Gamma-interferon activates a nuclear protein that binds to the gamma-interferon activation site of the thyroglobulin gene

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ABSTRACT

The release of interferon-gamma (IFN γ) has been demonstrated from the infiltrating T lymphocytes of the thyroid gland from patients with autoimmune thyroid disease (AITD). We have shown previously that IFN γ inhibited thyroglobulin (Tg) gene transcription, and that its action was mediated by an increase in intracellular calcium and inositol phosphates. In the present study, we tried to determine the specific site of action of IFN γ on the Tg gene. A 565 bp fragment (position -530 to +34) spanning the transcriptional start site of the human Tg promoter was ligated to the luciferase plasmid and transiently transfected into human thyrocytes. Stimulation with TSH (10 mIU/l) and IFN γ (500 IU/l) resulted in a twofold increase and a 60% reduction in the luciferase activity respectively, similar to the effect observed with the endogenous Tg gene. Deletion studies revealed that the region with the strongest suppression by IFN γ lay between 5' -388 to -258. Mobility gel shift experiments and DNA footprinting experiments demonstrated

that the action of IFN γ was mediated through a *trans*-acting protein which complexed to position -282 to -262 TTGAGCCTGTTCCCTC CAAA. Position -272 to -261 TTCCCTCCAA corresponded to the gamma-interferon activation site (GAS) consensus sequence TTNC^C_T NNNAA. The turnover time of the nuclear protein lasted for only 4 h although the suppressive effect of IFN γ on Tg gene transcription lasted for 48 h. The effect of IFN γ was lost when the thyrocytes were co-treated with genistein, a specific tyrosine kinase inhibitor.

The presence of the GAS in the promoter sequence of the Tg gene confirms the specific action of IFN γ in thyroid hormone metabolism. In conclusion, apart from its regulatory role in T cell development and perpetuation of the immune response in AITD, IFN γ may also play a role in altering cellular function of the thyrocytes by its action on the Tg gene promoter.

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INTRODUCTION

Autoimmune thyroid disease (AITD) is a common condition affecting between 2% and 4% of the population. The condition is characterized by lymphocytic infiltration of the thyroid gland together with the production of auto-antibodies against thyroid specific antigens, including thyroglobulin, thyroid peroxidase (TPO) and the thyrotropin (TSH) receptor. The intrathyroidal T lymphocytes are very important in the pathogenesis of AITD through thyroid antigen recognition as an essential step to B cell stimulation and for mediating the release of cytokines (Volpe 1993, Weetman & McGregor 1994). The clinical presentation of AITD depends on the spectrum of the cytokines

and antibodies produced by the activated lymphoid cells, as these cytokines and auto-antibodies are capable of altering the function and metabolism of the thyroid gland. Among the various cytokines, the action of gamma-interferon (IFN γ) has been the best studied. Functional IFN γ receptors have been identified on the thyrocytes, and thus IFN γ secreted from the infiltrating lymphocytes can exert a direct effect on the thyroid epithelial cells (Nishikawa *et al.* 1993). The macrophages and lymphocytes infiltrating the thyroid gland from patients with AITD have been shown to release IFN γ into the adjacent thyrocytes (Watson *et al.* 1994). Enhanced production of IFN γ by thyroid-derived T cell clones from patients with Hashimoto's thyroiditis has been demonstrated (Del-Prete *et al.* 1987).

Indeed, in thyroid sections from patients with either Hashimoto's thyroiditis or Graves' disease, class II MHC-positive thyrocytes were found immediately adjacent to aggregates of IFN γ -positive lymphocytes (Hamilton *et al.* 1991), suggesting that class II expression by the thyrocytes was secondary to the release of IFN γ by the infiltrating T cells. Furthermore, IFN γ inhibited proliferation of thyrocytes and altered the expression of the various thyroid specific proteins (Nagayama *et al.* 1987, Ashizama *et al.* 1989, Kung & Lau 1990, Kung *et al.* 1992, Watson *et al.* 1994). All these findings suggest that, apart from its role in the perpetuation of an autoimmune response in AITD, IFN γ may also have a direct effect on the cellular function of and the hormone production by the thyrocytes.

