Purification and characterization of thyroid transcription factor 2

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Thyroid transcription factor 2 binds to the promoters of both thyroglobulin and thyroperoxidase genes, two markers of thyroid tissue differentiation, and its binding modulates the activity of both promoters. In this paper we describe the purification of thyroid transcription factor 2 essentially to homogeneity and

demonstrate that it is a thyroid-specific DNA-binding protein. Furthermore, we provide a biochemical characterization suggesting that thyroid transcription factor 2 binds to DNA as a dimer and that it is a zinc-finger DNA-binding protein regulated *in vitro* by the redox state.

INTRODUCTION

Two of the most studied thyroid-specific proteins are thyroglobulin (Tg) and thyroperoxidase (TPO). The first is the precursor of the thyroid hormones tri-iodothyronine and thyroxine and the second is the enzyme that catalyses the coupling of iodine to Tg [1]. The tissue-specific expression of these thyroid-differentiation markers is regulated at the transcriptional level [1,2]. The promoters of both Tg and TPO genes in rat have been identified and are very similar in both the *cis* and *trans* elements [3,4]. Both have three binding sites for thyroid transcription factor 1 (TTF1) and both bind a second thyroid transcription factor, thyroid transcription factor 2 (TTF2). They also bind ubiquitous factors including UFA to the Tg promoter and UFB to the TPO promoter [3,4]. Recently Pax8, a paired domain transcription factor expressed in thyroid and kidney [5], has been demonstrated to be involved in both Tg and TPO gene regulation [6]. The binding site of Pax8 on both promoters overlaps with the TTF1-binding site proximal to the TATA box. The binding of the two factors at this site is mutually exclusive [6].

A schematic representation of the DNA–protein complexes on both Tg and TPO promoters is shown in Figure 1. The rat TTF1 gene has been cloned from a cDNA library obtained from a differentiated rat thyroid cell line (FRTL5) and it has been shown that TTF1 is a homeodomain containing transcription factor [7]. TTF1 is expressed in thyroid and lung [7]. Therefore it has been proposed that the thyroid-specific transcription of the Tg and TPO genes relies on the co-ordinated activity of the same transcription factors with a restricted tissue distribution and that the thyroid specificity of Tg and TPO gene expression depends on the fact that the exact combination of these factors is present only in the thyroid cell [4]. In fact, only the thyrocyte expresses TTF1, TTF2 and Pax8 at the same time. As TTF1 and Pax8 genes have been cloned [5,7], we know much more about these proteins than about TTF2. It binds on the K region of the Tg promoter and on the Z region of the TPO promoter (Figure 1) [3,4]. The functional importance of TTF2 binding to both promoters has been demonstrated by mutation experiments. In transfection assays in FRTL5 cells, both promoters containing a mutagenized TTF2-binding site showed reduced activity; Tg promoter activity was reduced twofold and that of the TPO promoter fourfold [3,4]. It has been shown also that TTF2-binding activity is modulated by hormones, insulin/insulin-like

growth factor I and thyrotropin [8,9]. Nuclear extracts of FRTL5 cells grown in the absence of these hormones are depleted of TTF2-binding activity. Furthermore, TTF2-binding activity disappears on transformation of FRTL5 cells with the *Ki-ras*, *Ha-ras* and poliovirus middle-T oncogenes. It has been proposed that this down-regulation contributes to the dedifferentiated phenotype of the transformed thyroid cells [10].

With the aim of characterizing it, we have purified TTF2 from calf thyroid nuclear extracts. We here present the purification scheme and data suggesting that it is a thyroid-specific zinc finger DNA-binding protein regulated by the redox state of the cell.

Figure 1 Schematic model of Tg and TPO promoters illustrating DNA–protein interactions

Thyroid-specific and ubiquitous factors are indicated as well as TTF2-binding sites K and Z on the Tg and TPO promoters respectively [3,4,6]. TTF2 is drawn as a dimer as proposed in this study. The arrangement of the ubiquitous factors TBP and TAFS on the TATA box is purely hypothetical. The transcription start sites of the two promoters are represented by arrows.

Abbreviations used: Tg, thyroglobulin; TPO, thyroperoxidase; TTF1, thyroid transcription factor 1; TTF2, thyroid transcription factor 2; DTT, dithiothreitol; NEM, *N*-ethylmaleimide.

