Multiple Regulatory Domains Control IRF-7 Activity in Response to Virus Infection*

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Recent studies implicate the interferon regulatory factors (IRF), IRF-3 and IRF-7, as key activators of Type 1 interferon genes, as well as the RANTES (regulated on activation normal T cell expressed) chemokine gene. Both IRF-3 and IRF-7 are regulated in part by virus-induced C-terminal phosphorylation, leading to nuclear translocation, stimulation of DNA binding, and transcriptional activities. Structure-function studies with IRF-7 suggested a complex organization of the C-terminal region, with a constitutive activation domain located between amino acids 150–246, an accessory inducibility region at the very end of IRF-7 between amino acids 467 and 503, and an inhibitory region (amino acids 341–467) adjacent to the C-terminal end that interferes with transactivation. Furthermore, an element that increases basal and virus-inducible activity is located between amino acids 278 and 305. A transcriptionally active form of IRF-7 was also generated by substitution of Ser-477 and Ser-479 residues with the phosphomimetic Asp. IRF-7, particularly IRF-7(S477D/S479D), was a strong transactivator of type 1 interferon and RANTES chemokine gene expression. Unlike wild type IRF-3, IRF-7 overexpression was able to stimulate interferon gene expression in the absence of virus infection. Using tagged versions of IRF-7 and IRF-3, the formation of homo- and heterodimers was detected by co-immunoprecipitation. These results demonstrate that IRF-3 and IRF-7 transcription factors possess distinct structural characteristics that impart complementary rather than redundant functional roles in cytokine gene activation.

Interferons (IFNs)† are a large family of multifunctional secreted proteins involved in antiviral defense, cell growth regulation, and immune activation (1). Virus infection induces the transcription and synthesis of multiple IFN genes (1–3); newly synthesized IFN interacts with neighboring cells through cell surface receptors and the JAK-STAT signaling pathway, resulting in the induction of over 30 new cellular proteins that mediate the diverse functions of the IFNs (4–7). Among the many virus- and IFN-inducible proteins are the growing family of interferon regulatory factors (IRFs), which now consists of nine members as well as several virally encoded IRFs (8). The presence of IRF-like binding sites in the promoter region of IFNα and IFNβ genes implicated the IRF factors as direct regulators of IFN gene induction (9–13). Within the IRF family, IRF-3 and IRF-7 have recently been identified as key regulators for the induction of IFNs (reviewed in Ref. 14).

IRF-3 is expressed constitutively in a variety of tissues and demonstrates a unique response to virus infection (15). Latent cytoplasmic IRF-3 is post-translationally modified and activated through phosphorylation of specific serine residues located in its C-terminal end following virus infection (16–19). Following virus infection, inducible phosphorylation of IRF-3 at the C terminus relieves an intramolecular association between two autoinhibitory domains, unmasking the N-terminal DNA binding and C-terminal IRF association domains (IAD). The conformational change in IRF-3 results in the formation of homodimers through the IAD. IRF-3 dimers translocate from the cytoplasm to the nucleus, associate with the CBP/p300 coactivator, and stimulate DNA binding and transcriptional activities (reviewed in Refs. 14 and 20). IRF-3 phosphorylation ultimately results in its degradation via the ubiquitin-proteasome pathway (16, 21). These biological features implicate IRF-3 as an important component of the immediate early response to virus infection (14, 20). Overexpression of IRF-3 significantly enhances virus-mediated expression of type I IFN genes and results in the induction of an antiviral state (22). Other studies demonstrated that transcription of the CC-chemokine RANTES was up-regulated by virus infection, mediated through IRF-3 activation and binding to overlapping ISRE-like elements in the −100 region of the RANTES promoter (23). IRF-7 was first described to bind and repress the Qp promoter region of the Epstein-Barr virus-encoded gene, EBNA-1, which contains an ISRE-like element (24, 25). Unlike IRF-3, IRF-7 is not expressed constitutively in cells. Rather, expression is induced by IFN, lipopolysaccharide, and virus infection. As with IRF-3, virus infection appears to induce phosphorylation of IRF-7 at its C terminus, a region that is highly homologous to IRF-3 C-terminal end (26, 27). IRF-7 also localizes to the cytoplasm in uninfected cells and translocates to the nucleus after phosphorylation (27, 28). Two groups have identified potential serine residues targeted for inducible phosphorylation by homology to IRF-3. Marié et al. (26) mutated the Ser-425/Ser-426 in the murine IRF-7, based on homology to Ser-385/Ser-386 in IRF-3. This mutant was not phosphorylated...
and did not activate IFNα gene expression (26). Sato et al. (27) generated a deletion mutant in which the region containing the potential sites of inducible phosphorylation between aa 411 and 453 was truncated. The mutant no longer translocated to the nucleus following virus infection, implicating inducible phosphorylation as a critical step for translocation.

