The EMBO Journal Vol.17 No.4 pp.1087–1095, 1998

Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300

Mitsutoshi Yoneyama, Wakako Suhara, Yukiko Fukuhara, Makoto Fukuda[1], Eisuke Nishida[1] and Takashi Fujita[2]

Department of Tumor Cell Biology, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113 and [1]Department of Biophysics, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan

[2]Corresponding author
e-mail: fujita@rinshoken.or.jp

It has been hypothesized that certain viral infections directly activate a transcription factor(s) which is responsible for the activation of genes encoding type I interferons (IFNs) and interferon-stimulated genes (ISGs) via interferon regulatory factor (IRF) motifs present in their respective promoters. These events trigger the activation of defense machinery against viruses. Here we demonstrate that IRF-3 transmits a virus-induced signal from the cytoplasm to the nucleus. In unstimulated cells, IRF-3 is present in its inactive form, restricted to the cytoplasm due to a continuous nuclear export mediated by nuclear export signal, and it exhibits few DNA-binding properties. Virus infection but not IFN treatment induces phosphorylation of IRF-3 on specific serine residues, thereby allowing it to complex with the co-activator CBP/p300 with simultaneous nuclear translocation and its specific DNA binding. We also show that a dominant-negative mutant of IRF-3 could inhibit virus-induced activation of chromosomal type I IFN genes and ISGs. These findings suggest that IRF-3 plays an important role in the virus-inducible primary activation of type I IFN and IFN-responsive genes.

Keywords: CBP/p300/IRF-3/phosphorylation/type I interferon/virus infection

Introduction

Infection by a variety of viruses triggers activation of a set of cellular genes including those of type I interferons (IFN-α and IFN-β). The secreted IFNs then transduce signals through the cognate receptor in an autocrine or paracrine manner to activate IFN-stimulated genes (ISGs), some of which are responsible for the versatile biological effects of IFNs including their antiviral activity. Hence the IFN system constitutes the primary defense against viral infections (DeMaeyer and DeMaeyer-Guignard, 1988; Sen and Lengyel, 1992).

Previous studies unequivocally demonstrated that the type I IFN signal is transduced by the activation of Jak family protein kinases and the signal transducers and activators of transcription (STAT) family of proteins, resulting in the generation of the transcriptional activator, ISG factor 3 (ISGF3). ISGF3 binds to an IFN-stimulated response element (ISRE) and participates in IFN responses (Darnell *et al.*, 1994).

However, the primary activation mechanism by virus infection remains to be elucidated. It has been shown that virus infection targets several promoter/enhancer elements. Virus-inducible IFN-β enhancer contains positive regulatory domain II (PRDII), a binding site for NF-κB which is required for the full activation of the gene, but NF-κB alone is not sufficient (Fujita *et al.*, 1989; Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989; Watanabe *et al.*, 1991). The other elements PRDI of IFN-β and VRE-α of IFN-α, which are related to each other, are shown to be activated through interaction with proteins belonging to the IRF family, namely IFN regulatory factor-1 (IRF-1) or ISGF3 (Miyamoto *et al.*, 1988; Harada *et al.*, 1989, 1996; Yoneyama *et al.*, 1996). However, these activators function secondarily as a result of *de novo* IRF-1 synthesis or autocrine activation by IFN (Watanabe *et al.*, 1991; Harada *et al.*, 1996; Yoneyama *et al.*, 1996). A distinct direct mechanism is suggested because PRDI is strongly activated by virus infection or by double-stranded RNA (dsRNA); however, much weaker activation is observed by IFN treatment (Watanabe *et al.*, 1991). Moreover, the virus-induced activation is sensitive to certain protein kinase inhibitors and does not require *de novo* protein synthesis (Zinn *et al.*, 1988; Goldfeld and Maniatis, 1989; Watanabe *et al.*, 1991), suggesting activation of a pre-existing factor by phosphorylation. ISRE-binding factors activated by dsRNA-induced biochemical modification were identified (DRAF1 and DRAF2) (Daly and Reich, 1993, 1995). However, their precise molecular nature and the activation mechanism remain to be elucidated.

IRF family proteins are candidates for the virus-induced ISRE/PRDI activation, since their potential DNA-binding domains are highly conserved. There are at least nine IRF family genes in human and mouse. Although IRF-1 and IRF-2 were identified as transcriptional activator and repressor, respectively (Miyamoto *et al.*, 1988; Harada *et al.*, 1989), the analysis of null mutant mice for either gene clearly showed that these gene products are dispensable for triggering of IFN gene expression (Matsuyama *et al.*, 1993). ISGF3γ (Veals *et al.*, 1992) was demonstrated to be essential for positive feedback regulation of IFN genes in certain cells (Harada *et al.*, 1996; Yoneyama *et al.*, 1996). However, a significant level of IFN production was demonstrated in the absence of the ISGF3γ gene (Harada *et al.*, 1996). ICSBP and IRF-4/pip/LSIRF were shown to be expressed exclusively in lymphoid cells (Driggers *et al.*, 1990; Eisenbeis *et al.*, 1995; Matsuyama *et al.*, 1995; Yamagata *et al.*, 1996). Although targeted disruption of these genes exhibited abnormalities in hematopoietic cell lineage, no significant alteration of the type I IFN system has been reported (Holtschke *et al.*, 1996; Mittrücker

© Oxford University Press

et al., 1997). IRF-3 was identified by primary sequence homology to the family and shown to be expressed ubiquitously in human tissues (Au *et al.*, 1995). A transient transfection assay demonstrated that IRF-3 augments the virus-induced activation of the IFNA4 gene enhancer, whereas no binding activity to PRDI was detected (Au *et al.*, 1995). These results suggest that IRF-3 has the potential to be activated directly by virus infection or dsRNA stimulation. The physiological significance of the other IRF family members, IRF-5, IRF-6 and IRF-7 (Zhang and Pagano, 1997), is still unclear.

Here we show that virus infection induces specific phosphorylation of IRF-3, which is present in the cytoplasm of uninfected cells, and results in induction of a transcription factor complex containing IRF-3/CBP/p300 in the nucleus which positively regulates IFN genes and ISGs.

Results

Ectopic expression of IRF-3 augments virus-induced activation of promoters containing IRF elements

To extend the finding that IRF-3 augments virus-induced gene activation (Au *et al.*, 1995), we transfected human IRF-3 expression vector with reporter constructs containing IFN enhancer or multimerized PRDI or PRDII to mouse L929 cells. Figure 1A shows that expression of IRF-3 alone did not activate either of the enhancers constitutively. However, IRF-3 significantly augmented Newcastle disease virus (NDV)-induced expression of reporters containing IFN-α, -β enhancer or multimerized PRDI, each of which contains IRF-binding sites. The virus-induced expression of the PRDII construct was unaffected by IRF-3 expression. In such cells, enhanced induction of endogenous mouse IFN-α and -β genes was coordinately observed (Figure 1B). Other IRFs, IRF-2, IRF-4, ICSBP and ISGF3γ, did not affect the gene expression significantly in similar assays (unpublished data).

