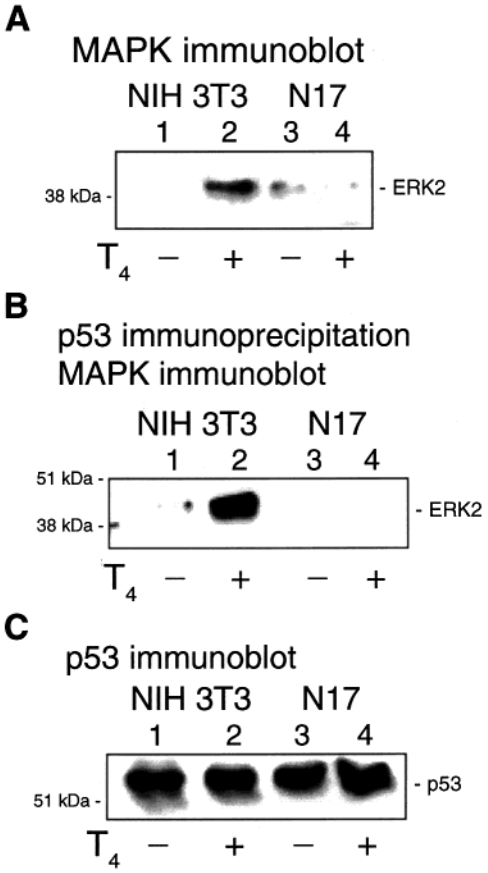

FIGURE 6: T_4 induction of p53−MAPK complex formation is not seen in dominant negative Ras cells. NIH 3T3 and corresponding dominant negative Ras (N17) cells were treated with T_4 for 30 min, and nuclei were prepared as described. (A) T_4 (10^{-7} M) caused nuclear accumulation of MAPK in NIH 3T3 cells (lane 2 compared with lane 1) but not in N17 cells (lane 4). (B) T_4 also caused nuclear complexing of p53 and MAPK in NIH 3T3 cells (lane 2), but this effect is clearly absent in N17 cells (lane 4). (C) Similar p53 content of all nuclear samples indicates that the changes seen in (B) were not the result of changes in p53 abundance.

inhibitor (*33*). Another source of p53, an immunoprecipitate from nuclei of untreated 293T cells, was exposed to activated MAPK, and a 53 kDa band is seen in lane 5, consistent with the phosphorylation of cellular p53 in vitro. Again, apigenin inhibited the MAPK effect.

Cellular Abundance of c-Jun in Resveratrol- and T_4-Treated Cells. Resveratrol (RV), a naturally occurring stilbene (*34*), induced accumulation of c-Jun protein in 293T cells (Figure 10). This confirmed preliminary studies of this resveratrol effect in other cell lines.[2] Measurement of c-Jun in resveratrol-treated cells allowed us to infer transcriptional activity of p53. In 293T cells exposed to resveratrol (RV, 100 μM, for 24 h), the levels of total p53 and c-Jun increased 1.5- and 6.5-fold, respectively (Figure 10, lanes 2). PD 98059 (PD, 30 μM) inhibited the RV effect on p53 by 32% and the effect on c-Jun by 20% (lanes 4). T_4, in the absence of RV, did not affect p53 or c-Jun abundance in these cells (not shown). Coincubation of cells with RV and T_4 for 24 h decreased the abundance of p53 by 24% but decreased c-Jun content by 67% (comparing lane 5 with lane 2). T_4 suppression of c-Jun content in RV-treated cells was reversed by coincubation with PD (30 μM, lane 7) although a comparable reduction in p53 level was not seen (comparing lanes 7 and 5). Thus, T_4 caused a reduction in RV-stimulated immediate−early gene product accumulation without causing a similar reduction in p53 content. This action of T_4 was blocked by an inhibitor of the MEK−MAPK signal transduction pathway.

