Thyroid hormone regulation of prohormone convertase 1 (PC1): regional expression in rat brain and *in vitro* characterization of negative thyroid hormone response elements

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Abstract

Most pro-neuropeptides are processed by the prohormone convertases, PC1 and PC2. We previously reported that changes in thyroid status altered anterior pituitary PC1 mRNA and this regulation was due to triiodothyronine (T₃)-dependent interaction of thyroid hormone receptor (TR) with negative thyroid hormone response elements (nTREs) contained in a large region of the human PC1 promoter. In this study, we demonstrated that hypothyroidism stimulated, while hyperthyroidism suppressed, PC1 mRNA levels in rat hypothalamus and cerebral cortex, but not in hippocampus. *In situ* hybridization was used to confirm real-time PCR changes and localize the regulation within the hypothalamus and cortex. Using a human PC1 (hPC1) promoter construct (with and without deletions in two regions that each contain a negative TRE) transiently transfected into GH3 cells, we found that T₃ negatively regulated hPC1 promoter activity, and this regulation required both of these two regions. Electrophoretic mobility shift assays (EMSAs) using purified thyroid hormone receptor α 1 (TR α 1) and retinoid X receptor β (RXR β) proteins demonstrated that RXR and TR α both bound the PC1 promoter. Addition of TR α 1/RXR β to the wild-type PC1 probe demonstrated binding as both homodimers and a heterodimer. EMSAs with oligonucleotides containing deletion mutations of the putative nTREs demonstrated that the proximal nTRE binds more strongly to TR and RXR than the distal nTRE, but that both regions exhibit specific binding. We conclude that there are multiple novel TRE-like sequences in the hPC1 promoter and that these regions act in a unique manner to facilitate the negative effect of thyroid hormone on PC1.

Journal of Molecular Endocrinology (2004) **33**, 21–33

Introduction

Prohormone convertases (PCs), are involved in the tissue-specific endoproteolytic processing of pro-hormones and neuropeptide precursors within the secretory pathway and are thought to be responsible for the cleavage of many prohormones at paired basic residues to generate bioactive hormones (Steiner 1998, Seidah & Chretien 1999). In mammals, seven prohormone and proprotein-processing

enzymes responsible for this cleavage have been molecularly characterized. PC1 and PC2 are specifically found in neural and endocrine cells equipped with a regulatory secretory pathway (Bloomquist *et al.* 1991, Day *et al.* 1992, Seidah & Chretien 1999) and are the likely enzymes involved in prohormone/pro-neuropeptide processing (Seidah & Chretien 1999). In the rat pituitary, PC1 is present primarily in the anterior lobe with lower levels in the posterior and intermediate lobes, while PC2 is found

predominantly in the intermediate lobe (Seidah *et al.* 1991). Pituitary PC1 and PC2 mRNA levels have been found to be regulated by dopamine agonists and antagonist (Birch *et al.* 1991, Bloomquist *et al.* 1991, Day *et al.* 1992).

The important role of PC1 and PC2 in hormonal biosynthesis has been elucidated by studies of PC1 and PC2 null mice and in a patient with defective PC1. PC1 null mice are short due to impaired pro-growth hormone-releasing hormone (proGHRH) processing and are hyperglycemic due to impaired proinsulin processing (Zhu *et al.* 2002*a,b*). PC2 null mice have absent proglucagon processing and impaired proinsulin processing and are hypoglycemic (Furuta *et al.* 1997). In addition, proenkephalin and prodynorphin processing are blocked in these mice (Berman *et al.* 2000, Laurent *et al.* 2004). A patient lacking PC1 had severe childhood-onset obesity, post-prandial hypoglycemia, infertility, and low levels of adrenocorticotrophic hormone (ACTH) and cortisol with elevated levels of pro-opiomelanocortin (POMC) (O'Rahilly *et al.* 1995, Jackson *et al.* 1997).

Thyroid hormone regulates target gene expression by binding with nuclear thyroid hormone receptors (TRs), which are ligand-activated transcription factors. Thyroid hormone acts through TRs at the transcriptional level to regulate many genes such as thyrotropin-releasing hormone (TRH), thyroid-stimulating hormone (TSH), growth hormone (GH) and α -myosin heavy chain (Lazar 1993). Triiodothyronine (T_3) mediates transcriptional regulation through interactions in the promoter region of target genes bearing consensus DNA sequences, referred to as thyroid hormone response elements (TREs) (Lazar 1993). Heterodimerization between TR and retinoid X receptor (RXR) usually augments the ligand-dependent stimulation or repression (Lazar 1993). However, in a few TREs, such as the one in human type-1 deiodinase promoter, RXR-independent mechanisms are involved in thyroid hormone regulation (Toyoda *et al.* 1995).