Cumulative molecular and biological results with IRF-3 and IRF-7 suggest a new model of IFN gene activation (reviewed in Ref. 14). Type I IFN genes can be subdivided into two groups: 1) immediate-early genes activated in response to virus infection by a protein synthesis-independent pathway (IFNβ and murine IFNα4); and 2) delayed-type genes, which include the other IFNα subtypes in which expression is dependent on de novo protein synthesis (26). Following virus infection, IRF-3, NF-κB, and ATF-2/C-Jun are post-translationally activated by inducer-mediated phosphorylation. These proteins cooperate to form a transcriptionally active enhancementosome at the IFNβ promoter, together with the CBP/p300 transcriptional coactivator and the chromatin-associated HMGB (high mobility group) protein (29–33). IRF-3 also up-regulates IFNα4 expression in murine cells (26). Secreted IFN produced from a subset of initially infected cells acts through an autocrine and paracrine loop, which requires intact IFN receptor and JAK-STAT pathways. IFN activation of the ISGF3 complex results in the transcriptional up-regulation of IRF-7 (26, 27). Viral infection activates IRF-7 through inducible phosphorylation, and phosphorylated IRF-7 participates together with IRF-3 in the transcriptional induction of immediate-early and delayed-type IFN genes (26, 27).

Because of the common and distinct biological features of IRF-3 and IRF-7, we sought to identify the structural features of IRF-7 that regulate transcriptional activation, dimerization, and cytoplasmic to nuclear translocation. Our results demonstrate that IRF-7 possesses distinct structural characteristics compared with IRF-3 that impart complementary rather than redundant functional roles to these proteins in cytokine gene activation.

MATERIALS AND METHODS

Plasmid Constructions and Mutagenesis—Wild type IRF-7 expression plasmids were prepared by cloning the IRF-7a DNA (polymerase chain reaction-amplified from pcDNA-IRF-7A, a gift from Drs. L. Zhang and J. Pagano) into the pFlag-CMV-2 (pFlag-IRF-7) or 5′-myc-pCDNA3 (myc-IRF-7) vector. The point mutations and deletion mutations of IRF-7 were generated by overlapped polymerase chain reaction mutagenesis with Vent DNA polymerase, and mutations were confirmed by sequencing. The IFNB-luciferase reporter was generated by cloning the EcoRI-FaqI fragment (−280 to +20, filled in with Klenow enzyme) from pUCl826 into the NheI site (filled in with Klenow enzyme) of the pGL3-basic vector (Promega). RANTES/pGL3 luciferase reporter was prepared by cloning the BglII-Sall fragment (−397 to +5, filled in with Klenow enzyme) from the RANTES/CAT (chloromycyclin acetyltransferase) reporter plasmid (23) into the NheI site (filled in with Klenow enzyme) of the pGL3-basic vector. IFNA1/pGL3 reporter (−140 to +90) were generated by cloning the polymerase chain reaction products from 293 cell genomic DNA into the SmaI site of the pGL3-basic vector. GFP-IRF-7 expression plasmids were generated by cloning cDNAs encoding wild type or mutated forms of IRF-7 into the downstream of enhanced green fluorescent protein in the pEGFP-C1 vector (CLONTECH).

Cell Culture, Transfections, and Luciferase Assays—All transfections for luciferase assays were carried out in human embryonic kidney 293 cells (from essential needs, MEM, Life Technologies, Inc.) supplemented with 10% fetal bovine serum, glutamine, and antibiotics. Subconfluent cells were transfected with 10 ng of pRLTK reporter (Renilla luciferase for internal control), 100 ng of pG-L3 reporter (firefly luciferase, experimental reporter), and 200 ng of expression plasmids by calcium phosphate coprecipitation method. The reporter plasmids were RANTES/pGL3, IFNB/pGL3, and IFNAI/pGL3, the transfection procedures were described previously (35). At 24 h after transfections, reporter gene activities were measured by the Dual-luciferase reporter assay following the instructions in the Promega Technical Manual.

