Thyroid hormone regulates the obesity gene *tub*

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Thyroid hormone T3/T4 is a major regulator of energy metabolism in vertebrates, and defects in thyroid status are frequently associated with changes in body weight. It is demonstrated here that thyroid hormone regulates *in vivo* and *in vitro* the *tub* gene, which when mutated in tubby mice causes obesity, insulin resistance and sensory deficits. Hypothyroidism in rats altered *tub* mRNA and protein in discrete brain areas. These changes could be attributed to thyroid hormone deficiency since T3/T4 treatment restored normal *tub* expression. T3 also upregulated *tub* mRNA within 4–6 h in neuronal cells in culture, suggesting that T3 is a positive regulator of *tub* gene expression. Thus, these results establish a novel pathway of T3 action and provide an important molecular link between thyroid status and the tubby-associated syndrome.

INTRODUCTION

Thyroid hormone (tri-iodothyronine, T3, and its precursor thyroxine, T4) plays an important role in development and physiology of vertebrate organisms, and altered T3/T4 levels manifest themselves in pleiotropic defects, e.g. in energy metabolism, body growth, neuronal development and in the cardiovascular system (Forrest, 1994; Freaque and Oppenheimer, 1995). T3 exerts its activity through binding to specific high affinity nuclear receptors (thyroid hormone receptors, TRs; Gronemeyer and Laudet, 1995; Mangelsdorf *et al.*, 1995). TRs are ligand-dependent transcription factors that regulate gene expression by interacting with specific response elements present in T3-responsive promoters. In mammals two TR genes exist, encoding TR α and

TR β , respectively. TR α and TR β have been inactivated in mice by gene targeted mutations, leading to defects e.g. in growth, bone maturation, heart rate, intestine development, body temperature and sensory functions (Forrest and Vennström, 2000; Ng *et al.*, 2001).

tub was initially identified in mouse as the gene involved in the tubby syndrome that includes late-onset obesity, insulin resistance, hearing loss and retinal degeneration (Kleyn *et al.*, 1996; Noben-Trauth *et al.*, 1996 and references therein). *tub* represents the founding member of a recently discovered multigene family that includes tubby-like protein 1, 2 and 3 (*tulp-1*, -2, -3) whose products contain a highly conserved C-terminal domain that shows no homology to other known proteins (Kleyn *et al.*, 1996; Noben-Trauth *et al.*, 1996; North *et al.*, 1997; Nishina *et al.*, 1998). Although highly homologous and exhibiting an overlapping expression profile, Tub and Tulp proteins seem to serve non-redundant roles in vertebrate organisms as demonstrated by gene inactivation experiments (Ikeda *et al.*, 2000; Stubdal *et al.*, 2000). Initial evidence has implicated Tub as an adapter protein in intracellular signaling of insulin receptor, while another study indicated that Tub might act as a transcription factor (Boggon *et al.*, 1999; Kapeller *et al.*, 1999). Additionally, targeted gene inactivation in mice demonstrated that the tubby phenotype results from the loss-of-function mutation of the *tub* gene (Stubdal *et al.*, 2000). Finally, the phenotype observed in tubby mutant and *tub*^{−/−} mice resembles certain human syndromes such as Usher, Alström and Bardet-Biedl (Kleyn *et al.*, 1996; Noben-Trauth *et al.*, 1996; Barsh *et al.*, 2000 and references therein), and mutations in the *tulp-1* gene in man have been implicated in retinitis pigmentosa, a hereditary

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Fig. 1. Thyroid hormone regulates *tub* expression in Purkinje cells of cerebellum in adult and neonatal rats. *In situ* hybridization micrographs of coronal cerebellar sections of control (A), hypothyroid (B) and T4-treated hypothyroid (C) adult animals, and of control (D) and hypothyroid (E) 10-day-old (P10) animals. Only Purkinje cells are labeled. (F) Background signal obtained by hybridizing sections of control adult brain with a sense riboprobe. EGL, external granular layer; ML, molecular layer; PCL, Purkinje cell layer; IGL, internal granular layer; wm, white matter. Bar in (F) corresponds to 200 μ m for (A)–(C) and (F), and 500 μ m for (D) and (E). Immunostaining of coronal cerebellar sections of control (G), hypothyroid (H) and T4-treated hypothyroid (I) adult animals with Tub-specific antibody. Bar in (G) corresponds to 50 μ m for (G)–(I).

disease of retina degeneration (see Ikeda *et al.*, 2000 for references).

