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Induction of Transcription Factor Interferon Regulatory Factor-1 by Interferon-γ (IFNγ) and Tumor Necrosis Factor-α (TNFα) in FRTL-5 Cells

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Abstract While it is well known that interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) play a role in the regulation of thyroid growth and differentiated functions, the cellular and molecular mechanisms involved in mediating the effects of IFNγ and TNFα on thyroid function are unknown. In the present study, we used FRTL-5 rat thyroid cells to examine the effects of IFNγ and TNFα on gene expression of transcription factor interferon regulatory factor-1 (IRF-1), which is involved in mediating the effects of these cytokines in a number of cell types. Northern blot analysis of FRTL-5 mRNA showed a single IRF-1 mRNA at 2.2 Kb. In quiescent FRTL-5 cells, IRF-1 mRNA levels were low but detectable by Northern analysis. Incubation of FRTL-5 cells with IFNγ or TNFα resulted in a dose- and time-dependent increase in IRF-1 mRNA levels. We have shown that TNF-α and IFN-γ act synergistically to block the TSH-induced increase in type I 5'-deiodinase (5'D-I) activity and 5'D-I gene expression in FRTL-5 rat thyroid cells. Incubation of FRTL-5 cells with IFNγ and TNFα in combination, however, did not synergistically increase IRF-1 mRNA levels. Electrophoretic mobility shift assay (EMSA) revealed that IFNγ induced the formation of a single complex to a IFNγ activation site (GAS) probe in a dose dependent manner. Several lines of evidence suggest that TNFα activates transcription factor nuclear factor-κB (NFκB) through activation of protein kinase C (PKC) or the hydrolysis of sphingomyelin to ceramide in a number of cell types. Here we demonstrate that hydrolysis of sphingomyelin to ceramide by sphingomyelinase (SMase), but not activation of PKC by 12-O-tetradecanoylphorbol 13-acetate (TPA), was involved in the activation of NFκB in FRTL-5 cells. Similarly, hydrolysis of sphingomyelin to ceramide, but not activation of PKC, resulted in an increased in IRF-1 mRNA levels in FRTL-5 cells. The present data demonstrate that IFNγ and TNFα increase IRF-1 mRNA levels in FRTL-5 cells through activation of GAS and NFκB binding proteins, respectively. Thus, our results suggest that upregulation of IRF-1 may play a role in mediating the effects of IFNγ and TNFα on thyroid function. Our results also suggest that the induction of IRF-1 mRNA by IFNγ and TNFα is not the cellular mechanism involved in the synergistic effect of these cytokines on thyroid function. J. Cell. Biochem. 74:211–219, 1999.

Key words: interferon-γ; tumor necrosis factor-α; thyroid

Interferon regulatory factor-1 (IRF-1) is a transcriptional activator originally shown to regulate interferon (IFN) inducible genes. Several lines of evidence, however, suggest that IRF-1 regulates of immune responses and apoptosis, and acts as an anti-oncogenic factor [Taniguchi et al., 1997]. Thus, activation of IRF-1 results in enhanced expression of inducible nitric oxide synthase (iNOS), class II transactivator (CIITA), and MHC class I and II antigens [Flodstrom and Eizirik, 1997; Gobin et al., 1997; Hobart et al., 1997]. Further, targeted disruption of the IRF-1 gene in mice results in reduced incidence and severity of antigen-induced autoimmune diseases [Tada et al., 1997] and a dominant negative mutant of IRF-1 prevents the expression of IFNγ inducible genes [Thornton et al., 1996]. Taken together, IRF-1 may play an important role in the pathogenesis and maintenance of autoimmune diseases including autoimmune thyroid disease (AITD).

IFNγ and tumor necrosis factor-α (TNFα) are pleiotropic cytokines involved in the regulation of thyroid growth and differentiated functions,

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and may contribute to the pathogenesis of AITD. Administration of TNFα to human or rodents results in decreased serum thyroid hormone concentrations, iodide uptake and TSH-induced T3 and T4 release [Ozawa et al., 1988; Pang et al., 1989; van der Poll et al., 1990]. In human thyroid cells, IFNγ blocks TSH-induced increases in TSH receptor (TSHR) content and mRNA levels, thyroid peroxidase (TPO), and thyroglobulin (Tg) [Nishikawa et al., 1993; Asakawa et al., 1992; Kung and Lau, 1990]. Further, IFNγ and TNFα decrease TSH-induced iodide uptake and release of T3 and T4 into the medium by human thyrocytes [Sato et al., 1990]. In FRTL-5 rat thyroid cells, IFNγ and TNFα decrease TSH-induced type I 5'- deiodinase (5′D-I) activity and gene expression of TPO, Tg, and 5′D-I [Ongphiphadhanakul et al., 1994; Pekary et al., 1994; Tang et al., 1995]. Further, studies show a synergistic effect of IFNγ and TNFα on the inhibition of thyroid cell growth, TPO, Tg, and 5′D-I gene expression [Tang et al., 1995; Zakarija and McKenzie, 1989; Weetman and Rees, 1988], and enhancement of class II major histocompatibility complex (MHC) antigens [Weetman and Rees, 1988; Zakarija et al., 1988].

