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Bipartite Nuclear Localization Signal Controls Nuclear Import and DNA-Binding Activity of IFN Regulatory Factor 3

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Accurate cellular localization plays a crucial role in the effective function of most signaling proteins, and nuclear trafficking is central to the function of transcription factors. IFN regulatory factor (IRF)3 is a master transcription factor responsible for the induction of type I IFN, which plays a crucial role in host antiviral innate immune responses. However, the mechanisms for control and regulation of IRF3 nuclear import largely remain to be elucidated. In our study, we identified a bipartite nuclear localization signal (NLS) in IRF3, with two interdependent basic clusters separated by a 7-aa linker. Our study further demonstrated that the bipartite NLS of IRF3 is also critical for IRF3 DNA-binding activity, indicating that the two functions of this region are integrated, which is in contrast to other IRFs. Furthermore, the IFN bioassay and infection studies suggest that IRF3 NLS is essential to the IRF3-mediated IFN responses and antiviral immunity. Overall, our results reveal a previously unrecognized bipartite NLS for IRF3 that contains both DNA-binding activity and nuclear import function, and they shed light on the regulatory mechanisms of IRF3 activation and IRF3-mediated antiviral responses. The Journal of Immunology, 2015, 195: 000–000.

Innate immunity contributes to the first line of host defense against microbial infection. Host cells have evolved a variety of mechanisms to recognize and eliminate invading pathogens. Recognition of pathogen is primarily mediated by pattern recognition receptors, including TLRs, RIG-I–like receptors, and NOD-like receptors, which invoke powerful cellular signaling cascades to stimulate the production of various cytokines (1). Upon virus infection, cytoplasmic RIG-I–like receptors and endosomal TLRs sense viral RNAs or DNAs and trigger rapid production of type I IFN (IFN-α/β), which confer resistance to virus infection through the induction of hundreds of IFN-stimulated genes (2–4). Thus, type I IFN plays an essential role in the host innate antiviral response.

The transcription activation of type I IFN has been studied extensively. The IFN-β gene promoter region contains at least four regulatory cis-elements: the positive regulatory domains (PRDs) I, II, III, and IV (5–8). The promoter region of IFN-α4 gene contains PRD I and PRD III–like elements (PRD-LEs), which bind IFN regulatory factor (IRF) family members (9–11). The PRD I and PRD III elements are activated by members of the IRF family, whereas the PRD II and PRD IV elements are recognized by NF-kB and ATF-2/c-Jun, respectively (12–14).

The IRF family contains nine mammalian members that play a critical role in diverse biological processes, including immunity, inflammation, and apoptosis (15). All of these members are most conserved in their N-terminal DNA-binding domain (DBD), which recognizes the IFN-stimulated response element (ISRE) (consensus sequence: 5’-GAAANNGAAAG/CT/C-3’), as found in the PRD and PRD-LEs of the IFN-α/β promoters (16, 17). Among IRFs, at least four members, IRF1, IRF3, IRF5, and IRF7, have been implicated as positive regulators of IFN-α/β gene transcription (18). IRF1 is the first identified IRF member and activates the type I IFN gene promoter (19). However, the induction of IFN-α/β mRNAs was normal in virus-induced Jurkat 1/− or Jurkat 5/− mouse embryonic fibroblasts (MEFs) (20, 21), which indicates that neither IRF1 nor IRF5 is essential for the induction of IFN-α/β gene. IRF3 and IRF7, which are highly homologous, were shown to play an essential role in the virus-induced expression of the type I IFN gene (22).

IRF3 is expressed constitutively in all cell types and resides in the cytoplasm prior to virus infection. Upon virus infection, it undergoes C-terminal phosphorylation and dimerization and then translocates into the nucleus, subsequently forming a complex with the coactivators CBP/p300 and binding to the PRD and PRD-LEs of the IFN-α/β promoters and ISRE sequences of targeted genes, including the cytokines RANTES and IP-10 (23–25). The biological effect of IRF3 transcriptional activity requires its ability to translocate into the nucleus. For this reason, the nuclear–cytoplasmic shuttling is a critical aspect of its regulation.

Eukaryotic cells have evolved with the genome restricted to the membrane-bound nucleus, which is separated from the cytoplasmic compartment. This separation coevolved with the regulatory mechanisms that control the nuclear–cytoplasmic trafficking of proteins and RNAs. Small molecules can diffuse through the nuclear pore complexes, but the passage of proteins >40 kDa relies on soluble receptors and carrier molecules that mediate movement across nuclear pore complexes (26). The passport-recognition signals on protein cargo are amino acid sequences corresponding to either nuclear localization signals (NLSs) or nuclear export signals (NESs) (27). Typical NLSs contain one (monopartite) or two (bipartite) clusters of basic residues. Monopartite NLSs, such as that of SV40 large-T Ag, have a single cluster of four or five basic residues (28), whereas bipartite NLSs, exemplified by nucleoplasmin, have a second basic cluster located ~10–12 residues downstream of the first cluster (29). The subcellular distribution...
of IRF family members has been investigated, and the key amino acids responsible for their nuclear import have been determined for most IRFs. However, the nature and function of the NLS of IRF3 have not been adequately characterized, and little is known about that of IRF7.

