Regulation of fibronectin by thyroid hormone receptors

Kwang-Huei Lin, Chia-yu Chen, Shen-Liang Chen, Chun-Che Yen, Ya-Hui Huang, Chung-hsuan Shih, Jiann-Jong Shen¹, Rong-Chi Yang² and Chia-Siu Wang³

Department of Biochemistry, Chang-Gung University, Taoyuan, Taiwan, Republic of China

¹School of Traditional Chinese Medicine, Chang-Gung University, Taoyuan, Taiwan, Republic of China

²Chinese Herbal Pharmacy, Chang Gung Memorial Hospital, Taoyuan, Taiwan, Republic of China

³Department of General Surgery, Chang Gung Memorial Hospital, Chiayi, Taiwan, Republic of China

(Requests for offprints should be addressed to K-H Lin; Email: khlin@mail.cgu.edu.tw)

Abstract

Thyroid hormones regulate growth, development, differentiation, and metabolic processes by interacting with and activating thyroid hormone receptors and associated pathways. We investigated the triiodothyronine (T_3) modulation of gene expression, in human hepatocellular carcinoma cell lines, via a PCR-based cDNA subtraction method. Here we present further data on one of the T_3 -upregulated genes, fibronectin (FN). We demonstrate that the induction of FN protein expression by T_3 in TR α 1 and TR β 1 over-expressing cells was time and dose-dependent at the mRNA and protein levels. Blockade of protein synthesis by cycloheximide almost completely inhibited the concomitant induction of FN mRNA by T_3 , indicating that T_3 indirectly regulates FN. Furthermore, nuclear-run on and FN promoter assay clearly demonstrated that the presence of T_3 can specifically increase the number of FN transcriptional initiations. In addition, we further confirmed that the up-regulation of FN by T_3 was mediated, at least in part, by transforming growth factor- β (TGF- β), because the induction of FN was blocked in a dose-dependent manner by the addition of TGF- β neutralizing antibody. In an effort to elucidate the signaling pathways involved in the activation of FN by T_3 , we demonstrated the involvement of the mitogen activated protein kinase/c-Jun N-terminal kinase/p38 MAPK (MAPK/JNK/p38) pathway. Although T_3 induces the expression of TGF- β , neither wild-type nor dominant-negative Smad3 or Smad4 over-expression affected the activation of FN by T_3 . Thus, we demonstrate that T_3 regulates FN gene expression indirectly at the transcriptional level, with the participation of the MAPK/JNK/p38 pathway and the TGF- β signaling pathway but independent of Smad3/4.

Journal of Molecular Endocrinology (2004) **33**, 445–458

Introduction

The subtractive cDNA hybridization method has been used to elucidate the regulation of molecular processes during hormone treatment, cell differentiation, embryonic development, drug application and malignant transformation. This technique is a powerful approach for the identification, cloning and detailed study of relevant subsets of differentially expressed genes of interest. Recently, an improvement to this technique has been used to selectively amplify differentially expressed target cDNA fragments and simultaneously suppress the amplification of non-target DNA (Kuang *et al.* 1998). This new procedure overcomes previous difficulties associated with differences in mRNA

abundance by incorporating a hybridization step to normalize the transcripts. We utilized the PCR-based cDNA subtraction method to examine the triiodothyronine (T_3) regulation of liver protein levels. Among these are genes including fibronectin (FN), fibrinogen, elongation factor, heat shock protein, protein disulfide isomerase, glucocorticoid receptor AF-1 specific elongation factor, and ribosomal protein L37 involved in metabolism or signal transduction. Several groups (Shirakami *et al.* 1986, Watzke *et al.* 1987) reported that thyroid hormones (THs) positively regulate plasma levels of FN by unknown mechanisms. In addition, Baumgartner-Parzer *et al.* (1997) also showed that hyperthyroidism is associated with elevated plasma levels of FN. Thus, we focused our study on the

mechanism of how FN was regulated by THs. This initial investigation, in hepatocellular carcinoma cell lines, demonstrated that FN was up-regulated by T_3 .

THs regulate growth, development, differentiation, and metabolic processes by interacting with TH receptors (TRs) that, in turn, bind to specific DNA sequences in the regulatory regions of target genes (Cheng 2000). TRs are members of the steroid hormone and retinoic acid superfamily of ligand-dependent transcription factors. Two TR genes, *TR α* and *TR β* , have been identified and mapped to human chromosomes 17 and 3 respectively (Lazar *et al.* 1994). Each gene encodes at least two TR isoforms (*TR α 1*, *TR α 2*, and *TR β 1*, *TR β 2*) that are generated as a result of alternate splicing and/or promoter choice (Wood *et al.* 1996). The mechanisms involved in the maintenance of liver-specific gene transcription by *TR α 1* and *TR β 1* have not yet been fully elucidated. HepG2, a well-differentiated hepatocellular carcinoma cell-line, has been well-characterized and has been reported to secrete all 15 known liver-specific plasma proteins. Thus, we used HepG2 to derive isogenic cell lines stably expressing high levels of wild-type *TR α 1* and β 1 (HepG2-*TR α 1* and -*TR β 1* cells) (Lin *et al.* 2000). These lines have proved to be a useful tool when investigating the target genes of THs.

FN mediates a wide variety of key interactions between cells and the extra-cellular matrix (ECM) and plays a significant role in cell adhesion, migration, growth, differentiation and the maintenance of normal cell morphology (Hynes 1985*a,b*, Pankov & Yamada 2002). FN is an important glycoprotein that usually occurs as a dimer composed of two, nearly identical, ~250 kDa subunits covalently linked near their C termini by a pair of disulfide bonds (Pankov & Yamada 2002). Although FN exists as a single gene, alternative splicing of a single pre-mRNA can generate as many as 20 variants in the human (Islami *et al.* 2001, Srebrow *et al.* 2002). These alternative splice products may contain at least three regions (ED-A, ED-B and IIICS) of the primary transcript (Borsi *et al.* 1990). Interestingly, it has been reported that transforming growth factor- β (TGF- β) preferentially increases the accumulation of the FN isoforms containing the ED-A sequence in cultured normal human fibroblasts (Balza *et al.* 1988). FN isoforms can be classified as either soluble plasma FN or as the less-soluble cellular FN (Sekiguchi *et al.* 1986).

