Thyroid hormone promotes the phosphorylation of STAT3 and potentiates the action of epidermal growth factor in cultured cells

Hung-Yun LIN†‡, Ai SHIH†, Faith B. DAVIS*† and Paul J. DAVIS*‡
*Molecular and Cellular Medicine Program, Department of Medicine, Albany Medical College, Albany, NY 12208, U.S.A., and †Veterans Affairs Healthcare Network Upstate New York at Albany, Albany, NY 12208, U.S.A.

We have examined the effects of l-thyroxine (T₄) on the activation of signal transducer and activator of transcription 3 (STAT3) and on the STAT3-dependent induction of c-Fos expression by epidermal growth factor (EGF). T₄, at a physiological concentration of 100 nM, caused tyrosine phosphorylation and nuclear translocation (i.e. activation) of STAT3 in HeLa cells in as little as 10–20 min. Activation by T₄ of STAT3 was maximal at 30 min (15 ± 4-fold enhancement; mean ± S.E.M.) in 18 experiments. This effect was reproduced by T₄–agarose (100 nM) and blocked by CGP 41251, genistein, PD 98059 and geldanamycin, inhibitors of protein kinase C (PKC), protein tyrosine kinase (PTK), mitogen-activated protein kinase (MAPK) kinase and Raf-1 respectively. Tyrosine-phosphorylated MAPK also appeared in nuclear fractions within 10 min of treatment with T₄.

In the nuclear fraction of T₄-treated cells, MAPK immunoprecipitate also contained STAT3. The actions of T₄ were similar in HeLa and CV-1 cells, which lack thyroid hormone receptor (TR), and in TR-replete skin fibroblasts (BG-9). T₄ also potentiated the EGF-induced nuclear translocation of activated STAT1 and STAT3 and enhanced the EGF-stimulated expression of c-Fos. Hormone potentiation of EGF-induced signal transduction and c-Fos expression was inhibited by CGP 41251, geldanamycin and PD 98059. Therefore the non-genomically induced activation by T₄ of STAT3, and the potentiation of EGF by T₄, require activities of PKC, PTK and an intact MAPK pathway.

Key words: c-Fos, signal transduction, thyroxine.

INTRODUCTION

The 92 kDa signal transducer and activator of transcription (STAT3) is involved in the transduction of cytokine and growth factor signals, such as those of epidermal growth factor (EGF) [1], granulocyte colony-stimulating factor [2], erythropoietin and interleukin 3 [3] and interleukin 6 [1,4]. When stimulated by EGF, STAT3 is tyrosine-phosphorylated at residue 705 and translocates to the nucleus, either as a homodimer or as a heterodimer with the 91 kDa STAT1α, where it binds to selected EGF-responsive DNA sequences [5]. STAT3 and STAT1α both contain a C-terminal serine residue (residue 727) that is subject to phosphorylation by the mitogen-activated protein kinase (MAPK) pathway [6,7], as well as by other serine kinases [6–8].

We have previously described the potentiation of antiviral and immunomodulatory effects of interferon γ (IFN-γ) by physiological concentrations of thyroid hormone in HeLa cells [9–12], which are known to contain no functional nuclear thyroid hormone receptor (TR) [13]. The several actions of IFN-γ are known to depend on the cytokine’s stimulation of the tyrosine phosphorylation cascade involving the IFN-γ receptor, Janus kinases 1 and 2, and tyrosine phosphorylation of the 91 kDa STAT1α [5]. We have established that physiological concentrations of thyroid hormone non-genomically cause the tyrosine phosphorylation and nuclear translocation of STAT1α [14]. We therefore postulated that thyroid hormone also causes the tyrosine phosphorylation and nuclear accumulation of STAT3, thus potentiating the cellular actions of EGF. The studies reported here support an action of thyroid hormone on the activation of STAT3, leading to potentiation by thyroid hormone of EGF-induced expression of c-Fos. Studies were conducted in HeLa cells, in CV-1 cells, which also lack TR [15], and in human skin fibroblasts (BG-9), which are TR-replete.