We have previously demonstrated that IFN γ inhibited thyroglobulin (Tg) gene transcription in human thyrocytes, and that the intracellular signaling pathway of its action was via mobilization of intracellular calcium and increased production of inositol phosphates (Kung *et al.* 1995). As Tg is the pro-thyroid hormone, inhibition of its production would result in hypothyroidism in AITD. The aim of the present study was to identify the specific site of action of IFN γ on the Tg gene. Understanding the role and action of IFN γ on the Tg gene may enable us to identify specific targets for intervention in AITD.

MATERIALS AND METHODS

Cells

Normal para-adenomatous human thyroid tissues obtained from subjects undergoing hemithyroidectomy were enzymatically digested and cultured in R.p.m.I-1640 medium supplemented with 10% fetal calf serum (Hinds *et al.* 1981). These cells had been confirmed previously to respond to TSH stimulation by the production of cAMP and release of tri-iodothyronine and Tg. For the stimulation experiment, the cells were resuspended in 1% bovine serum albumin at a concentration of 10^6 /ml. Recombinant human IFN γ (rhIFN γ , 500 IU/ml; Genzyme Diagnostics, Cambridge, MA, USA) was added to the culture for between 2 and 48 h. Bovine TSH (bTSH), forskolin, genistein and other chemicals were obtained from Sigma Chemicals Co. (St Louis, MO, USA).

Transient transfection study

A 565 bp fragment (position -530 to +34) spanning the transcriptional start site of the human

TABLE 1. Results of transient transfection with p Tg. luc in human thyrocytes

	Relative light units
p Tg. luc	4359
p Tg. luc+bTSH (10 mU/l)	17 330
p Tg. luc+IFN γ (500 IU/l)	2695
p Tg. luc+bTSH+IFN γ	2650
p Tg. luc+forskolin (10^{-5} M)	8108
Positive pGL-control luciferase plasmid	13 347
No transfection	191

Tg gene was subcloned into the pGL2-enhancer luciferase plasmid (Promega, Madison, WI, USA) between the KpnI and XhoI cloning sites. The sequence contains the cAMP-responsive region which is located within the first 250 bp of the promoter, as well as the TATA box (Christophe *et al.* 1985, 1989). For the transient transfection experiment, 2.5 μ g recombinant p Tg. luciferase (p Tg. luc) plasmid was introduced into the human thyroid cell culture by lipofectum-mediated transfection (Transfectam Assay, Promega). To monitor for transfection efficacy, the cells were co-transfected with 0.5 μ g β -galactosidase plasmid. Transfected cells were cultured in the presence or absence of rhIFN γ for 48 h. Cell extracts were prepared and the luciferase and β -galactosidase activities were determined. In each experiment, the results were adjusted for the β -galactosidase activity.

To determine the minimal sequence in the Tg promoter responsible for the action of IFN γ , exonuclease III was used to generate a set of 5' deletions from the Tg promoter fragment. The deleted DNA was subcloned into the luciferase plasmid for transient transfection experiments. For confirmation, all transfection studies were repeated three times.

Cell extracts

Nuclear extracts were prepared according to the method described by Dignam *et al.* (1983). Cells (10^9) were harvested from the culture and collected by centrifugation. The cell pellet was suspended in 10 mM Tris-HCl, pH 7.9, 1.5 mM MgCl $_2$, 10 mM KCl, 0.5 mM dithiothreitol at 4 °C for 10 min and again collected by centrifugation and broken down by Dounce homogenization in a small volume of the same buffer. The cell lysate was centrifuged at 25 000 *g* for 20 min to remove residual cytoplasmic material. The crude nuclei were further broken down by Dounce homogenization in the presence of 25% glycerol. Particulate matter was removed by

TABLE 2. Transient transfection with deletion mutants of Tg promoter

Construct	Relative light units	
	Without IFN γ	With IFN γ
— 530 ————— +34	8662	4181
— 470 ————— +34	7308	3642
— 388 ————— +34	7324	4591
— 258 ————— +34	7943	7690
— 530 ————— -171	919	917

centrifugation. The extract was collected for protein content estimation and stored at -80°C .

Gel retardation assays

DNA fragments of Tg promoter were incubated with nuclear extracts ($10\text{ }\mu\text{g}$) at 37°C for 10 min (Gel Shift Assay Systems, Promega). After 30 min incubation with $\gamma\text{-}^{32}\text{P}$ -labeled DNA fragment (5000–10 000 c.p.m.), free and bound DNA were separated by electrophoresis on 6% polyacrylamide gels. For competition experiments, the AP₂ consensus oligonucleotide (1.75 pmol) was added as the non-specific competitor.