A screen for T3-regulated genes by differential display identified *tub* as a potential TR target gene (see Supplementary data available at *EMBO reports* Online). This is interesting given the fact that T3 increases energy expenditure and that hypothyroidism increases the propensity to weight gain and eventually obesity in humans (Freake and Oppenheimer, 1995; Lowell and Spiegelman, 2000). Here we demonstrate that T3 regulates *tub* gene expression *in vivo* and *in vitro*, and thus provide a novel molecular link between thyroid status and the tubby-associated syndrome.

RESULTS AND DISCUSSION

Thyroid hormone regulates *tub* *in vivo*

To determine whether TR regulates *tub* gene expression *in vivo*, we analysed the effects of thyroid hormone administration and deprivation on *tub* expression in rat brain by *in situ* hybridization.

These experiments showed that *tub* mRNA is expressed in many regions of the adult rat brain, predominantly in neuronal populations (Figures 1 and 2, and data not shown). In line with previous reports in mice (Kleyn *et al.*, 1996; Sahly *et al.*, 1998), highest expression was found in cerebral cortex layers II and V, retrosplenial and piriform cortices, hippocampus, subiculum, hypothalamus and Purkinje cells of the cerebellum. In hypothyroid rats the regional pattern of expression was similar to that of control animals. However, most significantly in Purkinje cells, *tub* expression was lower in hypothyroid rats than in control (Figure 1A and B). To confirm that these differences were related to reduced levels of T3, a group of hypothyroid animals was treated with the pro-hormone thyroxine (T4), the major hormone produced in the thyroid gland, which crosses the blood–brain barrier more efficiently than T3 and is converted into the active T3 inside the cells. After four daily T4 injections, *tub* expression in Purkinje cells was restored and reached nearly the same level as in control (Figure 1C). Accordingly, immunohistochemical analysis of Purkinje cells revealed that Tub protein levels were

Fig. 2. Effect of thyroid hormone deprivation and of *in vivo* hormone treatment on *tub* expression in adult rat brain. *In situ* hybridization micrographs of coronal brain sections of control (A, D and G), hypothyroid (B, E and H) and T4-treated hypothyroid (C, F and I) animals. (A)–(C) Micrographs showing *tub* RNA expression in the subicular area (S), retrosplenial cortex (RS) and dentate gyrus (DG) of the hippocampus; fmj, forceps major corpus callosum. (D)–(F) Image of the somatosensory cortex. Note the higher signal in layers II and V, and subplate (sp) in hypothyroid animals (E) versus control (D) or T4-treated hypothyroid animals (F). (G)–(I) Labeled cells in the compact dorsomedial hypothalamic nucleus (DMC), surrounded by unlabeled cells in the diffuse dorsomedial hypothalamic nucleus (DMD). Bar in (D) corresponds to 500 μ m for (A)–(C) and to 200 μ m for (D)–(I).

reduced in hypothyroid animals and following T4 treatment restored to nearly normal levels (Figure 1G, H and I).