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MATERIALS AND METHODS

Nuclear extracts and band-shift assays

Calf tissues were obtained from the local slaughterhouse, immediately frozen in liquid nitrogen and stored at -80°C . Nuclear extracts were prepared as described by Lichtsteiner et al. [11], but using frozen calf tissue and homogenized using a standard blender. Protein concentration was determined using the Bio-Rad protein assay with BSA as standard. DNA-binding assays were performed as previously reported [12]. The oligonucleotide K used in the band-shift assays and to prepare the affinity column was prepared by annealing the Kt (5'-TGACTAGCAGAGAAAACAAAGTGA-3') and Kb (5'-TCACTTTGTTTCTCTGCTAGTCA-3') oligonucleotides in 10 mM Tris/HCl, pH 8, containing 1 mM EDTA and 0.1 M NaCl.

TTF2 purification

All purification steps were performed at $0-4^{\circ}\text{C}$. The resins and columns were from Pharmacia-LKB. Undialysed calf thyroid nuclear extracts were frozen in liquid nitrogen and stored at -80°C until processed. Nuclear proteins (10 mg) were extracted from about 30 g of frozen calf thyroid tissue and diluted to a conductance corresponding to 200 mM KCl with binding buffer [20 mM Hepes, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethanesulphonyl fluoride]. These proteins were loaded on 20 ml of Q Phast Flow matrix in an XK 26 column. After loading, the column was washed with 3 vol. of binding buffer containing 200 mM KCl. The bound proteins were eluted using a linear gradient from 0.2 to 1 M KCl in this buffer. Fractions containing TTF2-binding activity were determined by electrophoretic mobility-shift assays, pooled and dialysed against 0.2 M KCl in binding buffer. They were then loaded on to a 5 ml heparin-Sepharose CL-6B column. The bound proteins were eluted with a linear gradient of 0.2–1 M KCl in binding buffer. Active fractions were diluted to 200 mM KCl with binding buffer and loaded on to the sequence-specific affinity matrix (0.5 ml) prepared with oligonucleotide K as described previously [13]. This column was washed with 2 ml of binding buffer containing 200 mM KCl and the proteins were eluted with a 5 ml linear gradient from 0.2 to 1 M KCl in binding buffer. The active pool was diluted to 0.2 M KCl with binding buffer and loaded again on to the same affinity column. Fractions from the second affinity column containing TTF2-binding activity were pooled and stored at -80°C .

Materials

The materials used in the tissue culture media were obtained as reported by Civitareale et al. [12]. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was from NEN-DuPont. Diamide, 1,1'-azobis(*N,N*-dimethylformamide), and *N*-ethylmaleimide (NEM) were purchased from Sigma. Enzymes were from Pharmacia. Autoradiography was performed using Kodak X-R5 films.

RESULTS AND DISCUSSION

Thyroid-restricted distribution of TTF2

The oligonucleotide K, containing the sequence derived from the TTF2-binding site on the Tg promoter (see Figure 1 and the Materials and methods section), has been used in a band-shift assay to detect the presence of the factor in the nuclear extracts

Figure 2 Tissue distribution of TTF2

Oligo K, labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and polynucleotide kinase, was incubated with 5 μg of the indicated nuclear proteins: K indicates the probe alone; T, thyroid, Li, liver, Lu, lung and H, heart. All the extracts were tested for the presence of DNA-binding activity indicated by the ability to bind an AP1-binding site (results not shown). The TTF2–DNA complex is the major retarded band; the other minor bands are not characterized but are probably degradation products of TTF2.

Figure 3 TTF2 purification

(a) TTF2-purification scheme. (b) Band-shift assay of the last affinity-column fractions. The first lane shows oligo K alone, labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and polynucleotide kinase. The second lane shows the band-shift assay of the input of the column. The remaining lanes show band-shift assays of the column fractions. The activity is eluted at 500 mM KCl as indicated. (c) SDS/10% polyacrylamide gel stained with AgNO_3 . Relative positions of molecular-mass markers are indicated on the left.

of several bovine tissues. Binding activity corresponding to TTF2 is present only in the thyroid tissue (Figure 2). Although the analysis should be extended to other tissues, the data presented here suggest that TTF2-binding activity has a thyroid-restricted distribution.

Figure 4 Estimation of the molecular mass of native TTF2 by gel-filtration chromatography

Band-shift assays of the fractions from the gel-filtration chromatography are shown; 300 μ g of thyroid nuclear proteins were loaded on an f.p.l.c. Superdex 75 column and eluted in binding buffer containing 200 mM KCl. Lane 1, oligo K alone, labelled with [γ - 32 P]ATP and polynucleotide kinase; lane 2, band-shift assay with 5 μ g of thyroid nuclear proteins; lanes 3–19, assays of the column fractions. Bovine γ -globulin (158 kDa) and BSA (132 kDa as dimer and 66 kDa as monomer) were used as molecular-mass markers. The molecular mass corresponding to the fraction with TTF2 activity is indicated.