DNA footprinting assay

The Tg promoter fragment was labeled with T4 polynucleotide kinase and $20\text{ }\mu\text{g}$ nuclear protein extract was incubated with the labeled Tg promoter fragment at 37°C for 3 h. The top strand probe was prepared by removing the label from the 3' end of the fragment by digestion with XhoI. DNA footprinting was carried out with the Core Footprinting System (Promega). Sequencing was performed using T7 DNA polymerase (Sequenase Version 2.0 DNA Sequencing Kit, United States Biochemical, Amersham, IL, USA) and the DNA was loaded onto a 6% polyacrylamide sequencing gel.

RESULTS

Transfection study

In transient transfection experiments bTSH (10 mIU/l) increased luciferase activity by 2- to 4-fold and IFN γ decreased luciferase activity by 60%. These responses to bTSH and IFN γ were similar to those observed with the endogenous Tg

gene (Kung & Lau 1990), suggesting that the minimal promoter element contained the DNA signals necessary for TSH and IFN γ regulation. Forskolin (10^{-5} M) increased luciferase activity twofold. Table 1 shows the typical result from one set of experiments.

Systematic deletion experiments were performed to determine the minimal DNA sequence necessary for the action of IFN γ . The suppressive effect of IFN γ was lost when cells were transfected with mutant -258 to $+34$, suggesting that the minimal region required for the IFN γ response lay between position -388 and -258 (Table 2). Transfection with mutant -530 to -171 did not show any luciferase activity, as the regulatory elements of the Tg promoter lay within the first 250 bp of the 5' flanking region.

Gel shift assay

Gel shift assays demonstrated a mobility shift with Tg promoter sequence -316 to -199 (Fig. 1). Specific competition and mobility shift resulting in a retarded band was observed with nuclear extracts prepared from thyrocytes that had been incubated with IFN γ for 4 h (lane d). Nuclear extracts prepared from control thyrocytes did not demonstrate mobility shifting (lane b). Incubation with IFN γ for 2 h revealed a retarded band of weaker intensity (lane c), whereas incubation for 8 h (lane e) or longer (data not shown) did not demonstrate any mobility shift. In the presence of the non-specific competitor AP₂ consensus oligonucleotide, the specific retarded band persisted (lane f).

DNA footprinting experiment

This showed that the action of IFN γ was mediated via a *trans*-acting protein which complexed to the

FIGURE 1. Gel shift assays were carried out with ³²P-labeled probe (– 316 to – 199 of Tg promoter). Lane a, probe only; lanes b–e, nuclear extracts from IFNγ-treated cells, and 5 pmol specific competitor (– 316/– 199). Nuclear proteins from control thyrocytes (lane b), or incubation with IFNγ for 2 h (lane c), 4 h (lane d), 8 h (lane e). Lane f, non-specific competitor AP₂ oligonucleotide and nuclear extracts from thyrocytes incubated with IFNγ for 4 h. Arrow shows position of the retarded band.

DNA sequence – 282 to – 262 TTGAGCCTGT TCCCTCCAAA. Position – 272 to – 261 TTCC CTCCAA is homologous to the gamma-interferon activation site (GAS) consensus sequence TTNC^C_TNNNAA (Decker *et al.* 1991). Binding was visualized from nuclear extracts obtained from cells that were incubated with IFNγ for 2–4 h (Fig. 2). Nuclear extracts from cells incubated with IFNγ for a longer period did not demonstrate binding to the DNA, suggesting that the nuclear protein was turned on for only 4 h or less. Treatment with genistein, a specific tyrosine protein kinase inhibitor, resulted in loss of action of IFNγ (Fig. 3).

DISCUSSION

The results of this study demonstrated that the Tg gene contained the specific GAS in its promoter

FIGURE 2. DNA footprinting revealed protein complexing to DNA position – 282 to – 262 of the Tg promoter. Binding was observed from nuclear extracts of cells incubated with IFNγ for 4 h or less. AP₂ protein showed no complexing to the Tg promoter.

