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Loss of interferon alpha and interferon tau-induced antiviral protection in interferon regulatory factor-2 DNA-binding domain dominant negative mutants☆

Yaffa R. Rubinstein, Carol H. Pontzer *

Department of Cell Biology and Molecular Genetics, Bldg. 231, University of Maryland, College Park, MD 20742, USA

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Abstract

The role of the interferon regulatory factory (IRF) family of transcription factors in regulation of interferon alpha and interferon tau antiviral activity was investigated using a dominant negative mutant of IRF-2. The IRF-2 DNA binding domain (DBD), without the C-terminal regulatory region, was stably transfected into myeloid U937 cells. Expression of the IRF-2 DBD resulted in an increase in constitutive 2′5′ oligoadenylate synthetase (OAS) levels, indicative of an active repressive mechanism, but was not sufficient to protect cells from challenge with vesicular stomatitis virus. Treatment of the DBD clones with interferons alpha A and tau failed to upregulate 2′5′ OAS expression and did not elicit an antiviral response. While interferon alpha A was more sensitive than interferon tau to the inhibitory effects of the IRF-2 DBD, IRF-mediated gene induction is involved in successful interferon alpha and tau-induced anti-VSV activity.

Keywords: Interferons; Signal transduction; Dominant negative

1. Introduction

In addition to the well characterized JAK/STAT signal transduction pathway (Darnell et al., 1994), members of the interferon regulatory factor (IRF) family of transcription factors are induced and activated in response to interaction of type I interferons (IFNs) with their receptors (Harada et al., 1998; Pitha et al., 1998). These transcription factors bind to a 9 nt consensus sequence termed the IRF-E (Tanaka et al., 1993). The IRF family includes factors, such as IRF-1 and ISGF3γ, that act as transcriptional activators while others, such as IRF-2 and interferon consensus sequence binding protein, function as repressors (Pine et al., 1990; Weisz et al., 1992; Tanaka et al., 1993; Bluyssen et al., 1996). All members exhibit high homology in their N-terminal DNA-binding domains (DBD) (Pitha et al., 1998).

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* Corresponding author. Tel.: +1-301-405-5472; fax: +1-301-314-9489.

E-mail address: cp75@umail.umd.edu (C.H. Pontzer)

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While the transcriptional activity of STAT proteins is regulated by phosphorylation state, it is the relative protein levels of IRF-1:IRF-2, activator-to-repressor, that control IRF-1-mediated gene expression. In uninduced cells, IRF-2 is present at higher levels than is IRF-1, and genes that are under basal control of the IRF family are transcriptionally repressed (Watanabe et al., 1991; Harada et al., 1993). Upon exposure to IFNs, the IRF-1 gene is transiently induced, resulting in replacement of IRF-2 by IRF-1 on the IRF-E and the subsequent induction of many IFN-responsive genes (Miyamoto et al., 1988; Watanabe et al., 1991).

The presence of IRF-1 is critical for expression of some IFN-inducible genes, such as those for guanylate binding protein (GBP) and inducible nitric oxide synthase (Matsuyama et al., 1993). However, the role of the IRF family in transcriptional regulation of other genes, such as 2′5′ oligoadenylate synthetase (2′5′ OAS) and RNA-dependent protein kinase (PKR) remains uncertain. Induction of 2′5′ OAS and PKR by type I IFNs was not altered in embryonic fibroblasts from either IRF-$1^{-/-}$ or IRF-$2^{-/-}$ mice (Matsuyama et al., 1993). This suggested that their expression was independent of the IRF-1-mediated pathway of transcriptional activation. In contrast, the ability of IFNs to inhibit replication of encephalomyocarditis virus in the same IRF-$1^{-/-}$ embryonic fibroblasts was diminished (Kimura et al., 1994). It has been suggested that despite the lack of significant effect of IRF-1 on induction of these genes, their expression may be influenced by alterations in the IRF-1:IRF-2 balance during the cell cycle (Lamphier and Taniguchi, 1994). More recently, ectopic expression of IRF-1 was found to transactivate the 2′5′ OAS gene, and its effect was reversed by ectopic expression of IRF-2 (Coccia et al., 1999).