IFNγ activation sites (GAS) and NFκB binding sites are present in the promotor region of the IRF-1 gene [Taniguchi et al., 1997; Harada et al., 1994], and IFNγ and TNFα increased IRF-1 mRNA levels [Geller et al., 1993]. In the present study, we examined the effect of IFNγ and TNFα on IRF-1 mRNA levels in FRTL-5 rat thyroid cells. We found that IFNγ and TNFα increased IRF-1 mRNA levels in a time and dose dependent manner. Analysis of nuclear extracts in IFN or TNF-stimulated FRTL-5 cells revealed protein-DNA complexes to GAS and NFκB, respectively. Further, we found that TNFα-induced NFκB binding required hydrolysis of sphingomyelin to ceramide, but not the activation of protein kinase C. Thus, the presence of IRF-1 binding motifs in cytokine-responsive promoters suggests that IRF-1 activation may play a role in mediating the effects of TNFα and INFγ on thyroid function and in the pathogenesis of AITD.

MATERIALS AND METHODS

Materials

Recombinant rat IFNγ and recombinant mouse TNFα were obtained from the Genzyme Corporation (Cambridge, MA). Rat IRF-1 complementary DNA (cDNA), a 1.0 kilobase pair (kbp) fragment inserted in pBluescript SK-, was kindly provided by Dr. Li-Yuan Yu-Lee [Yu-Lee et al., 1990]. The oligonucleotide probe for rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Oncogene Science (Uniondale, NY). Other materials were purchased from the following sources: bacterial sphingomyelinase (SMase; Staphylococcus aureus), D,L-dithiothreitol (DTT), Coon's modified Ham's F-12, human transferrin, bovine insulin, hydrocortisone, somatostatin, glysyl-L-histidyl-L-lysine, bovine TSH, SDS, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, sodium orthovanadate, nonidet p-40 (NP-40), and fraction V of BSA from Sigma Chemical Co. (St. Louis, MO); 12-O-tetradecanoylphorbol 13-acetate (TPA) from Fluka (Ronkonkoma, NY); calf serum from Gibco BRL (Grand Island, NY); [^{32}P]α-dATP from New England Nuclear Corporation (Boston, MA); and poly (dI·dC) poly (dI·dC) from Pharmacia (Piscataway, NJ). All other chemicals and reagents were obtained from commercial sources and were of reagent or molecular biology grade.

Cell Culture

FRTL-5 cells were cultured in Coon's modified Ham's F-12 medium supplemented with 5% calf serum containing bovine TSH (l mU/ml), bovine insulin (10 µg/ml), glycyl-L-histidyl-L-lysine (2 ng/ml), somatostatin (10 ng/ml), and hydrocortisone (0.36 ng/ml; 6H) as previously described [Mori et al., 1996]. Cells were maintained throughout at 37°C in an atmosphere saturated with water and containing 95% air and 5% CO^2. After cells approached confluence, cells were made quiescent by incubation for 7 days in media devoid of TSH (5H). Culture medium was changed twice a week. On the day of experiment, cells were incubated with test reagents for indicated hours and harvested for experiments.

Isolation of Total Cellular RNA and Northern Blot Analysis

Cells were washed twice with ice cold PBS and total cellular RNA was isolated by the acid guanidium-thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi [1987], using materials obtained from Cinna/Biotex Lab, Inc. (Houston, TX). Total cellular RNA concentration was quantified by UV absorption at 260 nm. For Northern blot analysis,

equal amounts of RNA were fractionated by electrophoresis in 1.2% agarose gel containing 2.2 M formaldehyde and blotted on Duralon-UV filters (Stratagene, La Jolla, CA). Filters were cross-linked by UV and baked. After prehybridization for 6 h at 42°C in a solution containing 10% dextran sulfate, 50% deionized formamide, 1% SDS, 200 mg/ml denatured salmon sperm DNA, and 1 M NaCl, the filters were hybridized to a ^{32}P-labeled IRF-1 cDNA probe (kindly provided by Dr. Yu-Lee, Baylor College of Medicine, Houston, TX) overnight at 42°C. Blots were washed in 2 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate), 0.l% SDS three times at room temperature for 15 min and then in 0.5 × SSC, 0.1% SDS, for 30 min at 65°C. Filters were exposed to Fuji RX film (Fuji Photo Film Co., Tokyo, Japan) at −70°C using double fluorescent-intensifying screens.

Extraction of Soluble Nuclear Proteins

Soluble nuclear proteins were obtained as described by van Wijnen et al. [1992]. In brief, FRTL-5 cells were washed twice with ice-cold PBS, and harvested in 1 ml hypotonic buffer containing 10 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid; HEPES)-KOH, pH 7.9, 10 mM KC1, 0.1 mM EDTA, 0.l mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 ug/ml aprotinin, 10 µg/ml leupeptin, and 1 mM sodium orthovanadate. After incubation on ice for 15 min, cells were vortexed for 10 sec in hypotonic buffer containing 0.1% NP-40, followed by incubation on ice for 10 min. Nuclei were pelleted by centrifugation, washed twice with hypotonic buffer, and then the nuclear pellets were incubated at 4°C for 30 min in hypertonic buffer containing 20 mM HEPES-KOH, pH 7.9, 400mM KCI, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM sodium orthovanadate, and 20% glycerol. Supernatants were collected after centrifugation and stored at −80°C until use. Protein concentrations were measured by the Bradford method [Bradford, 1976].