Plasma FN is synthesized predominantly in the liver by hepatocytes and displays a relatively simple splicing pattern. On the other hand, cellular FN consists of a much larger and more heterogeneous group of FN isoforms, resulting from cell-type and species-specific splicing patterns.

The control of FN expression by T_3 and the specific isoforms of TR has not been investigated in a cellular context. We report here that T_3 up-regulates FN expression at the transcriptional and translational level in HepG2-*TR α 1* and -*TR β 1* cells. Furthermore, the effect of T_3 on the level of FN expression requires the *de novo* synthesis of cellular proteins. In addition, we found that the up-regulation of FN by T_3 is mediated, at least in part, by the TGF- β and/or MAP kinase (MAPK) signaling pathways. The MAP family is also the TGF- β downstream signaling mediator (Hocavar *et al.* 1999). Finally, we demonstrate that T_3 does not utilize the TGF- β signaling components, Smad3/4 in the control of FN expression.

Materials and methods

Cell culture

The human hepatoma cell line, HepG2, was obtained from the American Type Culture Collection (Manassas, VA, USA) and was routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum. *TR α 1* and β 1 over-expressing cell lines have been described previously (Lin *et al.* 2000). In this study, two HepG2-*TR α 1*- (#1 and #2) and one *TR β 1*-over-expressing clones were used. The serum was depleted of T_3 (Td) as described (Samuels *et al.* 1979). Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

Subtractive hybridization

Total RNA from HepG2-*TR α 1*#1, with or without T_3 treatment, was prepared using TRIzol (Life Technologies, Rockville, MD, USA). The PCR-based cDNA subtraction method was carried out according to the manufacturer's protocol (Clontech, Palo Alto, CA, USA). The subtracted cDNAs were ligated into the pGEM-T vector. Subsequently, the clones were screened for differentially expressed genes using the PCR-Select Differential Screening kit (Clontech).

Immunoblot analysis

Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% gel, and the separated proteins were transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ, USA). The membrane was gently shaken for 2 h at room temperature in 5% (w/v) nonfat dried milk in Tris-buffered saline (TBS), washed three times with TBS, and then incubated for 1 h with rabbit polyclonal antibodies to FN (1:1000 dilution in TBS) (Transduction, Lexington, KY, USA), or with mouse monoclonal antibody C4 to TR α 1 (1:1000 dilution in TBS) (kindly provided by S-Y Cheng, NCI, NIH, Bethesda, MD, USA) (Bhat *et al.* 1995). After further washing, the membrane was incubated for 1 h with horseradish peroxidase conjugated to affinity-purified antibodies to either rabbit (1:1000 dilution in TBS) or mouse (1:1000 dilution in TBS) immunoglobulin (Santa Cruz Biotechnology). Immune complexes were then visualized by chemiluminescence with an ECL detection kit (Amersham). The intensities of the immunoreactive bands were quantitated by analysis with Image Gauge software (Fuji Film, Tokyo, Japan).

Determination of the trans-activation activity of TRs

T_3 -dependent trans-activity of TRs was assayed in the various HepG2 cell lines as described previously (Kyriakis & Avruch 1996). Briefly, cells were transfected with a luciferase reporter plasmid (2 μ g) containing the Lys-TRE or FN 508 promoter (2 μ g, a gift from Kinichiro Oda, Science University of Tokyo, Noda, Japan) (Suzuki *et al.* 1998), along with a β -galactosidase plasmid (1 μ g) to control for transfection efficiency or TR expression vector. Transfected cells were subsequently incubated for 24 h in Td medium containing various concentrations of T_3 (Sigma, St Louis, MO, USA), after which the activities of luciferase and β -galactosidase in cell lysates were measured (Flores-Morales *et al.* 2002). The activity of luciferase was normalized against β -galactosidase activity.

Northern blot analysis

Total RNA was extracted from cells with the use of TRIzol Reagent. Equal amounts of total RNA (20 μ g) were analyzed on a 1.2% agarose-

formaldehyde gel as described (Lin *et al.* 2002). This was then blotted onto a nitrocellulose membrane and subjected to Northern blot analysis as described (Lin *et al.* 2000). A full-length FN cDNA fragment was amplified, labeled with [α - 32 P]dCTP (3000 Ci/mmol; Amersham) by the PCR and used as a probe. The membrane was subsequently re-probed with a 32 P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment to verify equal application of RNA to each lane. In some experiments cells were treated with T_3 and 10 μ g/ml cycloheximide (Sigma) simultaneously for 12 or 24 h, followed by total RNA isolation and Northern analysis.

Nuclear run-on assay

Sub-confluent HepG2-TR α 1#1 cells were treated with or without 100 nM T_3 for 3 h. Cells were subsequently washed twice with ice-cold PBS, collected, and centrifuged at 500 g for 5 min at 4 °C. The pellet was gently resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40, and allowed to swell and lyse on ice for 10 min. The lysate was recentrifuged at 500 g , and the resulting nuclear pellet was resuspended in 100 μ l labeling buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 140 mM KCl, 14 mM β -mercaptoethanol, 1 mM MnCl₂, and 20% glycerol. *In vitro* transcription was performed using the nuclear pellet (100 μ l) in labeling buffer with 1 mM creatine kinase, 10 mM phosphocreatine, 1 mM CTP, ATP, GTP, and 100 μ Ci [α - 32 P]UTP as described previously (Liao *et al.* 1995). The reaction was incubated in a shaking water bath at 30 °C for 30 min. Equal amounts (2 μ g) of purified, denatured full-length FN, human β -actin, and linearized pGEM-T cDNA were vacuum-transferred onto nylon membranes using a slot blot apparatus (Amersham). The membranes were baked and pre-hybridized, as described for Northern blots. The precipitated radio-labeled transcripts ($\sim 10^7$ cpm) were resuspended in 2 ml hybridization buffer containing 50% formamide, 5 $\times$ SSC, 2.5 $\times$ Denhardt's solution, 25 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, and 250 μ g/ml salmon sperm DNA. Hybridization of radio-labeled transcripts to the nylon membranes was carried out at 42 °C for 72 h. The membranes were then washed with 1 $\times$ SSC and 0.1% SDS for

1 h at 65 °C before auto-radiography for 24 h at – 80 °C.

Quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR)

To determine the expression of TGF- β , Q-RT-PCR was carried out. Total RNA was extracted from cells using TRIzol as described above. Subsequently, the first strand of cDNA was synthesized using the Superscript III kit for RT-PCR (Life Technologies). Real time Q-RT-PCR was performed in a 25 μ l reaction mixture containing 50 nM forward and reverse primers, 1 $\times$ SYBR Green reaction mix (Applied Biosystems, Foster City, CA, USA), and various amounts of template. Fluorescence emitted by SYBR Green was detected on line by the ABI PRISM 7000 sequence detection system (Applied Biosystems). All PCRs were carried out in duplicate on the same 96-well plate. For quantification of gene expression changes, the Δ Ct method was used to calculate relative-fold changes normalized against the ribosomal binding protein (RiboL35A) gene as described in User Bulletin number 2 (Applied Biosystems). The Ct is defined as the cycle at which fluorescence is determined to be statistically significant above background.

TGF- β protein assay

To determine the effect of T_3 on the expression of TGF- β protein, TGF- β 1 Emax ImmunoAssay System (Promega Corporation, Madison, WI, USA) was used. Briefly, HepG2-TR α 1#1 cells were treated with or without 10 to 100 nM T_3 for various times. The supernatant was collected for TGF- β determination according to the manufacturer's instructions.

Determination of the effect of exogenous Smad on FN expression

To determine the exogenous effect of Smad on the expression of FN, we transfected HepG2-TR α 1#1 cells ($1 \sim 2 \times 10^5$ per 60-mm dish) with wild-type or dominant-negative Smad plasmid using Lipofectamine (Gibco BRL). Twenty-four hours after transfection, the cells were lysed and Western blot analysis performed. Smad plasmids were a gift from Dr P T Dijke (Ludwig Institute, Uppsala, Sweden).

Results

Expression and trans-activation activity of TR α 1 and TR β 1 in HepG2 cell lines

We used a PCR-based cDNA subtraction method to identify the induction of genes by T_3 in HepG2 cells. FN was selected because the control mechanism of its expression by T_3 and TR has not been studied. To further investigate the molecular regulation by T_3 of the *FN* gene, we used isogenic HepG2 cell lines that stably express wild-type TR α 1 (HepG2-TR α 1 clones #1, #2) and TR β 1 (HepG2-TR β 1). As a control, HepG2 cells were transfected with the empty vector, yielding a cell line that expresses the Neo protein (HepG2-Neo cells). Prominent immunoreactive bands, corresponding to TR α 1 or TR β 1 were detected in HepG2-TR α 1#1, #2, and HepG2-TR β 1 using the monoclonal antibody C4 (Fig. 1). This antibody recognizes an epitope at the COOH-terminus of TR α 1 and TR β 1 (Bhat *et al.* 1995). Quantitation of the immunoreactive bands revealed that the abundance of TR α 1 protein in HepG2-TR α 1 cells was four- to sevenfold that in HepG2-Neo cells. Endogenous levels of TR in HepG2-Neo cells were only faintly observed after longer exposure times.

A Lys-luciferase reporter construct (containing the thyroid hormone response element (TRE) of the chicken lysozyme gene, a 6 nt inverted repeat), was used to compare the transcriptional activity of TR α 1 in HepG2-TR α 1#1 and #2 and of TR β 1 in HepG2-TR β 1 with that of the HepG2-Neo cells (Fig. 1B). HepG2-TR α 1#1 and HepG2-TR β 1 cells exhibited higher trans-activity (five- to sevenfold), which increased in a T_3 -dependent manner. TR α 1#2 displayed moderate trans-activation of the reporter construct (three-to fourfold), while the control HepG2-Neo cells exhibited a low level of trans-activity (Fig. 1B). These results indicate that the isogenic cell lines over-express TR α 1 or TR β 1 and the level of expression correlates well with their functional capacity to trans-activate the expression of downstream genes.

Effects of T_3 on the abundance of FN protein and mRNA in HepG2-TR α 1 and TR β 1 cell lines

Next, we were interested in the effect of the TRs on the level of FN protein expression when the HepG2 isogenic cell lines were incubated in media containing various levels of T_3 across different time

Figure 1 Expression and trans-activity of TR α 1 and TR β 1 in HepG2 cell lines. (A) Immunoblot analysis of TR α 1 and TR β 1 expression in isogenic HepG2 cell lines. Lysates (100 μ g protein) from HepG2-TR α 1#1, #2, TR β 1 (β 1) and HepG2-Neo (Neo) cells were subjected to immunoblot analysis with monoclonal antibody C4 as described in Materials and Methods. The positions of the 47 kDa TR α 1 protein or 55 kDa TR β 1 protein are indicated. Tubulin was used as an internal control (data not shown). (B) T_3 -dependent trans-activity of TRs in the various HepG2 cell lines. Cells were transfected with a luciferase reporter plasmid containing the Lys-TRE, as well as with a β -galactosidase plasmid to control for transfection efficiency. They were subsequently incubated for 24 h in T_3 -depleted (Td) medium containing the indicated concentrations of T_3 , after which the activities of luciferase and β -galactosidase in cell lysates were measured. The activity of luciferase was normalized on the basis of the activity of β -galactosidase. Data are from three independent experiments, each performed in duplicate. The ordinate indicates 'Fold Activation' and non-induced=1.

points (Fig. 2). T_3 significantly increased the abundance of FN in the HepG2-TR α 1#1, #2 and -TR β 1 stable cell lines as compared with the HepG2-Neo control cell line. FN levels increased approximately 1.5- to 2.5-fold after incubation of HepG2-TR α 1#1, #2, and TR β 1 cells with 10 nM T_3 for 24 h. In addition, 100 nM T_3 for 24 h gave a slightly greater and more significant induction

(2.2- to 2.8-fold) of FN protein. Moreover, after 48 h incubation in 100 nM T_3 , FN activation was further elevated ($\sim$ 2.5- to $\sim$ 4.2-fold). These results indicate that the effect of T_3 on the level of FN in TR α 1 and β 1 over-expressing cells was time- and dose-dependent. In addition, immunoblot analysis revealed that exposure of control HepG2-Neo cells, expressing endogenous levels of TR proteins, to 100 nM T_3 , resulted in little effect on the expression of FN protein (Fig. 2). Thus, the extent of FN protein induction by 100 nM T_3 correlated with the level of TR protein expression.