EXPERIMENTAL

Materials

l-Thyroxine (T₄), T₄–agarose and Protein A–agarose were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and EGF from Genzyme (Cambridge, MA, U.S.A.). CGP 41251, a protein kinase C (PKC) inhibitor, was a gift from Novartis Pharma AG (Basel, Switzerland). Genistein, an inhibitor of protein tyrosine kinase (PTK), was obtained from ICN Biochemicals (Costa Mesa, CA, U.S.A.), geldanamycin from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (National Institutes of Health, Bethesda, MD, U.S.A.), and PD 98059 from Calbiochem (La Jolla, CA, U.S.A.). HeLa cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.), CV-1 cells from Dr. William W. Chin and Dr. Paul M. Yen (Brigham and Women’s Hospital, Boston, MA, U.S.A.), and BG-9 cells (human skin fibroblasts) from Dr. Harshad R. Thacore (State University of New York at Buffalo School of Medicine and Biomedical Sciences, Buffalo, NY, U.S.A.).

Cell culture and preparation of nuclear fractions

Confluent cells grown in 100 mm culture dishes were placed in 0.25% fetal bovine serum-containing Dulbecco’s modified Eagle’s medium [9] for 48 h. Fetal bovine serum was depleted of thyroid hormone by the method of Samuels et al. [16], as
modified by Weinstein et al. [17]. The concentrations of total and free endogenous T₄ in this 0.25% hormone-depleted serum-supplemented medium were 23 pM and 10 fM respectively; the free T₄ concentration was below the limits of measurement [11]. After 48 h, cells were incubated with hormone 3,5,3'-triiodothyronine, EGF and/or other reagents for various periods as indicated. Stock solutions containing 100 µM T4 were prepared in 0.04 M KOH/4% (v/v) propylene glycol, and dilutions were made to a final concentration of 100 nM total T4 and 100 pM free T4 in culture medium. This physiological concentration of T4 was used in all experiments. T4-agarose was provided as a suspension in 0.5 M NaCl, containing approx. 6 mM T4, and was diluted in culture medium to a final T4 concentration of 100 nM. DMSO (0.1%, v/v) was the solvent for all inhibitors. This concentration of DMSO caused minimal activation of signal-transducing proteins.

Cell harvesting and preparation of nuclear extracts were then performed as follows: cultures were washed twice with ice-cold PBS, then lysed in hypotonic buffer [20 mM HEPES (pH 7.9)/10 mM KCl/0.1 mM Na₃VO₄/1 mM EDTA/10% (v/v) glycerol/1 mM PMSF/3 µg/ml apronitin/1 mg/ml peptatin/20 mM NaF/1 mM dithiothreitol] with 0.2% (v/v) Nonidet P40 on ice for 10 min. After centrifugation at 4°C and 14000 g for 1 min, supernatants were collected as cytoplasmic extracts. Nuclear extracts were prepared with the method of Wen et al. [6] by resuspension of the crude nuclei in high-salt buffer [hypotonic buffer containing 20% (v/v) glycerol and 420 mM NaCl] at 4°C for 30 min. The supernatants were collected after centrifugation at 4°C and 14000 g for 10 min.

Immunoprecipitation and immunoblotting

After normalization of protein content, immunoprecipitation was performed with polyclonal anti-phosphotyrosine (Transduction Laboratories, Lexington, KY, U.S.A.). After incubation overnight at 4°C with rocking, Protein A–agarose was added and samples remained for 1 h at 4°C with rocking. After two washes with hypotonic buffer containing 0.2% (v/v) Nonidet P40, immunoprecipitates were eluted with 2× sample buffer and proteins separated by discontinuous SDS/PAGE. In selected studies, immunoprecipitation was performed with monoclonal anti-MAPK (ERK2; Transduction) and Protein A–agarose. After PAGE, proteins were transferred to Immobilon membranes (Millipore Corporation, Bedford, MA, U.S.A.) by electroblotting. After being blocked with 5% (v/v) milk in Tris-buffered saline containing 0.1% (v/v) Tween, membranes were incubated with 1:1000 monoclonal anti-STAT1a (Transduction) overnight, 1:1000 monoclonal anti-STAT3 (Transduction), or 1:1000 monoclonal anti-MAPK (Transduction). Western blotting of nuclear fractions for expression of c-Fos was accomplished with 1:750 rabbit anti-c-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The secondary antibodies used in immunoblotting were either rabbit anti-mouse IgG (1:1000) or goat anti-rabbit IgG (1:1000) (Dako Corporation, Carpinteria, CA, U.S.A.). Immunoblots were detected by chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.) and analysed by digital imaging.