Limited studies of the regulation of processing enzymes by thyroid status have been performed in the rat pituitary. Rats made hypothyroid by thyroidectomy or 6-*n*-propyl-2-thiouracil (PTU) treatment had an increase in anterior pituitary PC1, PC2 (Day *et al.* 1992) and peptidylglycine α -amidating monooxygenase (PAM) (a processing enzyme involved in amidation) mRNA levels

(Ouafik *et al.* 1990), and a decrease in paired basic amino acid converting enzyme 4 (PACE4; a more ubiquitously distributed PC) mRNA levels (Johnson *et al.* 1994). Rats made hyperthyroid by daily injection of thyroxine (T_4) showed decreased anterior pituitary PC1, PC2 and PAM mRNA levels, and increased PACE4 mRNA levels (Day *et al.* 1992, Johnson *et al.* 1994).

We have previously shown that T_3 negatively regulates both PC1 and PC2 in the rat anterior pituitary and in GH3 cells (Li *et al.* 2000, 2001). We also localized regions on both human promoters which contain putative negative TREs (nTREs) (Li *et al.* 2000, 2001). In this paper, we first studied the *in vivo* regulation of PC1 mRNA by thyroid status in different regions of rat brain using *in situ* hybridization and real-time PCR. In order to address the mechanism of T_3 regulation of the PC1 gene at the promoter level, we used GH3 cells. This rat sommatotroph cell line expressing endogenous TRs (Hodin *et al.* 1989) has been widely used to study T_3 regulation of both endogenous (Misiti *et al.* 1998) and exogenous (Hooi *et al.* 1997) genes. We used human PC1 (hPC1) promoter-luciferase constructs and electrophoretic mobility shift assays (EMSAs) to identify two regions in the PC1 promoter that are both necessary for negative regulation by T_3 .

Materials and methods

Animals and treatments

Adult male Sprague–Dawley rats (8 weeks old, 250–270 g) were housed in a room with controlled light, temperature and humidity. Three groups of six animals each were treated as follows: control (euthyroid) group, implantation of placebo with normal chow; hyperthyroid group, implantation of thyroid hormone T_4 (L-thyroxine) pellet (15 mg) (Innovative Research of America, Sarasota, FL, USA) with normal chow; hypothyroid group, implantation of placebo with chow containing low iodine and 0.15% PTU (Harlan Teklad, Madison, WI, USA). Animals had free access to food and water; they were killed at 21 days after treatment and plasma was immediately collected.

Rat serum TSH levels were determined using a highly sensitive double-antibody method, developed by A F Parlow, Director of the National Hormone & Pituitary Program (Harbor–UCLA

Medical Center, Torrance, CA, USA; parlow@humc.edu), similar to that described for measuring mouse TSH (Schneider *et al.* 2001). The assay used highly purified rat TSH as the iodinated ligand, a guinea-pig anti-rat TSH at a final tube dilution of 1:500 000 as the primary antibody, and a partially purified extract of rat pituitary containing TSH as the reference preparation. Cross-reactivity of either highly purified rat follicle-stimulating hormone (FSH) or rat luteinizing hormone (LH) in this mouse TSH RIA was less than 1%. Displacement curves obtained by testing sera of hypothyroid rats in graded dilutions did not depart significantly from parallelism with displacement curves for the reference preparation. Recovery of exogenous rat TSH activity added to rat serum was 80–100%. All sera were assayed for TSH concentrations by a tester who had no knowledge of the treatment status of the rats.

Plasmid constructs and luciferase assay

The wild-type human PC1 (hPC1) promoter sequence and plasmid construct have been described previously (Li *et al.* 2001). Briefly, the wild-type hPC1 luciferase fusion gene expression plasmid was constructed by subcloning hPC1 promoter DNA comprising the region of –82 to +216 bp (numbers relative to the primary transcription start site (TSS)) into the pGL2-basic plasmid (Promega, Madison, WI, USA) which was then used for transfection into GH3 cells. Mutations of the proximal promoter elements were generated by the oligonucleotide-directed mutagenesis method, which was performed using *Pfu* DNA polymerase. Following temperature cycling and primer extension, the products were treated with DpnI and the desired mutations were then transformed into *Escherichia coli* XL 1-Blue supercompetent cells. Each hPC1 mutation was confirmed by sequencing.

Cell culture and transfection

GH3 cells (rat sommatotroph cells) were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Gibco) supplemented with 0.075% sodium

bicarbonate, 50 IU/ml penicillin-streptomycin. Cells were plated in growth medium in six-well plates and allowed to adhere overnight. The cells were then transfected with DNA (3 µg/well) of either wild- or mutation-type hPC1-luciferase, using the SuperFect transfection reagent (Qiagen) according to the manufacturer's protocol. The empty vector, pGL2-basic plasmid, was similarly transfected as a control. The medium was changed to DMEM serum-free medium for overnight incubation, and cells were treated with T₃ (10^{–8} M), or 9-*cis*-retinoic acid (9-*cis*-RA, 10^{–7} M) or all-*trans*-retinoic acid (all-*trans*-RA, 10^{–7} M) for 16 h and harvested in lysis buffer (Promega). Cell lysates were analyzed for luciferase activity using the dual-luciferase reporter assay system (Promega) following the manufacturer's protocol. This kit controls for transfection efficiency.

