Defective binding of IRFs to the initiator element of interleukin-1β-converting enzyme (ICE) promoter in an interferon-resistant Daudi subline

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Abstract To investigate mechanisms of interferon (IFN) resistance, we have established an IFN-resistant Daudi subline (Daudi^{res}), which is 1×10^4 times more resistant to IFN-α than parental cells. Among the IFN-inducible genes examined, only ICE mRNA expression was deficient in Daudi^{res} cells. We then analyzed the regulatory mechanisms of ICE transcription, and found that IFN-induced activation of the ICE promoter was dependent on the binding of IRFs to its initiator (Inr) element. Inr binding of IRFs was markedly diminished in Daudi^{res} cells, and forced expression of IRF-1 was able to activate the ICE promoter to the level of parental cells. These results suggest that IRFs and their target genes, as represented by ICE in this study, are involved in IFN resistance.

Key words: Interferon; Drug resistance; IRF; ICE; Initiator; Promoter

1. Introduction

Interferon is now widely used as a therapeutic reagent for cancer and virus infection, and its resistance is an important clinical problem [1]. A significant number of patients do not respond to IFN even at the time of initial therapy, and many patients become resistant after long-term treatment [1]. Although IFN resistance can be partially explained by the development of anti-IFN neutralizing antibody in the latter case, its underlying mechanisms are largely unknown. Insights into the molecular mechanisms of IFN resistance are necessary for better clinical application.

Recent studies have revealed that the mechanisms of IFN action are diverse. Binding of IFN-α/β to type I receptors activates JAK1 and TYK2 kinases that in turn phosphorylate cytoplasmic STAT (signal transducers and activators of transcription) proteins including STAT1α, STAT1β, and STAT2 [2]. The phosphorylated STAT proteins are translocated into the nucleus and form a complex called ISGF3 (interferon-stimulated gene factor 3) with p48 (ISGF3γ). ISGF3 complex binds to the IFN-stimulated response element (ISRE) in the promoter of a group of IFN-stimulated genes and induces their transcription. On the other hand, IFN-γ activates JAK1 and JAK2 kinases through type II receptor and induces phosphorylation of STAT1α. Upon phosphorylation, STAT1α dimerizes and binds to the γ-IFN-activated site (GAS) or GAS-like elements in the promoter of various target genes to activate their transcription. Via these signaling pathways, IFNs can induce expression of proteins with distinct functions, such as (2′-5′) oligoadenylate synthetase (2′-5′-AS) [3], 2′-5′-AS-dependent ribonuclease L (RNase L) [4], and double-stranded RNA-dependent protein kinase (PKR) [5], to exert many biological activities.

To investigate the mechanisms of IFN resistance, we have recently established a novel IFN-resistant cell line Daudi^{res} from human B-lymphoblastoid cell line Daudi. In contrast to the parental Daudi (hereinafter referred to as Daudi^{sen} for discrimination), the growth of Daudi^{res} cells is not inhibited by IFN. Using this cell line, we analyzed the expression of various genes involved in the signal transduction and function of IFN, and found that induction of interleukin-1β-converting enzyme (ICE) mRNA was deficient in Daudi^{res} cells. We then studied the molecular mechanisms of defective ICE expression in this cell line, and discussed the implications for IFN resistance.

2. Materials and methods

2.1. Establishment of an IFN-resistant Daudi cell line (Daudi^{res}) and cell culture

An IFN-resistant subline was established by long-term exposure of IFN-α (Sumitomo Pharmaceutical Co., Osaka, Japan) to Daudi^{sen} cells. Culture was started at 3×10^5 cells/ml in growth medium containing 100 IU/ml of IFN-α, and the medium was changed every 2 or 3 days to maintain viable cells at the concentration of 3×10^5 cells/ml in the presence of IFN. Growth of surviving cells was observed after 6 to 7 weeks, and the cells were subcloned in methylcellulose containing IFN-α to obtain a single cell clone. Regrowth assay revealed that the EC_{50} of Daudi^{sen} and Daudi^{res} was 10 IU/ml and 1×10^5 IU/ml, respectively, indicating that the Daudi^{res} cell line is 1×10^4 times more resistant to IFN-α than the parental Daudi^{sen} cell line.