Immunoblot Analysis of Flag-IRF-3 and Flag-IRF-7—To confirm expression of the transgenes, equivalent amounts of whole cell extract (20 μg) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% polyacrylamide gel. After electrophoresis, proteins were transferred to Hybond transfer membrane (Amersham Pharmacia Biotech) in a buffer containing 30 mM Tris, 200 mM glycine, and 20% methanol for 1 h. The membrane was blocked by incubation in phosphate-buffered saline containing 5% dried milk for 4 h and then probed with monoclonal Flag antibody M2 (Sigma) in 5% milk-phosphate-buffered saline at a dilution of 1:3000. These incubations were done at 4 °C overnight or at room temperature for 1–3 h. After four 10-min washes with phosphate-buffered saline, membranes were reacted with a peroxidase-conjugated secondary goat anti-mouse antibody (Amersham Pharmacia Biotech) at a dilution of 1:2500. The reaction was then visualized with the enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech) as recommended by the manufacturer.

Electrophoretic Mobility Shift Assay—Whole cell extracts were prepared 48 h after transfection with 5 μg of expression plasmids as indicated in individual experiments. Cells were washed in phosphate-buffered saline and lysed in 0.4% Triton X-100, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 0.5 μg/ml chymotrysin, and 0.25 μg/ml microcin. Equivalent amounts of whole cell extract (20 μg) were assayed for IRF-7 binding in gel shift analysis using 32P-labeled double-stranded oligonucleotide corresponding to the PRDI-PRDIII region of the IFNB promoter (′GAAACGTGAAAAGGCAAGGTGAAACTG′) Complexes were formed by incubating the probe with 20 μg each of the whole cell extracts or indicated amounts of recombinant proteins. The binding mixture (20 μl) contained 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 2 mM dithiothreitol, 0.5% glycerol, 0.5% Nonidet P-40, and 10 μg/ml of bovine serum albumin; 62.5 μg/ml of poly(dI-dC) was added to reduce nonspecific binding. After a 20-min incubation with probes, samples were loaded on a 5% polyacrylamide gel (60:1 cross-link) prepared in 0.5× Tris-boric acid-EDTA buffer. After running at 100–150 V for 3 h, the gel was dried and exposed to a Kodak film at −70°C overnight. To determine the specificity of protein-DNA complex formation, the cell extract was preincubated with anti-Flag antibody M2 (Sigma).

Immunoprecipitation and Immunoblot Analysis of Interactions between Different IRF-3 and IRF-7 Domains—293 cells were cotransfected with expression plasmids encoding wild type or mutated IRF-3 and IRF-7 as indicated. Whole cell extracts (200–300 μg) were prepared from co-transfected cells, and the extracts were incubated with 2 μl of anti-Myc antibody 9E10 cross-linked to 30 μl of protein A-Sepharose beads for 1 h at 4 °C. Immunoprecipitates were washed five times with lysis buffer and eluted by boiling the beads for 3 min in 1× SDS-sample buffer. Eluted proteins were separated by SDS-PAGE, transferred to Hybond transfer membrane, and incubated with anti-IRF-3, anti-Flag, or anti-Myc antibody (1:1000–1:3000). Immunocomplexes were detected by ECL, a chemiluminescence-based system.

Subcellular Localization of GFP-IRF-7 Proteins—To analyze the subcellular localization of wild type and mutated forms of IRF-7 proteins in uninfected and virus infected cells, the GFP-IRF-7 expression plasmids (5 μg) were transiently transfected into COS-7 cells by the calcium phosphate coprecipitation method. For virus infection, transfected cells were infected with Sendai virus (80 hemagglutinating units/ml for 2 h) at 24 h post-transfection. GFP fluorescence was analyzed in living cells with a Leica fluorescence microscope using a 40× objective.

RESULTS

Multiple Domain Structure of IRF-7—The IRF-7 transcription factor shares many structural features with the IRF-3 factor, including the N-terminal DNA binding domain common to all IRF members, a putative IRF association domain in the C-terminal half of the protein, and a stapled helix for dimerization. The involvement of the C-terminal end of the molecule between aa 471 and 487, which is the target of virus-inducible phosphorylation (26, 27), is key to the IRF-7 in the activation of delayed-type IFNA genes (26), we sought to analyze the structural features of IRF-7 as they relate to transactivation. We initially determined whether IRF-7 contained a long atypical
**Regulatory Domains of IRF-7**