Northern blot analysis was utilized to examine the response of *FN* mRNA expression to the exogenous addition of T_3 . In all three cell lines investigated, a 9 kb *FN* transcript was detected (Fig. 3A). Exposure to 100 nM T_3 for 24 h resulted in a significant induction in the expression of *FN* mRNA in HepG2-TR α 1#1, #2 and TR β 1 cells, with increases of 2.8-, 2.6- and 2.5-fold respectively. This suggests that the augmented expression of *FN* mRNA in response to T_3 corresponds to the amount of TR in the individual line. The highest levels of T_3 (100 nM) used in this experiment did not significantly increase the level of FN expression above that already displayed in the 10 nM T_3 experiment (Fig. 3B). These results indicate that *FN* gene expression may be very sensitive to the presence of small amounts of T_3 in the medium. Applying 10 nM T_3 for 24 h or 48 h, to the HepG2-TR β 1 cell line, increased FN levels 1.5- and 2.0-fold respectively. However, when the cells were exposed to 100 nM T_3 for 24 h or 48 h *FN* mRNA expression increased 2.5- and 3.5-fold respectively. This indicates that the action of T_3 in TR α 1 as compared with the TR β 1 stable cell line was slightly different. *FN* mRNA levels were less sensitive to the presence of T_3 in the TR β 1 compared with the TR α 1 stable cell line. Furthermore, T_3 had little effect on the abundance of *FN* mRNA in HepG2-Neo cells (Fig. 3). Thus, the effect of T_3 on the expression of FN protein appears to be mediated, at least in part, at the mRNA level.

Effects of T_3 and cycloheximide (CHX) on the abundance of FN mRNA

In an effort to further delineate the regulatory action of T_3 on the expression of *FN* mRNA, a protein synthesis inhibitor, CHX, was employed. Induction of *FN* mRNA expression by T_3 , in the presence or absence of CHX, was observed for

Figure 2 The effect of T_3 on FN protein expression in HepG2 cell lines. (A) HepG2-TR α 1#1 and #2, -TR β 1 (beta) and -Neo cells were incubated with medium in the absence or presence of T_3 for 24 or 48 h. Subsequently, cell lysates (100 μ g protein) were subjected to immunoblot analysis with polyclonal antibodies to FN. The position of the 250 kDa FN protein is indicated. Tubulin was used as an internal control. (B) The intensities of bands in (A) were quantified and the extent of T_3 -induced activation of FN expression was determined at each time point. In the key, #1-24 and #1-48 represents the data for HepG2-TR α 1#1 at 24 and 48 h incubation respectively. The other three cell lines are similarly represented in the key. Data are means $\pm$ S.E. of values from three independent experiments. Values are shown as fold induction of the Td-control. * P < 0.05, ** P < 0.01, T_3 -compared with Td-treated (Student's t -test).

both time periods in HepG2-TR α 1#1 cells. The transcriptional response of *FN* mRNA to T_3 over 12- and 24-h periods was greatly reduced in the presence of CHX (Fig. 4). Similar results were observed in the other two TR over-expressing cell lines (data not shown). These data indicate that blocking protein synthesis almost completely inhibits the induction of *FN* transcription by T_3 . It follows from this result that *de novo* protein synthesis may be required for this activation to occur.

T_3 induces the expression of *FN* at the transcriptional level

To further confirm that regulation of *FN* expression by T_3 occurred at the transcriptional level we

performed nuclear run-on experiments. T_3 induced an approximate 2.5-fold increase in *FN* mRNA transcription (Fig. 5), as observed in the Western and Northern blots. The expression levels of β -actin were used as an internal control, and pGEM-T vector as a negative control. The data clearly demonstrate that T_3 can specifically increase the number of *FN* transcripts in HepG2-TR α 1#1 cells.

To further support this conclusion, we carried out reporter assays. We used the *FN* 5'-flanking region encompassing nucleotides -508/+18 (Suzuki *et al.* 1998) and then placed it upstream of the luciferase reporter gene in pGL2. Using this reporter, we determined the effect of trans-activation of *FN* by T_3 . As shown in Fig. 6, at

Figure 3 The effect of T_3 on the abundance of *FN* mRNA in HepG2 cell lines. (A) HepG2-TR α 1#1 and #2, -TR β 1 (beta) and -Neo cell lines were incubated for 24 or 48 h in the absence or presence of T_3 , after which total RNA was isolated and subjected (20 μ g per lane) to Northern blot analysis with 32 P-labeled *FN* or *GAPDH* cDNA probes. The positions of the 9-kb *FN* and 1.0 kb *GAPDH* mRNAs are indicated. (B) The intensities of the *FN* mRNA bands shown in (A) were quantified, and the extent of the T_3 -induced increase in the abundance of *FN* transcripts was determined at each point. Data are means $\pm$ S.E. of values from three independent experiments. * P < 0.05, ** P < 0.01, T_3 - compared with Td-treated (Student's *t*-test). For description of key, see legend to Fig. 2.

100 nM T_3 , *FN* promoter had the highest activity (approximately 2.2-fold activation). Therefore, the *FN* promoter was sensitive to T_3 treatment.