RNA extraction and quantitative real-time reverse transcription (RT)-PCR

Total RNA was extracted from tissue using the RNeasy reagent (Ambion, Inc., Austin, TX, USA), following the manufacturer's protocol. To amplify PC1 and GAPDH cDNA, upstream and downstream primers were designed based on published cDNA sequences (Bloomquist *et al.* 1991, Winer *et al.* 1999). The sequences of the primers were as follows: 5'-agatcccgagaagaagtgtctc-3' (PC1-5'), 5'-ctcttggaactgaacgttttc-3' (PC1-3'), 5'-tgcaccaccaa ctgcttag-3' (GAPDH-5') and 5'-ggatgcagggatga ttgttc-3' (GAPDH-3').

Quantitative measurement of PC1 and GAPDH cDNA using total RNA was performed by QuantiTect SYBR Green RT-PCR kit according to the manufacturer's instructions (Qiagen). The real-time cycling conditions were as follows: reverse transcription at 50 °C for 30 min and after denaturation at 95 °C for 15 min, the cDNA products were amplified with 40 cycles, each cycle consisting of denaturation at 95 °C for 15 s and annealing/extension at 58 °C for 1 min. The accumulating DNA products were monitored by the iCycler iQ optical system (BioRad), with the data being stored continuously during the reaction. Product purity was confirmed by dissociation curve analysis and agarose gel electrophoresis in the presence of ethidium bromide. The calculations of the initial mRNA copy numbers in each sample were made according

to the cycle threshold (C_T) method (Livak & Schmittgen 2001).

Labeling of cRNA probes and *in situ* hybridization

For *in situ* hybridization, a construct containing bases 65 to 556 of rat PC1 (Bloomquist *et al.* 1991) in pBluescript II SK-vector (Stratagene, La Jolla, CA), a generous gift from Richard Mains (now at University of Connecticut) was used. Antisense and sense RNA probes were generated by transcription with T3 and T7 RNA polymerase, with a DIG RNA labeling kit (Roche). *In situ* hybridization was performed as previously described (Shen *et al.* 1997) with slight modifications. Briefly, sections were deparaffinized with xylene, digested with proteinase K, post-fixed with 4% paraformaldehyde, washed in phosphate buffer and treated with 0.2 M HCl. The sections were hybridized overnight at 42 °C and then treated with ribonuclease A for 30 min. *In situ* hybridization signals were detected immunohistochemically with alkaline phosphatase-conjugated anti-DIG antibody according to the manufacturer's instructions (DIG nucleic acid detection kit; Roche).

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as described previously (Li *et al.* 2001), using double-stranded oligonucleotide probes corresponding to the hPC1 promoter sequence from -20 to +30 bp (wild-type, Wt), and thyroid hormone receptor element deletion mutations (TRE Mut), and were radiolabeled using [γ - 32 P]ATP (6000 Ci/mmol, ICN; MP Biomedicals, Aurora, OH, USA) and T_4 polynucleotide kinase. EMSA reactions were incubated with purified TR α 1 (0.5 μ g, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or RXR β (0.5 μ g, Biomol, Plymouth Meeting, PA, USA) proteins in reaction buffer (25 mM HEPES, 0.5 mM EDTA, 50 mM KCl, 10% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethyl sulfonyl fluoride and 2 μ g poly di-dC) for 10 min on ice and subsequently, the incubation was continued with radiolabeled DNA probes for 30 min. For antibody supershift experiments, specific antibodies were added to mixtures 10 min after the addition of the radiolabeled DNA probes. Protein-DNA complexes were analyzed on 6% polyacrylamide gels that were run using

25 mM Tris, 190 mM glycine and 1 mM EDTA buffer, and were visualized by autoradiography.

Nuclear extract preparation and EMSAs

Nuclear extracts were prepared from GH3 cells according to Dignam (1990). Protein concentrations in nuclear extracts were determined by BioRad protein assay kit. EMSAs were performed as described above using 0.5 μ g GH3 nuclear proteins.

Results

Changes in PC1 mRNA expression in rat hypothalamus, hippocampus and cortex by thyroid status

We previously showed that rat anterior pituitary PC1 mRNA levels are increased in hypothyroidism and reduced in hyperthyroidism, compared with euthyroid levels (Li *et al.* 2001). We now sought to understand whether PC1 mRNA was similarly regulated in important neuropeptide-rich brain regions, such as the hypothalamus, cortex and hippocampus. In the current study, rats were made hypothyroid and hyperthyroid by a 3-week procedure slightly modified from our previous protocol (Li *et al.* 2001); hypothyroidism was induced using low-iodine/PTU chow instead of drinking water containing PTU, and hyperthyroidism was induced by the implantation of sustained-release T_4 pellets instead of injection of T_3 . The hypothyroidism of PTU-treated rats was confirmed by demonstrating increased plasma TSH levels ($P<0.0001$), and the hyperthyroidism of T_4 -treated rats was confirmed by demonstrating decreased plasma TSH levels ($P<0.005$) (Fig. 1A). There was no overlap in TSH levels between the euthyroid and hypothyroid animals or between the euthyroid and hyperthyroid animals.