Because of the important role of IFNs in antiviral protection and the clinical implication of this function, we were interested in investigating the involvement of the IRF family proteins in regulation of the antiviral activity of interferon alpha and interferon tau. We established a cellular model system for examination of IRF family-mediated transcriptional regulation of IFN inducible genes. The system involved cloning of the IRF-2 DBD, without the C-terminal regulatory domain. The IRF-2 DBD was stably transfected into U937 cells, creating a dominant negative mutant in which the ISRE was occupied by the transcriptionally inactive IRF-2 DBD. The effect of the DBD expression on the ability of interferon alpha and interferon tau treatment to elicit an antiviral state was examined.

2. Materials and methods

2.1. Cells

U937 human premonocyte lymphoma cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 50 μg/ml gentamicin sulfate, and 50 μM β-mercaptoethanol.

2.2. IFNs

Ovine IFNτ1mod (1×10^8 U/mg) was produced in our laboratory in a *Pichia pastoris* GS115 expression system and purified by DEAE and hydroxyl apatite chromatography as described previously (Van Heeke et al., 1996). Recombinant human IFNα-2a (IFN-αA) was purchased from either Lee Biomolecular (San Diego, CA) or PBL Biomedical Laboratories (New Brunswick, NJ) with specific activities of 1.3×10^6 and 3.8×10^8 U/mg, respectively.

2.3. Construction of the IRF-2 DBD expression vector

The IRF-2 DBD insert representing 129 amino acids of the IRF-2 N-terminus was generated by PCR using a plasmid expressing full length IRF-2 and the primers:

5′-AGA CTA CTGAG (*Xho*I) ACC ĀTG CCG GTG GAA AGG-3′

5′-CCA AAG ACA GAA AAĀ [His tag]

TAA (Stop) CTCGAG (*Xho*I) CCG CAT-3′

An expression plasmid for the dominant negative IRF-2 DBD was constructed by cloning the insert into the *Xho*I site of pCXN2, a 5.2 Kb vector based on puc-19 (Niwa et al., 1991).

2.4. Luciferase reporter assay

U937 cells (1×10^7) were cotransfected by electroporation with 10 μg of ISRE-luciferase reporter, 10 μg of the IRF-1 expression vector, pAct-1 (a gift from T. Taniguchi), with or without 0.01–10 μg of the IRF-2 DBD expression vector. The ISRE-luciferase reporter construct consisted of GBP $(ISRE)_3$ fused in front of the 40-bp basal promoter of the L^d gene and cloned into the pGL2 luciferase plasmid. The luciferase reporter without the ISRE insert and an unrelated retinoic acid-responsive reporter were used as controls. Cells were incubated for 18–20 h until harvest.

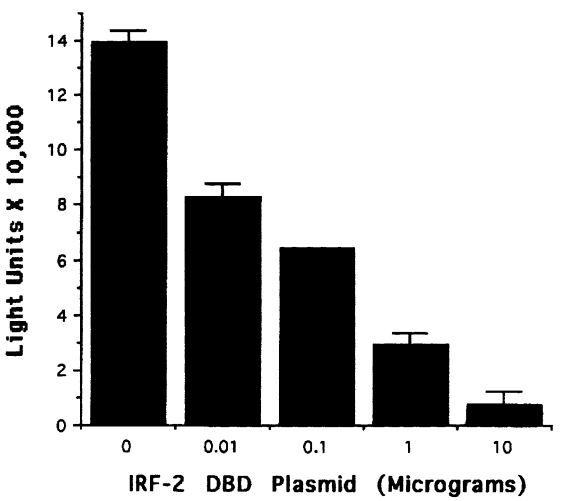

Fig. 1. Inhibition of ISRE promoter activity by the IRF-2 DBD. U937 cells (1×10^7) were cotransfected with 10 μg of ISRE-luciferase reporter construct consisting of GBP $(ISRE)_3$ fused in front of the 40-bp basal promoter of the L^d gene and cloned into the pGL2 luciferase plasmid, 10 μg of the IRF-1 expression vector, pAct-1, and the indicated amounts of the DBD expression vector. Cells were incubated for 18–20 h until harvest. Data represent the mean ± S.E. of two replicate experiments.