RESULTS

Promotion by T₄ of phosphorylation and nuclear uptake of STAT3

HeLa cells were treated with 100 nM T₄ for up to 180 min. The resulting total and free T₄ concentrations in the treatment medium were 100 nM and 100 pM respectively. Figure 1(A) shows the accumulation in nuclear fractions of tyrosine-phosphorylated STAT3 (activated STAT3) during exposure to T₄ from 10–180 min. In this representative blot, the enhancement of STAT3 (mean ± S.E.M.) after 30, 60, 120 and 180 min, as compared with untreated control cells, was 11±4, 3±0.8, 2±0.7 and 3±0.1 respectively. In experiments with HeLa, BG-9 and CV-1 cells, the fold increases in STAT3 activation with T₄ treatment for 30 min, as compared with untreated control cells, were 15±4 (n = 18 experiments), 23±6 (n = 8) and 5±2 (n = 3) respectively. A similar pattern of STAT1 activation in HeLa cells is seen in Figure 1(B), with 17-fold enhancement occurring at 30 min. In five experiments in HeLa cells there was 8±3-fold, 5±1-fold, 7±5-fold and 6±4-fold stimulation of STAT1 activation by T₄ after 30, 60, 120 and 180 min respectively.

In Figure 1(C) the effect of T₄–agarose on STAT3 activation in CV-1 cells is shown. T₄–agarose added to cells for 30 min stimulated the activation of STAT3 (2.7-fold; Figure 1C, lane 3) in this study. T₄–agarose containing 100 nM T₄, added for 30 min, also caused activation of STAT3 (2.5-fold; Figure 1C, lane 4) and a preparation of T₄–agarose washed three times with PBS and reconstituted at the same T₄–agarose concentration also activated STAT3 (2.7-fold; Figure 1C, lane 5). Protein A–agarose, added to the cell medium at the same agarose concentration as with T₄–agarose, had no effect on tyrosine phosphorylation and
were treated for 70 min with CGP 41251 (1–100 nM), an inhibitor that was not present in these experiments.

and this activation was inhibited

caused a 26-fold activation of STAT3 in BG-9 cells in 30 min, and the inhibition by CGP 41251 of T₄-induced STAT3 activation was 61±0.1%, 74±18% and 80±10% at 5, 50 and 100 nM respectively. (B) Shown is a 43-fold activation of STAT3 by T₄ in BG-9 cells, and 50% inhibition of that effect by 5 µg/ml genistein. In three HeLa cell experiments there was a 20±4-fold activation of STAT3 by T₄, and there were 53±25%, 89±10%, 94±5% and 99±0.4% inhibitions of T₄-induced STAT3 activation by 1, 5, 50 and 100 µg/ml genistein respectively.

nuclear translocation of STAT3 (Figure 1C, lane 2). These results indicate that T₄ exerts its effect through contact with a cell-surface site and that transport of T₄ into the cytoplasm is not necessary for hormone action. Similar results have been obtained in studies of the activation of STAT1α by T₄-agarose [14]. EGF was not present in these experiments.

Samples of TR-replete human skin fibroblasts (BG-9 cells) were treated for 70 min with CGP 41251 (1–100 nM), an inhibitor of the conventional, Ca²⁺-requiring PKC isoforms PKC-α, PKC-βII, PKC-γII and PKC-γ [18]; T₄ was added during the last 30 min. In the experiment shown in Figure 2(A), thyroid hormone caused a 26-fold activation of STAT3 in BG-9 cells in 30 min, and this activation was inhibited 14%, 81%, and 80% by 1, 50 and 100 nM CGP 41251 respectively. Genistein, a PTK inhibitor, also inhibited the effect of thyroid hormone. In the BG-9 cell study shown in Figure 2(B) there was a 43-fold activation of STAT3 by T₄ that was inhibited 39%, 50%, 76% and 98% by 1, 5, 50 and 100 µg/ml genistein respectively. Reported IC₅₀ values for CGP 41251 and genistein are 30 nM [18] and 10 µg/ml [19] respectively. These results provide evidence that the activation of STAT3 by T₄ is dependent on activities of PKC as well as PTK.