Effects of T_3 , TGF- β and MAP kinase on the abundance of FN protein

In an effort to elucidate the signaling pathways involved in the activation of *FN* by T_3 , we demonstrated the involvement of the TGF- β and/or MAPK/c-Jun N-terminal kinase/p38 MAP kinase (MAPK/JNK/p38) pathway. Our data indicate that treatment of cells with T_3 for various times induces TGF- β expression from 1.7- to 3.3-fold at the mRNA level (Table 1). Similarly,

treatment of cells with 10 to 100 nM T_3 for various times induces TGF- β expression from 1.5- to 3.8-fold at the protein level (Table 2). This is consistent with the report by Miller *et al.* (2001). We further confirmed that the induction of *FN* by T_3 was mediated by TGF- β (Fig. 7). Figure 7 indicates that the induction of *FN* by T_3 was blocked in a dose-dependent manner by the addition of TGF- β neutralizing antibody but not by the control antibody. Furthermore, it has been demonstrated that TGF- β signaling is mediated by members of the MAPK family in the induction of *FN* expression (Hocevar *et al.* 1999). To determine if the role of T_3 or recombinant TGF- β on the induction of *FN* in HepG2-TR α 1#1 cell lines is

Figure 4 The effect of cycloheximide (CHX) on the response of FN to T_3 -activation. (A) HepG2-TR α 1 #1 cells were treated as described in Fig. 3 with or without 10 μ g/ml CHX. After T_3 -activation for 12 or 24 h, total RNA was isolated and subjected (20 μ g per lane) to Northern blot analysis. (B) The intensities of the FN and GAPDH mRNA bands on blots were quantified, and the increase in abundance of FN transcripts was determined at each time point. The data from three independent experiments are displayed as a fold induction compared with those exposed to the control (Td) media. ** $P < 0.01$, no CHX compared with CHX-treated (Student's *t*-test).

mediated by MAPKs, we investigated the effect of several MAPK inhibitors. PD98059 is a selective inhibitor of MAPK kinase (MEK) 1/2 that blocks extracellular signal-regulated kinase (ERK) activation, whereas SB203580 inhibits both JNK and p38 MAPK, and SB202190 is a specific inhibitor of p38 MAPK. These inhibitors were applied

Figure 5 The effect of T_3 on FN gene transcription rate, measured in a nuclear run-on assay using HepG2-TR α 1 #1 cells. Nuclei were isolated after cells were treated with or without 100 nM T_3 for 3 h. These were subjected to *in vitro* transcription using the nuclear pellet as the template and hybridizing this product against denatured-linear plasmids that contain the cDNAs indicated at the left of the Figure. Actin, and pGEM-T were used as internal controls.

individually to HepG2-TR α 1#1 cells 3 h before the addition of 100 nM T_3 . After incubation for an additional 24 h, the induction of FN protein in the absence of inhibitors was $\sim$ threefold, as seen previously (Fig. 2). FN was also stimulated $\sim$ threefold by TGF- β . However, the activation of FN by T_3 or TGF- β was completely abolished after the addition of 15 μ M SB203580 or SB202190 (Fig. 8). In contrast, this inhibition was not observed with the application of the inhibitor PD98059 (10 μ M). These data indicate that activation of FN by T_3 or TGF- β is mediated, at least in part, by the JNK or p38, but not the MEK/ERK pathway.

Stimulation of FN by T_3 is not mediated by the Smad 3/4-dependent pathway

The MAPK family has been implicated in the events downstream of TGF- β signaling (Marais & Marshall 1996). TGF- β signaling is mediated by the phosphorylation and activation of either Smad2 or Smad3, which allows the formation of a heterodimeric complex with Smad4. This complex subsequently trans locates into the nucleus, where it acts on the promoters of target genes (Itoh *et al.* 2000). Therefore, we investigated the possible role of Smad3/4 in the JNK/p38-mediated activation of FN protein by T_3 . Both wild-type and dominant-negative forms of Smad3 and Smad4 were

Figure 6 T_3 -dependent trans-activity of TR β 1 in FN promoter. Cells were transfected with a luciferase reporter plasmid containing the FN 5'-flanking region encompassing nucleotides -508/+18 (Suzuki *et al.* 1998), TR β 1, as well as with a β -galactosidase plasmid to control for transfection efficiency. They were subsequently incubated for 24 h in T_3 -depleted (Td) medium containing the indicated concentrations of T_3 , after which the activities of luciferase and β -galactosidase in cell lysates were measured. The activity of luciferase was normalized on the basis of the activity of β -galactosidase. Data are from three independent experiments, each performed in duplicate. The ordinate indicates 'Fold Activation' and non-induced=1.

Table 1 Induction of TGF- β mRNA by 100 nM T_3 in HepG2-TR α 1#1 cells by Q-RT-PCR. Data are displayed as fold induction by T_3 (mean $\pm$ S.E. of three independent experiments)

	Hours after T_3 -treatment		
	6	12	48
TGF- β	1.7 $\pm$ 0.3	1.8 $\pm$ 0.2	3.3 $\pm$ 0.5

Table 2 Induction of TGF- β protein by 10 or 100 nM T_3 in HepG2-TR α 1#1 cells. Data are displayed as fold induction by T_3 (mean $\pm$ S.E. of three independent experiments)

	Hours after T_3 -treatment					
	6		12		48	
	10 nM T_3	100 nM T_3	10 nM T_3	100 nM T_3	10 nM T_3	100 nM T_3
TGF- β protein	1.5 $\pm$ 0.3	1.8 $\pm$ 0.2	1.6 $\pm$ 0.1	2.0 $\pm$ 0.2	2.6 $\pm$ 0.4	3.8 $\pm$ 0.5

transiently over-expressed in HepG2-TR α 1#1 cells to observe their effect on T_3 -mediated FN activation. Interestingly, neither the over-expression of wild-type nor dominant-negative Smad3 or Smad4 affected the activation of FN by T_3 (Fig. 9). This indicates that these components of the TGF- β signaling pathway are not involved in the T_3 -mediated transcriptional activation of FN.