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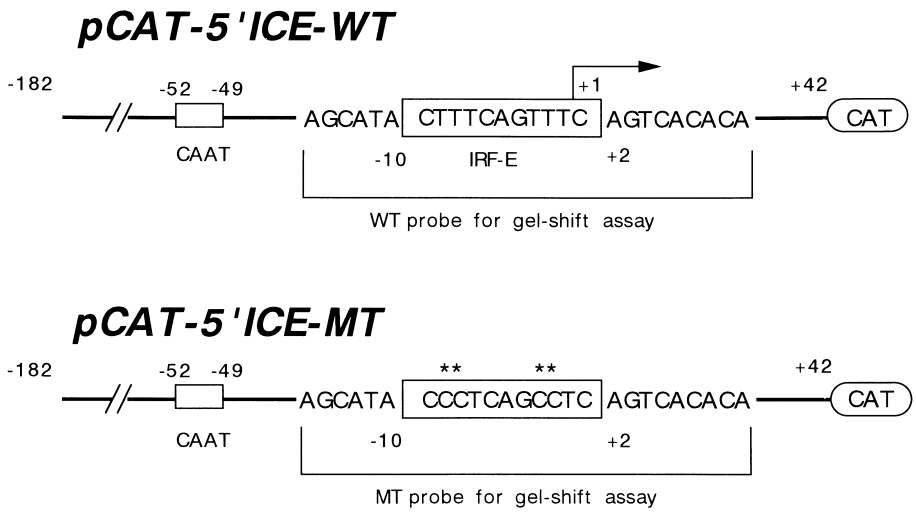

Fig. 1. Structure of the human ICE promoter and reporter plasmids used in this study. A 5′-untranslated region of the human ICE promoter (nucleotide positions −182 to +42) was subcloned into a promoterless CAT vector (pCAT-5′-ICE-WT). This segment includes a CAAT box (−52 to −49), the Inr/IRF-binding element (−10 to +1), and the transcription initiation site (+1). A mutant vector pCAT-5′-ICE-MT was generated by introducing a non-binding mutation into the IRF-E at the positions indicated by asterisks. The sequence between −16 and +10 of each construct was used as probes for gel-shift assays.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Semiquantitative RT-PCR was carried out as previously described [6]. The amplification cycles numbered 25 except for ICE (28 cycles). Information of the primer sequences is available on request.

2.3. Immunoblotting

Immunoblotting was performed with anti-STAT1 (E-23) and anti-STAT2 (C-20) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the standard method.

2.4. Gel-retardation assays

Nuclear extracts (5 μg) were incubated with approximately 0.5 ng (10 000 cpm) of (^{32}P)-labeled double-stranded oligonucleotides in the presence of 1 μg each of sonicated salmon sperm DNA and poly(dI-dC). The protein-DNA complexes were resolved on a 4% polyacrylamide gel in 0.25×Tris-borate-EDTA buffer.

Probes used in this study are as follows (sense strands are shown; consensus sequence is underlined, and mutated sites are shown in boldface): IRF element, 5′-AGCATACTTTCAGTTTCAGTCACACA-CA-3′; mutant IRF element, 5′-AGCATAC**CC**TCAG**CC**TCAGTCACACA-3′ (Fig. 1) [7]. The following antibodies (1 μg each) were included in the reaction mixture for antibody perturbation experiments: anti-IRF-1 (C-20), anti-IRF-2 (C-19), anti-STAT1 (E-23), anti-STAT2 (C-20), anti-ICSBP (C-19), anti-ISGF3γ (C-20) (all purchased from Santa Cruz Biotechnology).