Association of STAT3 and MAPK in nuclear fractions of T₄-treated cells

We have previously shown that treatment of HeLa cells with 100 nM T₄ leads to the complexing of activated MAPK and STAT1α in MAPK immunoprecipitates [20]. To determine whether MAPK also complexed with STAT1 on exposure of cells to T₄, we treated HeLa cells with T₄ for 10–120 min. Nuclear samples were then immunoprecipitated with antibody against MAPK; the immunoprecipitated proteins were separated by PAGE and immunoblotted with anti-phosphotyrosine, anti-STAT3 or anti-STAT1α. In Figure 3(A) an increase in tyrosine-phosphorylated MAPK induced by T₄ is seen in a representative immunoblot, with a maximal effect at 10 and 30 min (5-fold and 4.5-fold increases respectively). Immunoblotting of immunoprecipitated samples with anti-STAT3 (Figure 3B) or anti-STAT1α (Figure 3C) revealed that these signal transduction proteins co-precipitated with MAPK in the nuclear fractions during the same period. In the representative experiment shown in Figure 3, 2-fold and 5-fold enhancements respectively of STAT3 and STAT1α complexing with MAPK occurred in cells treated with T₄ for 30 min.

Because of the association of MAPK with STAT1α and STAT3, the contribution of the MAPK pathway to the activation and nuclear translocation of STAT3 in the absence of EGF was examined. PD 98059 is an inhibitor of MAPK kinase (MEK), with an IC₅₀ of 2 µM and a maximal effect at 10–100 µM [21]. HeLa cells were treated with T₄ with or without this inhibitor, for 30 min. In the experiment shown in Figure 4(A), T₄-induced tyrosine phosphorylation and nuclear translocation of STAT3 were inhibited 48%, and 88% by 0.3 and 30 µM PD 98059 respectively. Parallel experiments were conducted with geldanamycin, a benzoquinone ansamycin that at 1–2 µM binds heat shock protein 90, thus destabilizing Raf-1 and inhibiting the MAPK cascade [22]. BG-9 cells were treated with geldanamycin for 16 h and T₄ for the last 30 min. In the experiment shown in Figure 4(B), T₄-induced STAT3 activation (5.2-fold) was inhibited 84% and 98% by 0.5 and 10 µM geldanamycin respectively. These studies were performed in the absence of EGF.

Potentiation by T₄ of transduction of EGF signal

The interaction between EGF and T₄ was then investigated. HeLa cells were treated with 10–100 ng/ml EGF, with or without...
T	extsubscript{4}. In Figure 5 nuclear accumulations of activated STAT3, STAT1	extsubscript{x} and MAPK are seen with 100 nM T	extsubscript{4} (Figure 5, lanes 2: 6-fold, 3-fold and 14-fold increases over control cells respectively, in representative blots), with 10 ng/ml EGF (lanes 3: 2-fold, 3-fold and 3-fold increases respectively) and with 100 ng/ml EGF (lanes 4: 6-fold, 4-fold and 11-fold increases respectively). T	extsubscript{4} potentiated the nuclear translocation of the three tyrosine-phosphorylated signal transduction proteins in the presence of a submaximal concentration of EGF (10 ng/ml) (comparing lanes 3 and 5: 5-fold, 1.3-fold and 5-fold increases over the effect of EGF alone on STAT3, STAT1	extsubscript{x} and MAPK respectively). T	extsubscript{4} potentiated the effect of 100 ng/ml EGF on STAT3 activation (2-fold increase; Figure 5, lane 4 compared with lane 6), but not on STAT1	extsubscript{x} activation. There was a diminution in nuclear activated MAPK in cells treated with T	extsubscript{4} and EGF (100 rather than 10 ng/ml), suggesting the early stimulation of MAPK activation by the higher EGF concentration, followed by enhanced phosphatase activity and/or the return of MAPK to the cytoplasm.

The effect of MAPK pathway inhibition on STAT3 activation in response to 10 ng/ml EGF, with or without T	extsubscript{4}, was also examined. Figure 6(A) demonstrates an increased nuclear accumulation of tyrosine-phosphorylated STAT3 in BG-9 cells exposed to either T	extsubscript{4} or 10 ng/ml EGF (lanes 2 and 3 respectively) and 5-fold potentiation by T	extsubscript{4} of the EGF effect (lane 6) in a representative blot. Geldanamycin (0.5 and 10 μM) in fact enhanced the effect of EGF alone on STAT3 activation (2-fold and 3-fold increases respectively; Figure 6A, lanes 4 and 5), but partly inhibited the T	extsubscript{4} potentiation of EGF action on STAT3 in a dose-dependent manner (51% and 71% inhibitions respectively; lanes 7 and 8 compared with lane 6).