PC1 mRNA (determined by real-time PCR) increased significantly in the hypothalamus ($P<0.005$) and cerebral cortex ($P<0.05$) of hypothyroid rats compared with euthyroid animals (Fig. 1B). In contrast, no differences were detected in the mRNA levels of PC1 in hippocampus between hypothyroid and control rats ($P=NS$). On the other hand, in hyperthyroid rats, PC1 mRNA levels were significantly down-regulated in hypothalamus and cerebral cortex by T_4 compared with euthyroid

Figure 1 (A) Concentrations of plasma TSH in hypothyroid and hyperthyroid rats. Plasma TSH levels were measured by RIA. The data are represented as the means $\pm$ S.E.M. ($n=6$ /group). * $P<0.005$ vs control; ** $P<0.0001$ vs control. (B) Cortical, hypothalamic and hippocampal PC1 mRNA levels in hyperthyroid and hypothyroid rats as determined by real-time RT-PCR for each individual animal and corrected for GAPDH expression. Results are means $\pm$ S.E.M. ($n=5$ /group). * $P<0.05$; ** $P<0.005$.

animals ($P<0.05$). However, no significant change was detected in mRNA levels of PC1 in the hippocampus between hypothyroid and euthyroid rats ($P=NS$). These data indicate that there are brain region differences in thyroid hormone responsiveness for PC1.

Localization of PC1 mRNA expression in the rat hypothalamus, cortex and hippocampus

Thyroid hormones are essential for controlling a variety of metabolic and developmental processes, in particular brain regions (Bernal 2002). To determine the localization of PC1 mRNA in rat neuropeptide-rich brain regions, we used *in situ* hybridization with a non-radioactive cRNA probe to localize thyroid hormone regulation of PC1 expression in more detail (Fig. 2). Using a sense

probe, no hybridization signal was detected in hypothalamus (Fig. 2A), cortex (Fig. 2B) or hippocampus (Fig. 2C). Using an antisense probe, PC1 was clearly present in the granular layer of CA1, CA2 and CA3 and dentate gyrus (DG) of hippocampus (Fig. 2E), but no significant mRNA level changes occurred between the three groups. We found that in the cerebral cortex, PC1 mRNA was highly expressed in the retrosplenial (RS) agranular cortex and was abundant in the whole cerebral cortex region, including the motor cortex (M), auditory cortex, and somatosensory cortex (S) (Fig. 2E). Compared with control rats, *in situ* hybridization of the PTU-treated hypothyroid rats and the T_4 -treated hyperthyroid rats demonstrated a similar pattern in rat brain cerebral cortex, however the hypothyroid rats had higher expression than the control rats and the T_4 -treated rats had lower expression than the control rats (Fig. 2E, F and G). Additionally, PC1 mRNA expression in the hypothalamus was found in periventricular (Pe), paraventricular (Pa), and medial preoptic (MPO), as well as in the lateral preoptic (LPO) area, with the highest concentrations in the basal hypothalamus (Fig. 2H). Although similar PC1 mRNA expression patterns were found in the three groups, the expression levels of PC1 were higher in hypothyroid and lower in hyperthyroid rats (Fig. 2H, I and J).

T_3 and 9-*cis*-RA regulation of hPC1 promoter activity through two nTREs

We previously performed transient transfection assays in GH3 cells using a series of deletion hPC1 promoter constructs in the presence and absence of T_3 and reported that the minimal construct -82 to +19 bp exhibited both basal luciferase activity and down-regulation by T_3 (Li *et al.* 2001). We detected two TRE-like half-sites, which we named nTRE1 and nTRE2 in this region of the promoter. With the mutation of the central two and three nucleotides of two putative nTREs, the inhibition by T_3 treatment was only partially abolished using these mutated promoters (Li *et al.* 2001). These results led us to hypothesize that the T_3 inhibitory effect may involve more than these two TRE-like half-sites, and proposed that this region contains multiple TRE-like half-sites or other response elements that are directly or indirectly regulated by T_3 and which function in concert to mediate T_3