C-terminal transactivation domain as described for IRF-3 (34). To localize the putative IRF-7 transactivation domain, wild type IRF-7 and a series of IRF-7 deletion mutations were generated, co-transfected into 293 HEK cells together with an IFNA1 promoter construct, and examined for their ability to stimulate IFNA1 reporter gene activity (Fig. 1A). Co-expression of IRF-7 alone without virus induction resulted in a 40-fold stimulation of IFNA1 promoter activity. Deletion of 28 (IRF-7-(475)) or 97 (IRF-7-(416)) amino acids from the C-terminal region of IRF-7 reduced transactivation activity modestly to 15- and 24-fold, respectively, whereas cotransfection of IRF-7(aa 380) restored transcription to 55-fold. Further deletion to aa 340 and 246 resulted in a form of IRF-7 that activated transcription 100-fold. The next deletions to aa 200 and 150 essentially eliminated IRF-7 transactivation of the IFNA1 promoter.

Next, the effect of Sendai virus infection on IRF-7-stimulated IFNA1 expression was determined. In 293 cells cotransfected with vector alone, virus infection activated IFNA1 luciferase reporter gene activity up to 5.5-fold. Cotransfection of full-length IRF-7 enhanced virus-induced expression to 365-fold, whereas deletion of the C-terminal region of IRF-7 to a protein of 475, 416, or 380 aa totally abrogated this activity (Fig. 1A). Although deletion of IRF-7 to a protein of 340 or 246 aa resulted in a 100–120-fold stimulation of transcription, these deletions were not further activated by virus infection (Fig. 1A).

An internal deletion of IRF-7, which removed aa 247–467, activated transcription more than 1500-fold, to a level of activity that was much higher than seen with wild type IRF-7 irrespective of virus induction (Fig. 1B). When a smaller deletion (Δ247–415) of this region was generated, IRF-7 activity was reduced more than 100-fold, indicating that the region between aa 416 and 467 was an important component of the inhibitory domain. The next construct, Δ247–372, further decreased activity, indicating that the full C-terminal inhibitory region may be located between aa 372 and 467. Other smaller deletions of this region (Δ247–335 and Δ247–305) had no further effect on IRF-7 activity, because virtually all of the transactivation capacity of IRF-7 was blocked. Interestingly, analysis of the small internal deletion Δ247–278 revealed that both basal (13-fold) and virus-inducible activity (>200-fold) were significantly restored to IRF-7, which indicated that the region between aa 278 and 305 contributed to virus inducibility. Deletion of the constitutive activation domain (Δ151–246) resulted in an IRF-7 with weak activity (1.7-fold) but with about 10-fold inducibility by virus (Fig. 1B) because of the presence of the C-terminal composite element. Similar results were also obtained with these constructs in other cell types including COS-7, HEC1B, and U937 with RANTES, IFNB, and IFNA promoters (data not shown).

This deletion analysis suggested a complex structure to the C-terminal region of IRF-7, with a constitutive activation domain located between aa 150 and 246, an accessory inducibility region at the very end of IRF-7 between aa 468 and 503, and an inhibitory region adjacent to the C-terminal end, located between aa 341 and 467, which interferes with transactivation. Furthermore, a virus-activated domain (VAD), which increases basal and virus inducible activity, is located between aa 278 and 305.

**Generation of Constitutively Active Forms of IRF-7—**Like IRF-3, IRF-7 contains a serine-rich domain located in the C-terminal region between aa 471 and 487 (Fig. 2A) that is a target for virus-induced phosphorylation (26, 27). In the case of IRF-3, substitution of the Ser-Thr residues in the region aa 396–405 with the phosphomimetic Asp generated a constitutively active form of IRF-3 that activated the IFNB promoter over 100-fold (16). Given the sequence similarities between the C-terminal ends of IRF-3 and IRF-7 and the effect of C-terminal deletion on virus-induced IRF-7 activity (Fig. 1), we sought to generate a constitutively active form of IRF-7 by phosphomimetic amino acid substitution of different C-terminal serines (Fig. 2A). Substitution of Ser-477 and Ser-479 with the phosphomimetic Asp resulted in a form of IRF-7 that activated the IFNA promoter up to 200-fold (Fig. 2B). In contrast, the substitution of Ser-471 and Ser-472 or Ser-483 and Ser-487 with Asp dramatically reduced the transactivation activity of IRF-7.
Introduction of the phosphomimetic at other serine sites (Asp-475 and Asp-476, or Asp-475, Asp-476, Asp-483, and Asp-487, into IRF-7(S477D/S479D), IRF-7(S475D/S477D/S479D), and IRF-7(S475D/S476D/S477D/S479D/S483D/S487D)) did not further augment the transactivation activity of IRF-7(S477D/S479D) (Fig. 2B). The transactivation activity of these constitutively active forms of IRF-7 (S477D/S479D), IRF-7(S475D/S477D/S479D), and IRF-7(S475D/S476D/S477D/S479D/S483D/S487D) was further stimulated by virus infection (Fig. 2B). It is possible that sequences within the C-terminal region and the VAD (aa 278–305) are required for maximal response to virus infection. As predicted, substitution of Ser-477 and Ser-479 with alanine resulted in a form of IRF-7 that was unable to respond to virus infection (Fig. 2B). Together, these results are reminiscent of IRF-3 in that multiple serine residues were identified that affected IRF activity. Mutation of some residues caused a loss of IRF-7 activity that was not restored by mutation to a phosphomimetic amino acid, whereas other serine residues were inactivated by alanine substitution but constitutively activated by phosphomimetic substitution (34).