HeLa cells were treated with PD 98059 in the presence of T	extsubscript{4}, EGF or both. In the experiment shown in Figure 6(B), T	extsubscript{4} and EGF separately activated STAT3 4-fold and 2-fold respectively, and T	extsubscript{4} enhanced the EGF effect 11-fold. PD 98059 (0.3 and 30 μM) enhanced EGF-induced STAT3 activation 1.3-fold and 1.6-fold respectively (Figure 6B, lanes 4 and 5). However, the inhibition of thyroid hormone potentiation was 76% and 99% with the two inhibitor concentrations (Figure 6B, lanes 7 and 8 compared with lane 6). When HeLa cells were treated with PD 98059 for 16 h instead of 1 h, there was an inhibition of the EGF effect as well as of hormone potentiation (results not shown).

**Actions of T	extsubscript{4} and EGF on c-fos expression**

With the knowledge that T	extsubscript{4} potentiated signal transduction pathways involved in the actions of EGF, we investigated whether thyroid hormone potentiated the action of EGF on c-fos gene expression. HeLa cells were treated with 100 ng/ml EGF, with or without 100 nM T	extsubscript{4}, for 1 h. Nuclear fractions were prepared and immunoblotted for the presence of c-Fos protein. EGF increased the expression of c-Fos 23 ± 12-fold as compared with control cells in the three experiments shown (lanes 3), and the effect was potentiated by the addition of T	extsubscript{4} to the EGF incubation (Figure 7, lanes 4; 1.6 ± 0.2-fold enhancement).

To study the role of the MAPK pathway and PKC activity in T	extsubscript{4} potentiation of EGF-induced c-Fos expression, HeLa cells were pretreated with either geldanamycin, PD 98059 or CGP 41251 for 16 h, after which EGF (100 ng/ml) and/or T	extsubscript{4} were added for 1 h. Geldanamycin, at a low concentration of 0.5 μM, partly blocked the effect of EGF (Figure 7A, 24%, inhibition, comparing lanes 3 and 5) and fully inhibited c-Fos induction in the presence of EGF and T	extsubscript{4} (99.7%, inhibition, lane 6). A similar experiment with PD 98059 demonstrated that the T	extsubscript{4} potentiation of EGF-induced c-Fos was partly blocked by a low inhibitor concentration (Figure 7B, 39% inhibition, comparing lanes 4 and 6), whereas the action of EGF alone was not appreciably affected (lanes 3 and 5). In a study with CGP 41251 shown in Figure 7(C), the T	extsubscript{4} potentiation of EGF action was inhibited...
Figure 6  Geldanamycin and PD 98059 inhibit STAT3 activation by EGF and T₄

(A) BG-9 cells were treated for 16 h with 0.5 or 10 μM geldanamycin (GEL), then 10 ng/ml EGF and/or 100 nM T₄, for 30 min. T₄ (lane 2) and EGF (lane 3) both stimulated the activation of STAT3 in this representative blot, and T₄ potentiated the effect 5-fold (lane 6). In three BG-9 cell experiments T₄ potentiated the effect of EGF 3±1-fold, and geldanamycin enhanced the effect of EGF alone (1.3±0.3-fold and 1.9±0.7-fold increases with 0.5 and 10 μM geldanamycin respectively). In contrast, the two concentrations of geldanamycin inhibited BG-9 cell experiments T₄ potentiated the effect of EGF 3-fold in the same three experiments, and PD 98059 (0.3 and 30 μM) blocked the T₄ potentiation of the EGF effect by 63±7% and 85±8%, respectively.

77% by 2.5 nM CGP 41251 (comparing lanes 4 and 6), whereas cells treated with EGF alone were inhibited 60% in the experiment shown (comparing lanes 3 and 5). Additional studies were performed with 10 ng/ml EGF, and showed 8-fold enhancement of c-Fos expression by EGF, 2-fold potentiation of the EGF effect by T₄ in three experiments, and, as with the higher EGF concentration, greater inhibition of c-Fos expression in the presence of T₄ than in its absence. These findings support our premise that T₄ potentiation of the EGF effect is primarily dependent on the activity of the MAPK pathway, and that the effect of EGF, alone, does not depend to the same extent on this pathway.