2.5. Transfection and cloning of stable transfectants

U937 cells (1×10^7) were transfected with 50 μg of control pCXN2 (no insert), or pCXN2 containing the IRF-2 DBD insert by electroporation. Cells were selected with geneticin (200 μg/ml, Life Technologies) for 2 weeks and cloned by limiting dilution. A total of 40 IRF-2 DBD clones and twenty pCXN2 clones were isolated and screened for IRF-2 DBD expression by Western blot using polyclonal antisera raised against the purified IRF-2 DBD protein. From that pool, four clones expressing different levels of the IRF-2 DBD (#4, #13, #14, #16) and one control clone (#9) containing the empty vector were selected for further analysis.

2.6. Antiviral activity

U937 cells or 5×10^5/5 ml were treated for 24 h with no IFN, IFNα or IFNτ. Vesicular stomatitis virus (VSV; Indiana strain) was added at 10 PFU/cell, and the cells were cultured for an additional 48 h. Total viable cell counts were determined by trypan blue staining.

2.7. RNA blot analysis

Total RNA was extracted using RNAzol (Tel-Test, Friendswood, TX). RNA (12 μg) was electrophoresed through a 1.2% formaldehyde agarose gel and blotted into Hybond-N membrane (Amersham) in 20× SCC (3 M NaCl and 0.3 M sodium citrate, pH 7.0). Membranes were hybridized in QuikHyb buffer (Stratagene, La Jolla, CA) with ^{32}P-labeled probes at 68°C for 1 h. The 2′5′ OAS probe (generously provided by B.-Z. Levi, Benech et al., 1985) was labeled using the Prime-It RmT kit (Stratagene).

3. Results

The human premonocyte lymphoma cell line, U937 was transiently cotransfected with the IRF-2 DBD, an IRF-1 expression vector, and a construct of a luciferase reporter and an