IRF-7 Contains a Nuclear Export Sequence within the Inhibitory Domain—IRF-7 is localized predominantly in the cytoplasm, and viral infection stimulates IRF-7 translocation to the nucleus (28). To investigate the effect of deletion and point mutations on IRF-7 localization, different forms of IRF-7 were linked to GFP, transfected into COS-7 cells, and examined for Sendai virus-induced changes in subcellular localization (Fig. 3). IRF-7 localized mainly to the cytoplasm in uninfected cells and Sendai virus infection resulted in IRF-7 translocation to the nucleus within 8 h in ~50% of the cells (Fig. 3A). The IRF-7 form lacking the C-terminal end (aa 1–246) was analyzed in uninfected and Sendai virus-infected COS-7 cells at 8 h after infection. GFP fluorescence was analyzed in living cells with a Leica fluorescence microscope using 40× objective.

**Fig. 2. Transactivation of wild type and point mutations of IRF-7 in reporter gene assays.** A, schematic representation of IRF-7 point mutations. The following different domains are shown: DNA binding domain (DBD), constitutive activation domain (CAD), virus-activated domain (VAD), inhibitory domain (ID), and signal response domain (SRD). The sequence of aa 468–491 is amplified below the schematic diagram. The amino acids targeted for aspartic acid substitution are shown as larger letters, and point mutations are indicated below the sequence. B, activation of IFNA1-luciferase reporter gene by IRF-7. 293 cells were transfected with pRLTK control plasmid, IFNA1-pGL3 reporter plasmid, and various IRF-7 expression plasmids as indicated. Relative luciferase activity was measured as fold activation (relative to the basal level of reporter gene in the presence of pFLAG-CMV-2 vector after normalization with co-transfected Renilla luciferase activity). The values represent the average of three experiments done in duplicate with variability shown in the error bar. Relative induction (-fold) is also indicated on the top of the bar.

**Fig. 3. Virus-dependent cytoplasmic-nuclear translocation of IRF-7.** The subcellular localization of GFP-IRF-7 (A), GFP-IRF-7(1–246) (B), GFP-IRF-7(Δ247–467) (C), GFP-IRF-7(Δ416–467) (D), GFP-IRF-7(Δ247–415) (E), GFP-IRF-7(Δ247–305) (F), GFP-IRF-7(Δ417–440) (G), GFP-IRF-7(D477/479) (H), and GFP-IRF-7(A477/479) (I), was analyzed in uninfected and Sendai virus-infected COS-7 cells at 8 h after infection. GFP fluorescence was analyzed in living cells with a Leica fluorescence microscope using 40× objective.
failed to induce the nuclear translocation of IRF-7 (the VAD region between aa 278 and 305. Virus infection also IRF-7 (type.

PRDIII (5

plasmids. The 32P-labeled probe corresponds to the ISRE of the PRDIII-(S'-GAAAACGTAAAGGAGAAGTGAATA-3'). WT, wild type.

Fig. 4. Binding of IRF-7 to PRDIII. An electrophoretic mobility shift assay was performed on whole cell extracts (20 µg) derived from 293 cells transfected with various Flag-tagged IRF-7 expression plasmids. The 32P-labeled probe corresponds to the ISRE of the PRDII-(S'-GAAAACGTAAAGGAGAAGTGAATA-3'). WT, wild type.