DISCUSSION

We have shown that T₄ in physiological concentrations stimulates the tyrosine phosphorylation and nuclear translocation of STAT1α and enhances the activation of STAT1α induced by IFN-γ [14]. This thyroid hormone effect is blocked by inhibitors of PTK and PKC [14]; physiological concentrations of T₄ cause a greater response than physiological concentrations of T₃ [14].

In the present studies we have extended the role of T₄ in signal transduction to include the phosphorylation of STAT3. STAT3 also requires tyrosine phosphorylation (at residue 705, as compared with residue 701 for STAT1α) for activation and nuclear translocation and is susceptible to phosphorylation at Serine-727.

77% by 2.5 nM CGP 41251 (comparing lanes 4 and 6), whereas cells treated with EGF alone were inhibited 60% in the experiment shown (comparing lanes 3 and 5). Additional studies were performed with 10 ng/ml EGF, and showed 8-fold enhancement of c-Fos expression by EGF, 2-fold potentiation of the EGF effect by T₄ in three experiments, and, as with the higher EGF concentration, greater inhibition of c-Fos expression in the presence of T₄ than in its absence. These findings support our premise that T₄ potentiation of the EGF effect is primarily dependent on the activity of the MAPK pathway, and that the effect of EGF, alone, does not depend on the same extent on this pathway.

77% by 2.5 nM CGP 41251 (comparing lanes 4 and 6), whereas cells treated with EGF alone were inhibited 60% in the experiment shown (comparing lanes 3 and 5). Additional studies were performed with 10 ng/ml EGF, and showed 8-fold enhancement of c-Fos expression by EGF, 2-fold potentiation of the EGF effect by T₄ in three experiments, and, as with the higher EGF concentration, greater inhibition of c-Fos expression in the presence of T₄ than in its absence. These findings support our premise that T₄ potentiation of the EGF effect is primarily dependent on the activity of the MAPK pathway, and that the effect of EGF, alone, does not depend on the same extent on this pathway.

77% by 2.5 nM CGP 41251 (comparing lanes 4 and 6), whereas cells treated with EGF alone were inhibited 60% in the experiment shown (comparing lanes 3 and 5). Additional studies were performed with 10 ng/ml EGF, and showed 8-fold enhancement of c-Fos expression by EGF, 2-fold potentiation of the EGF effect by T₄ in three experiments, and, as with the higher EGF concentration, greater inhibition of c-Fos expression in the presence of T₄ than in its absence. These findings support our premise that T₄ potentiation of the EGF effect is primarily dependent on the activity of the MAPK pathway, and that the effect of EGF, alone, does not depend on the same extent on this pathway.

77% by 2.5 nM CGP 41251 (comparing lanes 4 and 6), whereas cells treated with EGF alone were inhibited 60% in the experiment shown (comparing lanes 3 and 5). Additional studies were performed with 10 ng/ml EGF, and showed 8-fold enhancement of c-Fos expression by EGF, 2-fold potentiation of the EGF effect by T₄ in three experiments, and, as with the higher EGF concentration, greater inhibition of c-Fos expression in the presence of T₄ than in its absence. These findings support our premise that T₄ potentiation of the EGF effect is primarily dependent on the activity of the MAPK pathway, and that the effect of EGF, alone, does not depend on the same extent on this pathway.

77% by 2.5 nM CGP 41251 (comparing lanes 4 and 6), whereas cells treated with EGF alone were inhibited 60% in the experiment shown (comparing lanes 3 and 5). Additional studies were performed with 10 ng/ml EGF, and showed 8-fold enhancement of c-Fos expression by EGF, 2-fold potentiation of the EGF effect by T₄ in three experiments, and, as with the higher EGF concentration, greater inhibition of c-Fos expression in the presence of T₄ than in its absence. These findings support our premise that T₄ potentiation of the EGF effect is primarily dependent on the activity of the MAPK pathway, and that the effect of EGF, alone, does not depend on the same extent on this pathway.

77% by 2.5 nM CGP 41251 (comparing lanes 4 and 6), whereas cells treated with EGF alone were inhibited 60% in the experiment shown (comparing lanes 3 and 5). Additional studies were performed with 10 ng/ml EGF, and showed 8-fold enhancement of c-Fos expression by EGF, 2-fold potentiation of the EGF effect by T₄ in three experiments, and, as with the higher EGF concentration, greater inhibition of c-Fos expression in the presence of T₄ than in its absence. These findings support our premise that T₄ potentiation of the EGF effect is primarily dependent on the activity of the MAPK pathway, and that the effect of EGF, alone, does not depend on the same extent on this pathway.