Discussion

We reported here the isolation of T_3 -responsive genes using a PCR-based cDNA subtraction method in HepG2 cell lines over-expressing TR α 1 protein. One gene (FN, the extra-cellular matrix component) that was significantly induced by T_3 was targeted because several groups (Shirakami *et al.* 1986, Watzke *et al.* 1987, Baumgartner-Parzer *et al.* 1997) have reported a significant positive correlation between plasma concentrations of FN and total THs. However, no detail of the regulation mechanism has been reported. HepG2, a well-differentiated hepatocellular carcinoma cell-line, secretes all 15 plasma proteins (Chang *et al.* 1983) and preserves many liver-specific functions. Thus, the HepG2 cell line serves as a suitable model system to study the cell type-specific and TR isoform-specific regulation of T_3 -target genes in the liver (Lin *et al.* 1997). The liver is the major target organ of THs (Cheng 2000) and is the main site of FN secretion (Pankov & Yamada 2002). However, the mechanisms of how TRs selectively maintain liver-specific gene transcription have not yet been elucidated. Furthermore, the use of cells over-expressing TRs enabled us to investigate the regulation of plasma protein levels in response to variations in the levels and isoforms of the TR receptors. Therefore, in this study we investigated the molecular regulation of FN by T_3 in isogenic HepG2 cell lines.

Figure 7 T₃-induced FN expression was blocked by the TGF-β antibody. (A) FN was induced by 100 nM T₃ in HepG2 TRα#1 cells for 24 h. Some other tubes were incubated simultaneously with T₃ and increasing volumes (4–12 μl) of the TGF-β or non-specific (NS) control antibody (Ab). Subsequently, cell lysates (100 μg protein) were subjected to immunoblot analysis with polyclonal antibodies to FN. Tubulin was used as an internal control. (B) The intensities of bands in (A) were quantified and the extent of T₃-induced activation of FN expression was determined in each lane. Data are means ±S.E. of values from three independent experiments. Values are shown as fold induction of the Td-control. **P*<0.05, ***P*<0.01, T₃-compared with TGF-β Ab-treated (Student's *t*-test).

Although this study investigated the induction of FN by T₃ in human hepatoma cell lines, similar results have also been observed in animal and primary cell culture models. Murata *et al.* (1990) showed that *FN* mRNA was decreased by half in thyroidectomized rats, while administration of physiological doses of thyroxine or T₃ for 5–6 days restored *FN* mRNA to control levels. Moreover, administration of pharmacological doses of thyroid hormones induced a further increase in the abundance of *FN* mRNA. Subsequently, Lee *et al.* (1992) demonstrated that TH promotes *FN* gene expression in primary cultured rat hepatocytes, yet

inhibits FN synthesis in cultured human skin fibroblasts. Furthermore, in hepatocytes, these authors demonstrated that TH directly enhanced *FN* gene expression without requiring *de novo* protein synthesis. In contrast, we demonstrate the need for *de novo* protein synthesis for the induction of FN by T₃ in HepG2 cell lines. The discrepancy between these results may be due, in part, to the previous authors using dot blot hybridization instead of the more sensitive Northern blot, to determine the effect of CHX on induction of *FN* mRNA by T₃ (see Fig. 5 of their paper) (Lee *et al.* 1992). This inconsistency cannot be commented on

Figure 8 T_3 - and TGF- β -induced FN expression was blocked by the JNK/p38 inhibitors, SB203580 and SB202190. (A) HepG2 TR α #1 cells were treated with the MAPK inhibitor PD98059 (10 μ M) and other pathway inhibitors SB203580 (15 μ M) and SB202190 (15 μ M) 3 h before the addition of 100 nM T_3 , prior to immunoblotting. Tubulin was used as an internal control. (B) The intensities of the immunoblot bands were quantified to determine the changes in FN abundance at each time point. The data are displayed as fold activation compared with those in Td media and are taken from three independent experiments. ** P < 0.01, T_3 /SB203580- or T_3 /SB202190-treated compared with T_3 - or T_3 /DMSO-treated; TGF- β /SB203580- or TGF- β /SB202190-treated compared with TGF- β /DMSO-treated (Student's t -test).

further as the previous authors did not present their dot blot data, although it would be very difficult to determine the effect of CHX on FN expression as their data indicate that T_3 did not induce FN mRNA expression (see Fig. 4 of their paper). Our data clearly show that FN mRNA induction by T_3 is in-directed, de novo protein synthesis required.

Additionally, studies in humans and animal models further support our findings. Watzke *et al.* (1987) studied the plasma concentration of FN in 13 untreated thyroid carcinoma patients after total thyroidectomy. FN levels were found to be significantly decreased compared with those of healthy volunteers. Following oral administration of L-thyroxine for 6 weeks, the same patients showed significantly increased levels of FN compared with untreated patients. In support of this data,

fibronectin expression in hypothyroid animals is reduced to about 40% in the midbrain compared with untreated animals (Calloni *et al.* 2001). Furthermore, *in vivo* animal studies demonstrated that THs promote the expression of the FN gene in the rat liver (Lee *et al.* 1992).

In this report we examined the potential role played by TGF- β in the enhanced expression of FN by T_3 . This pathway was investigated, as there is evidence of a close relationship between T_3 and TGF- β . A previous report (Miller *et al.* 2001) and ours have demonstrated that treatment with T_3 for 12 h or more induces TGF- β expression. Furthermore, an increased TGF- β plasma concentration was reported to be associated with high plasma T_3 levels in elderly patients with non-thyroidal illnesses (Corica *et al.* 1998). In addition, TGF- β signaling

Figure 9 Smad3/4 were not involved in T_3 -induced FN expression. (A) To determine the effect of Smad proteins, dominant-negative (DN) and wild type (Wt) Smad3 or Smad4 plasmids were transfected into HepG2 TR α #1 in the presence of T_3 . Tubulin was used as an internal control. (B) The intensities of the immunoblot bands were quantified and represented in a graphical format.

has been reported to be mediated by members of the MAPK family (Hocevar *et al.* 1999). The MAPK family incorporates the ERK pathway (Marais & Marshall 1996) and two stress activated pathways, the JNK and p38 pathways (Kyriakis & Avruch 1996, Woodgett *et al.* 1996). Consistent with our results, the JNK pathway is stimulated rapidly by TGF- β in a human fibrosarcoma cell line, and this activity is essential for TGF- β -mediated FN induction (Hocevar *et al.* 1999). Similar to our results, these authors demonstrated that Smad4 is not required for TGF- β -mediated FN induction. Interestingly, there are several activator protein 1 (AP1) binding sites in the *FN* promoter region, which are the target of downstream signaling following TGF- β activation (Hocevar *et al.* 1999). We speculate that T_3 induces TGF- β and the JNK/p38 pathway to phosphorylate AP1, allowing it to bind to the AP1 sites in the *FN* promoter and thus activate FN expression.