7(Δ416–467), localized to the nucleus in uninfected cells (Fig. 3, C and D). Within this region, a leucine-rich region was identified (Δ465LVLKLEPWLCRVHLΔ605) that represents a consensus for a nuclear export sequence. Other deletions (IRF-7(Δ247–415) or IRF-7(Δ247–305)) remained cytoplasmic and failed to undergo virus-mediated nuclear translocation of IRF-7 (Fig. 3, E–G), supporting the idea that nuclear translocation may be controlled by the VAD region between aa 278 and 305. Virus infection also failed to induce the nuclear translocation of IRF-7(Δ417–440). It is possible that the sequences in this region interact with nuclear proteins in virus-infected cells, mask the nuclear export sequence, and retain IRF-7 protein in nucleus. Furthermore, substitution of the Ser-477 and Ser-479 with alanine also completely inhibited IRF-7 nuclear translocation after virus infection (Fig. 3, E–G). Substitution of the Ser-477 and Ser-479 with the phosphomimetic Asp revealed mainly cytoplasmic and nuclear translocation (19). Substitution of the Ser-477 and Ser-479 with alanine abrogated the formation of the virus-induced slower migrating form of IRF-7(ΔPRDIII-I complex (Fig. 4, lanes 7 and 8). In IRF-7(Δ247–467) transfected cells, a slower migrating form of the IRF-7(ΔPRDIII-I complex (migrating to a position similar to the virus-induced IRF-7(ΔPRDIII-I complex) was detected in both uninfected and virus-infected cells. Supershift analysis demonstrated that this protein-DNA complex contained IRF-7 (data not shown). These results suggest that after virus activation, IRF-7 may associate with another co-activator and bind to DNA.

Dimerization of IRF-3 and IRF-7—As shown previously, IRF-3 formed homodimers in vivo following C-terminal phosphorylation in virus-infected cells as detected by co-immunoprecipitation (34). To determine whether IRF-7 also formed dimers in uninfected or virus-infected cells, co-immunoprecipitation experiments were performed in 293 HEK cells cotransfected with myc-tagged IRF-7 and Flag-tagged IRF-7. After immunoprecipitation of myc-tagged IRF-7 with anti-Myc antibody 9E10 from cell extracts, immunoblot analysis with anti-Flag antibody M2 indicated that Flag-tagged IRF-7 associated with myc-tagged IRF-7 in both uninfected and virus-infected cells (Fig. 5A, lanes 1 and 2). Flag-tagged IRF-7(D477/D479) also associated with myc-tagged IRF-7(Δ477/479) in the absence of virus infection (Fig. 5A, lane 3).

Interactions between distinct IRF-7 domains were investigated by co-immunoprecipitation using 293 HEK cells cotransfected with myc-tagged full-length IRF-7 and truncated IRF-7 and N- or C-terminally deleted Flag-tagged IRF-7 expression plasmids encompassing aa 1–246 and aa 247–503, respectively (Fig. 5B). After immunoprecipitation of myc-tagged IRF-7 from cell extracts, immunoblot analysis revealed that full-length IRF-7 and C-terminal deleted forms of IRF-7 co-precipitated with full-length IRF-7 (Fig. 5B, lanes 1 and 4); this interaction was not observed when immunoprecipitation was performed with pre-immune serum (data not shown). Both full-length and C-terminal IRF-7 were able to interact with Flag-tagged IRF-7(Δ247–503) (Fig. 5B, lanes 3 and 6), whereas the N-terminal domain of IRF-7-(1–246) only weakly associated with the C-terminal domain of IRF-7(Δ247–503) (Fig. 5B, lanes 5 and 9), indicating that the C-terminal domains mediated IRF-7 dimerization.

It was previously demonstrated that IRF-7 associated with IRF-3 in virus-infected cells (17). We next examined whether the N- or C-terminal regions of IRF-7 associated with IRF-3. Following transient co-transfection of 293 cells with myc-tagged IRF-3 and Flag-tagged IRF-7-(1–246) or Flag-tagged IRF-7(Δ247–503), immunoprecipitation of myc-IRF-3 was performed, followed by immunoblot analysis of the immunoprecipitate with anti-Flag antibody M2. As shown in Fig. 6A, IRF-3-associated with the C-terminal region of IRF-7(Δ247–503) in both uninfected and virus-infected cells (Fig. 6A, lanes 3 and 4) but did not interact with IRF-7-(Δ247–503) (Fig. 6A, lanes 1 and 2). To localize the region of IRF-3 that interacted with the C-terminal region of IRF-7, Flag-tagged wild type IRF-3, the constitutively active form IRF-3(S396D/S398D/S402D/T404D/S405D), and different IRF-3 deletions were tested for interaction with myc-tagged IRF-7. As shown in Fig. 6B, wild type IRF-3, IRF-3(S396D/S398D/S402D/T404D/S405D), IRF-3(1–394), and IRF-3(1–357) associated with IRF-7 (Fig. 6B, lanes 1–4). IRF-3(1–328) only weakly associated with IRF-7 (Fig. 6B, lane 5), whereas IRF-3(1–280) did not interact with IRF-7 (Fig. 6B, lane 6), thus demonstrating that the C-terminal part of IRF-3 between aa 328 and 357 is required for IRF-3 and IRF-7 heterodimerization.
DISCUSSION