Electrophoretic Mobility Shift Assay (EMSA)

The synthetic oligonucleotide probes used in EMSA were as follows; for the GAS element of IRF-1 promoter 5′-GATCCATTTCCCCGAAATGA-3′ and for NFκB 5′-CAACGGCAGGGGAATCTCCCTCTCCTT-3′. The double-stranded probes were end-labeled using the Klenow-DNA polymerase and [^{32}P]α-dATP. Nuclear proteins (5 µg) were incubated in a final volume of 20 µl with 40 fmol [^{32}P]-labeled probe at 22°C for 30 min in buffer containing 10 mM HEPES-KOH, pH 7.9, 100 mM KC1, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol, 0.05% NP-40 and 2 µg poly (dIodC). The nucleoprotein complexes were resolved by nondenaturing electrophoresis in a 5% polyacrylamide gel for 3 h at 4°C in buffer containing 45 mM Tris-HCl, pH 8.0, 45 mM boric acid, and 1 mM EDTA. Gels were dried and exposed to Fuji RX film at −80°C. For competition experiments, a 100-fold molar excess of the unlabeled oligonucleotides was added 15 min before incubation of nuclear extracts with radiolabeled probes.

Immunoprecipitation and Immunoblotting

Cells were solubilized on ice in 1 ml of lysis buffer containing 10 mM Tris-HCL, pH 7.6, 5 mM EDTA, 50 mM NaCl, 50 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM PMSF, 10 ug/ml aprotinin, and 10 ug/ml leupeptin. Insoluble material was pelleted at 10,000*g* for 30 min at 4°C. Clarified lysates were incubated for 24 h with anti-STAT1 at 4°C. Antibodies were captured by incubation for 60 min with protein A-Sepharose beads, washed, and subjected to SDS PAGE and transferred to nitrocellulose as previously described [DeVito et al., 1995]. Blots were blocked, probed with anti-phosphotyrosine antibodies, and tyrosine phosphorylated proteins were detected by enhanced chemiluminescence as previously described [DeVito et al., 1995].

RESULTS

To determine if IFNγ or TNFα increases IRF-1 gene expression in FRTL-5 cells, total RNA was isolated for Northern blot analysis. In quiescent FRTL-5 cells low or no expression of IRF-1 mRNA was detected (Fig. 1). In IFNγ and TNFα stimulated cells, however, a single IRF-1 mRNA at 2.2 Kb was easily detected. Incubation of FRTL-5 cells with low concentrations of IFNγ (6.25 U/ml) or TNFα (6.25 ng/ml) resulted in a rapid and marked increase in IRF-1 mRNA. IRF-1 mRNA levels peaked 1 to 2 h after stimulation with IFNγ or TNFα and remained elevated for at least 24 h. Reprobing of blots with a GAPDH probe verified that equal amounts of RNA had been loaded. As illustrated in Figure 2, incubation of FRTL-5 cells for 1 h with IFNγ

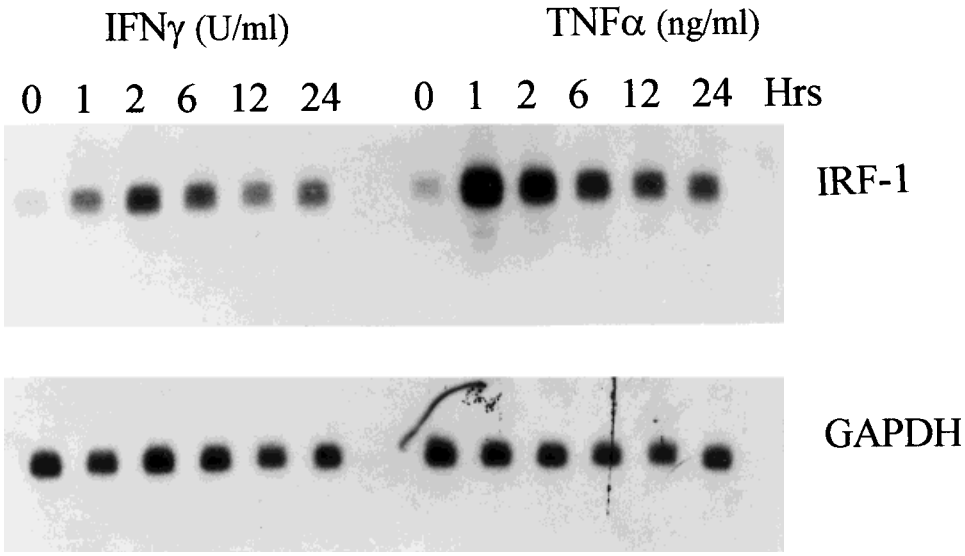

Fig. 1. Effect of IFNγ (12.5 U/ml) or TNFα (6.25 ng/ml) on IRF-1 mRNA levels in FRTL-5 cells. Cells were maintained in 5H medium plus 5% calf serum for 7 days and then stimulated with IFNγ or TNFα for indicated hours. Blots were hybridized with ^{32}P-labeled IRF-1 probe, then stripped and rehybridized with ^{32}P-labeled GAPDH probe The data presented are representative of three separate experiments.