Several other factors have been demonstrated to affect the expression of FN. Recombinant human interleukin-1 administered to rats was shown to increase plasma FN levels, concomitant with increased FN in the liver (Hagiwara *et al.* 1990). In addition, human cytomegalovirus (HCMV)-infected fibroblasts exhibited a progressive loss of cellular FN (Pande *et al.* 1990). These authors demonstrated that this decrease occurred at the transcriptional level and suggested that HCMV-encoded and/or -induced factors may induce these alterations. Vitamin A-deficient rats had increased levels of FN in their serum and it was demonstrated that regulation by vitamin A is at the level of transcription (Kim & Wolf 1987). Interestingly, all-trans retinoic acid (RA) induces FN expression, up to 130%, at the transcriptional level in bovine lens epithelial cells (Shanker & Sawhney 1996). Insulin-like growth factor-I (IGF-I) and insulin induce the level of FN in the culture media of smooth muscle cells (SMC) whereas only IGF-I upregulates FN in cell lysates from glomerular mesangial cells (Tamaroglio & Lo 1994). The liver regeneration process after surgery has been demonstrated to increase *FN* mRNA threefold (Caputi *et al.* 1995). Ehretsmann *et al.* (1995) reported that treatment of the human fibrosarcoma cell line HT-1080 with dexamethasone (DEX) results in the induction of FN protein and mRNA synthesis. These authors, through the use of nuclear run-on experiments, demonstrated that the DEX-dependent induction of FN occurs primarily at the post-transcriptional level. Vollmer *et al.* reported (1995) that FN is an estrogen-repressed protein in rat endometrial adenocarcinoma cells. This research indicates that different steroid hormones can affect the production of FN in the liver in different ways.

Our results have demonstrated that T_3 plays an important role in the expression of FN protein at the transcriptional and post-transcriptional level. The induction of FN by T_3 was demonstrated to be indirect as there was an absolute requirement for *de novo* protein synthesis. We further elucidated that FN activation by T_3 is mediated, at least in part, by the TGF- β , JNK/p38 pathway. In addition, we established that the TGF- β pathway, in particular Smad3 and Smad4, was not implicated in FN induction by T_3 . Further study is required to dissect the regulatory cascade induced by the T_3 activation of fibronectin expression.

Acknowledgements

This work was supported by grants from Chang-Gung University (CMRP 737, NMRP 092) and the National Science Council of the Republic of China (NSC 87–2316-B-182–002).