Figure 2 *In situ* localization of PC1 mRNA in rat brains of hyperthyroid, hypothyroid and euthyroid rats using non-radioactive DIG-labeled cRNA probes. (A, B and C) Hybridization with a sense PC1 cRNA probe in control rat hypothalamus (A), cortex (B) and hippocampus (C). (D) Hybridization with an antisense PC1 cRNA probe in control hippocampus (C). (E, F and G) Hybridization with an antisense PC1 cRNA probe in control cerebral cortex (E), T_4 -treated rat cortex (F) and PTU-treated rat cortex (G). (H, I and J) Localization on PC1 mRNA in control rat hypothalamus (H), T_4 -treated rat hypothalamus (I), PTU-treated rat hypothalamus (J). Abbreviations: Pe, periventricular hypothalamic nucleus; Pa, paraventricular hypothalamic nucleus; MPO, medial preoptic area; 3V, third ventricle; f, fornix; LPO, lateral preoptic area; CA1, 2 and 3, field CA of hippocampus; RS, retrosplenial granular cortex; M, motor cortex; S1, primary somatosensory cortex. Scale bars, 100 μ m.

regulation. To directly study this hypothesis, in the next series of experiments, we tested the regulation by T_3 , 9-*cis*-RA or all-*trans*-RA of hPC1 promoter construct -82 to +216 bp (higher promoter activity was present with this construct compared with using -82 to +19 bp) with either the nTRE1 or nTRE2 intact or deleted (Fig. 3A). 9-*cis*-RA binds primarily to the RXR, while all-*trans*-RA binds primarily to the retinoic acid receptor (RAR). Because mutations in the central nucleotide core of the two nTREs still led to significant negative

regulation by T_3 (Li *et al.* 2001), it was deemed necessary to delete regions of the promoter containing either nTRE1 and nTRE2, or both, in order to understand the relative contributions of each of these nTREs. Transfection of a construct with deletion of nTRE1 (PC1 Mut1) or nTRE2 (PC1 Mut2) alone showed substantial promoter activity, but there was still significant T_3 inhibition of hPC1 promoter luciferase activity (Fig. 3B; $P < 0.05$). However, transfection of a construct with deletion of both the nTRE1 and nTRE2 region

Figure 3 Effect of T_3 , 9-*cis*-RA and all-*trans*-RA on wild-type or deleted mutant human PC1 promoter (hPC1). (A) Schematic representations of hPC1 luciferase constructs (–82 to +216) wild-type (PC1 Wt), deletion mutant TRE1 (PC1 Mut1), deletion mutant TRE 2 (PC1 Mut2), and double-mutant TRE 1 and TRE 2 (PC1 dMut). (B) GH3 cells transfected with Wt or Mut hPC1 promoter luciferase construct and treated with or without T_3 (10^{-8} M) for 24 h. The data from triplicate measurements are expressed as means $\pm$ S.E.M. * P < 0.05 vs without T_3 . (C) GH3 cells transfected with Wt or Mut hPC1 promoter luciferase construct and treated with or without 9-*cis*-RA (10^{-7} M) for 24 h. The data from triplicate measurements are expressed as means $\pm$ S.E.M. * P < 0.05 vs without 9-*cis*-RA. (D) GH3 cells transfected with Wt or Mut hPC1 promoter luciferase construct and treated with or without all-*trans*-RA (10^{-7} M) for 24 h. The data from triplicate measurements are expressed as means $\pm$ S.E.M.

(PC1 dMut) showed significant promoter activity, yet absence of inhibition by T_3 treatment (P =NS). This indicates that the T_3 inhibitory effect involves multiple regions in this part of the promoter, more than the two discrete nTREs identified in our previous study.

In contrast to T_3 treatment, 9-*cis*-RA treatment resulted in 2-fold stimulation of the wild-type hPC1 promoter when transfected into GH3 cells (Fig. 3C; P < 0.05). Using the same single-region deletion constructs, significant stimulation by 9-*cis*-RA-treated promoter activity using both PC1 Mut1 or PC1 Mut2 constructs was still observed (P < 0.05),

in a pattern similar to the stimulation using the wild-type construct. But when both the nTRE1 and nTRE2 regions were deleted (PC1 dMut), 9-*cis*-RA stimulation was completely abolished (P =NS). These data indicate that the full nTRE1 and nTRE2 regions of the hPC1 promoter contain important *cis*-acting DNA elements for negative regulation by T_3 and positive regulation by 9-*cis*-RA and this full region is involved in the transcriptional regulation of this important target gene.

On the other hand, when all-*trans*-RA (10^{-7} M) was added to PC1-promoter (–82/+216 bp region

(A) Wt: -20CTGGGAAGGGCTGGGGCTCCACTCAGCCTGGAGACCGAAGCGCTTCACT₊₂₉
Mut 1: -20CTGGGAAGGGGCCTGGAGACCGAAGCGCTTCACT₊₂₉
Mut 2: -20CTGGGAAGGGCTGGGGCTCCACTCAGCGCTTCACT₊₂₉
dMut: -20CTGGGAAGGGGCGCTTCACT₊₂₉

of the hPC1 promoter)-transfected GH3 cells, no stimulatory effects were noted (Fig. 3D). These results indicate that RAR does not interact with this region of the PC1 promoter.