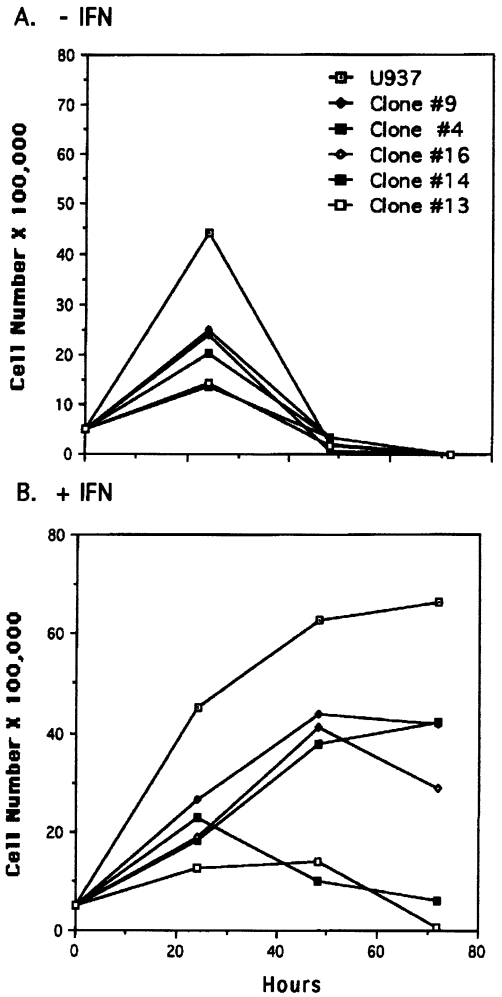

Fig. 2. Time course of IFN-αA antiviral activity in IRF-2 DBD clones. Parental U937 cells, the control clone (# 9), and IRF-2 DBD clones, all at 0.5×10^6/5 ml, were untreated or treated for 24 h with 1000 U/ml IFN-αA and challenged with VSV at 10 PFU/cell for the indicated times. Representative data from one of three replicate experiments are presented as total viable cell counts. At 48 h, $P = 0.32$ and $P < 0.001$ by analysis of variance for − IFN and + IFN, respectively. Multiple comparison tests of + IFN data indicated that U937 cells were significantly ($P < 0.05$) different from all groups except clone # 16 by Least Significant Difference, and U937 cells and clones # 9, # 4 and # 16 were significantly ($P < 0.05$) different from clones # 14 and # 13 by Scheffe's F-test.

IFN-stimulated response element (ISRE) which contains the IRF-E. The IRF-2 DBD inhibited induction by IRF-1 of ISRE-dependent luciferase activity (Fig. 1). Further, the amount of IRF-2 DBD transfected was inversely related to IRF-1-induced luciferase activity. Expression of the IRF-2 DBD did not inhibit activity of an unrelated retinoic acid responsive reporter (data not shown). Therefore, the IRF-2 DBD binds to the IRF-E and inhibits gene activation in response to IRF-1.

To examine IRF family regulation of IFN-α and IFN-τ-induced antiviral activity, stable IRF-2 DBD transfectants were produced. Four clones expressing different levels of the IRF-2 DBD (# 13 > # 14 > # 16 > # 4) and one control clone (# 9) containing the empty vector were selected for further study. Thus, dominant negative mutants were created in which the IRF-E was occupied by the transcriptionally inactive IRF-2 DBD. The ability of these clones to exhibit an antiviral response to IFN-αA was examined by challenge with VSV. In the absence of IFN-αA treatment the controls as well as the DBD clones had been killed by 48 h (Fig. 2A). When the parental U937 cells were exposed to 1000 U/ml IFN-αA, they were protected from the cytopathic effect of VSV (Fig. 2B). However, their growth rate was retarded by one doubling time relative to controls without either IFN treatment or virus infection (data not shown). This is consistent with the known growth inhibitory activity of type I IFNs (Harada et al., 1998). IFN treatment alone did not affect cell viability because no decrease in viability was observed in the absence of VSV challenge (data not shown). In the presence of both IFN and virus, the control clone transfected with the empty vector and the low DBD-expressing clones # 4 and # 16 exhibited slightly reduced viable cell numbers relative to uninfected U937 cells. This reduction in viability despite IFN-αA treatment was much more pronounced in the high DBD-expressing clones # 13 and # 14. Thus, the IRF-2 DBD clones were not responsive to the antiviral effect of IFN-αA.

We wished to assess whether or not this lack of responsiveness was peculiar to IFN-αA or was extended to other type I IFNs as well. Antiviral protection is induced by both IFN-α and IFN-τ in the parental U937 cell line[1]. IFN-τ is a relatively

[1] Rubinstein, Y.R., Maguire, T.E., Shi, J. and Pontzer, C.H. Differential Cytotoxicity and Gene Induction by Interferon Alpha and Interferon Tau (manuscript submitted).

newly discovered type I IFN, originally identified as a pregnancy recognition signal (Imakawa et al., 1987; Pontzer et al., 1988). IFN-τ has been shown to bind to the common type I IFN receptor (Stewart et al., 1987), but it is unusual in that it can be used at high concentrations without exhibiting the cytotoxicity that is associated with IFN-α (Soos et al., 1995; Subramaniam et al., 1995). IFN-α afforded greater antiviral protection than did IFN-τ in the control clone. In contrast, the two high-expressing IRF-2 DBD stable transfectants exhibit reduced responsiveness to both IFNs (Fig. 3). The IRF-2 DBD, therefore, inhibited antiviral activity of IFN-αA to a greater extent than that of IFN-τ.