The present structure-function studies have identified multiple regulatory domains in the C-terminal region of IRF-7 that contribute to transactivation. A constitutive activation domain was identified between aa 150 and 246 adjacent to the conserved DNA binding region of IRF-7. An accessory inducibility region at the very end of IRF-7 between aa 468 and 503 was also identified as the target for virus-inducible C-terminal phosphorylation. An inhibitory region that interferes with transactivation is immediately adjacent to the C-terminal end, located between aa 372 and 467 but primarily within the region of aa 416–467. Furthermore, an element that increases basal and virus-inducible activity is located between aa 278 and 305 and is adjacent to the constitutive activation domain. We also generated a constitutively active form of IRF-7 by substitution of the Ser-477 and Ser-479 residues with the phosphomimetic Asp. IRF-7, particularly IRF-7(S477D/S479D), was a strong transactivator for IFNA1 (Fig. 2) and RANTES chemokine (data not shown) gene expression; and, unlike wild type IRF-3, IRF-7 was able to stimulate gene expression in the absence of virus infection. Internal deletion analysis of the C-terminal domain of IRF-7 also generated a very strong constitutive form of IRF-7 by removing the region between aa 247 and 467. The transactivation activity of IRF-7(Δ247–467) was about 40-fold higher than wild type IRF-7 in unstimulated cells. Interestingly, a deletion that removed IRF-7(Δ247–415) displayed weak transcriptional activity and was not inducible by virus infection, thus implicating aa 416–467 as an essential functional component of IRF-7. In parallel, experiments using GFP-IRF-7 fusion proteins indicate that this region contains an efficient nuclear export signal; when deleted, IRF-7(Δ416–467) is constitutively localized to the nucleus and is unable to stimulate transactivation.

IRF-7 was first described as a protein that bound to and repressed the Qp promoter region of the Epstein-Barr virus-encoded ERNA-1 gene, which contains an ISRE-like element in its promoter (24). Recent studies have demonstrated that IRF-7 plays an important role in the activation of interferon-α gene expression in virus-infected cells (26–28). Like IRF-3,
transactivation activity of IRF-7 is regulated by phosphorylation of IRF-7 at its C terminus, which is highly homologous to the IRF-3 C-terminal end (26, 27). Virus-induced phosphorylation of IRF-7 resulted in the cytoplasm-to-nucleus translocation of the phosphorylated protein (27, 28). A possible explanation for the inability of IRF-7 to stimulate transcription is that nuclear IRF-7 is not targeted for virus-induced phosphorylation as a consequence of sequestration in the nucleus. Another possibility is that a second inhibitory domain is located between aa 341 and 415, which may inhibit IRF-7 DNA binding and/or transactivation activity. The deletion mutants truncated at aa 340 and 246 exhibit a robust transcriptional response (>100-fold compared with 24-fold for IRF-7(1–416)), supporting the idea that a second inhibitory domain is located between aa 341 and 415.

We previously demonstrated that IRF-3 contains two autoinhibitory domains that interact to generate in uninfected cells a closed conformation that masks the C-terminal IAD and the DNA binding domain of IRF-3 and prevents DNA binding and homodimerization in uninfected cells (34). Virus-inducible phosphorylation of IRF-3 relieved the intramolecular interaction between the two autoinhibitory domains, unmasking the IAD and the DNA binding domain. The conformational change in IRF-3 resulted in homodimerization of IRF-3 (34). In this paper, we show that intermolecular dimerization of IRF-7 can be detected in both uninfected and virus-infected cells (Fig. 3). Furthermore, previous studies indicated that IRF-7 associates with IRF-3 in uninfected or virus-infected cells (17). Here, we demonstrate that the IRF-3 and IRF-7 interaction is mediated through the C-terminal region of both IRF-7 and IRF-3. The fact that IRF-7 has constitutive DNA binding and transactivation activity, whereas the DNA binding and transactivation activity of IRF-3 must be activated by virus infection, suggests that dimerization of IRF-3 and/or IRF-7 plays an important role in the activation of target gene expression. It also appears that phosphorylation of the C-terminal domain has different functions in IRF-3 and IRF-7. With IRF-3, phosphorylation contributes to the generation of an open conformation that mediates activation and dimerization, whereas with IRF-7, homodimers form in unstimulated cells and phosphorylation appears to act primarily to relieve the negative regulatory effect of the inhibitory domain on the constitutive activation domain. Since IRF-7(1–246) failed to form homodimers but constitutively localized to the nucleus, the homodimerization of IRF-7 does not appear to be required for nuclear localization.

