Identification of a Phorbol Ester-responsive Element in the Interferon-γ Receptor 1 Chain Gene*

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Human monocytic leukemia THP-1 cells differentiate into macrophage-like cells when treated with 12-O-tetradecanoylphorbol-13-acetate (TPA). During this process, interferon-γ (IFN-γ)-inducible expression of human leukocyte antigen-DRα is markedly enhanced. The enhancement of human leukocyte antigen-DRα expression is at least due to the TPA-dependent induction of the IFN-γ receptor 1 chain and IFN-γ receptor 2 chain genes. Here we have studied the mechanism of TPA-induced up-regulation of the IFN-γ receptor 1 chain gene. Reporter gene analyses of 5′-deletion constructs of the IFN-γ receptor 1 gene (IFNGR1) promoter indicated that the critical region for control of transcription and the TPA-responsive element (TRE) were present in the −128 to −109 base pair (bp) region. We confirmed that this region of the IFNGR1 promoter was responsive to TPA-induced signals by using a reporter construct whose promoter consisted of the −128 to −109 bp fragment and the minimal herpes simplex virus thymidine kinase promoter. Moreover, a supershift assay indicated that Sp1 bound to this TRE in TPA-treated THP-1 cells. These results suggest that in TPA-treated cells the binding of Sp1 to the TRE of the IFNGR1 promoter causes the up-regulation of this gene.

Macrophages can recognize and ingest many types of extracellular bacteria, thereby destroying the bacteria, and at the same time present bacterial peptides to CD4 T cells. This can lead to the generation of armed effector CD4 T cells specific for the ingested microorganism. An important function of these armed effector T cells is to enhance the ability of the macrophages to kill the ingested bacteria, many of which have evolved strategies for surviving and proliferating inside phagocytic cells. The induction of antimicrobial activity in macrophages is known as macrophage activation (1–3), and many of the intracellular events leading to this have been determined. The macrophage-activating factor interferon-γ (IFN-γ)1 binds to IFN-γR and activates a Janus kinase (Jak)/Stat signaling pathway consisting of Jak1 and Jak2 as well as the transcription factor signaling transducer and activator of transcription 1 (STAT1). STAT1 binds to the cytoplasmic portion of the ligand-activated IFN-γR via the Src homology 2 domain and is phosphorylated by Jak on a single tyrosine residue (Tyr-701). This phosphorylation results in the Src homology 2 domain-mediated formation of a dimer of STAT1, which is then translocated to the nucleus to bind to the IFN-γ-responsive element leading to transcriptional activation of IFN-γ-responsive genes (4–7).

Major histocompatibility complex class II molecules play a key role in macrophage activation by presenting peptides derived from bacteria to CD4 T cells (8). Expression of major histocompatibility complex class II genes is induced by IFN-γ in macrophages (9, 10) and is mediated via the induction of the class II transactivator (CIITA) by the Jak/STAT pathway (11–13).

We have previously observed that the induction of the HLA-DRα gene by IFN-γ was significantly enhanced in TPA-activated THP-1 cells (14) and showed that this was due to an increase in levels of IFN-γR1 and IFN-γR2 following the TPA treatment (15). Here we have investigated the mechanism of the TPA-induced increase in IFN-γR1 expression and have identified an essential TPA-responsive cis-element in the promoter region of IFNGR1, which is activated by Sp1 in response to TPA treatment.

EXPERIMENTAL PROCEDURES

Cell Culture and Cytokine Treatment—THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells were plated at 1 × 10⁶ cells/ml and treated with or without 10 ng/ml TPA for 24 h and then with 100 units/ml IFN-γ for 15, 30, and 60 min. The human embryonic kidney cell line HEK293S was maintained in Eagle’s minimal essential medium supplemented with 10% fetal calf serum. Cells were plated at 1 × 10⁵ cells/ml and treated with or without 10 ng/ml TPA for 24 h.

Reagents—Anti-STAT1, anti-phospho-STAT1 (Tyr-701) and anti-phospho-STAT1 (Ser-727) antibodies were purchased from New England Biolabs Inc., Cell Signaling Technology, and Upstate Biotechnology, respectively. Anti-IFN-γR1 and anti-Sp1 were products of Santa Cruz Biotechnology.

Western Blot Analysis—Cells (1 × 10⁶ cells) were harvested, washed twice with phosphate-buffered saline, pelleted, resuspended in 200 μl of buffer D (20 mM Heps, pH 7.4, 20% glycerol, 0.1 mM NaCl, 0.2 mM EDTA, 0.2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and disrupted by sonication. Homogenates were centrifuged at 15,000 rpm for 10 min, and the resultant supernatants were used as cell lysates. Protein concentrations of the cell lysates were determined by the Bradford method (16). The proteins in the cell lysates were then subjected to SDS-polyacrylamide gel electrophoresis and electrotransfered onto a polyvinylidene difluoride membrane. The membrane was probed with affinity-purified polyclonal rabbit antibody against either STAT1 (1:1000), Tyr-701 phospho-STAT1 (1:1000), Ser-727 phospho-STAT1 (1:1000), or IFN-γR1 (1:1000). Following hybridization, the membrane was washed and incubated for 30 min with peroxidase-conjugated anti-rabbit IgG and subsequently developed by chemiluminescence using the ECL Western blotting system (Amersham Pharmacia Biotech).