A mechanistic explanation for the reduction in IFN-induced antiviral state in the IRF-2 DBD clones was provided by examination of IFN-induced 2′5′ OAS gene expression. 2′5′ OAS plays an important role in the antiviral function of

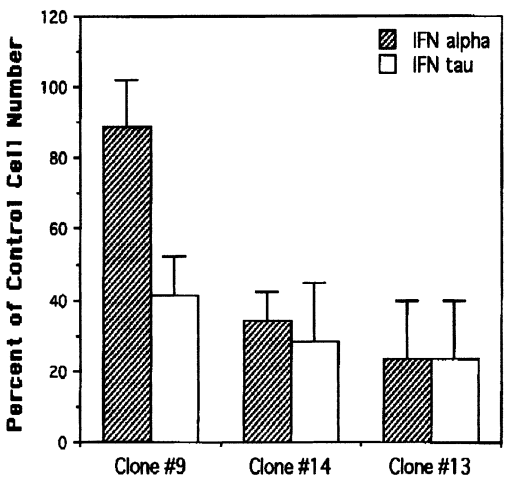

Fig. 3. Antiviral response of IRF-2 DBD clones to IFN-τ. Cells were untreated or treated with 1000 U/ml of either IFN-αA or IFN-τ for 24 h and infected with VSV for 48 h. Control cultures did not receive IFN. Representative data from one of three replicate experiments are presented as percent of control cell number ± S.D. Control cell numbers were $5.3 \times 10^6 \pm 0.4 \times 10^6$, $3.5 \times 10^6 \pm 1.2 \times 10^6$ and $1.7 \times 10^6 \pm 0.1 \times 10^6$ for the control clone #9 and clones #14 and #13, respectively. IFN-αA treated control clone #9 was significantly different from IFN-αA treated IRF-2 DBD clones #14 and #13 by Student's t-test with $P < 0.001$. IFN-αA and IFN-τ treatment of control clone #9 were significantly different as assessed by Student's t-test with $P < 0.05$.

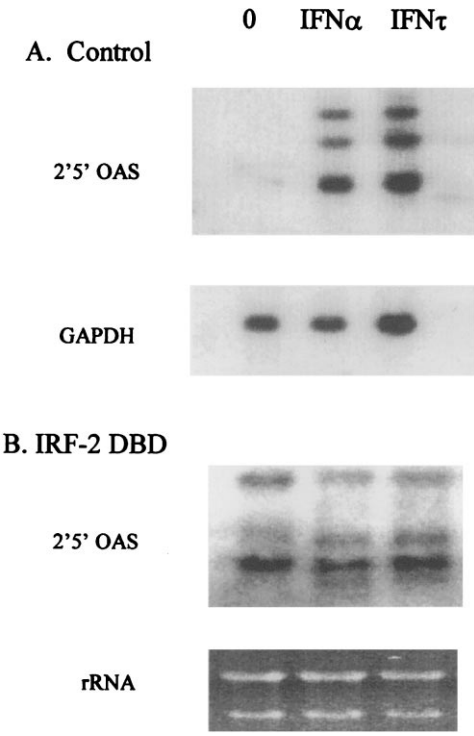

Fig. 4. Type I IFN-induced expression of 2′5′ OAS. U937 cells (A) or the IRF-2 DBD clone #14 (B) were treated with either no IFN, represented by '0', or IFN-α or IFN-τ at 1000 U/ml for 5 h. Total RNA was extracted using RNAzol (Tel-Test, Friendswood, TX). A total of 12 μg of RNA was electrophoresed through a 1.2% formaldehyde/agarose gel and blotted into Hybond-N membrane (Amersham) in 20× SCC (3 M NaCl and 0.3 M sodium citrate, pH 7.0). The 2′5′ OAS probe (generously provided by B.-Z. Levi, Benech et al., 1985) was labeled using the Prime-It RmT kit (Stratagene). Membranes were hybridized in QuikHyb buffer (Stratagene, La Jolla, CA) with the ^{32}P-labeled probe at 68°C for 1 h. The figure is representative of two independent experiments with virtually identical results. The three bands represent 3.6, 1.8, and 1.6 kb mRNAs.