Characterization of virus-induced DNA-binding factor VA-IRF which contains IRF-3 and CBP/p300

To correlate the gene activation by IRF-3 and the DNA-binding activity to the enhancer, whole cell extracts prepared from L929 cells or stable transformants expressing hemagglutinin (HA)-tagged human IRF-3 were subjected to electrophoretic mobility shift assay (EMSA) using the PRDI probe (Figure 2A, lanes 1–4). NDV infection induced ISGF3 (Figure 2A, arrow), as diagnosed by reactivity to antibodies to the ISGF3 subunits (Yoneyama *et al.*, 1996). The extract prepared from the transformant infected with NDV exhibited an additional complex that migrated slowly in the gel (lane 4, arrowhead). This complex was also detected with the ISRE probe (lane 8) and contained ectopically expressed IRF-3 as shown by reactivity to anti-human IRF-3 and anti-HA antibodies (lanes 15 and 16); thus we refer to the factor as virus-activated IRF (VA-IRF). Untransfected L929 cells exhibit a similar virus-inducible DNA-binding activity (lane 6) which corresponds to endogenous mouse VA-IRF containing mouse IRF-3, as shown by supershift by anti-mouse IRF-3

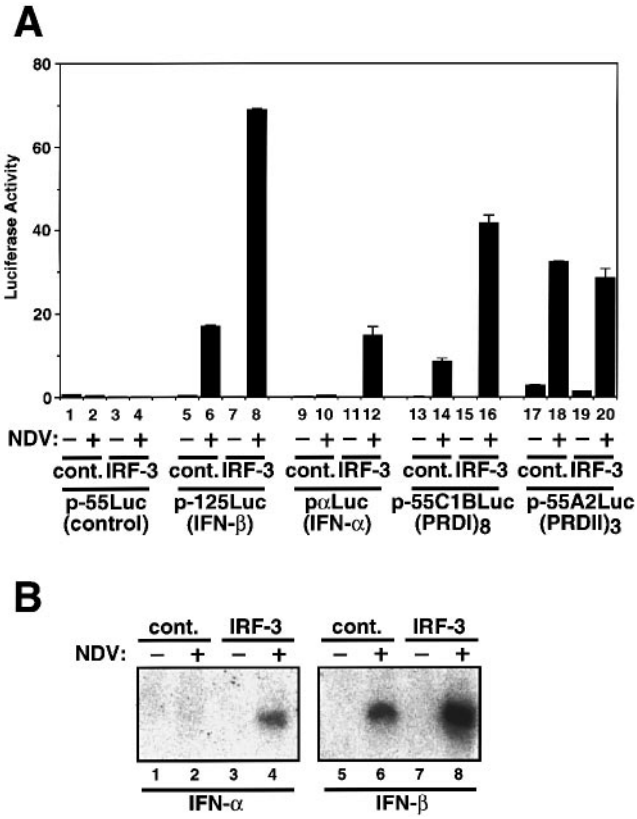


Fig. 1. IRF-3 augments NDV-induced activation of type I IFN genes. (**A**) Ectopic expression of IRF-3 can up-regulate virus-induced activation of type I IFN enhancers. L929 cells were transiently transfected with reporter constructs containing control promoter (p-55Luc; lanes 1–4), IFN-β enhancer (p-125Luc; lanes 5–8), IFN-α enhancer (pαLuc; lanes 9–12), repeated PRDI (p-55C1BLuc; lanes 13–16) or repeated PRDII (p-55A2Luc; lanes 17–20), with either control vector pEF-BOS (cont.; lanes 1, 2, 5, 6, 9, 10, 13, 14, 17 and 18) or expression plasmid for HA-tagged human IRF-3, pEF-HAIRF-3 (IRF-3; lanes 3, 4, 7, 8, 11, 12, 15, 16, 19 and 20). Cells were mock treated (–) or infected with NDV (+) for 12 h, and subjected to luciferase assay. Error bars show the standard error for triplicate transfections. (**B**) Expression of IRF-3 can augment NDV-induced activation of endogenous type I IFN genes. L929-derived M1 cells, which are stably expressing N-terminal truncated ISGF3γ (Yoneyama *et al.*, 1996), were transiently transfected with pEF-BOS (cont.; lanes 1, 2, 5 and 6) or pEF-HAIRF-3 (IRF-3; lanes 3, 4, 7 and 8), and mock treated (–; lanes 1, 3, 5 and 7) or infected with NDV (+; lanes 2, 4, 6 and 8) for 12 h. Total RNA (10 μg) was subjected to Northern blotting using mouse IFN-α (lanes 1–4) or IFN-β (lanes 5–8) cDNA as probes. Since M1 cells lack an autocrine amplification mechanism for type I IFN gene expression, the virus-induced expression of the IFN-α gene was barely detected in this assay (lane 2) (Yoneyama *et al.*, 1996).

antibody (lane 12). The specific antibodies to IRF-1, IRF-2, ISGF3γ, ICSBP, STAT1 and STAT2 failed to react with VA-IRF *in vitro* (our unpublished observation).

The relative molecular mass of the DNA-binding complex was analyzed by glycerol gradient sedimentation. IRF-3 extracted from unstimulated cells sedimented at ~50 kDa whereas IRF-3 from virus-infected cells sedimented at >300 kDa with VA-IRF binding activity (unpublished data). The result suggested that IRF-3 complexed with other molecule(s) such as co-activator(s) in the virus-infected cells. Indeed, the DNA-binding activity of VA-IRF was completely abolished by adding antibodies to the co-activators CBP and p300 (Figure 2A, lane 19). Since the inhibition was partial with

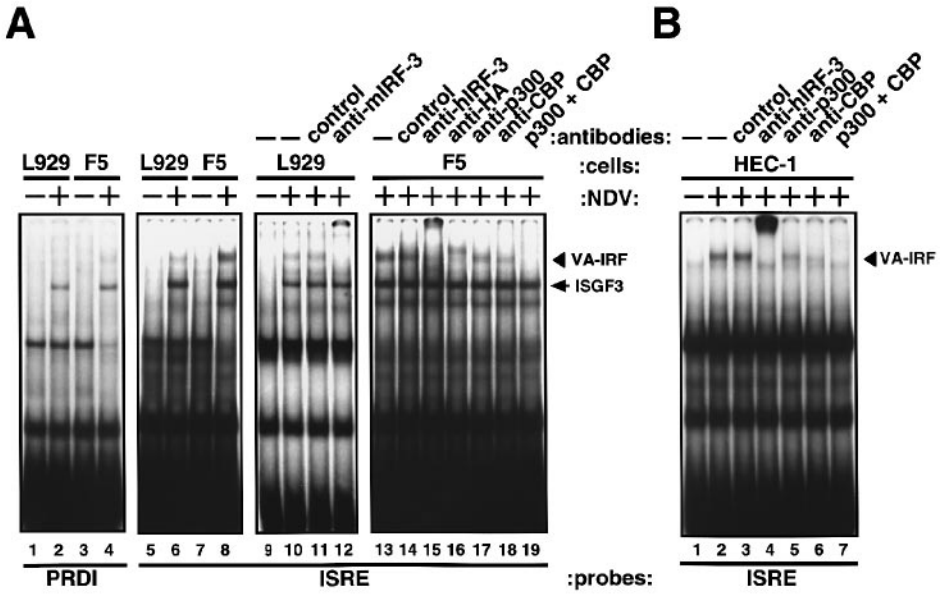


Fig. 2. Virus-inducible DNA-binding complex VA-IRF contains IRF-3 and CBP/p300 co-activator. (**A**) Characterization of VA-IRF in L929 cells. L929 cells (lanes 1, 2, 5, 6 and 9–12) or L929-derived transformant expressing HA-tagged IRF-3 (F5: lanes 3, 4, 7, 8, 13–19) were mock treated (–: lanes 1, 3, 5, 7 and 9) or infected with NDV (+: lanes 2, 4, 6, 8 and 10–19) for 12 h, and crude extracts were subjected to EMSA using PRDI (lanes 1–4) or ISRE (lanes 5–19) oligonucleotides as probes. The indicated antibodies [control normal rabbit serum, lanes 11 and 14; anti-mouse IRF-3 antiserum (anti-mIRF-3), lane 12; anti-human IRF-3 antiserum (anti-hIRF-3), lane 15; anti-HA antibody, lane16; anti-p300 antiserum, lane17; anti-CBP antiserum, lane 18; both anti-p300 and anti-CBP, lane 19] were added to extracts before the binding reaction for 1 h. The arrow and arrowhead indicate the complex of ISGF3 and VA-IRF, respectively, with ^{32}P-labeled probe. NDV-infected L929 cells exhibited a weak but reproducible VA-IRF with the PRDI probe under certain conditions. (**B**) Detection of endogenous VA-IRF in human HEC-1 cells. HEC-1 cells were mock-treated (lane 1) or NDV-infected (lanes 2–7) for 12 h, and subjected to EMSA as described in (A) using ISRE probe. The indicated antibodies [control serum, lane 3; anti-human IRF-3 antiserum (anti-hIRF-3), lane 4; anti-p300 antiserum, lane 5; anti-CBP antiserum, lane 6; both anit-p300 and anti-CBP, lane 7] were added.

either antibody alone (lanes 17 and 18), VA-IRF is a mixture of at least two different molecular species. The virus-induced association of IRF-3 with p300 was also confirmed by co-immunoprecipitation (see Figure 4E). The involvement of CBP/p300 is consistent with the report that overexpression of E1A, which interacts with these proteins, blocked IFN-β gene induction by dsRNA (Ackrill *et al.*, 1991).