2.5. Reporter plasmids

A 5′-untranslated region of the human ICE promoter (nucleotide positions −182 to +42 relative to the transcription start site) was subcloned into pCAT-basic vector (Promega, Madison, WI, USA), which was then used as a reporter plasmid pCAT-5′-ICE-WT. Mutated reporter plasmid, pCAT-5′-ICE-MT, was prepared by PCR-based site-directed mutagenesis [8] using 5′-AGCATAC**CC**T-CAG**CC**TCAGTCACACA-3′ as a mutagenesis primer (mutated sites are in boldface) (Fig. 1).

2.6. Expression plasmids

IRF-1 and IRF-2 expression plasmids were constructed by ligating full-length cDNAs into pCR3.1 vector (Invitrogen).

2.7. Transient transfection and CAT assay

Reporter plasmids were linearized and transfected into cells by a Gene Pulser (Bio-Rad Laboratories) [9]. The cells were split equally into two flasks and cultured for 24 h after adding 250 IU/ml of IFN-α to one flask. For cotransfection assays, cells were transfected with 30 μg of reporter plasmid, 4 μg of β-galactosidase expression plasmid (pSV-β-gal, Promega), and 8 μg of IRF-1 expression plasmid. CAT and β-galactosidase activities were determined after 24 h.

3. Results

3.1. Expression of IFN-inducible molecules in Daudi^{sen} and Daudi^{res} cells

In an attempt to understand the molecular basis of the IFN resistance of Daudi^{res} cells, we screened for expression of the molecules involved in signal transduction of IFN (IFN receptor, STAT1 and STAT2) and their functions (2′-5′-AS, RNase L, PKR and ICE) in IFN-treated Daudi^{sen} and Daudi^{res} cells by semiquantitative RT-PCR. As shown in Fig. 2a, IFN-α was able to induce or enhance the expression of a number of IFN-inducible genes in Daudi^{res} cells as well as in Daudi^{sen} cells. Immunoblotting also revealed that there was no significant difference in the amounts of STAT1 and STAT2 proteins between the two cell lines (Fig. 2b). Among the IFN-inducible genes examined, however, induction of ICE mRNA was completely defective in Daudi^{res} cells, suggesting that the loss of ICE induction plays a role in the IFN resistance of this cell line. This prompted us to analyze the mechanisms of transcriptional activation of the ICE gene by IFN.

3.2. IFN-α-induced binding of IRF-1 and IRF-2 in the Inr element of human ICE promoter

Transcriptional regulation of the ICE gene is not fully understood. Human ICE promoter has a CAAT box at the position of 52 bp relative to the transcription start site, but lacks a classical TATA motif [7]. Instead of the TATA box, it possesses the sequence TTTCAGTTTC encompassing the transcription start site (Fig. 1). This sequence is highly conserved between human and mouse, and matches the consensus of the initiator (Inr) element, PyPyCANPyPyPyPy [10], suggesting that transcription of the TATA-less ICE gene is primarily driven by the Inr element. Furthermore, upon inspection of this element, we have found that its core sequence (TTCAGTTT) conforms to the consensus of IRF-binding element (IRF-E), TTCNNTTT [11]. This led us to test whether