TTF2 purification

TTF2 was purified by a combination of anion-exchange and affinity chromatography (Figure 3a). Heparin–Sephacrose CL-6B is an unspecific affinity step for DNA-binding proteins. The active fractions eluted from heparin–Sephacrose CL-6B were applied to a TTF2 sequence-specific affinity-chromatography column. This step was repeated twice. Assay of the fractions from the second affinity column shows that TTF2-binding activity is eluted at 500 mM KCl (Figure 3b). This fraction, on a silver-stained protein gel, shows a single band with a molecular mass of approximately 60 kDa (Figure 3c). As it has been previously demonstrated by Southwestern analysis that this is the molecular mass of TTF2 [4], we are confident that the 60 kDa band is TTF2. The yield of TTF2-specific activity at each purification step was not determined as activity was tested using a different amount of unspecific DNA competitor, poly(dI-dC) ranging from 1 mg/ml in the assay for crude nuclear extract to none in the fraction in the last step of purification. Quantification of activity by the gel mobility-shift assay was therefore approximate, as at each purification step the amount of TTF2–probe complex depended not only on the amount of active factor, but also on the concentration of poly(dI-dC) in the assay.

TTF2 binds DNA as a dimer

To estimate the molecular mass of TTF2 in native conditions, calf thyroid nuclear extract was chromatographed on a gel-filtration column. Figure 4 shows the assay of the f.p.l.c. Superdex 75 (Pharmacia) fractions. Under native conditions, the molecular mass of TTF2 was found to be about 110 kDa, with an assumed gyration radius of a globular molecule. We obtained the same result using glycerol gradient sedimentation (not shown). As under denaturing conditions, the molecular mass of TTF2 is 60 kDa [4], these results suggest that native TTF2 binds DNA as a dimer. The dimer could be either a homodimer or a heterodimer with subunits of similar sizes. Denatured TTF2 in the Southwestern experiment was able to bind to DNA [4]. There are two possible explanations for this: (i) the monomer is also able to bind

Figure 5 Requirement of Zn^{2+} for TTF2-binding activity

Band-shift assay with oligo K, labelled with [γ - 32 P]ATP and polynucleotide kinase, in the presence of 5 μ g of (a) thyroid nuclear proteins (TNP) and (b) purified TTF2 as indicated. The concentration of $ZnSO_4$ and 1,10-phenanthroline was 10 μ M and 5 mM respectively.

to DNA; (ii) although less likely, during the renaturation step in the Southwestern experiment a homodimer formed on the filter.

Binding of TTF2 to DNA requires zinc

During purification of TTF2 we noticed that its binding activity to DNA was lost during prolonged dialysis and this phenomenon was greatly decreased by adding micromolar concentrations of Zn^{2+} to the dialysis buffer (results not shown). Therefore we tested whether TTF2 binding required Zn^{2+} . In Figure 5 a band-shift assay is shown in which treatment with a chelator such as 1,10-phenanthroline [14] inhibited TTF2 binding in both calf thyroid nuclear extract and the purified fraction. In the presence of 1,10-phenanthroline, TTF2-binding activity was recovered when 10 μ M Zn^{2+} was added to the assay. The binding activity was not recovered when the same amount of other metals was used (results not shown), thus indicating that TTF2 has a specific requirement for Zn^{2+} ions in order to bind to DNA. This experiment suggests that this factor is a zinc-finger DNA-binding protein. If this is confirmed by subsequent cloning and sequencing of the TTF2 gene, the thyroid-specific activity of the Tg and TPO promoters should provide a good experimental system for studying the co-ordinated action of different classes of transcription factor. In fact, both promoters bind TTF1 (a homeo-domain transcription factor), Pax8 (a paired-domain protein) and possibly a zinc-finger factor, TTF2.

TTF2-binding activity is reversibly inactivated by oxidizing reagents and requires thiol groups

Thyroid nuclear extract was treated with reagents that oxidize free thiol groups and reagents that reduce oxidized thiol groups. Diamide is an oxidizing agent and NEM alkylates thiol groups [15–18]. Figure 6 (lanes 1–6) shows that the TTF2–DNA complex was inhibited by the presence of 1 mM diamide in the assay. To determine whether the oxidizing agent irreversibly inhibited binding, a reducing agent, DTT, was added. The presence of 5 mM DTT restored the TTF2–DNA complex (Figure 6, lane 5).