A human endometrial adenocarcinoma cell line, HEC-1, lacks functional type I IFN receptor, therefore autocrine induction of ISGF3 is not observed (Fuse *et al.*, 1984; Daly and Reich, 1993). HEC-1 cells, however, can respond to virus infection or dsRNA stimulation to activate genes regulated by ISRE (Daly and Reich, 1993, 1995), with simultaneous induction of endogenous human VA-IRF as diagnosed by antibody reactivity (Figure 2B), indicating that the induction is not mediated by autocrine IFNs.

Virus-induced nuclear translocation and phosphorylation of IRF-3

Next, to explore the activation mechanism of VA-IRF, the subcellular localization of IRF-3 and its modification were tested (Figure 3). In uninduced cells, IRF-3 was restricted to the cytoplasm and NDV infection resulted in nuclear translocation as shown by immunostaining (Figure 3A). The nuclear translocation was also induced by poly(rI): poly(rC) treatment in the presence of cycloheximide (Figure 3A). To explore biochemical modification of IRF-3, its phosphorylation was determined (Figure 3B). IRF-3 appears to be constitutively phosphorylated; however, virus infection resulted in a dramatic increase in ^{32}P incorporation with a concomitant change in mobility in SDS gels. This is due to induction of additional phosphorylation on specific residues as shown in Figure 3C, particularly generation of a major phosphopeptide 5 (Figure 3C). Phosphoamino acid analysis revealed that the inducible phosphorylation occurs on serine residues (Figure 3D, see below).

Functional domains of IRF-3

To delineate the structure–function relationship of IRF-3, we generated a series of mutants (described in Figure 4A) and characterized them. IRF-3$^{58–427}$, which lacks a functional DNA-binding domain, failed to induce VA-IRF DNA binding and repressed the IFN-β enhancer activity induced by virus (Figure 4B, lanes 5 and 6). However, the mutant can be phosphorylated inducibly (Figure 4D, lanes 5 and 6), associate with p300 (Figure 4F, lanes 5 and 6) and translocate to the nucleus (Figure 4F).

This mutant, when fused to the heterologous DNA-binding region of Gal4, activated the promoter containing UAS_G in a virus infection-dependent manner (Figure 4B, lanes 25 and 26). Although parental Gal4 is constitutively nuclear (unpublished data), the fusion protein exhibited dramatic translocation from cytoplasm to nucleus upon virus infection (Figure 4F). The result shows that the region 58–427 is sufficient to confer a responsiveness to the signal induced by virus infection.

The deletion of C-terminal residues 375–427 (IRF-3$^{1–374}$) resulted in constitutive cytoplasmic IRF-3 (Figure 4F), no detectable generation of VA-IRF binding (Figure 4C, lanes 7 and 8), no inducible phosphorylation (Figure 4D, lanes 7 and 8), no association with p300 (Figure 4E, lanes 7 and 8) and no transactivation (Figure 4B, lanes 7

and 8). Of the seven serine residues within this region (Figure 4A), six were replaced by alanine either singly or in different combinations, except the C-terminal serine which is not conserved between human and mouse. The mutants behaved essentially indistinguishably from the wild-type, except the point mutants S385A and S386A, which exhibited a phenotype identical to IRF-$3^{1–374}$ (Figure 4 and unpublished data). Further, these mutants were not phosphorylated on the specific residues by virus infection as diagnosed by mobility change on SDS–PAGE (Figure