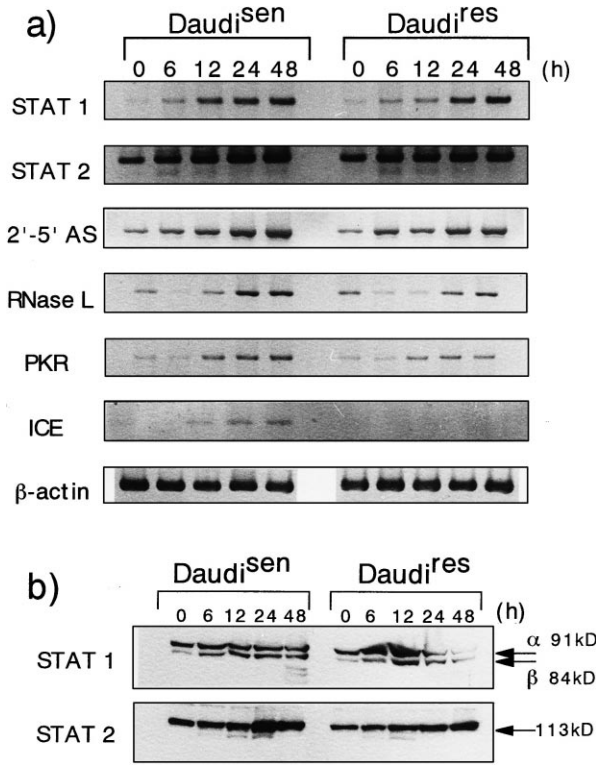

Fig. 2. Expression of the molecules involved in signal transduction and function of IFN in Daudi^{sen} and Daudi^{res} cells. The cells were cultured in the absence or presence of 250 IU/ml of IFN-α for up to 48 h. Total cellular RNA and whole cell lysates were prepared at the indicated time points, and subjected to semiquantitative RT-PCR (a) and immunoblotting (b). Data shown are representatives of three independent experiments.

IFN-induced activation of the ICE gene is regulated by the binding of IRFs to its Inr element.

We first examined whether IRF proteins actually bound to the Inr/IRF-E of ICE promoter in an IFN-dependent manner. As shown in Fig. 3, three specific complexes, designated as A, B and B′, were detectable by gel-retardation assays with oligonucleotide probe corresponding to the Inr/IRF-E of ICE promoter; other bands (indicated by asterisks) were considered to be non-specific, because they were not detected in a reproducible manner. Then, we identified the nature of each complex by antibody perturbation experiments. Anti-IRF-1 antibody eliminated band A and B′, which resulted in the appearance of shifted bands (dashed arrows). Anti-IRF-2 removed a significant amount of the complex B to the position indicated by a solid arrow (Fig. 3b). In contrast, the complexes were unaffected by antibodies against STAT proteins (STAT1 and STAT2) and other IRF family proteins (ICSBP and ISGF3γ) (data not shown).

To negate the possibility that other factors bind to this site, we carried out the same experiments with the mutated probe. The mutation was designed to retain the consensus of the Inr element but lose the ability to bind IRF proteins (Fig. 1). Therefore, this probe could render other Inr-binding proteins, such as TAFII150 [12], YY1 [13] and USF [14], visible if they bound to the ICE promoter in addition to IRFs. No DNA-binding complex was observed in IFN-treated Daudi^{sen} extracts when the mutant probe was used (Fig. 3c), indicating that IRFs are the sole molecules which bind to the Inr element of the ICE promoter along with IFN-induced ICE mRNA expression. However, it is still possible that other members of the IRF family are the components of the complex, since B and B′ were not completely eliminated even by the combination of anti-IRF-1 and anti-IRF-2 (data not shown).

We then examined the effects of IFN on IRF binding to the ICE promoter. As shown in Fig. 3a, a small amount of IRF-1 (B′) was observed in both cell lines before IFN treatment, suggesting a role for IRF-1 in basal-level transcription of