Figure 6 TTF2-binding activity is affected by the redox state

Band-shift assay with oligo K, labelled with [γ - 32 P]ATP and polynucleotide kinase, in the presence of 5 μ g of thyroid nuclear proteins (TNP). The presence of the reagents is indicated above the lanes. (+) indicates, as described in the text, that the diamide only in this case was added to the proteins after the DNA probe. [+] indicates that, in order to remove DTT, the thyroid nuclear proteins were dialysed against 80 mM KCl in binding buffer without DTT. The NEM concentration used in the experiment shown in lane 11 was 0.5 mM.

In previous experiments, diamide and/or DTT were added at the same time as the nuclear extract and before the labelled DNA probe. However, when the proteins were incubated with the probe before the addition of diamide, the oxidizing agent was no longer able to inhibit the binding. Hence the action of diamide on TTF2 takes place only on the free protein and not when it is complexed with DNA. It is worth pointing out that 1 mM DTT is present in the buffer of the thyroid nuclear extract and that addition of 5 mM DTT, in the band-shift assay, slightly stimulated TTF2 binding (Figure 6, lanes 2 and 3). This stimulation was more evident when 5 mM DTT was added to the assay with the DTT-depleted thyroid extract (Figure 6, lanes 7 and 8). These experiments show that TTF2, like other mammalian transcription factors, binds to the target DNA sequence in its reduced form and binding is reversibly inhibited by oxidation.

The presence of an important thiol group in this protein is shown by the experiment with NEM (Figure 6, lanes 9–11). This alkylating agent inhibited binding of TTF2 to DNA, indicating once more that the presence of thiol groups within the TTF2 molecule is crucial for its activity.

It is worth mentioning that, although all cell types produce oxygen radicals constitutively [19,20], in thyroid cells these radicals are involved in one of the most important biochemical reactions, i.e. the coupling of iodine to Tg, and this reaction is catalysed by TPO [21]. Hence the redox state, particularly in thyroid cells, must be tightly controlled. It has been demonstrated that H_2O_2 regulates the activity of TPO [22] and consequently Tg iodination. As TTF2 modulates TPO gene expression [4], a molecular explanation of this phenomenon could reside, at least in part, in TTF2 sensitivity to the redox environment. Experiments are in progress to address this question. Oxidation inhibits binding to DNA of other redox-regulated transcription factors [15–18,20,23]. This sensitivity is usually due to cysteine residues directly affecting the binding of the factor to DNA. Several zinc-finger proteins are regulated by the redox state and, among transcription factors, the glucocorticoid receptor is activated by

thioredoxin reductase [24] and the *Caenorhabditis elegans* lin-11 is also a redox-sensitive DNA-binding protein [25].

A characteristic property of TTF2 is that many different agents can induce its DNA-binding activity. In fact, it has been demonstrated that TTF2 is involved in the hormonal regulation of both Tg and TPO gene expression [8,9]. Its binding activity is regulated positively by cyclic AMP and negatively by Ca^{2+} [9]. In this paper, we show that the redox state also modulates TTF2-binding activity *in vitro*. Therefore it appears that this transcription factor may play a crucial role, not only in thyroid-specific transcription, but also in signal transduction between extra- and intracellular signals and thyroid gene expression.

An attractive hypothesis about the role of TTF2 is that, in addition to modulating thyroid-specific expression, it may be required to initiate transcriptional competence of the thyrocyte. Consistent with this hypothesis is the finding that TTF1 and Pax8 genes are expressed, during thyroid development, at day 10.5 post coitum whereas the expression of Tg and TPO starts with a delay of 5 days [26]. Thus the factors required to maintain thyroid gene transcription, namely TTF1 and Pax8, are unable to initiate it *de novo*. Among several possible explanations of this phenomenon, our hypothesis envisages that additional factors may be required to establish thyroid-specific gene transcription. Such a factor could be TTF2. If this hypothesis is correct, the pre-thyroid cell should already be determined at day 10.5 post coitum. This step is likely to be determined by the expression of TTF1 and Pax8 [26], but to become a differentiated thyrocyte it needs TTF2 to be expressed, which may occur at about day 15 post coitum. The presence of TTF2 would then allow expression of the thyroid-differentiation markers. A similar role has been proposed for NF κ B in plasmacytoma cells; this factor is required for establishment but not maintenance of κ gene transcription [27].

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