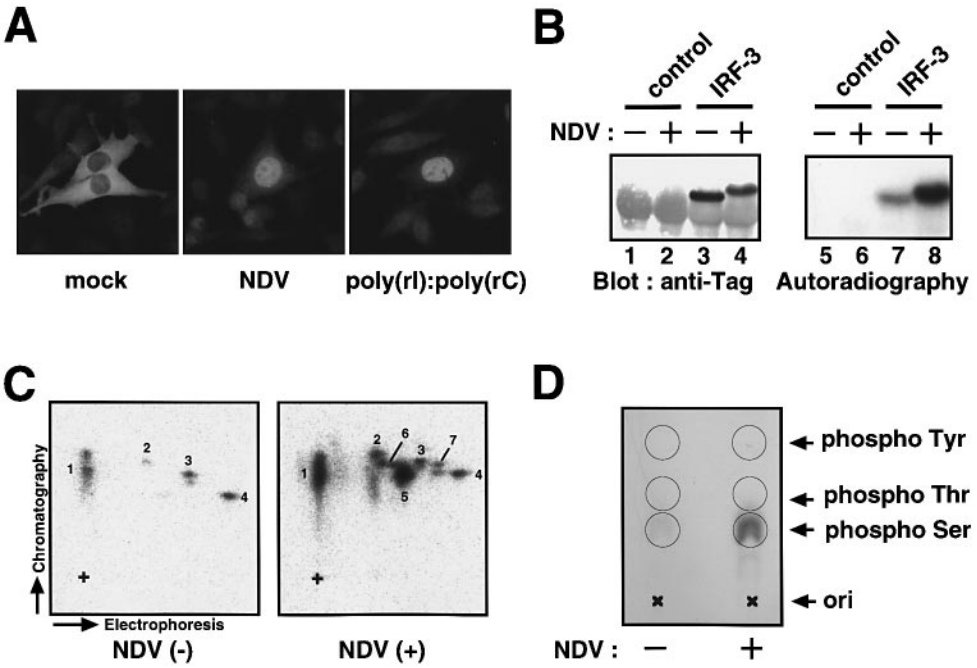


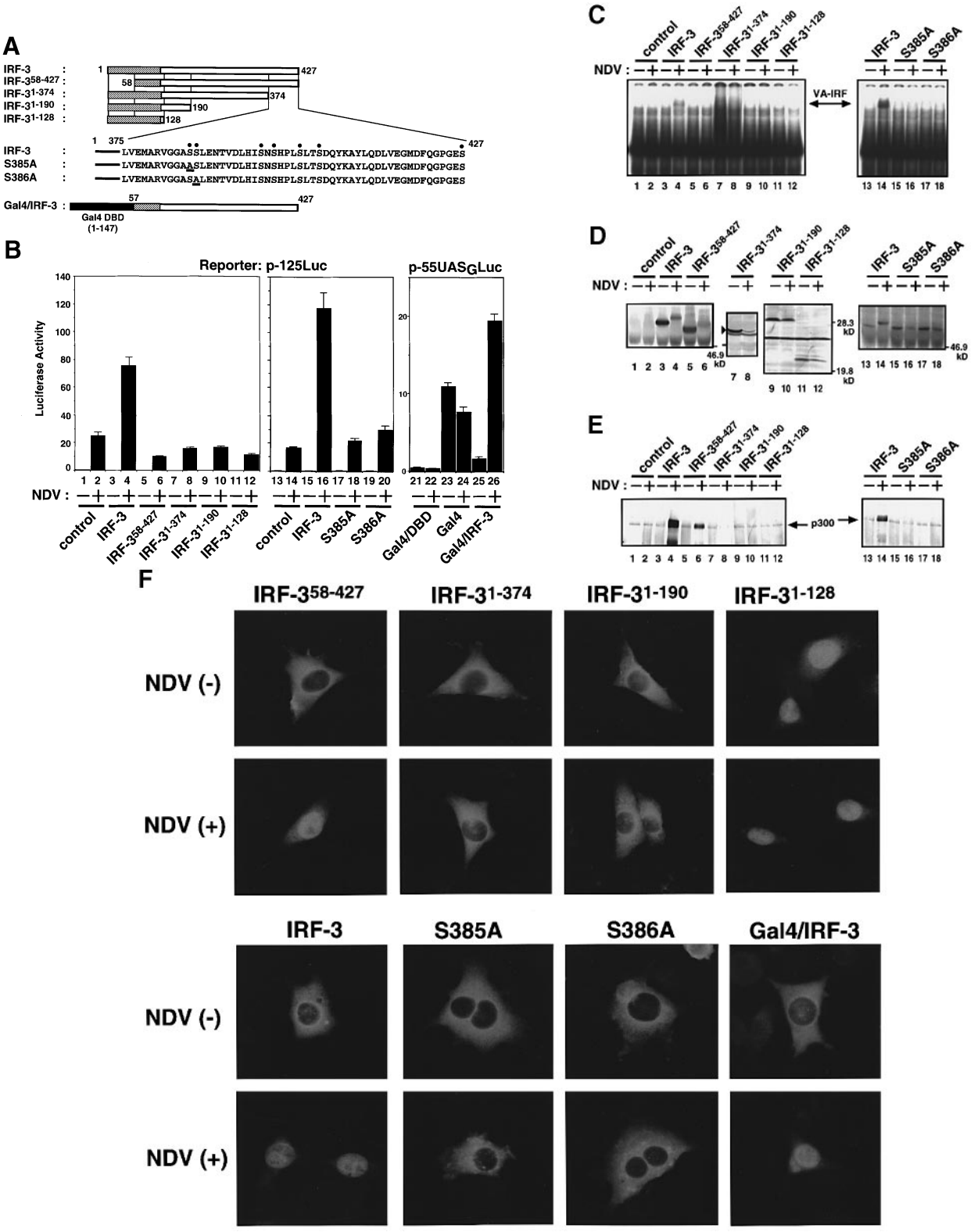
Fig. 3. Nuclear translocation and serine phosphorylation of IRF-3 induced by virus infection. (**A**) Virus or poly(rI):poly(rC) stimulation induces nuclear translocation of IRF-3. L929 cells transiently transfected with the expression plasmid for p50 epitope-tagged IRF-3 (pEF-p50IRF-3) were mock treated, infected with NDV or treated with poly(rI):poly(rC), fixed and stained with anti-p50 epitope monoclonal antibody. Anti-mouse immunoglobulin antibody labeled with FITC was used to visualize IRF-3. (**B**) Virus infection induces phosphorylation of IRF-3. L929 cells transfected with pEF-BOS (control: lanes 1, 2, 5 and 6) or pEF-p50IRF-3 (IRF-3: lanes 3, 4, 7 and 8) were mock treated (–) or infected with NDV (+) and cultured for 12 h in the presence of [^{32}P]phosphate. IRF-3 was immunoprecipitated with anti-hIRF-3 antibody, separated on SDS–PAGE, blotted to Immobilon membrane (Millipore) and detected by Western blotting using anti-p50 epitope antibody (lanes 1–4). Phosphorylation of IRF-3 was detected by autoradiography (lanes 5–8). (**C**) Virus infection induces phosphorylation of IRF-3 at specific residue(s). The ^{32}P-labeled IRF-3 prepared from mock-treated (–) or NDV-infected (+) cells was digested with trypsin and subjected to two-dimensional separation on thin-layer cellulose plates. The phosphorylated peptides were visualized by autoradiography. Several spots were detected: spots 1–4, the constitutively phosphorylated peptides; spot 5, the major phosphorylated peptide by virus infection; spots 6 and 7, the minor phosphorylated peptides. The crosses indicate the sample origins. (**D**) IRF-3 is phosphorylated on serine residues. The ^{32}P-labeled IRF-3 excised from lanes 3 and 4 in (B), respectively, were hydrolyzed, separated by thin-layer chromatography with unlabeled markers and visualized by autoradiography. The positions of unlabeled phosphoamino acids detected by ninhydrin staining are indicated. The crosses indicate the sample origins.

Fig. 4. Characterization of functional domains of IRF-3. (**A**) IRF-3 mutants. The DNA-binding domains of IRF-3 and Gal4 are shown as shadowed and closed boxes, respectively. The seven serine residues are shown by dots, and the substituted amino acids in S385A and S386A are underlined. (**B**) Reporter activation by the IRF-3 mutants. L929 cells were transiently transfected with the indicated expression constructs, together with the reporter plasmids (p-125Luc: lanes 1–20; p-55UAS$_G$Luc: lanes 21–26). The expression constructs were: control vector (pEF-BOS; lanes 1, 2, 13 and 14), HA-tagged full-length IRF-3 (IRF-3; lanes 3 and 4), p50-tagged full-length IRF-3 (IRF-3; lanes 15 and 16), HA-tagged deletion mutants (IRF-$3^{58–427}$, lanes 5 and 6; IRF-$3^{1–374}$, lanes 7 and 8; IRF-$3^{1–190}$, lanes 9 and 10; IRF-$3^{1–128}$, lanes 11 and 12) or p50-tagged point mutants (S385A, lanes 17 and 18; S386A, lanes 19 and 20), Gal4/DBD (lanes 21 and 22), full-length Gal4 (Gal4; lanes 23 and 24) and Gal4/IRF-3 (lanes 25 and 26). Luciferase activity was examined at 12 h after mock (–) or NDV stimulation (+). Error bars show the standard error for triplicate transfections. (**C**) DNA-binding activity of IRF-3 mutants. The expression constructs for control (control; lanes 1 and 2), HA-tagged wild-type (IRF-3; lanes 3 and 4), p50-tagged wild-type (IRF-3; lanes 13 and 14) or mutant IRF-3 (IRF-$3^{58–427}$, lanes 5 and 6; IRF-$3^{1–374}$, lanes 7 and 8; IRF-$3^{1–190}$, lanes 9 and 10; IRF-$3^{1–128}$, lanes 11 and 12; S385A, lanes 15 and 16; S386A, lanes 17 and 18) were transiently transfected into 293T cells, which have no endogenous VA-IRF activity. After mock (–) or NDV stimulation (+), crude extract was prepared and subjected to EMSA using ISRE oligonucleotide as a probe. (**D**) Phosphorylation of IRF-3 mutants. L929 cells were transiently transfected with the indicated expression constructs, then mock treated (–) or infected with NDV for 12 h (+). Wild-type IRF-3 (lanes 3, 4, 13 and 14), IRF-$3^{58–427}$ (lanes 5 and 6), S385A (lanes 15 and 16) and S386A (lanes 17 and 18) were immunoprecipitated with anti-hIRF-3 antibody and detected by immunoblotting using anti-Tag antibodies. IRF-$3^{1–374}$ (lanes 7 and 8; arrowhead), IRF-$3^{1–190}$ (lanes 9 and 10) and IRF-$3^{1–128}$ (lanes 11 and 12) were detected by immunoblotting with anti-HA antibody. A mobility change due to the virus-induced phosphorylation was observed with wild-type IRF-3 and IRF-$3^{58–427}$. (**E**) Complex formation of IRF-3 mutants with p300. Expression constructs for control (lanes 1 and 2), HA-tagged wild-type IRF-3 (lanes 3 and 4), p50-tagged IRF-3 (lanes 13 and 14) or mutant IRF-3 (IRF-$3^{58–427}$, lanes 5 and 6; IRF-$3^{1–374}$, lanes 7 and 8; IRF-$3^{1–190}$, lanes 9 and 10; IRF-$3^{1–128}$, lanes 11 and 12; S385A, lanes 15 and 16; S386A, lanes 17 and 18) were transiently transfected into 293T cells together with the expression construct for human p300, and cells were mock treated (–) or infected with NDV (+) for 12 h. IRF-3 was immunoprecipitated with anti-Tag antibody, and co-precipitated p300 was detected by immunoblotting with anti-p300 antibody. (**F**) Cellular localization of IRF-3 mutants. L929 cells transiently expressing wild-type or mutant IRF-3 were mock treated or infected with NDV and were stained with anti-HA, anti-p50 tag or anti-Gal4(DBD) (Santa Cruz Biotechnology, Inc.) antibodies.