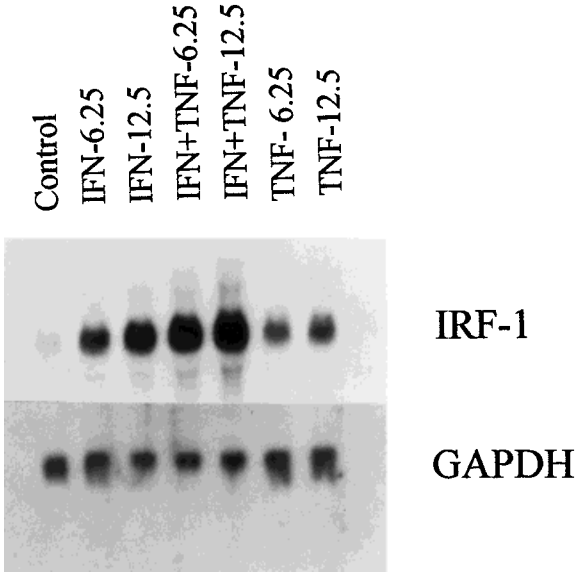

Fig. 2. Effect of IFNγ TNFα or IFNγ and TNFα in combination on IRF-1 mRNA levels in FRTL-5 cells. Cells were maintained in 5H medium plus 5% calf serum for 7 days and then stimulated with IFNγ (6.25 or 12.5 U/ml) or TNFα (6.25 or 12.5 ng/ml) for 60–120 min. Blots were hybridized with ^{32}P-labeled IRF-1 probe, then stripped and rehybridized with ^{32}P-labeled GAPDH probe. The data presented are representative of three separate experiments.

or TNFα resulted in a dose-dependent increase in IRF-1 mRNA levels. It is well known that IFNγ and TNFα act synergistically to modulate thyroid function [Tang et al., 1995]. When FRTL-5 cells were incubated with IFNγ and TNFα in combination, however, no synergistic increase in IRF-1 mRNA levels was found (Fig. 2). EMSAs were used to determine if IFNγ or TNFα activated nuclear protein binding to GAS or NFκB specific probes in FRTL-5 cells. In quiescent FRTL-5 cells, no GAS binding activity was detected (Fig. 3). Incubation of FRTL-5 cells with IFNγ, but not TNFα, resulted in a dose dependent formation of a single GAS complex. Addition of a 100-fold excess of unlabeled oligonucleotide to the binding reaction abolished the formation of IFNγ-induced nucleoprotein complex with radiolabeled probes (data not shown). Whereas NFκB binding elements are present in the promotor region of the IRF-1 gene, TNFα did not potentiate IFNγ-induced activation of GAS binding in FRTL-5 cells (data not shown). IFNγ regulates cell function through the tyrosine phosphorylation of signal transducers and activators of transcription (STAT) Stat1α, the subsequent formation of Statlα homodimers and translocation into the nucleus, where they bind to specific DNA response elements in IFNγ responsive genes [Darnell et al., 1994]. Consistent with observations in nonthyroidal cells, we found that IFNγ induced the rapid tyrosine phosphorylation of STAT1α in FRTL-5 cells (Fig. 4).

The hydrolysis of sphingomyelin to ceramide and/or activation of PKC may be involved in

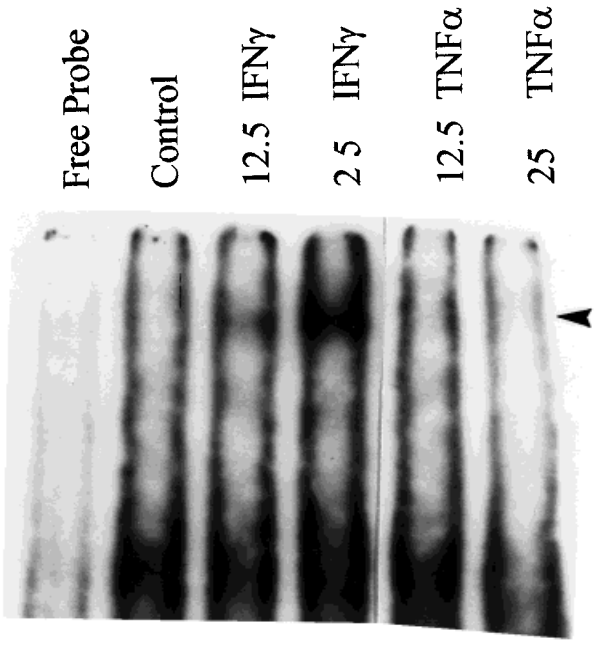

Fig. 3. Effect of IFNγ or TNFα on GAS binding activity analyzed by EMSA in FRTL-5 cells. Cells were maintained in 5H medium plus 5% calf serum for 7 days and then stimulated with IFNγ (12.5 or 25 U/ml) or TNFα (12.5 or 25 ng/ml) for 30 min. Nuclear proteins were extracted and then incubated with ^{32}P-labeled double stranded oligonucleotide containing GAS. Nucleoprotein complexes were resolved by non denaturing polyacrylamide gel electrophoresis. The data presented are representative of three separate experiments.

mediating the effects of TNFα in a variety of cell types, including FRTL-5 cells [Wiegmann et al., 1992; Mori et al., 1996]. Accordingly, EMSA was used to determine if hydrolysis of sphingomyelin to ceramide or the activation of PKC induces nuclear NFκB binding in FRTL-5 cells. In quiescent FRTL-5 cells, low levels of NFκB binding activity were detected as two bands (Fig. 5). In cells incubated with SMase (0.1 U/ml), hydrolysis of sphingomyelin to ceramide resulted in a marked increase in NFκB binding activity 30 min after stimulation. In contrast, activation of PKC by TPA (10 nM) failed to induce NFκB binding. Addition of a 100-fold excess of unlabeled oligonucleotides to the binding reaction completely decreased both bands in SMase-stimulated FRTL-5 cells. These results suggested that hydrolysis of sphingomyelin to ceramide, but not activation of PKC, is involved in mediating the effects of TNFα on IRF-1 mRNA levels in FRTL-5 cells. To test this hypothesis, we determined whether IRF-1 mRNA levels were increased in response to activation of PKC or hydrolysis of sphingomyelin to ceramide in FRTL-5 cells. As shown in Figure 6, hydrolysis of sphingomyelin to ceramide, but not activation of PKC, resulted in a marked increase in IRF-1 mRNA levels. Further, TPA did not potentiate the SMase-induced increase in IRF-1 mRNA levels.