References

- Balza E, Borsi L, Allemanni G & Zardi L 1988 Transforming growth factor beta regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts. *FEBS Letters* **228** 42–44.
- Baumgartner-Parzer SM, Wagner L, Reining G, Sexl V, Nowotny P, Muller M, Brunner M & Waldhausl W 1997 Increase by tri-iodothyronine of endothelin-1, fibronectin and von Willebrand factor in cultured endothelial cells. *Journal of Endocrinology* **154** 231–239.
- Bhat MK, McPhie P & Cheng SY 1995 Interaction of thyroid hormone nuclear receptor with antibody: characterization of the thyroid hormone binding site. *Biochemical and Biophysical Research Communications* **210** 464–471.
- Borsi L, Castellani P, Risso AM, Leprini A & Zardi L 1990 Transforming growth factor-beta regulates the splicing pattern of fibronectin messenger RNA precursor. *FEBS Letters* **261** 175–178.
- Calloni GW, Alvarez-Silva M, Vituri C & Trentin AG 2001 Thyroid hormone deficiency alters extracellular matrix protein expression in rat brain. *Developmental Brain Research* **126** 121–124.
- Caputi M, Melo CA & Baralle FE 1995 Regulation of fibronectin expression in rat regenerating liver. *Nucleic Acids Research* **23** 238–243.
- Chang C, Lin Y, O-Lee TW, Chou CK, Lee TS, Liu TJ, P'Eng FK, Chen TY & Hu CP 1983 Induction of plasma protein secretion in a newly established human hepatoma cell line. *Molecular and Cellular Biology* **3** 1133–1137.
- Cheng SY 2000 Multiple mechanisms for regulation of the transcriptional activity of thyroid hormone receptors. *Reviews in Endocrine and Metabolic Disorders* **1** 9–18.
- Corica F, Allegra A, Corsonello A, Buemi M, Rubino F, Bonanzinga S, Ruello A & Ceruso D 1998 Increased transforming growth factor-beta1 plasma concentration is associated with high plasma 3,3',5'-tri-iodothyronine in elderly patients with nonthyroidal illnesses. *European Journal of Endocrinology* **138** 47–50.
- Ehretsmann CP, Chandler LA & Bourgeois S 1995 A nuclear post-transcriptional mechanism mediates the induction of fibronectin by glucocorticoids. *Molecular and Cellular Endocrinology* **110** 185–194.
- Flores-Morales A, Gullberg H, Fernandez L, Stahlberg N, Lee NH, Vennstrom B & Norstedt G 2002 Patterns of liver gene expression governed by TRbeta. *Molecular Endocrinology* **16** 1257–1268.
- Hagiwara T, Suzuki H, Kono I, Kashiwagi H, Akiyama Y & Onozaki K 1990 Regulation of fibronectin synthesis by interleukin-1 and interleukin-6 in rat hepatocytes. *American Journal of Pathology* **136** 39–47.
- Hocevar BA, Brown TL & Howe PH 1999 TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. *EMBO Journal* **18** 1345–1356.
- Hynes R 1985a Molecular biology of fibronectin. *Annual Review of Cell and Developmental Biology* **1** 67–90.
- Hynes RO 1985b Fibronectins: a family of complex and versatile adhesive glycoproteins derived from a single gene. *The Harvey Lectures* **81** 133–152.
- Islami D, Shoukir Y, Dupont P, Campana A & Bischof P 2001 Is cellular fibronectin a biological marker for pre-eclampsia? *European Journal of Obstetrics, Gynecology, and Reproductive Biology* **97** 40–45.
- Itoh S, Itoh F, Goumans MJ & Ten Dijke P 2000 Signaling of transforming growth factor-beta family members through Smad proteins. *European Journal of Biochemistry* **267** 6954–6967.
- Kim HY & Wolf G 1987 Vitamin A deficiency alters genomic expression for fibronectin in liver and hepatocytes. *Journal of Biological Chemistry* **262** 365–337.
- Kuang WW, Thompson DA, Hoch RV & Weigel RJ 1998 Differential screening and suppression subtractive hybridization identified genes differentially expressed in an estrogen receptor-positive breast carcinoma cell line. *Nucleic Acids Research* **26** 1116–1123.
- Kyriakis JM & Avruch J 1996 Protein kinase cascades activated by stress and inflammatory cytokines. *Bioessays* **18** 567–577.
- Lazar J, Desvergne B, Zimmermann EC, Zimmer DB, Magnuson MA & Nikodem VM 1994 A role for intronic sequences on expression of thyroid hormone receptor alpha gene. *Journal of Biological Chemistry* **269** 20352–20359.
- Lee J, Murata Y, Seo H, Menjo M, Torii S & Matsui N 1992 The effect of thyroid hormone on fibronectin messenger ribonucleic acid levels in primary cultured rat hepatocytes. *Endocrinology* **130** 2733–2738.
- Liao JK, Zulueta JJ, Yu FS, Peng HB, Cote CG & Hassoun PM 1995 Regulation of bovine endothelial constitutive nitric oxide synthase by oxygen. *Journal of Clinical Investigation* **96** 2661–2666.
- Lin K, Chen S, Zhu XG, Shieh H, McPhie P & Cheng S 1997 The gene regulating activity of thyroid hormone nuclear receptors is modulated by cell-type specific factors. *Biochemical and Biophysical Research Communications* **238** 280–284.
- Lin KH, Shieh HY & Hsu HC 2000 Negative regulation of the antimitastatic gene Nm23-H1 by thyroid hormone receptors. *Endocrinology* **141** 2540–2547.
- Lin KH, Wang WJ, Wu YH & Cheng SY 2002 Activation of antimitastatic Nm23-H1 gene expression by estrogen and its alpha-receptor. *Endocrinology* **143** 467–475.
- Marais R & Marshall CJ 1996 Control of the ERK MAP kinase cascade by Ras and Raf. *Cancer Survey* **27** 101–125.
- Miller LD, Park KS, Guo QM, Alkharouf NW, Malek RL, Lee NH, Liu ET & Cheng SY 2001 Silencing of Wnt signaling and activation of multiple metabolic pathways in response to thyroid hormone-stimulated cell proliferation. *Molecular and Cellular Biology* **21** 6626–6639.
- Murata Y, Seo H, Sekiguchi K, Imai T, Lee J & Matsui N 1990 Specific induction of fibronectin gene in rat liver by thyroid hormone. *Molecular Endocrinology* **4** 693–699.
- Pande H, Terramani T, Tressel T, Churchill MA, Hawkins GG & Zaia JA 1990 Altered expression of fibronectin gene in cells infected with human cytomegalovirus. *Journal of Virology* **64** 1366–1369.
- Pankov R & Yamada KM 2002 Fibronectin at a glance. *Journal of Cell Science* **115** 3861–3863.
- Samuels HH, Stanley F & Casanova J 1979 Depletion of L-3,5,3'-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. *Endocrinology* **105** 80–85.
- Sekiguchi K, Klos AM, Kurachi K, Yoshitake S & Hakomori S 1986 Human liver fibronectin complementary DNAs: identification of two different messenger RNAs possibly encoding the alpha and beta subunits of plasma fibronectin. *Biochemistry* **25** 4936–4941.
- Shanker G & Sawhney R 1996 Retinoic acid: identification of specific receptors through which it may mediate transcriptional regulation of fibronectin gene in bovine lens epithelial cells. *Cell Biology International* **20** 613–619.

- Shirakami A, Hirai Y, Takeichi T, Nishino H, Inomoto T, Watanabe S, Shigeiyo T, Kawauchi S, Saito H & Saito S 1986 Changes in plasma fibronectin levels in thyroid diseases. *Hormone and Metabolism Research* **18** 345–348.
- Srebrow A, Blaustein M & Kornblihtt AR 2002 Regulation of fibronectin alternative splicing by a basement membrane-like extracellular matrix. *FEBS Letters* **514** 285–289.
- Suzuki M, Oda E, Nakajima T, Sekiya S & Oda K 1998 Induction of Sp1 in differentiating human embryonal carcinoma cells triggers transcription of the fibronectin gene. *Molecular and Cellular Biology* **18** 3010–3020.
- Tamaroglio TA & Lo CS 1994 Regulation of fibronectin by insulin-like growth factor-I in cultured rat thoracic aortic smooth muscle cells and glomerular mesangial cells. *Experimental Cell Research* **215** 338–346.
- Vollmer G, Hopert AC, Ellerbrake N, Wunsche W & Knuppen R 1995 Fibronectin is an estrogen-repressed protein in RUCA-I rat endometrial adenocarcinoma cells. *Journal of Steroid Biochemistry and Molecular Biology* **54** 131–139.
- Watzke H, Schwarz HP & Weissel M 1987 Fibronectin during thyroid hormone replacement therapy. *Thrombosis Research* **46** 347–353.
- Wood WM, Dowding JM, Bright TM, McDermott MT, Haugen BR, Gordon DF & Ridgway EC 1996 Thyroid hormone receptor beta2 promoter activity in pituitary cells is regulated by Pit-1. *Journal of Biological Chemistry* **271** 24213–24220.
- Woodgett JR, Kyriakis JM, Avruch J, Zon LI, Zanke B & Templeton DJ 1996 Reconstitution of novel signalling cascades responding to cellular stresses. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **351** 135–141; discussion 142.

Received 2 May 2004

Accepted 29 June 2004

Made available online as an Accepted Preprint 8 July 2004