Plasmid Construction—First we constructed a luciferase reporter gene containing the IFNGR1 promoter −840 to +28 bp sequence. The DNA fragment consisting of the −840 to +28 bp sequence was synthesized by

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1 The abbreviations used are: IFN-γ, interferon-γ; IFN-γR, IFN-γ receptor; IFNGR, IFN-γ receptor gene; TPA, 12-O-tetradecanoylphorbol-13-acetate; STAT1, signal transducers and activators of transcription 1; Jak, Janus kinase; HLA-DR, human leukocyte antigen-DR; CIITA, class II transactivator; TRE, TPA-responsive element; HSV, herpes simplex virus; bp, base pair(s); TK, thymidine kinase.

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with IFN-γ for 30 and 60 min compared with cells that had not undergone pretreatment (Fig. 1A, lanes 7 and 8). There was only minimal phosphorylation on Ser-727 in TPA-pretreated cells, and this was not affected by treatment with IFN-γ (Fig. 1B, lanes 5–8). Lysates were also immunoblotted with an anti-STAT1 antibody that recognizes both phosphorylated and non-phosphorylated forms of STAT1. An increased amount of STAT1 was detected when TPA-pretreated cells were stimulated with IFN-γ for 30 and 60 min (Fig. 1C, lanes 7 and 8). However, in THP-1 cells that were treated with IFN-γ but not with TPA, a small amount of STAT1 production and Tyr-701 phosphorylation could only be detected when 300 μg of the lysates were used for immunoblotting (data not shown). These observations seem to reflect not only the IFN-γ-inducible phosphorylation of STAT1 but also the TPA-facilitated production of STAT1 molecules.

Since activation of IFN-γ-Rs by IFN-γ increases the production of STAT1 and its phosphorylation on Tyr-701 (12, 19), we examined the expression of IFN-γ-R1 in THP-1 cells under various conditions (15). The expression of IFN-γ-R1 was significantly increased by treatment with TPA but not with IFN-γ (15). These results possibly explain why pretreatment of THP-1 cells with TPA results in enhanced STAT1 production and phosphorylation on Tyr-701 following treatment with IFN-γ.

Identification of the TRE in the Promoter of IFNGR1—We next investigated the mechanism of transcriptional control of IFNGR1 by TPA. To identify a putative cis-acting element responsive to TPA in the IFNGR1 promoter, we constructed luciferase reporter genes for the 5′ promoter fragment of each construct shown as a shaded area. On the right, the corresponding promoter activities are shown as the relative intensity of the luciferase activity in THP-1 cells obtained by transient transfection experiments. Relative luciferase activities were defined as 1.0 and the luciferase activities with respect to the relative values given in the figure were as follows. A relative value of 1.0 was equivalent to a normalized relative value of 1.25 (−840 to +1), 0.53 was equivalent to 0.37 (−540 to +1), 0.61 was equivalent to 0.76 (−240 to +1), 0.53 was equivalent to 0.66 (−160 to +1), 0.28 was equivalent to 0.35 (−128 to +1), and 0.08 was equivalent to 0.11 (−109 to +1). These values are the averages of triplicate determinations. The assays with these constructs were performed independently three times with similar results.

Identification of the TRE in the IFNGR1 promoter. On the left, the structure of each construct is shown as a shaded area. The nucleotide sequence of the deleted region is shown, and the 5′ promoter fragment of each construct is shown as a shaded area. On the right, the corresponding promoter activities are shown as the relative intensity of the luciferase activity in THP-1 cells obtained by transient transfection experiments. Relative luciferase activities were defined as mentioned in the legend to Fig. 2. The open columns show the results of treatment with TPA. The closed columns show the results of treatment with IFN-γ. A relative value of 1.0 was equivalent to a normalized relative value of 1.28 (−840 to +1/TPA), 0.2 was equivalent to 0.32 (−128 to +1/TPA), 8.9 was equivalent to 11.4 (−128 to +1/TPA), 0.0078 was equivalent to 0.01 (−109 to +1/TPA), and 0.085 was equivalent to 0.11 (−109 to +1/TPA). These values are the averages of triplicate determinations. The assays with these constructs were performed independently three times with similar results.
provides an important clue to help solve the pathogenesis of autoimmune disease, such as Grave’s disease in which the number of HLA-DR molecules is anomalously increased (20). In the present study, we have previously shown that treatment of THP-1 cells with TPA results in increased levels of IFN-γ-IRα that lead to an increase in STAT1 and CIITA, resulting in increased HLA-DR expression (15). Thus, elucidation of the mechanism resulting in STAT1 and CIITA, resulting in increased HLA-DR expression (15).