DISCUSSION

Transcription factor IRF-1 plays an important role in the regulation of IFNγ- and TNFα-induced cellular effects in a variety of tissues. A number of studies in non-thyroidal tissues demonstrate that IRF-1 is involved in the modulation of cellular responses to IFNγ, cell growth, susceptibility to oncogenic transformation, apoptosis, and development of the T cell immune response [Taniguchi et al., 1997]. Further, several lines of evidence indicate that IRF-1 is involved in expression of iNOS [Flodstrom and Eizirik, 1997], CIITA, and class I and II MHC antigens [Gobin et al., 1997; Hobart et al., 1997]. In addition, the incidence and severity of type II collagen-induced arthritis are significantly reduced in mice lacking IRF-1 gene [Tada et al., 1997]. Thus, IRF-1 seems to be a key molecule in the pathogenesis of autoimmune diseases and may play a role in AITD.

In spite of well known actions of IFNγ and TNFα on thyroid function, the cellular and molecular events activated by these cytokines remain poorly understood in the thyroid. Studies in a variety of cell types, however, indicate that TNFα binds to two cell surface receptors, TNF-R1 and -R2 [Heller and Kronke, 1994]. Further, most of TNFα-induced responses are mediated by the activation of TNF-R1 [Wiegmann et al., 1992] and involves interactions among a number of signal transduction systems. Two of the best understood systems include TNF-R1-induced activation of protein kinase C (PKC) and hydrolysis of sphingomyelin to ceramide catalyzed by sphingomyelinase (SMase) [Wiegmann et al., 1992] resulting in the activation of NFκB [Yang et al., 1993; Schutze et al., 1992]. Consistent with studies in other tissues, TNFα induces the activation of NFκB in a human papillary thyroid carcinoma cell line [Pang et al., 1992]. Further, we have recently shown that activation of the ceramide pathway is involved in the regulation of 5'D-I in FRTL-5 cells [Mori et al., 1996]. We have now clearly shown that IFNγ and TNFα induced a rapid and marked increase in IRF-1 mRNA

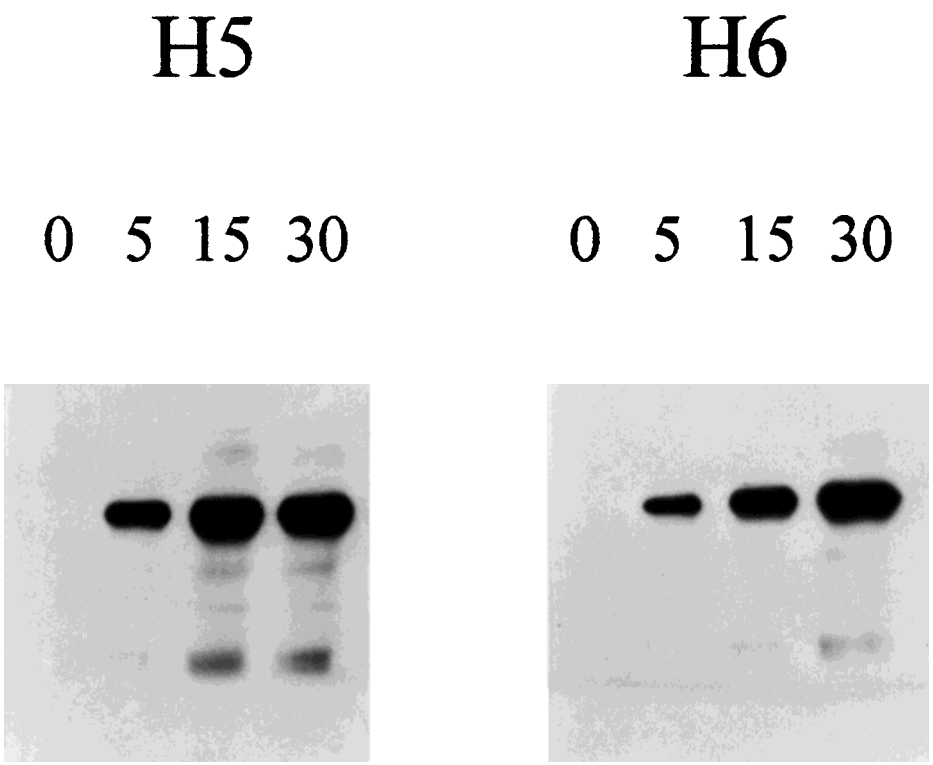

Fig. 4. Effect of INFγ on tyrosine phosphorylation of STAT1α in FRTL-5 cells. Cells were maintained in 5H or 6H medium plus 5% calf serum for 7 days and then stimulated with IFN (6.25 U/ml) for 10 min. Cell extracts were immunoprecipitated with anti-STAT1α and Western blot analysis was performed using an anti-phophotyrosine.

levels, peaking 1 to 2 h after stimulation, and remaining elevated for at least 24 h. Consistent with our results, a similar time course of IFNγ- or TNFα-induced increases in IRF-1 mRNA levels was observed in rat hepatocytes [Geller et al., 1993] and in retinoic acid stimulated NB4 cells, a human promyelocytic leukemia cell line [Matikainen et al., 1996]. In contrast, in Nb2 rat T cell lymphoma cells, prolactin induces two peaks of IRF-1 mRNA levels, a rapid and transient peak at 1 h and a second sustained peak 12 h after stimulation [Schwarz et al., 1992].