DISCUSSION

Results of the present studies support a role for T_4 in activation of MAPK leading to serine phosphorylation of p53. p53 has been described as a substrate for several serine kinases at multiple phosphorylation sites (*4*). Recently added to these kinases are Chk2, a checkpoint kinase (*35*), and MAPK (*12*, *36*, and present report). MAPK has been previously reported to phosphorylate several serines of p53 (*12*, *36*), including serine-15 (*36*), although thyroid hormone has not been implicated in the modulation of such MAPK activity. Phosphorylation has been shown to enhance transcriptional activity of p53 (*4*, *37*) and to decrease its transcriptional activity (*38*). It is not clear why there are a variety of kinases, including T_4-directed MAPK activity, that serine-phosphorylate this protein. Existence of multiple phosphorylation sites may make p53 more specific or selective for promoter sites (*39*).

Our studies provide a new mechanism by which serine phosphorylation of this oncogene suppressor protein may be

[2] H.-Y. Lin, unpublished observations.

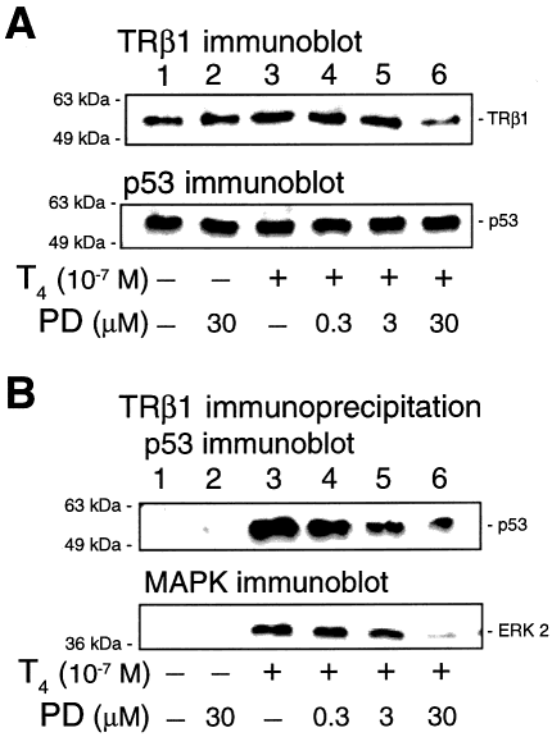

FIGURE 7: Stimulation by T_4 of nuclear co-immunoprecipitation of TRβ1 and p53 in 293T cells is inhibited by PD 98059. Cells were incubated with T_4 (10^{-7} M), with or without PD 98059 (PD, 0.3, 3, or 30 μM) as described for Figure 4. (A) Nuclear fractions were immunoblotted with monoclonal antibodies to TRβ1 or p53. Nuclear TRβ1 increased with T_4 treatment (upper panel, lane 3 compared with lane 1), and this effect was inhibited by PD, 30 μM (lane 6). The abundance of nuclear p53 did not change with either T_4 or PD treatment (lower panel). (B) Nuclear fractions were immunoprecipitated with polyclonal anti-TRβ1, and the resulting proteins were immunoblotted with anti-p53 or anti-MAPK (ERK2). T_4 induced nuclear complexing of TRβ1 and p53 (upper panel, lane 3), which was inhibited 30% and 70% by PD, 0.3 and 3 μM, respectively (upper panel, lanes 4 and 5). PD, 30 μM, had no effect in the absence of T_4 (lane 2) but blocked the T_4 effect (lane 6). Nuclear complexing of TRβ1 and MAPK in a T_4-treated sample was also evident (lower panel, lane 3), and 3 μM PD inhibited the T_4 effect by 50% as shown in lane 5.