4D, lanes 13–18). These results strongly suggest that phosphorylation of these residues is critical for triggering virus-induced signal and for the subsequent events.

IRF-3 contains functional NES

Further deletion of the residues 191–427 (IRF-$3^{1–190}$) resulted in the same phenotype as IRF-$3^{1–374}$, namely it had a constitutive cytoplasmic localization and was transcriptionally inactive (Figure 4). Additional removal of residues 129–190 (IRF-$3^{1–128}$), which leaves a potential DNA-binding domain, resulted in a constitutively nuclear IRF-3 (Figure 4F); however, the mutant did not associate with p300 (Figure 4E, lanes 11 and 12) and did not exhibit transactivation (Figure 4B, lanes 11 and 12). The results



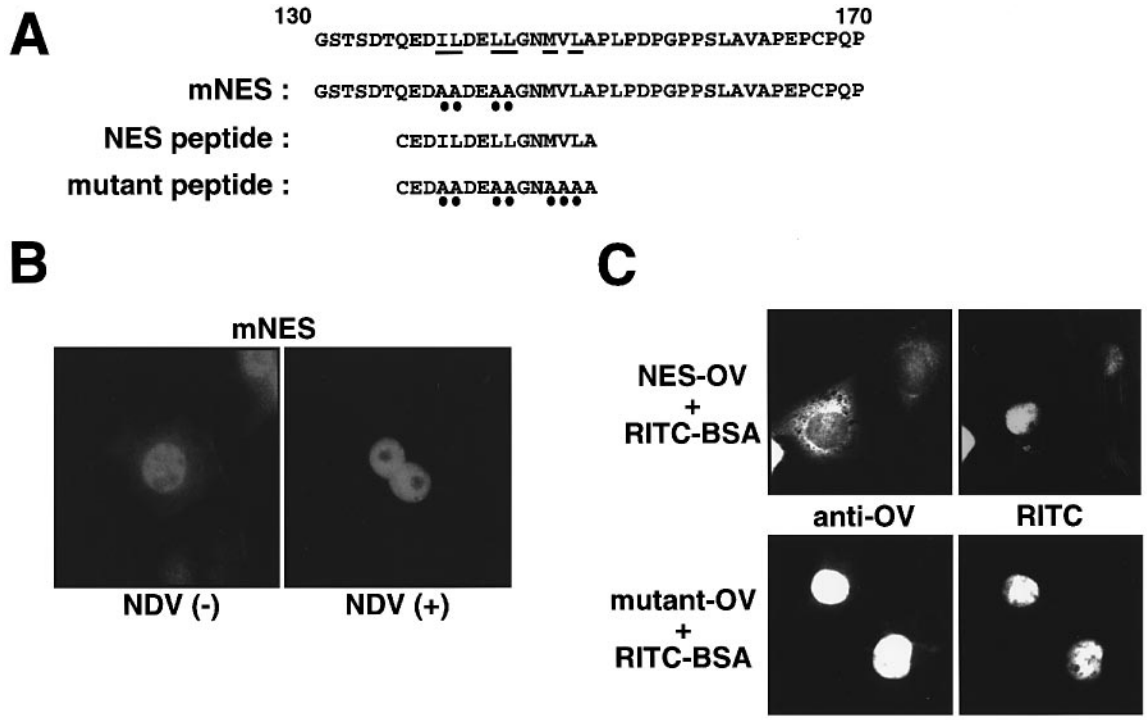


Fig. 5. IRF-3 contains a functional NES. (**A**) The primary sequence of IRF-3 between residues 130 and 170. The hydrophobic NES-like residues are underlined. NES mutant (mNES) was generated from the full-length p50 epitope-tagged IRF-3 by the Kunkel method. The sequences of synthetic NES and the mutant peptides are also shown. The mutated amino acid residues are shown by dots. (**B**) mNES constitutively localizes in the nucleus. mNES was transiently expressed in L929 cells, mock infected (–) or infected with NDV (+), and stained with the anti-epitope antibody. (**C**) The NES peptide but not the mutant peptide functions as an NES. The NES and the mutant peptides were conjugated to ovalbumin (OV) by sulfo-SMCC to yield NES-OV and mutant-OV, respectively. The conjugates and the RITC-labeled BSA were co-injected into the nuclei of 3Y1 cells. Cells were fixed at 1 h after injection, and the localization of OV and BSA was determined by anti-OV staining and RITC fluorescence, respectively.

suggested that the region 129–190 determines the cytoplasmic localization of IRF-3 in unstimulated cells.

This region contains a stretch of hydrophobic amino acids resembling the nuclear export signal (NES) identified in certain cytoplasmic proteins (Figure 5A) (Fischer *et al.*, 1995; Wen *et al.*, 1995; Fukuda *et al.*, 1996). The mutation of these residues resulted in a constitutively nuclear IRF-3 (mNES, Figure 5B). The tetradecamer peptides derived from the putative NES and its mutant were synthesized and conjugated to ovalbumin. The conjugates were subjected to nuclear export assay in living cells. The NES peptide conjugate but not the mutant conjugate was exported efficiently to the cytoplasm (Figure 5C). In a similar assay, residues 128–190 fused to GST are also exported specifically (unpublished data). IRF-3 export from the nucleus was sensitive to leptomycin B (unpublished data), indicating that the export is mediated by exportin 1/CRM1 (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997). The above results indicate that the hydrophobic residues function as authentic NESs and are responsible for the cytoplasmic localization of IRF-3 in uninduced cells.

Trans-dominant negative mutant of IRF-3 represses virus-induced but not IFN-induced gene activation

The above results suggest that IRF-$3^{58–427}$ can function as a competitive inhibitor for the formation of functional VA-IRF. To test the effect of IRF-$3^{58–427}$ on endogenous IFN genes and ISGs, we generated a stable L929 transformant overexpressing IRF-$3^{58–427}$ (L-IRF-$3^{58–427}$) (Figure 6A). The viral induction of endogenous IFN-α, -β genes was significantly suppressed (Figure 6B, lanes 3 and 6 of IFN-α and IFN-β). The residual induction of the IFN-β gene (lane 6 of IFN-β) is probably due to the κB motif (PRDII) which is absent in the IFN-α enhancer. The two ISGs, 2′–5′ oligoadenylate synthetase (2–5AS) and GBP-1, are activated by IFN treatment in both the parental and the L-IRF-$3^{58–427}$ cells (lanes 2 and 5 of 2–5AS and GBP-1). However, viral induction was again significantly suppressed in the L-IRF-$3^{58–427}$ cells (lanes 3 and 6 of 2–5AS and GBP-1). The repression was incomplete, probably because low levels of IFN (particularly IFN-β) are secreted by L-IRF-$3^{58–427}$ cells and secondarily activated the genes. These results strongly suggest that VA-IRF participates in the activation of type I IFN genes and ISGs in virus-infected cells.

Discussion

The above results highlight a novel gene activation pathway induced by virus infection or dsRNA, which is characteristic of low constitutive activity. This is because IRF-3 is strictly under negative control in unstimulated cells: IRF-3 is retained in the cytoplasm by NESs and, presumably due to its conformation, its DNA-binding activity is masked.