We recently have shown that hydrolysis of sphingomyelin to ceramide is involved in mediating the effects of TNFα in FRTL-5 cells [Mori et al., 1996]. Here we show that hydrolysis of sphingomyelin to ceramide, but not activation of PKC, is involved in the activation of NFκB and an elevation of IRF-1 mRNA levels in FRTL-5 cells. Thus, hydrolysis of sphingomyelin to ceramide may play a role in the induction of IRF-1 gene expression by TNFα in the thyroid. Consistent with the present findings, studies in HL-60 cells and the human leukemic cell lines, K62 and Jurket, demonstrate that the hydrolysis of sphingomyelin to ceramide, but not activation of PKC, is a critical cellular event involved in TNFα-induced activation of NFκB [Meichle et al., 1990; Yang et al., 1993]. In contrast, studies in human FS-4 and murine L929 cell lines have shown that activation of PKC by TPA increases IRF-1 mRNA levels [Fujita et al., 1989]. Taken together, these studies suggest that the cellular mechanisms involved in regulation of IRF-1 gene expression by IFNγ and TNFα may be tissue and/or growth factor specific.

A variety of cytokines and hormones use cytoplasmic signal transducers and activators of transcription (STAT) to regulate expressions of specific genes. IFNγ binding to the cell surface receptor leads to tyrosine phosphorylation of two protein kinases which are members of the Janus kinase (Jak) family, JAK-1 and JAK-2 [Darnell et al, 1994]. Tyrosine phosphorylation of Jak-1 and -2 then results in the tyrosine phosphorylation of Stat1α, and the subsequent formation of Statlα homodimers and their translocation into the nucleus, where they bind specific DNA response elements on IFNγ responsive genes [Darnell et al., 1994]. In the present study, we demonstrated that IFNγ increases

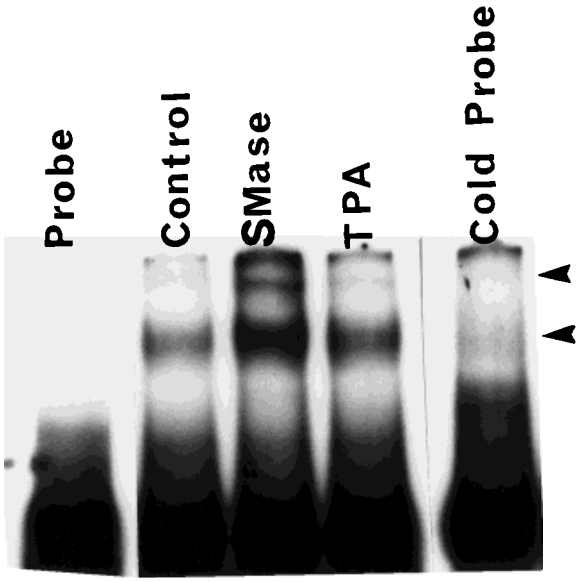

Fig. 5. Effect of TPA or SMase on NFκB binding activity analyzed by EMSA in FRTL-5 cells. Cells were maintained in 5H medium plus 5% calf serum for 7 days and then stimulated with TPA (10 nM) or SMase (01 U/ml) for 30 min. Nuclear proteins were extracted and then incubated with ^{32}P-labeled double stranded oligonucleotide containing NFκB binding consensus. Nucleoprotein complexes were resolved by nondenaturing polyacrylamide gel electrophoresis. The data presented are representative of three separate experiments.

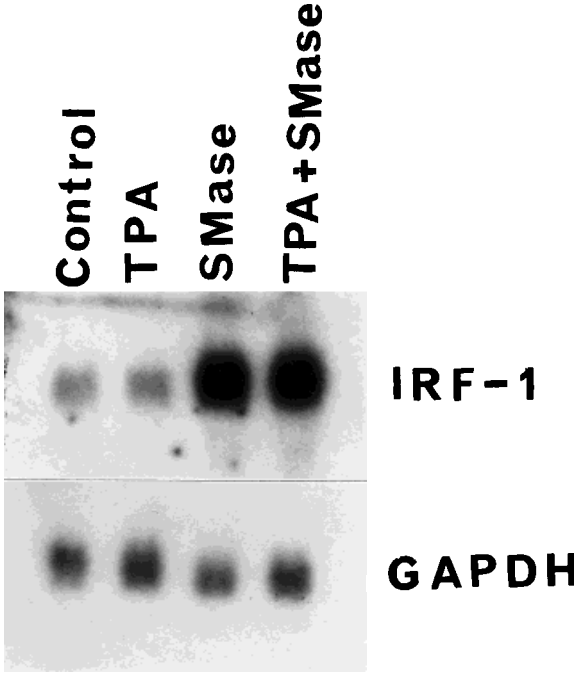

Fig. 6. Effect of TPA or SMase on IRF-1 mRNA levels in FRTL-5 cells. Cells were maintained in 5H medium plus 5% calf serum for 7 days and then stimulated with TPA (10 nM) or SMase (01 U/ml) for 120 min. Blots were hybridized with ^{32}P-labeled IRF-1 probe, then stripped and rehybridized with ^{32}P-labeled GAPDH probe. The data presented are representative of three separate experiments.

tyrosine phosphoralation of STAT1, GAS binding, and IRF-1 mRNA levels in FRTL-5 cells.