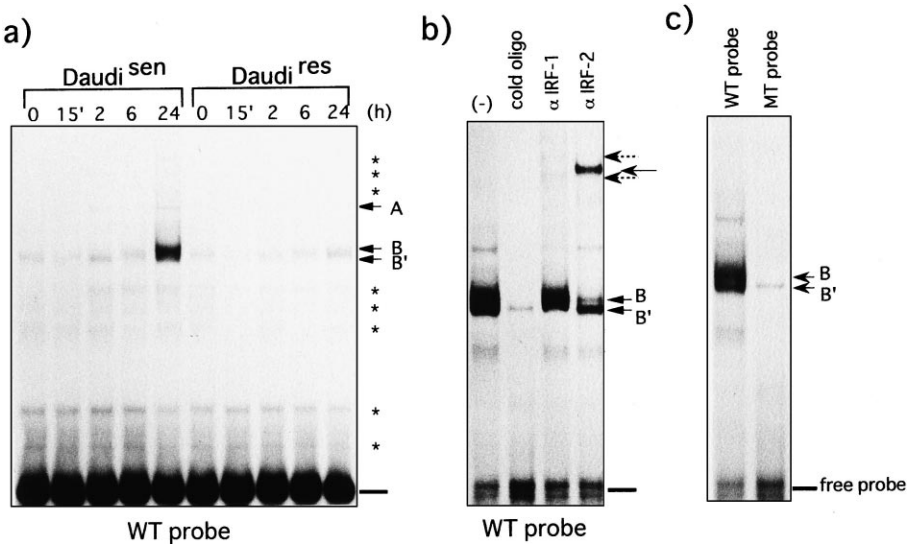

Fig. 3. Binding of IRF proteins to the Inr element of ICE promoter. a: Nuclear extracts were prepared from IFN-treated cells at the indicated time points and incubated with (^{32}P)-labeled oligonucleotides corresponding to the Inr element of the ICE promoter. Specific complexes are indicated by A, B and B′ and non-specific bands are indicated by asterisks. b: Nuclear extracts from Daudi^{sen} cells after 24 h treatment with IFN-α were incubated with WT probe in the absence (−) or the presence of specific antibodies against IRF-1 and IRF-2, or a 100-fold molar excess of the unlabeled oligonucleotides (cold oligo), and subjected to native acrylamide gel electrophoresis. c: Gel-shift assays were performed with either WT probe or MT probe.

ICE. IFN-α increased IRF-1 binding (A and B′) in Daudi^{sen} cells after 2 h along with striking induction of IRF-2 (B) which showed a peak of intensity at 24 h. In contrast, IRF-1 binding was not enhanced and much less IRF-2 was induced in IFN-treated Daudi^{res} cells, suggesting that the defective binding of IRFs is responsible for the lack of ICE induction in this cell line.

3.3. IFN activated the ICE promoter through the binding of IRFs to its Inr element

Next, we tried to clarify the functional significance of the binding of IRFs to the ICE promoter. For this purpose, we first examined whether IFN-α could activate transcription from the ICE promoter. The 222 bp fragment of the ICE promoter, which was defined as a minimal promoter in our pilot experiments (data not shown), was subcloned into a promoterless CAT vector (pCAT-5′-ICE-WT, see Fig. 1 for the structure) and used as a reporter plasmid. We also generated a mutated vector pCAT-5′-ICE-MT by introducing a non-binding mutation into the IRF-E. These plasmids, with empty pCAT-basic vector as a negative control, were transiently transfected into Daudi^{sen} cells, and intracellular CAT activity was measured after 24 h of culture with or without IFN-α. As anticipated, IFN-α was able to increase the activity of wild-type promoter 5.5-fold relative to the untreated cells (Fig. 4a). IFN-α failed to activate the mutated promoter, indicating that IFN-induced transcription of the ICE gene is totally dependent on the binding of IRFs to its Inr element. In addition, the basal activity of the ICE promoter is also likely to be regulated by the same mechanism, since the CAT activity of pCAT-5′-ICE-MT was significantly lower than that of pCAT-5′-ICE-WT in untreated cells.

We then carried out the same experiments with IFN-resistant Daudi^{res} cells. As shown in Fig. 4b, IFN-induced activation of the wild-type promoter was significantly reduced in Daudi^{res} cells ($P < 0.01$ vs. Daudi^{sen} cells by Student's t-test). The mutated promoter was also unresponsive to IFN-α in Daudi^{res} cells. These results clearly show that binding of IRFs is essential for basal and IFN-induced transcriptional activation of the ICE gene, and also suggest that the defect in this system underlies IFN resistance in Daudi^{res} cell line.