EMSAs using wild-type and mutant oligonucleotides with TR α 1 and RXR β proteins

To further determine whether the TR/RXR binding sites of the hPC1 promoter act as a unique region interacting with the TR α 1 and RXR β proteins, and if T₃ can cause a change in protein binding to these regions, we performed EMSA experiments using this region of the PC1 promoter. The EMSAs were performed using a series of radiolabeled oligonucleotide probes. The wild-type (Wt) oligonucleotide probe contained sequences from -20 to +29 bp of the PC1 promoter, which contain both nTRE1 and nTRE2. The deleted mutant-type 1 (Mut1) oligonucleotide probe contained sequences from -20 to +29 bp of the PC1 promoter without sequences from -10 to +5. The deleted mutant-type 2 (Mut2) oligonucleotide probe contained sequences from -20 to +29 bp of the PC1 promoter without sequences from +6 to +19. The deleted double-mutant (dMut) oligonucleotide probe contained sequences from -20 to +29 bp of the PC1 promoter without sequences from -10 to +19 (Fig. 4A). In all experiments, the amount of probe added to each lane was normalized by the number of counts. When TR α 1 was added to the reactions with Wt PC1 probe, predominantly a TR homodimer was formed as indicated by the smaller arrow in Fig. 4B (lane 5). Addition of both TR α 1/RXR β proteins to the Wt PC1 probe resulted in strongly binding complex (larger arrow) consisting of an RXR homodimer and a TR plus RXR heterodimer (lane 6). It is noteworthy that the effects of RXR were much stronger in the current paper than in our prior publication (Li *et al.* 2000, 2001), which we postulate may reflect a difference in protein preparation supplied by the company. The intense TR/RXR complex was reduced with decreasing

amounts of RXR added in a dose-dependent manner (lanes 2–4). As expected, homodimer binding between the wild-type probe and TR was not affected by the dose of RXR (lanes 2–4). In contrast to the Wt probe, the Mut1 probe exhibited substantially decreased TR/RXR complex binding and mildly decreased TR homodimer binding (the same number of counts of each probe were added to each lane) (lanes 7–8) compared with the Wt probe. The Mut2 probe exhibited essentially no TR homodimer binding and substantially decreased TR/RXR complex binding (lanes 9–10) compared with the Wt probe (lanes 5–6). We observed that the dMut did not form specific protein complexes with either TR alone or TR plus RXR (lanes 11 and 12) (see Fig. 4D, lanes 22–24, which demonstrates that the faint binding in the heterodimer region with the dMut is not specific).

To confirm the specificity of the interactions between the RXR protein and the PC1 promoter probe, we tested the effect of 9-*cis*-RA and T₃ using EMSAs. As shown in Fig. 4C, 9-*cis*-RA increased the binding of RXR β alone to the PC1 probe (compare lane 3 with lane 1). Similarly, 9-*cis*-RA increased the binding of RXR β plus TR to the PC1 probe (compare lane 4 with lane 2). In contrast, T₃ did not affect the binding of RXR β to the PC1 probe (compare lane 5 with lane 1). These results confirm the specificity of the binding between RXR and the PC1 promoter and agree nicely with our studies showing 9-*cis*-RA increases PC1 promoter activity (Fig. 3C).

To test if the binding of the TR/RXR proteins to the PC1 promoter probe was specific, we examined the effects of T₃ or TR or RXR antibodies on the protein–probe complex (Fig. 4D). As can be seen in lanes 1 and 2, both TR (small arrow) and RXR (large arrow) form homodimer bands with the Wt probe, with stronger binding using RXR compared with TR. The band complex (indicated by the larger arrow) most probably represents binding of an RXR homodimer and a TR plus RXR heterodimer to the probe. The addition of T₃ to the Wt probe in the presence of

Figure 4 Binding of TR α 1 and RXR β proteins to wild-type (Wt) and mutant (Mut) oligonucleotides. (A) Sequences of Wt and Mut oligonucleotide. (B) EMSAs using purified TR α 1 and RXR β proteins binding to Wt and mutant probes. Components of the EMSAs are indicated at the top of the figure. (C) Effect of 9-*cis*-RA and T₃ on EMSAs using purified TR α 1 and RXR β proteins binding to the Wt hPC1 probe. (D) Effect of T₃ and RXR β Ab and TRAb on EMSAs using purified TR α 1 and RXR β proteins binding to Wt and mutant probes. Components of the EMSAs are indicated at the top of the figure.

TR plus RXR caused a dramatic reduction in this complex (Fig. 4D, lane 4). The addition of anti-TR α antibody (TRAb) reduced the TR homodimer band, but had little effect on the complex (lane 5), while the addition of anti-RXR β antibody (RXRAb) reduced the complex band of TR plus RXR (lane 6). As indicated in Fig. 4D, lanes 7–12, the binding of the Mut1 probe to TR, RXR or TR plus RXR revealed quite similar binding patterns compared with the Wt probe. The addition of the Mut2 probe also revealed similar but substantially reduced binding (lanes 13–18) compared with the Wt. However, the dMut probe failed to exhibit specific TR or RXR homodimer binding, or heterodimer complexes (lanes 19–24). Thus, from these EMSA experiments, we conclude that both TR and RXR bind more strongly to the nTRE2 region than to the nTRE1 region, but TR and RXR are capable of some binding to both these regions, which is absent in the double mutation. These results indicate that the TR/RXR binding sites of the hPC1 promoter involve both regions containing the nTREs, which both act in concert to mediate T₃ and RXR regulation. Thus, both our transfection experiments and our EMSA data support the finding that both broad regions in the hPC1 promoter are needed to exert the full negative effect of T₃ and the positive effect of 9-*cis*-RA.