IFNγ and TNFα act synergistically to block TSH-induced increases in 5'D-I, Tg, and TPO mRNA levels in FRTL-5 cells [Tang, 1995]. Further, we have reported that IFNγ did not potentiate the inhibitory effect of activation of PKC or the hydrolysis of sphingomyelin to ceramide on TSH-induced 5'D-I activity or mRNA levels in FRTL-5 cells [Mori et al., 1996]. In the present study, incubation of FRTL-5 cells with TNFα and IFNγ together did not synergistically increase IRF-1 mRNA levels in FRTL-5 cells. In contrast, IFNγ and TNFα synergistically increased IRF-1 mRNA levels in mouse fibroblasts, and the synergy depended, in part, on cooperation between Stat1α and NFκB [Ohmori et al., 1997]. Several lines of evidence, however, suggest that a variety of transcription factors may be involved in the synergy between IFNγ and TNFα. In the THP-1 human monocytic cell line, synergistic induction of IL-6 gene by IFNγ and TNFα involves cooperation between IRF-1 and NFκB p65 homodimers with concomitant changes in Sp1 activity [Sanceau et al., 1995]. Thus, the cellular mechanisms which mediate the synergistic interaction of IFNγ and TNFα may be tissue specific and remain unknown in the thyroid.

In summary, our results clearly demonstrate that IFNγ increases IRF-1 mRNA levels through activation of Stat1α in FRTL-5 cells. On the other hand, ceramide production followed by the activation of NFκB is involved in the TNFα-induced increase in IRF-1 mRNA in these cells. Thus, these findings suggest that IRF-1 may play a role in mediating the effects of IFNγ and TNFα on thyroid function, and possibly in the pathogenesis of AITD. Further, the synergistic effects of TNFα and IFNγ on thyroid function is not the result of an enhanced IRF-1 gene expression in the thyroid.

REFERENCES

Asakawa H, Hanafusa T, Kobayashi T, Takai S, Kono N, Tarui S. 1992. Interferon-γ reduces the thyroid peroxidase content of cultured human thyrocytes and inhibits its increase induced by thyrotropin. J Clin Endocrinol Metab 74:1331–1335.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium-thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Darnell JE, Kerr IM, Stark GR. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264:1415–1421.

DeVito WJ, Stone S, Shamgochian M. 1995. Prolactin induced expression of glial acidic protein and tumor necrosis factor-alpha at a wound site in the rat brain. Mol Cell Endocrin 108:125–130.

Flodstrom M, Eizirik DL. 1997. Interferon-γ-induced interferon regulatory factor-1 (IRF-1) expression in rodent and human islet cells precedes nitric oxide production. Endocrinology 138:2747–2753.

Fujita T, Reis LFL, Watanabe N, Kimura Y, Taniguchi T, Vilcek J. 1989. Induction of the transcription factor IRF-1 and interferon-β mRNAs by cytokines and activators of second-messenger pathways. Proc Natl Acad Sci USA 86:9936–9940.

Geller DA, Nguyen D, Shapiro RA, Nussler A, Di Dilvivo M, Freeswick Wang SC, Tweardy DJ, Simmons RL, Billiar TR. 1993. Cytokine induction of interferon regulatory factor-1 in hepatocytes. Surgery 114:235–242.

Gobin SJP, Peijnenburg A, Keijsers V, van der Elsen PJ. 1997. Site α is crucial for two routes of INFγ-induced MHC class I transactivation: the ISRE-mediated route and a novel pathway involving CIITA. Imminity 6:601–611.

Harada H, Takahashi E, Itoh S, Harada K, Hori T, Taniguchi T. 1994. Structure and regulation of human interferon regulatory factor 1 (IRF-1) and IRF-2 genes: implications for a gene network in the interferon system. Mol Cell Biol 14:1500–1509.

Heller RA, Kronke M. 1994. Tumor necrosis factor receptor-mediated signaling pathways. J Cell Biol 126:5–9.

Hobart M, Ramassar V, Goes N, Urmson J, Halloran PF. 1997. IFN regulatory factor-1 plays a central role in the regulation of the expression of class I and II MHC genes in vivo. J Immunol 158:4260–4269.

Kung AWC, Lau KS. 1990. Interferon-γ inhibits thyrotropin-induced thyroglobulin gene transcription in cultured human thyrocytes. J Clin Endocrinol Metab 70:1512–1517.

Matikainen S, Ronni T, Hurme M, Pine R, Julkunen I. 1996. Retinoic acid activates interferon regulatory factor-1 gene expression in myloid cells. Blood 88:114–123.

Meichle A, Schutze S, Hansel G, Brunsing D, Kronke M. 1990. Protein kinase C-independent activation of nuclear factor kB by tumor necrosis factor. J Biol Chem 265:8339–8343.

Mori K, Stone S, Braverman LE, DeVito WJ. 1996. Effects of ceramide and protein kinase C on the regulation of type I 5'-deiodinase in FRTL-5 rat thyroid cells. Endocrinology 137:4944–4999.

Nishikawa T, Yamashita S, Namba H, USA T, Tominaga T, Kimura H, Izumi M, Nagataki S. 1993. Interferon-γ inhibition of human thyrotropin receptor gene expression. J Clin Endocrinol Metab. 77:1084–1089.

Ohmori Y, Schreiber RD, Hamilton TA. 1997. Synergy between interferon-γ and tumor necrosis factor-α in transcriptional activation is mediated by corporation between signal transducers and activators of transcription-1 and nuclear factor kB. J Biol Chem 272:14899–14907.