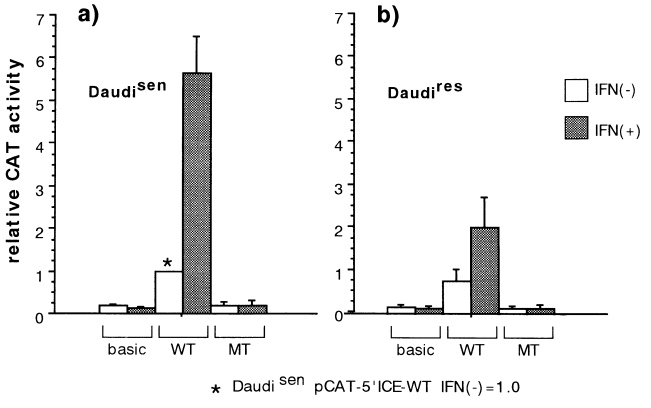

Fig. 4. IFN-α enhanced ICE promoter activity in an IRF-dependent manner. Reporter plasmids, pCAT-5′-ICE-WT (WT) and pCAT-5′-ICE-MT (MT), were transfected into Daudi^{sen} (a) and Daudi^{res} (b) cells. Empty pCAT-basic vector was used as a negative control (basic). The cells were split equally into two fractions, and cultured in the absence (open bars) or presence (shaded bars) of 250 IU/ml of IFN-α. CAT activity was measured after 24 h. Relative CAT activity was determined with the value obtained from untreated Daudi^{sen} cells transfected with pCAT-5′-ICE-WT set at 1.0 (shown by an asterisk). The mean ± S.D. (bar) of five independent experiments is shown.

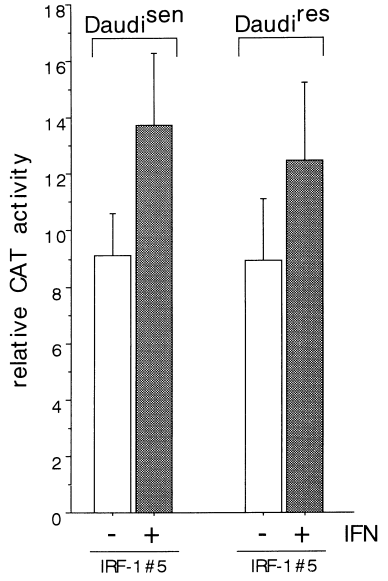

Fig. 5. Effect of IRF-1 overexpression on ICE promoter activity. IRF-1 expression plasmid was transfected into Daudi^{sen} and Daudi^{res} cells with pCAT-5′-ICE-WT and pSV-β-gal vectors. Empty pCR3.1 vector was used for mock transfection. The cells were split equally into two fractions and IFN-α was added to one (shaded bars). Relative CAT activity was determined as the fold increase against the value obtained from untreated mock-transfected Daudi^{sen} cells. All values were normalized to β-galactosidase activity. The mean ± S.D. (bar) of five independent experiments is shown.

3.4. Forced IRF-1 expression resulted in ICE promoter activation in the Daudi^{res} cell line

Finally, we investigated whether ectopic IRF expression could activate the ICE promoter in the Daudi^{res} cell line. As shown in Fig. 5, ICE promoter activity was increased in Daudi^{res} cells to the level of that in Daudi^{sen} cells when IRF-1 was overexpressed. Under this condition, IFN was able to slightly enhance the promoter activity in both Daudi^{sen} and Daudi^{res} cells. In contrast, forced expression of IRF-2 resulted in remarkable suppression of ICE promoter activity (data not shown).