IRF-7 appears to be regulated at two distinct levels; transcription of the IRF-7 gene is induced by IFN and lipo polysaccharide treatment (26, 28), whereas the transactivation function of IRF-7 is modulated by C-terminal phosphorylation by virus infection (26–28). Substitution of Ser-425 and Ser-426 with Ala (two serine residues with a sequence context similar to the Ser-385 and Ser-386 of IRF-3) in murine IRF-7 totally blocked the virus-induced phosphorylation and transactivation activities (26). However, substitution of the corresponding serine residues in human IRF-7 (Ser-471 and Ser-472) with the phosphomimetic Asp did not increase but rather decreased its transactivation activity. In contrast, substitution of two downstream serine residues at Ser-477 and Ser-479 with Asp generated a constitutively active form of IRF-7, a result similar to that obtained with IRF-3 (34). Phosphorylation of the Ser/Thr cluster between aa 395 and 407 of IRF-3, but not the Ser-385 and Ser-386 residues, plays an important role in IRF-3 DNA binding and transactivation activity, suggesting that Ser-385 and Ser-386 of IRF-3 may represent an important site for the interaction with CBP/P300 coactivator. These residues may also be involved in the interaction with the kinase(s) that ultimately phosphorylate IRF-3 at the downstream Ser/Thr sites (34). The parallel results with IRF-3 and IRF-7 suggest that both transcription factors may be phosphorylated by the same or a similar kinase.

Substitution of the Ser/Thr cluster in the C-terminal region of IRF-3 with the phosphomimetic Asp (IRF-3(S396D/S398D/S402D/T404D/S405D)) generated a constitutively active form of IRF-3 with constitutive DNA binding and transactivation activities, dimer formation, association with CBP/P300 coactivators, and nuclear localization (16, 34). The transactivation potential of IRF-3(S396D/S398D/S402D/T404D/S405D) is not further induced by virus infection (23). Although the substitution of serine residues in the C-terminal region of IRF-7 to Asp generated constitutively active forms of IRF-7 (IRF-7(S477D/S479D), IRF-7(S475D/S476D/S477D/S479D) and IRF-7(S475D/S476D/S477D/S483D/S487D)), the transactivation activity of these mutants was further induced by virus infection (Fig. 2B). These results indicate that additional residues may be required for response to virus-mediated activation; it is possible that sequences within VAD (aa 278–305) are also involved in the maximal response to virus infection. As shown in Fig. 1B, IRF-7(Δ247–305) and IRF-7(Δ247–325), in which the VAD region is deleted, are transcriptionally inert; with IRF-7(Δ247–278), basal and virus-induced transactivation activity was restored. However, the VAD region alone is not sufficient to respond to virus infection because the transcriptional activity of the C-terminal deletion mutants was not induced by virus infection (Fig. 1A). These results suggest that VAD (aa 278–305) is able to collaborate with the C-terminal signal response domain for maximal response to virus infection.

Despite the highly inducible expression of the human IFNA promoter by IRF-7 in 293 HEK cells, the inducibility of these promoter constructs by Sendai virus infection alone in 293 HEK cells was quite low. One possibility is that IRF-7 is not expressed in 293 HEK cells, because it has been shown that the expression of IRF-7 is restricted to the lymphoid cell types. Consistent with this result, recent experiments indicate that IRF-7 mRNA is not present in 293 HEK cells but is nonetheless inducible by virus infection (37). In fact, reconstitution of IRF-7 production in human fibroblastic cells by ectopic expression resulted in the activation of endogenous IFNA gene expression (38). In conclusion, we have demonstrated that despite an overall similarity in structure between IRF-3 and IRF-7, both transcription factors possess unique functional characteristics and share complementary rather than redundant roles in the activation of Type 1 interferon genes.

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