FIGURE 3. DNA footprinting; co-treatment with genistein (50 μg/ml) resulted in loss of action of IFNγ, and no binding of the nuclear extracts to the Tg promoter was observed. GEN, genistein; CON, control.

region and that the action of IFNγ was mediated through a *trans*-acting protein. Among the few thyroid-specific proteins, the Tg gene is the best

characterized (Musti *et al.* 1986). The molecular mechanisms involved in the tissue-specific and hormone-dependent expression of the Tg gene have been studied both in primary cells and in transgenic animals (Civitareale *et al.* 1989, Ledent *et al.* 1990). Using nuclear run-on experiments and measurement of steady state mRNA levels, we had previously demonstrated that IFN γ exerted an inhibitory action on Tg gene transcription (Kung & Lau 1990). However, Nishikawa *et al.* (1993) suggested that this effect of IFN γ on the Tg gene was probably secondary to the negative effect of IFN γ on TSH receptor expression. The results obtained from the present study confirmed that IFN γ had a direct specific effect on the Tg gene which could be inhibited by genistein, a specific tyrosine protein kinase inhibitor. We further showed that the action of IFN γ on the human Tg gene was mediated by a *trans*-acting protein, similar to the action of IFN γ on the lymphoid cells. Whether this *trans*-acting protein is the same gamma-interferon activation factor (GAF) as described for the lymphoid cells remains to be confirmed (Decker *et al.* 1991). In the lymphoid cell lines, partial purification of GAF revealed a prominent 91 kDa protein, and this GAF activity was inhibited by antibodies to STAT 1 α (Khan *et al.* 1993). The STAT (signal transducers and activators of transcription) proteins are activated through phosphorylation of tyrosine residues of the Jak-1 and -2 kinases (Igarashi *et al.* 1993, Muller *et al.* 1993). These proteins are then translocated to the nucleus where, by themselves or in combination with other DNA binding proteins, they bind to the GAS and stimulate transcription (Darnell *et al.* 1994). We had previously also demonstrated that the effect of IFN γ on the mobilization of intracellular calcium and the production of inositol phosphates in human thyrocytes were inhibited by genistein (Kung *et al.* 1995), suggesting that the signal transduction mechanism for IFN γ was the same for both lymphoid and non-lymphoid cells. However, whereas IFN γ produced a stimulatory action on the responsive genes in the lymphoid cells, IFN γ exerted an inhibitory effect on the Tg gene. Whether this difference lies in the transcriptional factor that differs between lymphoid cells and the thyroid epithelial cells or whether the Tg gene also binds other proteins which modulate the promoter activity remains to be elucidated. Although the induction of the transcriptional protein in the thyrocytes lasted for only 4 h, the effect of the IFN γ -induced inhibition of Tg transcription continued for much longer and lasted for 48 h as reported previously (Kung & Lau 1990). The same phenomenon was

observed in the responsive genes in the lymphoid cells.

Apart from its action on the Tg gene, IFN γ had also been reported to inhibit the transcription of TPO and TSH receptor gene (Nagayama *et al.* 1987, Ashizama *et al.* 1989) by a reduction of steady-state mRNA level. The signaling mechanism of IFN γ for these two genes has not been described and it is not certain whether these genes also contain the GAS in their promoter region. Searching the 5' flanking sequence of the human TPO gene revealed two regions in the distal 5' area, -2475 to -2466 and -1994 to -1985, showing some degree of homology to the minimal sequence of the GAS (Kimura *et al.* 1989). Whether these two regions are important in the control of TPO gene transcription and whether they are the sites of IFN γ action remain to be confirmed. No GAS region could be identified from the published sequence of the TSH receptor 5' flanking region.

At present, the pathogenesis and the initiation of AITD is far from well understood. Although early data suggested that aberrant expression of class II antigen by thyroid epithelial cells may be the initiating process of AITD (Bottazzo *et al.* 1983), recent studies found that expression of class II antigen alone, without the presence of a co-stimulatory second signal, actually conferred peripheral tolerance and led to T cell anergy (Miller & Morahan 1992, Weetman 1995). Whether IFN γ has a role in the initiation of AITD is uncertain. Nevertheless, the presence of the GAS in the promoter sequence of the Tg gene confirms a specific action of IFN γ in thyroid hormone metabolism. The clarification of the cellular action of IFN γ on the Tg gene enables us to have a better understanding of how these cytokines affect the cellular function of and the hormone production by the thyrocytes. The identification of the activation signals and biological function of these cytokines on the thyroid-specific antigens may enable us to develop alternative immuno-modulatory agents for AITD.

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