77% by 2.5 nM CGP 41251 (comparing lanes 4 and 6), whereas cells treated with EGF alone were inhibited 60% in the experiment shown (comparing lanes 3 and 5). Additional studies were performed with 10 ng/ml EGF, and showed 8-fold enhancement of c-Fos expression by EGF, 2-fold potentiation of the EGF effect by T₄ in three experiments, and, as with the higher EGF concentration, greater inhibition of c-Fos expression in the presence of T₄ than in its absence. These findings support our premise that T₄ potentiation of the EGF effect is primarily dependent on the activity of the MAPK pathway, and that the effect of EGF, alone, does not depend on the same extent on this pathway.

77% by 2.5 nM CGP 41251 (comparing lanes 4 and 6), whereas cells treated with EGF alone were inhibited 60% in the experiment shown (comparing lanes 3 and 5). Additional studies were performed with 10 ng/ml EGF, and showed 8-fold enhancement of c-Fos expression by EGF, 2-fold potentiation of the EGF effect by T₄ in three experiments, and, as with the higher EGF concentration, greater inhibition of c-Fos expression in the presence of T₄ than in its absence. These findings support our premise that T₄ potentiation of the EGF effect is primarily dependent on the activity of the MAPK pathway, and that the effect of EGF, alone, does not depend on the same extent on this pathway.

77% by 2.5 nM CGP 41251 (comparing lanes 4 and 6), whereas cells treated with EGF alone were inhibited 60% in the experiment shown (comparing lanes 3 and 5). Additional studies were performed with 10 ng/ml EGF, and showed 8-fold enhancement of c-Fos expression by EGF, 2-fold potentiation of the EGF effect by T₄ in three experiments, and, as with the higher EGF concentration, greater inhibition of c-Fos expression in the presence of T₄ than in its absence. These findings support our premise that T₄ potentiation of the EGF effect is primarily dependent on the activity of the MAPK pathway, and that the effect of EGF, alone, does not depend on the same extent on this pathway.

77% by 2.5 nM CGP 41251 (comparing lanes 4 and 6), whereas cells treated with EGF alone were inhibited 60% in the experiment shown (comparing lanes 3 and 5). Additional studies were performed with 10 ng/ml EGF, and showed 8-fold enhancement of c-Fos expression by EGF, 2-fold potentiation of the EGF effect by T₄ in three experiments, and, as with the higher EGF concentration, greater inhibition of c-Fos expression in the presence of T₄ than in its absence. These findings support our premise that T₄ potentiation of the EGF effect is primarily dependent on the activity of the MAPK pathway, and that the effect of EGF, alone, does not depend on the same extent on this pathway.

77% by 2.5 nM CGP 41251 (comparing lanes 4 and 6), whereas cells treated with EGF alone were inhibited 60% in the experiment shown (comparing lanes 3 and 5). Additional studies were performed with 10 ng/ml EGF, and showed 8-fold enhancement of c-Fos expression by EGF, 2-fold potentiation of the EGF effect by T₄ in three experiments, and, as with the higher EGF concentration, greater inhibition of c-Fos expression in the presence of T₄ than in its absence. These findings support our premise that T₄ potentiation of the EGF effect is primarily dependent on the activity of the MAPK pathway, and that the effect of EGF, alone, does not depend on the same extent on this pathway.
it is the activation of MAPK by T$_4$ that is responsible for the Serine-727 phosphorylation of STAT3 and that this effect contributes significantly to the potentiation of EGF action by T$_4$.

It is apparent that MAPK and STAT3 are associated in the nuclear fraction of cells exposed to thyroid hormone. That STAT proteins might form complexes with non-STAT proteins has been shown by others in the case of ISGF3, a STAT1–STAT1β–STAT2 complex that also includes a DNA-binding protein (p48) [5]. In the STAT3–MAPK complex that we describe, however, the non-STAT protein (MAPK) has the presumptive function of phosphorylating a serine residue on STAT3.