Interestingly, T3 deprivation had an opposite effect on *tub* expression levels in other brain areas. In hypothyroid animals, *tub* expression was higher in subiculum and retrosplenial cortex (Figure 2A and B) and in cortical layers II and V (Figure 2D and E). T4 treatment restored normal *tub* expression in all these areas (Figure 2C and F). The same result was obtained when *Tub* protein expression was analysed by immunohistochemistry (data not shown). There were only marginal differences in *tub* expression in ventral and dorsomedial hypothalamic nuclei (Figure 2G, H and I), which play a role in body weight control and feeding behavior (Kleyn *et al.*, 1996; Schwartz *et al.*, 2000). No differ-

ences in *tub* mRNA and protein expression were found in other regions such as the piriform cortex or the hippocampus.

Importantly, *tub* is one of the few genes found to be under thyroid hormone control in the adult brain while most target genes identified so far are exclusively thyroid dependent in the early postnatal period (reviewed in Bernal and Guadano-Ferraz, 1998). Consequently, we next analysed the influence of congenital hypothyroidism on *tub* expression. The major effect was found in the cerebellum. Like adult rats, hypothyroid 10-day-old animals showed reduced *tub* expression in Purkinje cells (Figure 1D and E). Differences in other brain regions were similar to but less apparent than those observed in adult rats. Taken together, these results indicated that *tub* expression in Purkinje cells is upregulated

Fig. 3. T3 upregulates *tub* expression. **(A)** *tub* expression in 911 cells was analysed by RT-PCR. *tub*-specific signals after treatment with 9cRA (lane 2; 6 h), T3 (lane 3, 4 h; lane 4, 6 h) or T3 plus 9cRA (lane 5, 6 h) were quantified by Phosphorimager, normalized for GAPDH control and plotted relative to untreated cells that were arbitrarily set as 100 (lane 1). Average values of three experiments with standard deviation are shown. **(B)** Northern blot analysis of *tub* expression in PC12 cells in response to T3 and 9cRA as indicated (lanes 5–8; 0.001, 0.01, 0.1 and 1 μM 9cRA, respectively). Signals were quantified as in (A) and average values of two independent experiments with duplicate values are shown. **(C)** Northern blot analysis of TRα1 and TRα2 expression in PC12 cells (total RNA). Control, brain poly(A)⁺ RNA. Probe was a 1 kb *Xba*I fragment of TRα cDNA that detects both TRα1 and TRα2. Exposure times were 24 h and 4 days for brain and PC12 cells, respectively. **(D)** TRα1 expression in PC12-TRα1 cells is induced by doxycyclin treatment (16 h) as revealed by western blotting using TR-specific antibody. No TRα1 induction was seen in unmodified PC12 Tet-On cells (not shown). PC12-TRα1 cells were also analysed for *tub* expression by northern blotting as indicated; equal amounts of total RNA were loaded per lane. **(E)** TRα1 expression was induced in PC12-TRα1 cells as in (D) followed by T3 or T3 plus 9cRA treatment as indicated, and *tub* expression was analysed by northern blotting. Signals were quantified and plotted relative to untreated cells as in (A).

by thyroid hormone *in vivo* while in other brain areas *tub* is regulated in a complex regional and temporal-specific fashion.

T3 upregulates *tub* expression *in vitro*

To gain further insight into the regulation of *tub* by T3, a panel of cell lines and primary cells representing neuronal and glial cell types was screened for *tub* expression. Rat PC12 cells, representing

neuronal/chromaffin progenitor cells, and human embryonic retina cells (911 cells; Fallaux *et al.*, 1996) were found to express *tub* (Figure 3 and data not shown), which is in accordance with the prominent *tub* expression in adrenal medulla and retina, respectively, in mice (Sahly *et al.*, 1998).