Ongphiphadhanakul B, Fang SL, Tang K-T, Patwardhan NA, Braverman LE. 1994. Tumor necrosis factor-α decreases thyrotropin-induced 5'-deiodinase activity in FRTL-5 rat thyroid cells. Eur J Endocrinol 130:502–507.

Ozawa M, Sato K, Han DC, Kawakami M, Tsushima T, Shizume K. 1988. Effects of tumor necrosis factor-α/cachectin on thyroid hormone metabolism in mice. Endocrinology 123:1461–1467

Pang X-P, Hershman JM, Mirell CJ, Pekary AE. 1989. Impairment of hypothalamic-pituitary-thyroid function in rats treated with human recombinant tumor necrosis factor-α (Cachectin). Endocrinology. 125:76–84.

Pang X-P, Ross NS, Park M, Juillard GJF, Stanley TM, Hershman JM. 1992. Tumor necrosis factor-α activates nuclear factor kB and induces manganous superoxide dismutase and phosphodiesterase mRNA in human papillary thyroid carcinoma cells. J Biol Chem 267:12826–12830.

Pekary AE, Berg L, Santini F, Chopra I, Hershman JM. 1994. Cytokines modulate type I iodothyronine deiodinase mRNA levels and enzyme activity in FRTL-5 rat thyroid cells. Mol Cell Endocrinol 101:R31–R35.

Sanceau J, Kaisho T, Hirano T, Wietzerbin J. 1995. Triggering of the human interleukin-6 gene by interferon-γ and tumor necrosis factor-α in monocytic cells involves corporation between interferon regulatory factor-1, NFkB, and Sp1 transcription factors. J Biol Chem 270:27920–27931.

Sato K, Satoh T, Shizume K, Ozawa M, Han DC, Imamura H, Tsushima T, Demura H, Kanaji Y, Ito Y, Obara T, Fujimoto Y. 1990. Inhibition of 125I organification and thyroid hormone release by interleukin-1, tumor necrosis factor-α, and interferon-γ in human thyrocytes in suspension culture. J Clin Endocrinol Metab 70:1735–1743.

Schutze S, Potthoff K, Machleidt T, Berkovic D, Wiegmann K, Kronke M. 1992. TNF activates NK-kB by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown. Cell 71:765–776.

Schwarz LA, Stevens AM, Hrachovy JA, Yu-Lee L-Y. 1992. Interferon regulatory factor-1 is inducible by prolactin, interleukin-2 and concanavalin A in T cells. Mol Cell Endocrin 86:103–110.

Tada Y, Ho A, Matsuyama T, Mak TW. 1997. Reduced incidence and severity of antigen-induced autoimmune disease in mice lacking interferon regulatory factor-1. J Exp Med 185:231–238.

Tang K-T, Braverman LE, DeVito WJ. 1995. Tumor necrosis factor-α and interferon-γ modulate gene expression of type I 5'-deiodinase, thyroid peroxidase and thyroglobulin in FRTL-5 thyroid cells. Endocrinology 136:881–888.

Taniguchi T, Lamphierm MS, Tanaka N. 1997. IRF-1: The transcription factor linking the interferon response and oncogenesis. Biochim Biophys Acta 1333:M9–M17.

Thornton AM, Ogryzko VV, Dent A, Sharf R, Levi B-Z, Kanno Y, Staudt LM, Howard BH, Ozato K. 1996. A dominant negative mutation of an IFN regulatory factor family protein inhibits both type I and type II IFN-stimulated gene expression and antiproliferative activity of IFNs. J Immunol 157:5145–5154.

van der Poll T, Romijn JA, Wiersinga WM, Sauerwein HP. 1990. Tumor necrosis factor: a putative mediator of the euthyroid syndrome in man. J Clin Endocrinol Metab 71:1567–1572.

van Wijnen AJ, van den Ent FMI, Lian JB, Stein JL, Stein GS. 1992. Overlapping and CpG methylation-sensitive protein-DNA interactions at the histon H4 transcriptional cell cycle domain: distinction between two human H4 gene promoters. Mol Cell Biol 12:3273–3287.

Weetman AP, Rees AJ. 1988. Synergistic effects of recombinant tumour necrosis factor and interferon-gamma on rat thyroid cell growth and Ia antigen expression. Immunology 63:285–289.

Wiegmann K, Schutze S, Kampen E, Himmler A, Machleidt T, Kronke M. 1992. Human 55-kDa receptor for tumor necrosis factor coupled to signal transduction cascades. J Biol Chem 267:17997–18001.

Yang Z, Costanzo M, Golde DW, Kolesnick RN. 1993. Tumor necrosis factor activation of the sphingomyelin pathway signals nuclear factor κB translocation in intact HL-60 cells. J Biol Chem 268:20520–20523.

Yu-Lee L-Y, Hrachovy JA, Stevens AM, Schwarz LA. 1990. Interferon-regulatory factor 1 is an immediate-early gene under transcriptional regulation by prolactin in Nb2 T cells. Mol Cell Biol 10:3087–3094.

Zakarija M, Hornicek FJ, Levis S, McKenzie JM. 1988. Effects of γ-interferon and tumor necrosis factor-α on thyroid cells: induction of class II antigen and inhibition of growth stimulation. Mol Cell Endocrin 58:129–136.

Zakarija M, McKenzie JM. 1989. Influence of cytokines on growth and differentiated function of FRTL5 cells. Endocrinology 125:1260–1265.