EMSAs using PC1 promoter oligonucleotide with GH3 nuclear extracts

In order to confirm that the binding of the wild-type oligonucleotide hPC1 promoter with purified TR α 1 and RXR β proteins reflects the binding within GH3 cells, we performed EMSAs using GH3 cell nuclear extracts to the PC1 promoter probe. As shown in Fig. 5, a complex that probably represents binding of an RXR homodimer and a TR plus RXR heterodimer to the probe was observed (lane 2). This binding (primarily the lower band of the complex) was decreased with the addition of T₃ (lane 3) and increased by the addition of 9-*cis*-RA (lane 5). TR α 1 (lane 4) and RXR (lane 6) antibody diminished the intensity of the complex and caused the appearance of a less mobile band complex (supershift). These results confirm the specificity of binding to TR and RXR proteins in GH3 nuclear extracts.

Figure 5 The binding of the GH3 nuclear extracts to the Wt hPC1 probe. The effect of T₃ and 9-*cis*-RA as well as RXR β Ab and TRAb on EMSAs using GH3 nuclear extracts is shown.

Discussion

PC1, as a member of the prohormone convertases family, is an important processing enzyme which usually makes the earlier cleavages of prohormones (Zhou *et al.* 1993). In combination with PC2, which often makes the later cleavages, PC1 expression determines the ratio of many inactive prohormone precursors to active hormones (Li *et al.* 1999, Nie *et al.* 2000). Baseline expression of PC1 and PC2 in various brain regions has been well studied (Schafer *et al.* 1993, Pu *et al.* 1996), but regulation of expression of PC1 and PC2 following various pharmacological manipulations has been limited to the pituitary. Schafer *et al.* (1993), using *in situ* hybridization, found that in general, PC2 was more widely expressed than PC1 in the central nervous system (CNS), although many regional variations were detected. The finding that some brain regions express PC1, some express PC2 and some express both convertases (Schafer *et al.* 1993) suggests that PC1 and PC2 may act both independently and in combination for the activation of neuropeptides in important brain regions. In the present study, we found that basal brain expression of PC1 was of a

similar pattern to that reported by Schafer *et al.* (1993). We then demonstrated that PTU-induced hypothyroidism stimulated, and T₄-induced hyperthyroidism suppressed, PC1 mRNA expression in the rat hypothalamus and cerebral cortex, as measured by real-time PCR and localized by *in situ* hybridization. The regulation was specific to certain brain regions, as we did not detect regulation by thyroid hormone status in the hippocampus. These results led us to postulate that the regulation of PC1 by thyroid hormone would be expected to alter levels of bioactive neuropeptides in important brain regions and may explain some of the profound central effects of altered thyroid hormone status. In another study, we have found that thyroid hormone treatment regulates PC1 and PC2 protein expression in various brain regions in a similar manner as the gene expression described here (Shen *et al.*, unpublished observations). This leads to blunted proTRH processing in selective brain regions of hyperthyroid rats and increased proTRH processing in hypothyroid rats (Shen *et al.*, unpublished observations).

Thyroid hormones are essential for mammalian growth and development. They also exert specific effects on several organ systems, including cardiovascular, reproductive, central and peripheral nervous systems (Herman-Bonert & Friedman 2001). A number of studies have indicated that thyroid hormones have important physiological functions, not only during brain maturation, but also in the adult vertebrate brain (Bernal 2002). Multiple genes in the CNS are regulated by thyroid hormone status (Anderson *et al.* 2000). One example is the neuronal gene, RC3, whose expression was influenced by early neonatal hypothyroidism as well as thyroid hormone treatment in adult rat brain (Iniguez *et al.* 1992). Additionally, chronic thyroxine treatment down-regulated the noradrenergic cyclic AMP generating system in rat cerebral cortex (Schmidt & Schultz 1985). Thus, our finding that thyroid hormone status regulates PC1 expression in brain regions is consistent with the effects of thyroid status on the expression of other genes.