In IRF3, a leucine-rich sequence (ILDELLGNNVL) was identified to function as an NES spanning aa 139–149 (25). A previous study defined the local generalization of the IRF3 NLS domain but lacked a detailed characterization of it (30). In our study, we demonstrated that IRF3 contains a bipartite NLS that controls both DNA-binding activity and nuclear import. First, analysis of GFP-IRF3 fusion proteins shows that IRF3 contains the necessary sequences within or flanking the DBD (aa 64–130) to specify its nuclear import. Further mutagenesis determined the basic amino acids responsible for IRF3 nuclear import. Our studies showed that the bipartite NLS of IRF3 is characterized by the basic amino acid clusters 77KR78 and 86RK87, which may occupy the minor binding site and major binding site of importin-α, respectively. Mutation of 77KR78 partially abrogates the nuclear import ability, whereas mutation of 86RK87 totally disrupts the nuclear import ability. Immunofluorescence imaging assays reinforced our results. Our study further demonstrated that the bipartite NLS of IRF3 is also critical for IRF3 DNA-binding activity, indicating that the two functions of this region are integrated. Overall, our results revealed a previously unrecognized bipartite NLS for IRF3 that contains both DNA-binding activity and nuclear import ability, which is unique among the IRF family proteins.

Materials and Methods

Cell culture and reagents

IRF3+/−, IRF7−/− MEFs were provided by Dr. Ping-hui Feng (University of Southern California, Los Angeles, CA). Cells were grown in DMEM supplemented with 10% FCS. Sendai virus (SeV) and GFP-containing vescicular stomatitis virus (VSV) (VSV-GFP) were supplied by Dr. Hong-Bing Shu (Wuhan University). Mouse monoclonal anti-Flag, anti-HA (Sigma), rabbit polyclonal anti-IRF3, anti-LMN1, anti-GAPDH, and anti-tubulin (Proteintech) were purchased from the indicated manufacturers.

Constructs

The IFN-β promoter luciferase reporter plasmid was provided by Dr. Hong-Bing Shu. Mammalian expression plasmids for GFP-tagged IRF3 and its truncated forms were constructed by standard molecular biology techniques.

Site-directed mutagenesis technique

The site-specific mutants of IRF3 were constructed using the site-directed mutagenesis kit (Vazyme). The primers for the mutations are as follows: RK68/70Q Fwd: 5′-GATGCTTCCGGGAACATGACCGACCGACG-IC3′; RK68/70Q Rev: 5′-AGCAACCTCCTCCGGGAACATGACCGACCGACG-TC3′; RK77/78N Fwd: 5′-CTGGCAACCTCCTCCGGGAACATGACCGACCGACG-TC3′; RK77/78N Rev: 5′-CTGGCAACCTCCTCCGGGAACATGACCGACCGACG-TC3′; R81L Fwd: 5′-CTGGCAACCTCCTCCGGGAACATGACCGACCGACG-TC3′; R81L Rev: 5′-AGCAACCTCCTCCGGGAACATGACCGACCGACG-TC3′.

Protein expression and purification

IRF3 1–115 or IRF3 1–115, coupled with mutation KR77/78NG, R81L, or K105Q Rev: 5′-AGAAGGGTTGCTTTTAGCAGAGG-3′ and 5′-AGCAACCTCCTCCGGGAACATGACCGACCGACG-TC3′, were inserted in frame to the GST coding sequence in the bacterial expression vector pGEX-6P-1. Escherichia coli BL21 (DE3) cells (500 ml at OD600 nm = 1.2) harboring the recombinant expression vectors were incubated with 0.4 mM isopropyl-β-D-thiogalactopyranoside at 16°C for 24 h. Cells were harvested by centrifugation and suspended in 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 2 mM DTT, 1 mM PMSF, 0.02% Nonidet P-40, 2 mM leupeptin, 10 mM MgCl2, 150 mM NaCl, 0.4 mg/ml lysozyme, and 40 μg/ml DNase I. Cells were lysed for 60 min. A total of 5 ml bacterial supernatant was mixed with 200 μl 50% (v/v) glutathione-arose beads and incubated for 60 min at 4°C with gentle rotation. The agarose beads were washed four times with 5 ml ice-cold PBS. The recombinant proteins were cleaved and eluted through incubation with 100 μl 50 mM Tris (pH 8), 2 mM DTT, and GST-tagged human rhinovirus (HRV 3C) protease (50 μl) at 4°C for 5 h. Supernatants containing the recombinant proteins were collected by centrifugation. The purity and quantity of the recombinant proteins were examined by SDS-PAGE, followed by Coomassie blue staining.

