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

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We have examined the effects of L-thyroxine (T_4) 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). T_4 , 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 T_4 of STAT3 was maximal at 30 min (15 ± 4 -fold enhancement; mean $\pm$ S.E.M.) in 18 experiments. This effect was reproduced by T_4 -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 T_4 .

In the nuclear fraction of T_4 -treated cells, MAPK immunoprecipitate also contained STAT3. The actions of T_4 were similar in HeLa and CV-1 cells, which lack thyroid hormone receptor (TR), and in TR-replete skin fibroblasts (BG-9). T_4 also potentiated the EGF-induced nuclear translocation of activated STAT1 α 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 T_4 of STAT3, and the potentiation of EGF by T_4 , 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 STAT1 α , where it binds to selected EGF-responsive DNA sequences [5]. STAT3 and STAT1 α 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 STAT1 α [5]. We have established that physiological concentrations of thyroid hormone non-genomically cause the tyrosine phosphorylation and nuclear translocation of STAT1 α [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 (T_4), T_4 -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 Womens' 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

Abbreviations used: EGF, epidermal growth factor; IFN- γ , interferon γ ; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PKC, protein kinase C; PTK, protein tyrosine kinase; STAT1 α and STAT3, signal transducers and activators of transcription 1 α and 3; T_4 , L-thyroxine; TR, thyroid hormone receptor.

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modified by Weinstein et al. [17]. The concentrations of total and free endogenous T_4 in this 0.25% hormone-depleted serum-supplemented medium were 23 pM and 10 fM respectively; the free T_3 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 μ M T_4 were prepared in 0.04 M KOH/4% (v/v) propylene glycol, and dilutions were made to a final concentration of 100 nM total T_4 and 100 pM free T_4 in culture medium. This physiological concentration of T_4 was used in all experiments. T_4 -agarose was provided as a suspension in 0.5 M NaCl, containing approx. 6 mM T_4 , and was diluted in culture medium to a final T_4 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 Na_3VO_4 /1 mM EDTA/10% (v/v) glycerol/1 mM PMSF/3 μ g/ml aprotinin/1 mg/ml pepstatin/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 $\times$ 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-STAT1 α (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 T_4 of phosphorylation and nuclear uptake of STAT3

HeLa cells were treated with 100 nM T_4 for up to 180 min. The resulting total and free T_4 concentrations in the treatment medium were 100 nM and 100 pM respectively. Figure 1(A) shows the accumulation in nuclear fractions of tyrosine-phos-

Figure 1 T_4 causes tyrosine phosphorylation and nuclear translocation of STAT3 and STAT1 α

HeLa cells were treated with 100 nM T_4 for the durations indicated. Nuclear extracts were prepared and immunoprecipitated with anti-phosphotyrosine antibody. The immunoprecipitated proteins were separated by PAGE and immunoblotted with antibody against STAT3 or STAT1 α . The time-dependent nuclear accumulation of STAT3 (A) and STAT1 α (B) in HeLa cells is seen in representative blots. CV-1 cells were treated for 30 min with 100 nM or 100 nM T_4 bound to agarose. (C) Nuclear accumulation of activated STAT3 in CV-1 cells treated for 30 min with T_4 (lane 3 of this representative blot; 6 ± 3 -fold increase in two experiments). T_4 -agarose (T_4 -ag) also activated STAT3 (lane 4; 5 ± 2 -fold increase in two experiments) as did T_4 -agarose that had been previously washed three times with PBS (lane 5; 7 ± 5 -fold increase in two experiments). Cells treated with Protein A-agarose demonstrated no activation of STAT3 (pA-ag, lane 2).

phorylated STAT3 (activated STAT3) during exposure to T_4 for 10–180 min. In this representative blot, the enhancement of STAT3 was 15-fold in 30 min, as compared with untreated control cells. In two experiments the fold increases in activated STAT3 (means $\pm$ S.E.M.) after 30, 60, 120 and 180 min, as compared with untreated control samples, were 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 T_4 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 α 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 α activation by T_4 after 30, 60, 120 and 180 min respectively.