4. Discussion

The mechanisms of IFN resistance have been investigated by many researchers using IFN-resistant cell lines. Loss of receptor expression and function was shown to be responsible for IFN resistance in H82 (human lung cancer cell line) [15], Capan 2 (human pancreatic carcinoma cell line) [16], and IFN-resistant variants of Daudi and HeLa [17]. In addition, defects in the expression and activation of STAT1 and STAT2 were reported to be a major cause of IFN resistance in some cell lines [17–19]. To further investigate the mechanisms of IFN resistance, we have established a novel IFN-resistant cell line Daudi^{res} from IFN-sensitive Daudi cells (Daudi^{sen}) by long-term exposure to IFN-α. It was shown that signal transduction from IFN receptors to STAT was not severely impaired in Daudi^{res} cells unlike in the other cell lines de-

scribed above. Among the IFN-inducible genes examined, only for the ICE gene was mRNA induction defective in Daudi^{res} cells.

Then, we investigated the mechanisms of ICE induction by IFN-α. Transcriptional regulation of the ICE gene is not fully understood, although genomic cloning has already been done in both human and mouse [7]. Human ICE promoter lacks a classical TATA motif and, instead, has an Inr element, which may be responsible for transcription of the ICE gene. In this study, we found that binding of IRFs to this element coincided with ICE mRNA induction by IFN-α. Mutation at the Inr/IRF-E, which prevents IRF binding but retains Inr function, completely abrogated IFN-induced activation of the ICE promoter. This indicates that IFN-induced transcriptional activation of the ICE gene is completely dependent on IRF binding to its Inr element. Our finding explains why ICE mRNA is defective in mitogen-stimulated T-lymphocytes from IRF-1 knock out mice [20].

In TATA-less promoters, the Inr element is critical in positioning RNA polymerase II, and is believed to initiate transcription according to two different models [10]. In one model, RNA polymerase II recognizes the Inr element directly and forms a transcription competent complex with TBP, TFIIB, and TFIIF. In the alternative model, Inr element-binding proteins interact with components of the general transcriptional machinery to assemble a transcription competent complex. Two Inr-binding proteins, cap-site-binding factor [21] and TFII-I [22], start transcription from the adenovirus major late promoter according to the latter model. In this study, we have found that IRF-1 acts as an Inr-binding protein and plays a crucial role in IFN-induced transcription of the ICE gene. To our knowledge, this is the first report to demonstrate IRF-1 acts as an Inr-binding protein for a native promoter. Recently, Wang et al. [23] reported that recombinant IRF-1 physically associated with TFIIB and cooperatively regulated IFN-responsive promoter activity. This finding supports our notion very well.

Binding of IRFs to the ICE promoter was markedly diminished in IFN-resistant Daudi^{res} cells, and ectopic IRF-1 expression was able to activate the ICE promoter to the level in Daudi^{sen} cells. How is the loss of IRF binding implicated in IFN resistance? Although the defect in ICE expression may affect IFN resistance in some manner, the antiproliferative effect of IFNs is not solely due to the induction of apoptosis, especially in Daudi cells in which apoptosis is hardly induced (Iwase, S. et al., unpublished data). Therefore, it is possible that, in addition to ICE, IRFs regulate the transcription of other genes which play crucial roles in the antiproliferative action of IFNs, and their defect is primarily responsible for IFN resistance of IRF-deficient cells. An earlier study of Ozes and Taylor [24] provides a good example of such a scenario. They have described that indoleamine 2,3-dioxygenese is essential for growth inhibition of human cervical carcinoma cell line ME180 by IFN-γ, and its induction is completely dependent on IRF-1. IFN-γ-mediated induction of IRF-1 and, subsequently, indoleamine 2,3-dioxygenese is defective in the IFN-resistant variant of ME180. We are now trying to identify other IRF-dependent genes implicated in IFN resistance of Daudi^{res} cells. Taken together, these results suggest that multiple interrelated mechanisms are involved in IFN resistance, and a comprehensive approach is required to elucidate the molecular basis of the resistance in each case.

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