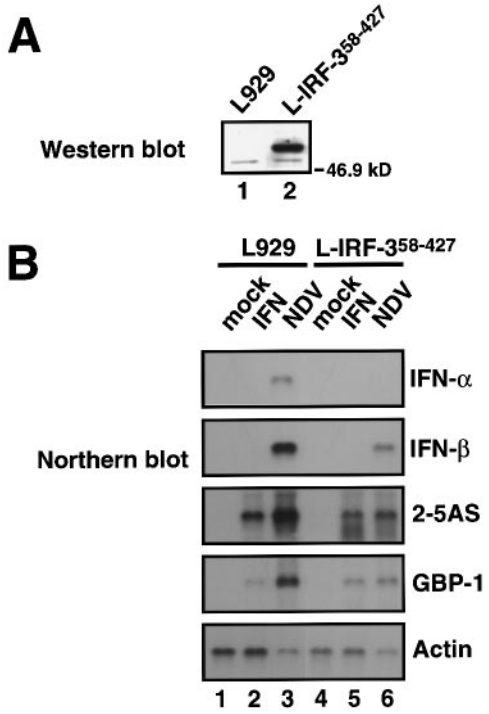


Fig. 6. IRF-3$^{58–427}$ can function as a dominant-negative inhibitor for virus-induced expression of endogenous IFN and ISGs.
(**A**) Ectopically expressed IRF-3$^{58–427}$. A L929-derived transformant expressing IRF-3$^{58–427}$ was established (L-IRF-3$^{58–427}$). Crude extracts from L929 cells (lane 1) and L-IRF-3$^{58–427}$ cells (lane 2) were subjected to Western blotting with anti-Tag anitibody. (**B**) Induction of endogenous mRNA after IFN or NDV treatment. L929 cells (lanes 1–3) and the transformants (lanes 4–6) were mock treated (lanes 1 and 4), stimulated with IFN-β (10^3 U/ml, 6 h; lanes 2 and 5) or infected with NDV (12 h; lanes 3 and 6), and the total RNA was subjected to Northern blot analysis using IFN-α, IFN-β, 2–5AS, GBP-1 and actin probes as indicated.

Upon stimulation by virus infection or dsRNA treatment, a series of dramatic events occur on IRF-3: phosphorylation on the specific residues, association with co-activator CBP/p300, nuclear translocation and induction of its sequence-specific DNA-binding activity.

Phosphorylation of IRF-3 on specific serine residues

It has not yet been elucidated completely which signal is actually generated by virus infection. Because poly(rI): poly(rC) and other dsRNA have been known as potent non-viral inducers in cell culture (Torrence and De Clercq, 1981), viral dsRNA generated in the cytoplasm is probably a direct stimulus for the specific phosphorylation of IRF-3. Thus the dsRNA-dependent kinase (PKR) is a candidate for the inducible phosphorylation of IRF-3. However, the amino acid sequence surrounding the serine residue which is phosphorylated by PKR *in vivo* (ILLSELSRR; phosphoserine is underlined, Colthurst *et al.*, 1997) exhibited little homology to the sequence surrounding the critical serines of IRF-3 (ARVGGASSLEN). Moreover, the observation that cells with a disrupted PKR gene can be induced to activate type I IFN genes by virus infection (Yang *et al.*, 1995) suggests the presence of other kinase(s). This issue remains to be investigated further.

Mechanism of VA-IRF formation in the nucleus

Of the NES-containing proteins identified so far, IRF-3 is the first example that conditionally accumulated in the nucleus. Although little is known about the NES regulation, similar mechanisms are likely to be operating in other systems in which nuclear import/export is physiologically controlled (Shibasaki *et al.*, 1996). Nuclear translocation, association with CBP/p300 and generation of DNA-binding activity take place after phosphorylation of IRF-3. Because these three events were observed simultaneously, we are unable to determine their precise order. We speculate that although IRF-3 is exported continuously from the nucleus, it may have an opportunity to encounter CBP/p300 in the nucleus (Eckner *et al.*, 1994). The phosphorylated IRF-3 associates strongly with CBP/p300, then its export from the nucleus is prevented by an unknown mechanism. The association of CBP/p300 with IRF-3 may alter the conformation of its DNA-binding domain, then induce specific DNA binding of VA-IRF. Unmasking of IRF-3 DNA binding has been reported when the DNA-binding domain was artificially fused to the C-terminus of GST (Au *et al.*, 1995). We do not rule out the possibility of the direct participation of CBP/p300 in DNA binding, because the association with CBP/p300 is indispensable for DNA binding of IRF-3. It is also intriguing to speculate that the intrinsic protein acetyltransferase activity of CBP/p300 may play a role in the unmasking of DNA binding (Gu and Roeder, 1997) and/or masking the NES function.

Activation of transcription by VA-IRF

Finally, a potent transcription factor complex VA-IRF is accumulated in the nucleus and participates in the activation of genes including the type I IFN genes. It has been proposed that IFN-β enhancer forms an enhanceosome with transcription factors and HMG I(Y) after induction (Thanos and Maniatis, 1995). In view of the present findings, CBP/p300, which has associated histone acetylase activity (Ogryzko *et al.*, 1996), may have an active role in converting the chromatin from the inert to the activated conformation, which allows recruitment of different transcription factors to maximize gene activation.

Physiological implications

When virus infection occurs, the biochemical signal initiated in the cytoplasm is transmitted to the nucleus by the generated VA-IRF as mentioned above. In addition to this direct activation, indirect mechanisms, which also target ISGs and are mediated by secreted IFN, come into operation, thus the initial signal is maximally transmitted. These include post-translational activation of ISGF3 and *de novo* synthesis of IRF-1 and ISGF3γ (Kawakami *et al.*, 1995). ISGF3 is dedicated solely to the IFN signal and is not activated directly by virus infection (Harada *et al.*, 1996; Yoneyama *et al.*, 1996). Induction of IRF-1 takes place at the level of transcription by IFN-γ activation factor (GAF), which is also induced by type I IFN (Pine *et al.*, 1994). Interestingly, up-regulation of IRF-3 either by IFN or virus has not been reported and its protein level even decreases after virus infection (Figures 3B and 4D). IRF-3 peptide is detectable in all the cell lines tested and its level changes depending on growth conditions

(unpublished data). This suggests that cytoplasmic IRF-3 in uninduced cells may have other biological functions such as growth regulation.

Materials and methods

Cells, DNA transfection and microinjection

L929, HEC-1 and 293T cells were maintained in Eagle's minimun essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). DNA transfection of L929 cells, NDV infection and stimulation with poly(rI):poly(rC) were performed as described previously (Yoneyama *et al.*, 1996). For orthophospate labeling, the transfected cells were washed with phosphate-depleted MEM after NDV infection and cultured with [^{32}P]orthophosphate (0.5 mCi/ml) in phosphate-depleted MEM for 12 h. To obtain stable transformants expressing HA-tagged wild-type or mutant IRF-3, L929 cells were transfected with expression constructs (50 μg) with the selectable marker, pCDM8neo (0.5 μg), and selected in medium containing G418 (1 mg/ml). 293T cells (2×10^6 cells) were transfected with 10 μg of plasmids by the calcium phosphate method, divided into two aliquots and treated with or without NDV for 12 h. 3Y1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Microinjection into the nucleus of 3Y1 cells was performed as described previously (Fukuda *et al.*, 1996).

Plasmid constructs

Human IRF-3 cDNA was isolated by PCR with the oligonucleotides corresponding to the published sequence (Au *et al.*, 1995), digested with *Bam*HI at 20 nucleotides downstream of the start codon and inserted into the *Xba*I site of pEF-BOS with oligonucleotides encoding the HA-tagged or p50-tagged N-terminal sequence of IRF-3 to generate pEF-HAIRF-3 or pEF-p50IRF-3. The p50 epitope is residues 5–18 of human p50, the 50 kDa subunit of NF-κB. The amino acid sequence of the cloned human IRF-3 is consistent with the published sequence except for two residues, a substitution of arginine for proline at residue amino acid 174 and threonine for serine at residue 427. We confirmed that IRF-3 with Pro174 exhibited the identical phenotype to IRF-3 with Arg174. Since the amino acid at 427 was not conserved between human and mouse, cDNA with Thr427 was used in all experiments. The deletion mutants for IRF-3 were obtained by insertion of the appropriate oligonucleotides at *Nde*I (IRF-3$^{58–427}$), *Pma*cI (IRF-3$^{1–374}$), *Apa*I (IRF-3$^{1–190}$) or *Acc*III (IRF-3$^{1–128}$) sites of HA-tagged IRF-3 cDNA. A point mutation at two serine residues (S385A and S386A) and NES (mNES) was introduced into p50 epitope-tagged cDNA by the Kunkel method. The Gal4 DNA-binding domain (DBD) (1–147) was linked to the *Nde*I site of IRF-3 cDNA with the appropriate oligonucleotides (Gal4/IRF-3). GST–human IRF-3 was obtained by insertion of the *Nde*I–*Xba*I fragment of human IRF-3 cDNA into the *Sma*I site of pGEX-4T-2 (Pharmacia). To obtain GST–mouse IRF-3, mouse IRF-3 was isolated by PCR, digested with *Hin*cII and inserted into the *Sma*I site of pGEX-4T-1 (Pharmacia). Reporter constructs p-55Luc, p-125Luc, pαLuc, p-55C1BLuc and p-55A2Luc were described elsewhere (Yoneyama *et al.*, 1996). Reporter plasmid containing the UAS_G sequence was obtained by substituting the κB motif (*Sal*I–B*am*HI fragment) of p-55A2Luc with the corresponding oligonucleotides.