PC12 and 911 cells were then analysed for *tub* expression in response to T3. Cells were treated with T3 to activate TR or with T3 plus 9-*cis*-retinoic acid (9cRA) to also activate RXR, the

obligate heterodimeric partner of TR, and *tub* mRNA levels were monitored by RT–PCR and northern blot hybridization. 911 cells express TR α 1 and low levels of *tub* mRNA and protein (Figure 3A and data not shown), and *tub* expression in response to hormone was determined by RT–PCR. Importantly, T3 effectively induced *tub* expression within 4–6 h, which was even further enhanced by T3 plus 9cRA treatment (Figure 3A). In PC12 cells, *tub* mRNA levels remained virtually unaffected by T3 treatment (Figure 3B). This is most likely because of low levels of TR α 1 and TR β , and high expression of the non-hormone-binding TR α 2 isoform (Figure 3C and data not shown; Muñoz *et al.*, 1993), which is believed to counteract TR function (Forrest, 1994). In addition, 9cRA caused a dose-dependent downregulation of *tub*, possibly through formation of TR α 2–RXR heterodimers that might repress *tub* expression (Figure 3B). This downregulation by 9cRA was also seen in the presence of T3, indicating that the low level of endogenous hormone-activated TR α 1 is unable to relieve *tub* repression. Therefore, to increase TR α 1 activity, PC12 cells were generated that conditionally express TR α 1 from a tetracyclin-dependent expression vector.

PC12 Tet-On cells expressing the tetracyclin-controlled trans-activator were infected with retrovirus containing TR α 1 under the control of a tetracyclin response element. PC12-TR α 1 cells effectively expressed TR α 1 in response to the tetracyclin derivative doxycycline in a dose-dependent fashion (Figure 3D). Additionally, unliganded TR α 1 repressed *tub* transcription, in line with the *tub* downregulation observed in Purkinje cells of hypothyroid rats (Figure 1B and E, and Figure 3D). Downregulation of transcription by unliganded TR has been reported in several systems (see Bartunek and Zenke, 1998 and references therein). PC12-TR α 1 cells were then treated with T3 or T3 plus 9cRA, and 6 h later *tub* expression was analysed by northern blotting. Interestingly, increasing levels of hormone-activated TR α 1 elevated *tub* mRNA (Figure 3E). Ligand-activated TR α 1 also relieved the 9cRA-induced downregulation of *tub* (Figure 3B and E) possibly by alleviating the effect of TR α 2. Collectively, these results demonstrate that T3 upregulates *tub* expression.

Regulation of *tub* by T3 is interesting for several reasons: (i) TR and *tub* are coexpressed in various regions in the brain (Mellström *et al.*, 1991; Strait *et al.*, 1991; Forrest, 1994; Kleyn *et al.*, 1996; Sahly *et al.*, 1998) and, as also demonstrated here, in neuronal cells. (ii) TR α ^{−/−} mice and the tubby mutant or *tub*^{−/−} mice exhibit an overlapping phenotype. For example, TR β ^{−/−} mice show sensory defects in vision and hearing as do tubby mutant or *tub*^{−/−} mice (Forrest *et al.*, 1996; Kleyn *et al.*, 1996; Noben-Trauth *et al.*, 1996; Stubdal *et al.*, 2000; Ng *et al.*, 2001). This indicates that TR and *tub* may be interconnected and/or part of the same biochemical pathway(s). (iii) Defects in T3 metabolism and/or function (e.g. hypothyroidism) often manifest themselves in obesity as does inactivation of *tub* in mice.

Tub protein is specifically phosphorylated in response to insulin, thus supporting a role of Tub in signaling from insulin receptor (Kapeller *et al.*, 1999; Schwartz *et al.*, 2000). Our results suggest that T3, by affecting Tub expression, might influence insulin signaling. Yet another study suggested that Tub acts as a transcription factor that directly affects gene expression (Boggon *et al.*, 1999). Our observation that in PC12 and NIH 3T3 cells Tub protein is found both at the cytoplasmic membrane and in the nucleus (see Supplementary data) would

be compatible with Tub acting as an adapter molecule in insulin receptor signaling and/or as a transcription factor.

tub levels were very low in Purkinje cells of adult hypothyroid animals and induced by T3/T4 treatment as demonstrated here on the RNA and protein level. Thus, *tub* expression and its regulation by T3 in these cells might be important for *tub*-associated functions. Interestingly, recent evidence demonstrated that Purkinje cells express leptin receptor (Udagawa *et al.*, 2000); yet the importance of these two obesity-related pathways in Purkinje cells is presently unknown.