Thyroid hormone facilitation of brain development is based on the assumption that thyroid hormone action proceeds along a nuclear pathway generally similar to that operating in other tissues (Oppenheimer & Schwartz 1997). TRs bind to the TREs located upstream from the promoters of

target genes to regulate their expression transcriptionally (Harvey & Williams 2002). In most cases, TRs are transcriptional repressors in the absence of their cognate hormone (T₃) and are turned into activators upon ligand binding (Zhang & Lazar 2000). We previously identified two potential nTRE half-sites on the PC1 promoter (Li *et al.* 2001). Mutation of the core two nucleotides of these half-sites still leads to significant negative regulation of PC1 promoter activity by T₃. We hypothesized that the negative transcription mediated by T₃ on this region thus involves more than the two putative nTRE half-sites, and may also involve the activation of co-repressors or the suppression of other co-activators.

Recent studies have demonstrated that RXR heterodimerization can augment T₃-mediated gene regulation to increase TR/DNA interactions (Force *et al.* 1994, Harbers *et al.* 1996). However, the magnitude of the augmentation varies significantly, especially when different cell lines were used. Both 9-*cis*-RA and T₃-stimulated rGH promoter, and the combination of T₃ and 9-*cis*-RA exerted additive effects on this promoter in GH3 cells (Li *et al.* 2001). In our study, using the hPC1 promoter transiently transfected into GH3 cells, we found that T₃ negatively regulated hPC1 promoter activity, while 9-*cis*-RA stimulated the activity, indicating that binding to RXR abrogates, rather than augments the effects of T₃ on TR. All-*trans*-RA had no effect on promoter activity indicating that RAR does not interact with this region of the PC1 promoter.

In our previous study, after mutation of the central two and three nucleotides of the two putative nTREs, substantial residual inhibition by T₃ was found. These results led us to hypothesize that the T₃ inhibitory effect may involve more than these two TRE-like half-sites, and proposed that this region contains multiple sites which either directly or indirectly mediate T₃ regulation. In the present study, deletional analysis of hPC1 promoter constructs in transient transfection assays coupled with EMSAs demonstrate that the broad region from -10 to +19 bp of the hPC1 promoter is probably responsible for the negative regulation by T₃. However, the fact that deletion of nTRE2 in the EMSAs (Fig. 4B) led to the most dramatic decrease in binding to both TR and RXR provides evidence that the region between +5 and +19 on the hPC1 promoter is most crucial for binding to TR and RXR. This region is near the TSS, similar

to the observation for nTREs described by others (Sato *et al.* 1996). Transcriptional repression by T₃ can occur by binding of TRs to specific nTREs near the TSS to sterically interfere with components of transcription initiation machinery (Oppenheimer & Schwartz 1997). Although the primary TSS is deleted from the double mutant, it is noteworthy that the double mutant still had greater than 50-fold more activity than the control (promoterless) construct, indicating that the transcriptional machinery is still present in this promoter. This suggests that alternative TSS in the PC1 promoter can be used, as suggested by Jansen *et al.* (1995).

The fact that in the double mutant, there was no specific binding to either TR or RXR (the non-specific binding seen in lane 20 and 21 of Fig. 4D was not supershifted by TR or RXR Ab or reduced by T₃), provides evidence that there are no other TR or RXR binding sites in this region of the PC1 promoter. Our findings suggest that multiple nuclear factors may be involved in T₃ suppression of the hPC1 promoter, and indicate that the full nTRE1 and nTRE2 regions of the hPC1 promoter contain important *cis*-acting DNA elements to recruit transcriptional cofactors (corepressors and coactivators) to the promoter to facilitate transcriptional regulation of the target genes.

In summary, this paper demonstrates that thyroid status regulated PC1 expression in the hypothalamus and cortex. These results suggest that this prohormone convertase is involved in neuropeptide precursor processing not only in pituitary, but also in neuropeptide-rich brain regions. We also demonstrated that nTRE2 binds more strongly to TR and RXR than nTRE1, however, both regions exhibit some specific binding. Binding to the PC1 promoter oligonucleotide occurred with GH3 nuclear extracts suggesting that this binding can occur in intact cells. These results indicate that there are multiple elements in this region of the hPC1 promoter that TR and RXR interact with either directly or indirectly, and that these regions act in concert in a unique manner to facilitate the negative effect of thyroid hormone on PC1.

Acknowledgments

We wish to thank Monica Ferrini (Harbor-UCLA Medical Center) for her assistance with interpreting the *in situ* hybridization results, Richard Mains

(University of Connecticut) for supplying us with the rat PC1 plasmid and Erik Jansen (N.V. Organon, The Netherlands) for supplying us with the PC1 promoter construct.

Funding

T C F and X S are supported by a Center of Clinical Research Excellence grant (U54 RR14616-01) to Charles R Drew University of Medicine & Sciences. G A B is supported by Merit Review funds from the Department of Veterans Affairs and NIH Grant CA8936401. This work was also supported by a Thyroid Research Advisory Council (Knoll Pharmaceutical Company) grant SYN-0400-02 to T C F and an RCMI Program grant G21 RR03026-13 to Charles R Drew University of Medicine & Sciences. There is no conflict of interest that could prejudice the impartiality of this research

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Received in final form 10 March 2004

Accepted 30 March 2004