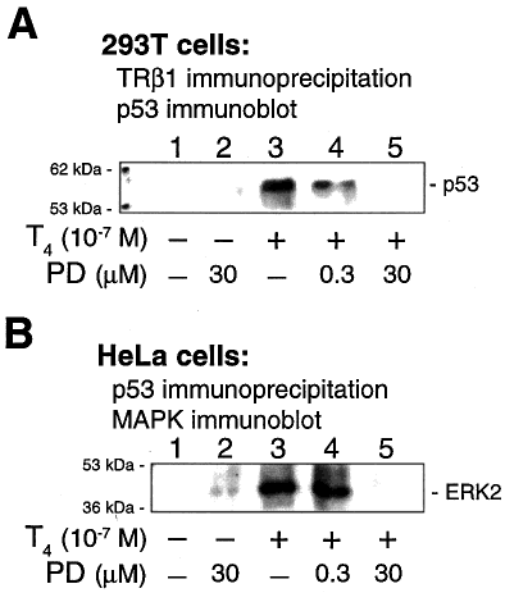

FIGURE 8: T_4-stimulated nuclear complexing of p53 with MAPK is not dependent on the presence of TRβ1 in the nucleus. (A) 293T cells with endogenous TR were incubated with T_4, with or without PD, 0.3 or 30 μM, as described for Figure 4. Nuclei were immunoprecipitated with monoclonal anti-TRβ1, and precipitates were immunoblotted with anti-p53 or anti-MAPK. T_4 caused nuclear complexing of TRβ1 and p53 (lane 3), which was inhibited by PD (lanes 4 and 5). These findings duplicate the results shown in the upper panel of Figure 7B, although the TRβ1 and p53 antibodies were different in the two studies. (B) HeLa cells, lacking functional TRβ1, were treated as in (A), the nuclei were immunoprecipitated with anti-p53, and resulting proteins were immunoblotted with anti-MAPK. T_4-induced nuclear co-immunoprecipitation of these proteins occurred in HeLa cells (lane 3), and this effect was inhibited by PD. Comparison of panel B of this figure with Figure 4A reveals similar results and indicates that the presence or absence of functional TRβ1 does not influence the T_4 effect on nuclear co-immunoprecipitation of p53 and activated MAPK.

controlled, since rapid activation of MAPK by L-thyroxine is shown here to be an upstream step in the phosphorylation of p53. In T_4-treated cells containing nuclear complexes of MAPK and p53, the p53 is found to be serine-phosphorylated, and inhibition of the MAPK cascade by PD blocks both the association of MAPK and p53 and the serine phosphorylation of the latter. There are multiple serine residues on p53, and the present studies do not identify a single specific site at which MAPK may work, although specific phosphorylation of serine 15 is demonstrated. The sequence PLS is present at amino acids 13−15 and represents a site suitable for MAPK phosphorylation (*28*). In addition, we report here that constitutively activated MAPK can, in vitro, phosphorylate a recombinant p53-GST fusion protein, as well as immunoprecipitable p53 recovered from 293T cells.

Transactivator proteins, among which are p53 and the nuclear superfamily of hormone receptors, form complexes in the cell nucleus (*18*, *19*). Hormone receptors (*40*) and transactivators such as the STAT proteins (*41*) may form homodimers or heterodimers or more complicated associations with coactivator or corepressor proteins (*42*). Additional proteins may bind to the transactivators that stabilize the nucleoprotein complex ["enhanceosome" (*43*)] assembled on DNA or, if the enhanceosome is formed away from DNA, foster its recruitment to DNA. Protein phosphorylation may importantly influence the formation and activity of these enhanceosomes (*43*). We have recently reported (*17*) that MAPK can participate in nucleoprotein complexes involving TR. p53 and the nuclear T_3 receptor TRβ1 have been reported to form a heterodimer that decreases the transcriptional activity of each protein (*18*, *19*), although a mechanism for regulation of the association has not been described. In the observations presented here, we confirm the existence of nuclear TR−p53 complexes and show that the complexes include MAPK. Further, the rapid formation of this TR−p53−MAPK complex is fostered by T_4 alone. We have recently reported that T_4 directs formation of a TR−MAPK complex and serine phosphorylation of the hormone receptor protein (*17*). A consequence of MAPK-induced serine phosphorylation of TR is dissociation of the TR and corepressor proteins (*17*). That T_4-activated MAPK is capable of serine-phosphorylating both TR and p53 is not, therefore, surprising. We have also recently described the promotion by T_4 of the action of MAPK on STAT proteins and the biological consequences that attend this action (*13*, *14*, *16*).