While T3/T4 upregulated *tub* expression *in vivo* and *in vitro* in some cells (Purkinje and 911 cells, respectively) its effects on *tub* in other cell types and brain areas were more complex. A distinct regional and temporal-specific regulation of genes by T3 is not without precedence and has been reported previously (Bernal and Guadano-Ferraz, 1998; Alvarez-Dolado *et al.*, 1999). This might be because T3 cooperates with other factors in regulating TR target gene expression, or because hormone action is modulated by region- and cell-specific proteins. Different expression levels of coactivators and corepressors that mediate TR transcriptional activity or the expression of TR isoforms (like TR α 2 in PC12 cells) are candidates to modulate T3 effects (Forrest, 1994). Another possible factor regulating hormonal effects is the selective expression of enzymes that are responsible for the generation of T3 from T4 and their catabolism to inactive metabolites in different brain regions (Escamez *et al.*, 1999 and references therein).

The impact of thyroid hormone on energy metabolism is well established and deficiencies in thyroid status are frequently associated with increased body weight. The finding reported here that T3 regulates the *tub* gene uncovers a novel pathway of how thyroid hormone might regulate body weight and might help to devise new strategies for treatment of the obesity syndrome.

METHODS

Induction of hypothyroidism. Adult-onset hypothyroidism in rats was induced by surgical thyroidectomy on P40 followed by treatment with 2-mercapto-1-methylimidazole (MMI, 0.02%; Sigma; 20 days), and animals were sacrificed on P60. At least three animals per experimental group were analysed. Thyroxine (T4; Sigma) was administered as single daily intraperitoneal injections of 1.8 µg/100 g body weight for 4 days and rats were killed 24 h after the last T4 injection. Neonatal hypothyroidism was induced as described (Alvarez-Dolado *et al.*, 1999).

In situ hybridization and immunohistochemistry. Coronal brain sections (25 µm) were analysed by *in situ* hybridization as described (Alvarez-Dolado *et al.*, 1999). Rat *tub* cDNA (DDBJ/EMBL/GenBank accession No. U92546) was used as template for riboprobes. For immunohistochemistry, tissue sections were incubated overnight with anti-Tub antibody (Santa Cruz Biotechnology Inc.), or as control, goat IgG, at 4°C. Sections were then incubated with a secondary biotinylated rabbit anti-goat antibody followed by ABC peroxidase detection system (Vector Laboratories).

Cells and cell culture. PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 5% horse serum and 5% fetal calf serum (FCS), 2 mM glutamine and 100 U/ml penicillin/streptomycin. 911 cells (Fallaux *et al.*, 1996) were grown in DMEM plus 10% FCS but no antibiotics. Ligand treatments were

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carried out with serum samples that were extensively deprived from T3 and retinoids (Bartunek and Zenke, 1998).

Analysis of *tub* expression by northern blotting and RT-PCR. TR α 1 cDNA (Sap et al., 1986) was introduced into pRev-TRE vector (Clontech), virus stocks were generated in Phoenix-ECO packaging cells and used for infection of PC12 Tet-On cells (Clontech). After hygromycin selection PC12-TR α 1 cells were treated with doxycyclin (16 h, 0.1 or 1 μ g/ml; Sigma) followed by treatment with T3 (0.1 μ M; Sigma) or T3 plus 9cRA (0.1 μ M and 1 μ M, respectively; Sigma). Total RNA was extracted and analysed by northern blotting with rat *tub* cDNA probe.

tub expression in 911 cells was analysed by semiquantitative RT-PCR (27 cycles) with *tub*-specific primers (positions 1290–1622). GAPDH-specific cDNA was amplified accordingly (20 cycles). PCR fragments were then analysed by Southern blotting using rat *tub* or mouse GAPDH as probes.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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