IFNs. It is activated by dsRNAs to synthesize 2′5′ oligoadenylates, which in turn, activate RNase L, resulting in degradation of viral and cellular RNAs (Chebath and Revel, 1992). Both IFN-α and IFN-τ induced significant 2′5′ OAS expression in U937 cells (Fig. 4). In DBD clones we observed higher constitutive 2′5′ OAS expression. However, this higher basal expression was not sufficient to produce an effective antiviral response in the untreated IRF-2 DBD clones. Ele-

vated 2′5′ OAS may be a contributing factor to the slow growth rate of the DBD clones, because overexpression of this gene has been shown to inhibit cell proliferation (Rysiecki et al., 1989). Further, 2′5′ OAS was not upregulated in response to either IFN-α or IFN-τ, which is consistent with blockage of the IRF-E and the observed lack of IFN protection of the IRF-2 DBD clones from the cytopathic effect of VSV.

4. Discussion

Since in the dominant negative mutant, overexpression of the IRF-2 DBD alone, lacking the repressor function, would compete with wild type IRF-2, the observed up-regulation of 2′5′ OAS gene expression in the IRF-2 DBD clones could be attributable to abrogation wild-type IRF-2 repression. In the uninduced state, this should result in lack of repression of transcriptional activation by constitutive enhancers having binding sites positioned nearby in the promoter. In fact, negative control of IFN-inducible genes in the absence of IFN induction has been previously suggested as a mechanism of transcriptional regulation by Taniguchi (Harada et al., 1993; Taniguchi et al., 1995). It was postulated that the high IRF-1 levels induced by viruses would be required to displace IRF-2, if it were indeed bound, and elicit an antiviral response. Our data suggest that this basal negative transcriptional regulation of 2′5′ OAS by IRF-2 may be operative. Alternatively, promoter accessibility may be changed upon DBD binding (Wolffe, 1994). The promoter region of the p69 form of the enzyme does contain binding sites for multiple activators, including AP-1 and ets-1, in addition to an ISRE and two IRF-E-like sites which interact cooperatively (Wang and Floyd-Smith, 1998). Upon IFN induction, the IRF-2 DBD would compete with IRF-1 for binding to the IRF-E. Subsequent expression of genes for which IRF-1-mediated transcriptional activation is redundant would still increase, while transcription of those absolutely or solely dependent on IRF-1 would not. Since IFNs are without effect on 2′5′ OAS induction and protection from virus, IRF-mediated gene induction must be critical to a successful antiviral response to VSV.

Since overexpression of just the IRF-2 DBD results in an increase in the basal level of 2′5′ OAS, we have identified active repression by IRF-2 which keeps 2′5′ OAS transcriptionally silent. The IRF-1:IRF-2 balance, though, is only one component of a complex system, and this system is susceptible to modulation by IFN treatment. Since IFN-αA induced antiviral activity showed a greater dependence on IRF-mediated signaling than did induction by ovine IFN-τ1mod, there may be differences in the mechanisms employed by individual IFN subtypes to induce their antiviral state. Interestingly, the upregulation of IRF-2 which follows IRF-1 induction by IFNs has been suggested, in the case of IFN-τ, to facilitate its maternal recognition of pregnancy function by silencing transcription of the estrogen receptor gene in the uterine endometrium (Spencer et al., 1998). Viruses may alter the system and IFN-inducible gene regulation, blocking IFN responsiveness through virally-encoded IRFs. Like the IRF-2 DBD, vIRFs have been shown to down-modulate promoter activity of IFN-inducible genes (Gao et al., 1997; Pitha et al., 1998). The IRF-2 DBD dominant negative mutants should serve as a important tool to further decipher the function of the IRF family in regulation of antiviral activity.

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