Antibodies and peptides

To generate the anti-human and anti-mouse IRF-3 antibodies, the recombinant GST–human or mouse IRF-3 was expressed in *Escherichia coli*, purified by glutathione–Sepharose 4B (Pharmacia) and used to immunize rabbits. Anti-p50 monoclonal antibody was established by Dr H.Hanai (Kyowa Hakko Kogyo Co., Ltd). The monoclonal antibody reacts strongly with the tagged IRF-3 but the reaction with human p50 is below the detectable level. Anti-p300, anti-CBP (Santa Cruz Biotechnology, Inc.) and anti-ovalbumin antibodies (Cappel) are commercial products. The peptides corresponding to the indicated sequences in Figure 5A were synthesized, conjugated to ovalbumin with the cross-linking reagent sulfo-SMCC (Calbiochem) and purified by gel filtration as described previously (Fukuda *et al.*, 1996).

Preparation of cell extracts and luciferase assay

Prepration of cell extracts and the luciferase assay were performed as described previously (Yoneyama *et al.*, 1996).

EMSA

The oligonucleotides containing self-complementary sequences for PRDI of the IFN-β gene or ISRE of the ISG15 gene were used as probes. The sequences of the PRDI probe and the methods for EMSA were described previously (Yoneyama *et al.*, 1996). The oligonucleotide for ISRE probe is: 5′-GAGAGGGAAACCGAAACTGAATTAGCTTTCAGTTTCGGTTTCCCTCT-3′ (the ISRE is underlined).

Immunohistochemistry

Immunostaining of transfected cells was performed as described previously (Watanabe *et al.*, 1997). Briefly, transfected cells were plated on plastic chamber slides, fixed with 4% paraformaldehyde at 48 h after transfection, permeabilized with 0.2% Triton X-100 and treated with primary antibody in phosphate-buffered saline containing 0.05% Tween-20 (PBST) at room temperature for 1 h. After washing with PBST, cells were treated with secondary antibodies and washed. Staining of injected 3Y1 cells was performed as described previously (Fukuda *et al.*, 1996).

Immunoprecipitation and Western blotting

Immunoprecipitation was performed as shown previously (Watanabe *et al.*, 1997). For phosphate-labeled IRF-3, the binding reaction was performed in RIPA buffer (50mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with sodium orthovanadate (1 mM), phenylmethylsulfonyl fluoride (PMSF; 1 mM), leupeptin (20 μg/ml) and bovine serum albumin (2 %). In the case of the co-precipitation experiment for p300, the lysis buffer (20 mM HEPES, pH 7.9, 50 mM NaCl, 10 mM EDTA, 2 mM EGTA, 0.1% NP-40, 10% glycerol, 1 mM dithiothreitol, 10 mM sodium orthovanadate, 1 mM PMSF and 20 μg/ml leupeptin) was used for binding. Western blotting was performed as described previously (Watanabe *et al.*, 1997).

Phosphopeptide mapping and phosphoamino acid analysis

The ^{32}P-labeled IRF-3 was excised from the SDS–PAGE gel, eluted, digested with trypsin and subjected to two-dimensional separation on thin-layer cellulose plates. The first dimension of separation was electrophoresis in pH 1.9 buffer (2.2% formic acid, 7.8% acetic acid), and the second dimension was chromatography in phospho chromatography buffer (37.5% *n*-butanol, 25% pyridine and 7.5% acetic acid) (Boyle *et al.*, 1991). For phosphoamino acid analysis, the bands containing ^{32}P-labeled IRF-3 were excised from the Immobilon membrane and hydrolyzed directly in 6 M HCl for 2 h at 95°C using a capillary tube. The hydrolyzed amino acids were separated by thin-layer chromatography with unlabeled markers and visualized by autoradiography (Mori *et al.*, 1991).

Northern blotting

Northern blotting was performed as described previously (Yoneyama *et al.*, 1996). To detect murine IFN-α, IFN-β, 2–5AS and GBP-1 mRNA, we used the *Bam*HI–*Bgl*II fragment from pMGβ3-1 (Yoneyama *et al.*, 1996), the *Hin*dIII–*Eco*RI fragment from pBR327(*Hin*dIII)/chrMu-IFNα1/pGS3 (Yoneyama *et al.*, 1996), the *Eco*RI fragment from pMA25 (Matsuyama *et al.*, 1993) and the *Bam*HI–*Eco*RI fragment from pSP65mGBP-1 (Cheng *et al.*, 1991), respectively.

Acknowledgements

We thank Drs D.Livingston and I.Kitabayashi for p300 cDNA, Dr N.Hanai for anti-p50 epitope antibody, Dr M.Yoshida for leptomycin B and Drs B.Mayer and E.Barsoumian for critical reading of the manuscript. This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, Nippon Boehringer Ingelheim Co., Ltd, Kyowa Hakko Kogyo Co., Ltd and Toray Industries Inc.

References

Ackrill,A.M., Foster,G.R., Laxton,C.D., Flavell,D.M., Stark,G.R. and Kerr,I.M. (1991) Inhibition of the cellular response to interferons by products of the adenovirus type 5 E1A oncogene. *Nucleic Acids Res.*, **19**, 4387–4393.

Au,W.-C., Moore,P.A., Lowther,W., Juang,Y.-T. and Pitha,P.M. (1995) Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes. *Proc. Natl Acad. Sci. USA*, **92**, 11657–11661.

Boyle,W.J., van der Geer,P. and Hunter,T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.*, **201**, 110–149.

Cheng,Y.S., Patterson,C.E. and Staeheli,P. (1991) Interferon-induced guanylate-binding proteins lack an N(T)KXD consensus motif and bind GMP in addition to GDP and GTP. *Mol. Cell. Biol.*, **11**, 4717–4725.

Colthurst,D.R., Campbell,D.G. and Proud,C.G. (1987) Structure and regulation of eukaryotic initiation factor eIF-2. *Eur. J. Biochem.*, **166**, 357–363.

Daly,C. and Reich,N.C. (1993) Double-stranded RNA activates novel factors that bind to the interferon-stimulated response element. *Mol. Cell. Biol.*, **13**, 3756–3764.

Daly,C. and Reich,N.C. (1995) Characterization of specific DNA-binding factors activated by double-stranded RNA as positive regulators of interferon α/β-stimulated genes. *J. Biol. Chem.*, **270**, 23739–23746.

Darnell,J.E.,Jr, Kerr,I.M. and Stark,G.R. (1994) Jak–STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*, **264**, 1415–1421.

DeMaeyer,E. and DeMaeyer-Guignard,J. (1988) *Interferons and Other Regulatory Cytokines*. John Wiley and Sons, New York.

Driggers,P.H., Ennist,D.L., Gleason,S.L., Mak,W.-H., Marks,M.S., Levi,B.-Z., Flanagan,J.R., Appella,E. and Ozato,K. (1990) An interferon γ-regulated protein that binds the interferon-inducible enhancer element of major histocompatibility complex class I genes. *Proc. Natl Acad. Sci. USA*, **87**, 3743–3747.

Eckner,R., Ewen,M.E., Newsome,D., Gerdes,M., DeCaprio,J.A., Lawrence,J.B. and Livingston,D.M. (1994) Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes Dev.*, **8**, 869–884.