In Figure 1(C) the effect of T_4 -agarose on STAT3 activation in CV-1 cells is shown. T_4 added to cells for 30 min stimulated the activation of STAT3 (2.7-fold; Figure 1C, lane 3) in this study. T_4 -agarose containing 100 nM T_4 , added for 30 min, also caused activation of STAT3 (2.5-fold; Figure 1C, lane 4) and a preparation of T_4 -agarose washed three times with PBS and reconstituted at the same T_4 -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 T_4 -agarose, had no effect on tyrosine phosphorylation and

Figure 2 Effect of PKC and PTK inhibitors on T₄ activation of STAT3

BG-9 cells were treated for 70 min with 1–100 nM CGP 41251 (CGP) or 1–100 μg/ml genistein (GEN); 100 nM T₄ was added for the last 30 min of that period. T₄-induced tyrosine phosphorylation and nuclear translocation of STAT3 are seen in lanes 2 of two representative blots. **(A)** T₄ activated STAT3 26-fold in the blot shown. In three HeLa cell experiments T₄ activated STAT3 10 ± 7-fold 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-βI, PKC-βII 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 STAT3 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

Figure 3 Co-immunoprecipitation of MAPK with STAT3 and STAT1α in HeLa cells treated with T₄ for 10–120 min

Nuclear extracts of cells were immunoprecipitated with anti-MAPK antibody; the immunoprecipitated proteins were separated by PAGE. **(A)** T₄ stimulated the nuclear accumulation of tyrosine-phosphorylated MAPK, with maximal effects at 10 and 30 min (4 ± 0.4-fold and 7 ± 3-fold increases in two experiments). STAT3 **(B)** and STAT1α **(C)** co-precipitated with MAPK, with a maximal effect in the same period. The greatest increase of STAT3 occurred in 30 min (2 ± 0.1-fold in three experiments) and that of STAT1α also occurred in 30 min (4 ± 1-fold in two experiments).

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.

Potential 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_4 . In Figure 5 nuclear accumulations of activated STAT3, STAT1 α and MAPK are seen with 100 nM T_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_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 α and MAPK respectively). T_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 α activation. There was a diminution in nuclear activated MAPK in cells treated with T_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_4 , was also examined. Figure 6(A) demonstrates an increased nuclear accumulation of tyrosine-phosphorylated STAT3 in BG-9 cells exposed to either T_4 or 10 ng/ml EGF (lanes 2 and 3 respectively) and 5-fold potentiation by T_4 of the EGF effect (lane 6) in a representative blot. Geldanamycin (0.5 and 10 μ M) 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_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_4 , EGF or both. In the experiment shown in Figure 6(B), T_4 and EGF separately activated STAT3 4-fold and 2-fold respectively, and T_4 enhanced the EGF effect 11-fold. PD 98059 (0.3 and 30 μ M) 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_4 and EGF on *c-fos* expression

With the knowledge that T_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_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_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_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_4 were added for 1 h. Geldanamycin, at a low concentration of 0.5 μ M, 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_4 (99.7 % inhibition, lane 6). A similar experiment with PD 98059 demonstrated that the T_4 potentiation of EGF-induced c-Fos was partly blocked by a low inhibitor

Phosphotyrosine immunoprecipitation STAT3 immunoblot

Figure 4 MAPK pathway inhibitors block the activation of STAT3 by T_4 .

(A) An increase in HeLa cell nuclear tyrosine-phosphorylated STAT3 in response to treatment with 100 nM T_4 for 30 min is seen in lane 2. In three studies, T_4 activated STAT3 5 ± 0.6 -fold and PD 98059 (PD, 30 min treatment) inhibited the T_4 effect by $60 \pm 6\%$ and $83 \pm 4\%$ at concentrations of 0.3 and 30 μ M respectively (lanes 3 and 4). (B) Partial inhibition of the T_4 effect on STAT3 activation was again seen with geldanamycin (GEL, 16 h treatment) in BG-9 cells. In five experiments T_4 induced STAT3 activation 11 ± 5 -fold. Geldanamycin blocked the T_4 -induced STAT3 activation by $44 \pm 18\%$ and $71 \pm 13\%$ at concentrations of 0.5 and 10 μ M respectively. Treatment with geldanamycin for 30–60 min did not cause inhibition (results not shown).

Figure 5 Thyroid hormone enhances the activation of STAT1 α , STAT3 and MAPK in HeLa cells treated with 10 or 100 ng/ml EGF

T_4 (100 nM) and EGF were added to cells for 30 min for STAT3 studies, and for 1 h for STAT1 α and MAPK studies. Representative blots are shown. In two experiments T_4 activated STAT3, STAT1 α and MAPK 6-fold, 3-fold and 11-fold respectively. EGF (10 ng/ml) activated STAT3, STAT1 α and MAPK 2-fold, 3-fold and 3-fold respectively; at 100 ng/ml, EGF stimulated these effects 21-fold, 4-fold and 10-fold respectively. The growth factor effect on STAT3, STAT1 α and MAPK activation in two experiments was enhanced by T_4 7-fold, 4-fold and 3-fold respectively with 10 ng/ml EGF, and 2-fold (STAT3) and 1.5-fold (STAT1 α) with 100 ng/ml EGF. Activation of MAPK decreased 93 % when T_4 was added to 100 ng/ml EGF.