**Fig. 4. TPA responsiveness of the −128 to −109 bp fragment linked to the minimal TK promoter.** On the left, the structure of each construct is shown. On the right, the corresponding promoter activities are shown as the intensity of the luciferase activity in 293T cells obtained by transient transfection experiments. The corresponding reporter genes were transfected into 293T cells and incubated with (open columns) or without (closed columns) TPA. The cell lysates (20 μg of proteins) were used to measure the luciferase activity. The experiments were repeated three times, and bars represent the S.D. values.

**Fig. 5. Characterization of a protein that binds to the TRE of the IFNγR1 promoter.** THP-1 cells were treated with 10 ng/ml TPA for 1 day, and then nuclear extracts were prepared from the cells. The nuclear extracts (1.5 μg) were incubated without (lane 1), 1 μg (lane 2), or 2 μg (lane 3) of anti-Sp1 IgG (α-Sp1) for 30 min at 25 °C prior to the addition of 32P-labeled TRE. An arrow indicates the DNA–protein complex.

We have been studying the regulatory mechanisms of the IFN-γ-inducible HLA-DRα gene for several years. We have previously shown that treatment of THP-1 cells with TPA results in increased levels of IFN-γ-IRα that lead to an increase in STAT1 and CIITA, resulting in increased HLA-DRα expression (15). Thus, elucidation of the mechanism resulting in the TPA-induced increase in IFN-γ-IRα may provide an important clue to help solve the pathogenesis of autoimmune disease, such as Grave’s disease in which the number of HLA-DR molecules is anomalously increased (20). In the present study, we first examined the effect of IFN-γ on phosphorylation of Tyr-701 and Ser-727 of STAT1 in TPA-treated THP-1 cells. Steinmüller et al. (21) have reported that staurosporine, a specific inhibitor of Tyr-701 phosphorylation (22, 23), blocks induction of CIITA by IFN-γ. Van Wagner et al. (24) have demonstrated that H7, an inhibitor of Ser-727 phosphorylation (25), abrogates IFN-γ-induced expression of CIITA and major histocompatibility complex class II genes. These observations suggest that phosphorylation of Tyr-701 and Ser-727 of STAT1 leads to CIITA expression, which is a key factor in the induction of HLA-DRα by IFN-γ. Consequently, results shown in Fig. 1, A and B, together with these observations suggest that, in TPA-treated THP-1 cells that contain increased levels of IFN-γ-IRα, enhanced
expression of ILA-DRα (14) is due to the increase in the phosphorylation of Tyr-701 and Ser-727 of STAT1 following treatment with IFN-γ. Recently Kovarik et al. (26) have shown that Ser-727 of STAT1 is phosphorylated through the p38 mitogen-activated protein kinase pathway by using SB 203580, a specific p38 mitogen-activated protein kinase inhibitor. Furthermore, it has been known that TPA activates p38 mitogen-activated protein kinase (27, 28), and this activation is inhibited by SB 203580 (28). These observations taken together with our results shown in Fig. 1A suggest that STAT1 is phosphorylated on Ser-727 by p38 mitogen-activated protein kinase following activation by TPA.

IFN-γ binds to a heterodimeric receptor composed of IFN-γR1, which is able to bind to the ligand with high affinity (29), and IFN-γR2, which is required for signal transduction (30). Our previous observations (15) indicate that the expression of IFN-γR1 is increased by TPA treatment alone, which is in good agreement with the observations of Mao et al. (31). Binding of IFN-γ to the IFN-γR complex results in phosphorylation of Tyr-701 of STAT1 (19). Thus, the present results (Fig. 1A) together with our previous results (15) suggest that in the TPA-pretreated cells, the enhanced phosphorylation of Tyr-701 of STAT1 following treatment with IFN-γ may be due to the increase in the levels of IFN-γRs.

We also investigated the transcriptional control mechanisms associated with the TPA-induced up-regulation of IFNGR1. To identify the transcriptional regulatory element that responds to TPA, we performed deletion analysis of the promoter region of IFNGR1 reporter constructs in THP-1 cells. As shown in Fig. 2, the DNA region located between positions −128 and −109 plays an important role in the regulation of IFNGR1 expression. Furthermore, we used this cis-element (−128 to −109 bp) and the minimal HSV TK promoter to construct a reporter gene and confirmed that this region was a TPA-responsive element (Fig. 4). TPA-responsive elements have been well characterized and confirmed that this region was a TPA-responsive element (32–34). Sp1 has been shown to be an important factor for Sp1 (36). The IFNGR1 promoter also lacked a TATA sequence motif (37). Sp1 has been shown to interact with several transcription factors such as Oct-1 (44), TATA-binding protein (45), nuclear factor-Y (46), SREBP-2, Elf-1, and transforming growth factor-β-inducible early gene (47). Furthermore, other groups have demonstrated that phosphorylation and glycosylation of Sp1 also regulate its activity (48, 49). Therefore, identification of how Sp1 activity is controlled and its role in the regulation of IFNGR1 expression will require extensive work in the future.

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