The foregoing discussion has dealt with the serine phosphorylation of STAT3 and STAT1α. What is the mechanism by which T$_4$ can promote the tyrosine phosphorylation of these signal transduction proteins? Although MAPK itself is a serine-threonine kinase, it is activated by MEK, a tyrosine kinase thought to act exclusively on MAPK. PD 98059 inhibits MEK and in our studies it blocked the T$_4$-directed phosphorylation and nuclear uptake of STAT3 as well as the potentiation by T$_4$ of EGF-induced c-Fos expression. Geldanamycin, an upstream inhibitor of the MAPK pathway at Raf-1, produced a similar inhibition. These results support the possibility that MEK has a substrate alternative to MAPK, namely STAT3.

We have shown here that the inhibition of PKC blocks the activation (tyrosine phosphorylation) of STAT3 that is induced by T$_4$. It is known that PKC-α can activate the MAPK pathway [24] and we have reported that thyroid hormone can activate PKC [25]. We have also implicated PKC in non-genomic effects of T$_4$, that result in the potentiation of actions of IFN-γ [10–12, 14]. We propose that the ability of CGP 41251, an inhibitor of conventional PKC isoforms α, β, and γ, to block T$_4$ hormone’s activation of STAT3 is the result of inhibition of hormone activation, via PKC, of the MEK–MAPK pathway, probably at Raf-1. This results in inhibition of the action of MEK on the tyrosine phosphorylation of STAT3.

In other studies of signal transduction, we have shown that tetraiodothyroacetic acid blocks the activation of STAT1α by iodothyronine [14], but that tetraiodothyroacetic acid itself is not an agonist in such activation. We have also reported that iodothyroacetic acid inhibits the binding of T$_4$ to human erythrocyte membranes [26]. That agarose–T$_4$ represept the action of T$_4$, in the present studies also supports the hypothesis that T$_4$ stimulates signal transduction through an interaction with a plasma membrane-binding site. We have established that nuclear TR is not involved in kinase activation, because the actions of T$_4$ on STAT proteins and MAPK have been demonstrated in cells that lack functional TR (HeLa and CV-1). The signal transduction sequence in the hormonally directed activation of STAT3 is thus thought to be: (1) binding of hormone at the cell surface, (2) activation of PKC, (3) activation of the MAPK pathway, including MEK, (4) tyrosine phosphorylation of STAT3 by MEK, and (5) MEK-directed activation of MAPK and serine phosphorylation of STAT3.

Despite the ability of iodothyronines in the absence of EGF to induce the nuclear uptake of phosphorylated STAT1α and STAT3, thyroid hormone alone does not reproduce either the biological actions of EGF, such as c-fos gene expression, or the antiviral action of IFN-γ [9–11]. This probably reflects the primacy in initiation of signal transduction via the IFN-γ receptor, or the EGF receptor and its endogenous PTK activity, in contrast with the effect of T$_4$ on signal transduction that is mediated by MAPK and, presumably, by MEK. The redundancy within the STAT signalling pathway has been established by serine phosphorylation that is complementary to tyrosine phosphorylation to achieve maximal gene activation by STAT proteins [6]. We have shown this complementary effect in the activation of STAT1α when T$_4$ potentiates the antiviral [11,27] or immunomodulatory [14] activities of IFN-γ and, in the present studies, with the potentiation by thyroid hormone of the EGF-induced activation of STAT3 and expression of c-Fos protein.

The actions of thyroid hormone on STAT proteins are not universal within the STAT family. For example, the nuclear translocation of STAT2 is unaffected by T$_4$ (H.-Y. Lin, unpublished work). However, the present studies involving STAT3 raise the possibility that the activities of other cytokines or growth factors that require STAT3 activation, or that of STAT1α, might be enhanced by the signal-transducing effects of thyroid hormone.

This work was supported in part by the Office of Research Development, Medical Research Service, Department of Veterans Affairs (to P.J.D.), and by a grant from the Candace King Weir Foundation.

REFERENCES

16 Samuels, H. H., Stanley, F. and Casanova, J. (1979) Endocrinology (Baltimore) 105, 80–85
17 Weinstein, S. P., Watts, J., Graves, P. N. and Haber, R. S. (1990) Endocrinology (Baltimore) 126, 1421–1429
20 Davis, F., Gordindier, J., Lin, H.-Y., Martino, L. and Davis, P. (1997) Thyroid 7 (suppl. 1), S-109
27 Davis, F., Lin, H.-Y., Martino, L. and Davis, P. J. (1997) Cytokine 9, 926