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_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 EGF 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 potentiation by T₄ by 27 ± 15% and 52 ± 10% in three experiments (as shown in lanes 6, 7 and 8). (B) HeLa cells were treated for 1 h with 0.3 or 30 μM PD 98059 (PD), then with EGF and/or T₄ for 30 min. Results are displayed as in (A). In three similar experiments, T₄ activated STAT3 8 ± 2-fold, and EGF activated STAT3 5 ± 2-fold. PD 98059 (0.3 and 30 μM) enhanced the EGF effect on STAT3 activation 1.1 ± 0.2-fold and 1.5 ± 0.1-fold respectively. T₄ enhanced the EGF-induced activation of STAT3 5 ± 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

Figure 7 Inhibition by geldanamycin, PD 98059 and CGP 41251 of EGF-induced and T₄-potentiated c-Fos expression

HeLa cells were treated with geldanamycin (GEL, 0.5 μM), PD 98059 (PD, 0.3 μM) or CGP 41251 (CGP, 2.5 nM) for 16 h, and EGF (100 ng/ml) with or without T₄ (100 nM) for the last 1 h. Nuclear extracts were prepared and proteins were separated by PAGE. Western blotting with anti-c-Fos was then performed. Representative blots from four experiments with each inhibitor are shown. There was an increase in c-Fos protein with EGF (23 ± 12-fold increase in all lanes 3; 29 ± 7-fold increase in 12 experiments) that was potentiated by T₄ (1.6 ± 0.2-fold in all lanes 4; 1.5 ± 0.1-fold increase in 12 experiments). (A) Geldanamycin (0.5 μM) inhibited the effect of EGF (by 24% in this Figure; 39 ± 16% inhibition in four experiments), and blocked the potentiation by T₄ of EGF (by 99% in this Figure; 91 ± 4% decrease in four experiments). (B) In four studies with PD 98059 (0.3 μM) there was 53 ± 11% inhibition of the T₄ potentiation by the inhibitor (39% in this Figure) and a 23 ± 18% inhibition of the effect of EGF alone (little inhibition in this Figure). (C) The potentiation by T₄ of EGF-induced c-Fos (lower band) was inhibited 85 ± 3% by 2.5 nM CGP 41251 in four experiments (77% in this Figure), whereas the effect of EGF alone was inhibited 59 ± 11% in four experiments (60% in this Figure).

Serine-727. T₄ or T₄-agarose in the absence of EGF promoted the tyrosine phosphorylation and nuclear uptake of STAT3 as well as that of STAT1α. The effect of thyroid hormone on STAT3 in the absence of EGF was inhibited by agents that block activities of PKC and PTK. A mechanism by which T₄-driven tyrosine phosphorylation of STAT3 can be affected by PKC inhibition is discussed below. In the presence of EGF, T₄ potentiated the phosphorylation and nuclear transport of STAT3 induced by EGF. Similar findings were obtained in HeLa and CV-1 cells, which lack functional TR [15], and in BG-9 cells, which are TR-replete, indicating the non-genomic nature of this thyroid hormone action.

We also found that T₄, in the absence of EGF, promoted the nuclear translocation of tyrosine-phosphorylated MAPK. EGF alone also increased the phosphorylation and nuclear uptake of MAPK; the effect of EGF on MAPK was enhanced by T₄. STAT3 has been shown by others to undergo Serine-727 phosphorylation by the MAPK pathway [23], as well as by a mechanism independent of that pathway [7]. This serine phosphorylation has been regarded as essential for maximal transcriptional activity of STAT3 [6]. We have previously shown that 100 nM T₄ causes the tyrosine phosphorylation and nuclear translocation of MAPK [20] and STAT1α [14]. We believe that

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 α -STAT1 β -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 STAT1 α . 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 I, β II 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 STAT1 α by iodothyronine [14], but that tetraiodothyroacetic acid itself is not an agonist in such activation. We have also reported that tetraiodothyroacetic acid inhibits the binding of T_4 to human erythrocyte membranes [26]. That agarose- T_4 reproduces 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 STAT1 α 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, presumptively, by MEK. The redundancy within the STAT signalling pathway has been es-

tablished 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 STAT1 α 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 STAT1 α , might be enhanced by the signal-transducing effects of thyroid hormone.

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