Eisenbeis,C.F., Singh,H. and Storb,U. (1995) Pip, a novel IRF family member, is a lymphoid-specific, PU.1-dependent transcriptional activator. *Genes Dev.*, **9**, 1377–1387.

Fischer,U., Huber,J., Boelens,W.C., Mattaj,I.W. and Lührmann,R. (1995) The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell*, **82**, 475–483.

Fornerod,M., Ohno,M., Yoshida,M. and Mattaj,I.W. (1997) CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell*, **90**, 1051–1060.

Fujita,T., Miyamoto,M., Kimura,Y., Hammer,J. and Taniguchi,T. (1989) Involvement of a *cis*-element that binds an H2TF-1/NFκB like factor(s) in the virus-induced interferon-β gene expression. *Nucleic Acids Res.*, **17**, 3335–3346.

Fukuda,M., Gotoh,I., Gotoh,Y. and Nishida,E. (1996) Cytoplasmic localization of mitogen-activated protein kinase kinase directed by its NH_2-terminal, leucine-rich short amino acid sequence, which acts as a nuclear export signal. *J. Biol. Chem.*, **271**, 20024–20028.

Fukuda,M., Asano,S., Nakamura,T., Adachi,M., Yoshida,M., Yanagida,M. and Nishida,E. (1997) CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature*, **390**, 308–311.

Fuse,A., Ashino-Fuse,H. and Kuwata,T. (1984) Binding of ^{125}I-labeled human interferon to cell lines with low sensitivity to interferon. *Gann*, **75**, 379–384.

Goldfeld,A.E. and Maniatis,T. (1989) Coordinate viral induction of tumor necrosis factor α and interferon β in human B cells and monocytes. *Proc. Natl Acad. Sci. USA*, **86**, 1490–1494.

Gu,W. and Roeder,R.G. (1997) Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell*, **90**, 595–606.

Harada,H., Fujita,T., Miyamoto,M., Kimura,Y., Maruyama,M., Furia,A., Miyata,T. and Taniguchi,T. (1989) Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. *Cell*, **58**, 729–739.

Harada,H. *et al*. (1996) Regulation of IFN-α/β genes: evidence for a dual function of the transcription factor complex ISGF3 in the production and action of IFN-α/β. *Genes to Cells*, **1**, 995–1005.

Holtschke,T. *et al*. (1996) Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. *Cell*, **87**, 307–317.

Kawakami,T., Matsumoto,M., Sato,M., Harada,H., Taniguchi,T. and Kitagawa,M. (1995) Possible involvement of the transcription factor ISGF3γ in virus-induced expression of the IFN-β gene. *FEBS Lett.*, **358**, 225–229.

Lenardo,M.J., Fan,C.-M., Maniatis,T. and Baltimore,D. (1989) The involvement of NF-κB in β-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. *Cell*, **57**, 287–294.

Matsuyama,T. *et al*. (1993) Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. *Cell*, **75**, 83–97.

Matsuyama,T. *et al*. (1995) Molecular cloning of LSIRF, a lymphoid-specific member of the interferon regulatory factor family that binds the interferon-stimulated response element (ISRE). *Nucleic Acids Res.*, **23**, 2127–2136.

Mittrücker,H.W. *et al*. (1997) Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. *Science*, **275**, 540–543.

Miyamoto,M., Fujita,T., Kimura,Y., Maruyama,M., Harada,H., Sudo,Y., Miyata,T. and Taniguchi,T. (1988) Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-β gene regulatory elements. *Cell*, **54**, 903–913.

Mori,A., Aizawa,H., Saido,T.C., Kawasaki,H., Mizuno,K., Murofushi,H., Suzuki,K. and Sakai,H. (1991) Site-specific phosphorylation by protein kinase C inhibits assembly-promoting activity of microtubule-associated protein 4. *Biochemistry*, **30**, 9341–9346.

Ogryzko,V.V., Schiltz,R.L., Russanova,V., Howard,B.H. and Nakatani,Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell*, **87**, 953–959.

Ossareh-Nazari,B., Bachelerie,F. and Dargemont,C. (1997) Evidence for a role of CRM1 in signal-mediated nuclear protein export. *Science*, **278**, 141–144.

Pine,R., Canova,A. and Schindler,C. (1994) Tyrosine phosphorylated p91 binds to a single element in the ISGF2/IRF-1 promoter to mediate induction by IFNα and IFNγ, and is likely to autoregulate the p91 gene. *EMBO J.*, **13**, 158–167.

Sen,G.C. and Lengyel,P. (1992) The interferon system: a bird's eye view of its biochemistry. *J. Biol. Chem.*, **267**, 5017–5020.

Shibasaki,F., Price,E.R., Milan,D. and McKeon,F. (1996) Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature*, **370**, 370–373.

Stade,K., Ford,C.S., Guthrie,C. and Weis,K. (1997) Exportin 1 (Crm1p) is essential nuclear export factor. *Cell*, **90**, 1041–1050.

Thanos,D. and Maniatis,T. (1995) Virus induction of human IFNβ gene expression requires the assembly of an enhanceosome. *Cell*, **83**, 1091–1100.

Torrence,P.F. and De Clercq,E. (1981) Interferon inducers: general survey and classification. *Methods Enzymol.*, **78**, 291–299.

Veals,S.A., Schindler,C., Leonard,D., Fu,X.-Y., Aebersold,R., Darnell,J.E.,Jr and Levy,D.E. (1992) Subunit of an alpha-interferon-responsive transcription factor is related to interferon regulatory factor and Myb families of DNA-binding proteins. *Mol. Cell. Biol.*, **12**, 3315–3324.

Visvanathan,K.V. and Goodbourn,S. (1989) Double-stranded RNA activates binding of NF-κB to an inducible element in the human β-interferon promoter. *EMBO J.*, **8**, 1129–1138.

Watanabe,N., Sakakibara,J., Hovanessian,A.G., Taniguchi,T. and Fujita,T. (1991) Activation of IFN-β element by IRF-1 requires a post-translational event in addition to IRF-1 synthesis. *Nucleic Acids Res.*, **19**, 4421–4428.

Watanabe,N., Iwamura,T., Shinoda,T. and Fujita,T. (1997) Regulation of NFKB1 proteins by the candidate oncoprotein BCL-3: generation of NF-κB homodimers from the cytoplasmic pool of p50–p105 and nuclear translocation. *EMBO J.*, **16**, 3609–3620.

Wen,W., Meinkoth,J.L., Tsien,R.Y. and Taylor,S.S. (1995) Identification of a signal for rapid export of proteins from the nucleus. *Cell*, **82**, 463–473.

Yamagata,T., Nishida,J., Tanaka,T., Sakai,R., Mitani,K., Yoshida,M., Taniguchi,T., Yazaki,Y. and Hirai,H. (1996) A novel interferon regulatory factor family transcription factor, ICSAT/Pip/LSIRF, that negatively regulates the activity of interferon-regulated genes. *Mol. Cell. Biol.*, **16**, 1283–1294.

Yang,Y.L., Reis,L.F.L., Pavlovic,J., Aguzzi,A., Schäfer,R., Kumar,A., Williams,B.R.G., Aguet,M. and Weissmann,C. (1995) Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J.*, **14**, 6095–6106.

Yoneyama,M., Suhara,W., Fukuhara,Y., Sato,M., Ozato,K. and Fujita,T. (1996) Autocrine amplification of type I interferon gene expression mediated by interferon stimulated gene factor 3 (ISGF3). *J. Biochem.*, **120**, 160–169.

Zhang,L. and Pagano,J.H. (1997) IRF-7, a new interferon regulatory factor associated with Epstein–Barr virus latency. *Mol. Cell. Biol.*, **17**, 5748–5757.

Zinn,K., Keller,A., Whittemore,L.A. and Maniatis,T. (1988) 2-Aminopurine selectively inhibits the induction of beta-interferon, c-*fos*, and c-*myc* gene expression. *Science*, **240**, 210–213.

Received October 20, 1997; revised November 28, 1997; accepted December 1, 1997