To determine whether T_4-promoted complexing of p53 and MAPK may affect the transcriptional activity of p53, we induced p53-dependent c-*jun* expression with resveratrol in

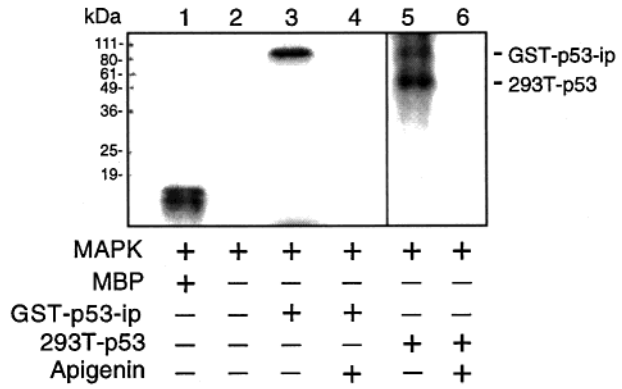

FIGURE 9: Purified activated MAPK phosphorylates recombinant p53. Recombinant human p53 (1−393) as an 80 kDa GST fusion protein (2 μg) was immunoprecipitated with anti-p53 and then resuspended and incubated for 30 min at 30 °C with activated MAPK (5 units/mL), 20 μM ATP, and 0.05 μCi of [γ-^{32}P]ATP; the resulting proteins were separated by PAGE. Also included were a sample with myelin basic protein (MBP) and MAPK as a positive control (lane 1) and a sample containing MAPK alone (lane 2). In lane 3 a phosphorylated band at 80 kDa is seen, indicating phosphorylation of GST-p53-ip by MAPK. In the lane 4 sample apigenin (25 μM) was added to GST-p53-ip and activated MAPK, and inhibition by apigenin of phosphorylation is seen. A p53 immunoprecipitate from 293T cells (293T-p53) was exposed to activated MAPK, in the absence of T_4, and a ^{32}P-labeled band consistent with nuclear p53 is seen in lane 5. Again, apigenin blocked this phosphorylation (lane 6).

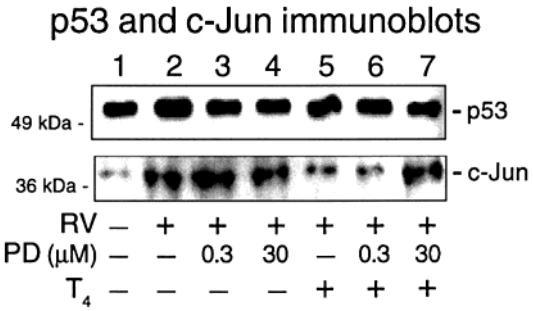

FIGURE 10: T_4 decreases abundance of c-Jun protein in resveratrol-treated cells. 293T cells were treated with 100 μM resveratrol (RV) for 24 h to induce p53 activation of c-*jun* immediate−early gene expression. There was a 1.5-fold increase in p53 with RV treatment (upper panel, lane 2) and a 6.5-fold increase in c-Jun with RV treatment, demonstrated by immunoblot (lower panel, lane 2). PD 98059 (PD) inhibited the RV effect on p53 and c-Jun content by 32% and 20%, respectively (lanes 4). In the presence of T_4 (10^{-7} M for 24 h) the effect of RV on c-Jun expression was inhibited by 67% (comparing lane 5 with lane 2, lower panel), although p53 content was reduced by only 24% (lane 5, upper panel). PD blocked this suppression by T_4 of RV-induced c-Jun expression (lower panel, lane 7 compared with lane 5). This finding is consistent with the premise that T_4, in causing serine phosphorylation of p53 by MAPK, suppresses p53 activation of c-Jun expression.

293T cells and then showed that T_4 decreased the abundance of c-Jun protein in such cells. This indicates that T_4-induced serine phosphorylation of p53 results in decreased transcriptional activity of the oncogene suppressor protein. Previous studies of serine phosphorylation of p53 from other laboratories have described increased (*4*, *37*) and decreased (*38*) transcriptional activity of p53 to result from phosphorylation.

How T_4 nongenomically activates MAPK has been documented elsewhere (*13*). It involves (1) a G-protein-coupled receptor (GPCR) that responds to T_4 in preference to T_3 at physiological concentrations of each analogue, (2) activation of protein kinase C, and (3) an intact MAPK signal transduction cascade that includes Ras, Raf-1, and MEK, as confirmed in the present studies. By means of dominant negative Ras and antisense MEK experiments, we show in the present studies that T_4 activation of MAPK and serine phosphorylation of p53 require Ras and MEK. Eicosanoids may also act via the MAPK pathway and a GPCR (*44*), illustrating again that both nonpeptide and peptide hormones may activate GPCRs (*45*).

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