EMSAs

For the IRF3-DNA complex, binding reactions were assembled at 2°C in a total volume of 5 μl in buffer 10 mM HEPES (pH 8), 500 mM NaNO3, 100 mM NaCl, 5 mM MgCl2, 5% glycerol, and 1 mM DTT. Binding reactions were loaded onto an actively running 7% polyacrylamide gel in TBE-0.5 mM EDTA, 0.5 mM MgCl2, and 100 mM NaCl, incubated with 0.4 mM isopropyl-β-D-thiogalactopyranoside at 16°C for 24 h. Cells were washed with PBS three times, and fresh medium was added. The supernatants were removed, and medium containing 0.75% agar was overlaid. At 2–3 d postinfection, cells were fixed with 4% formaldehyde for 10 min and then permeabilized with 0.2% Triton X-100 in PBS for 15 min. After blocking cells with 1% BSA in PBS for 30 min, the coverslips were incubated with the indicated primary Abs and FITC-goat anti-rabbit IgG or TRITC-goat anti-mouse IgG sequentially, and observed with a Leica confocal microscope using a 3100 oil-immersion objective.

Detection of cytokine production

The concentrations of IFN-β in culture supernatants were measured with a mouse IFN-β ELISA kit (BioLegend), according to the manufacturer’s instructions.

VSV plaque assay

IRF3+/−, IRF7−/− MEFs (2 × 105) were transfected with the indicated plasmids for 24 h prior to VSV infection. At 1 h postinfection, cells were washed with PBS three times, and fresh medium was added. The supernatants were harvested at 24 h, diluted 1:10–1:105, and used to infect confluent BHK21 cells plated on 24-well plates. At 1 h postinfection, the supernatants were removed, and medium containing 0.75% agar was overlaid. At 2–3 d postinfection, cells were fixed with 4% formaldehyde.
for 20 min and stained with 0.2% crystal violet. Plaques were counted, averaged, and multiplied by the dilution factor to determine viral titers (PFU/ml).

Native-PAGE
Native-PAGE was performed using an 8% acrylamide gel without SDS. The gel was prerun with 25 mM Tris-HCl (pH 8.4) and 192 mM glycine, with or without 0.5% deoxycholate, in a cathode and anode chamber, respectively, for 30 min at 40 mA on ice. Samples in the native sample buffer (50 mM Tris-HCl [pH 6.8], 15% glycerol) were applied on the gel and electrophoresed for 60 min at 35 mA on ice, followed by immunoblotting.

Data collection and statistics
All experiments were repeated at least three times, and data from parallel treatments were acquired. The significance of the difference between control and experimental groups was tested using an unpaired two-tailed Student t test. Fluorescent imaging analysis was performed in a blind fashion.

Results
Mapping of the NLS domain of IRF3
It is well known that IRF3 is predominantly localized in the cytoplasm prior to virus infection and is imported into the nucleus following infection. A previous study identified two basic amino acid residues (77KR78) as the NLS of IRF3 (30). However, during our work on the function and regulation of IRF3, we found, as expected, that the composition and function of IRF3 are much more complicated than anticipated. Therefore, we started to systematically characterize the nature and function of IRF3.

We first analyzed the subcellular distribution and the NLS domain of endogenous IRF3 by nucleus–cytoplasm extraction and immunofluorescence assays. As shown in Fig. 1A, prior to virus infection, IRF3 was predominantly localized in the cytoplasm, whereas a weak band of IRF3 was detected in the nucleus; upon SeV infection, IRF3 began to translocate into the nucleus. The same results were obtained in the immunofluorescence assay. As shown in Fig. 1B, in uninfected cells, IRF3 localized exclusively to the cytoplasm; SeV infection resulted in translocation of IRF3 to the nucleus within 12 h. These results are consistent with previous observations (31).

IRF3 contains both NLS and NES, and the predominant cytoplasmic localization of IRF3 before virus infection implies that the NES is constitutively active, and nuclear export is more dominant than nuclear import. To define the NLS domain of IRF3, we inactivated the NES of IRF3 to avoid its interference. Point mutations of the NES of IRF3, the IL139/140MM modification (designated as IRF3-NES), abolished the nuclear export function of IRF3 (25). As shown in Fig. 1D, in uninfected cells, GFP-IRF3 was mainly localized in the cytoplasm, whereas GFP-IRF3(-NES) was found in the nucleus and cytoplasm, indicating that IRF3 could enter and stay in the nucleus when the NES was deficient. We then truncated IRF3 from both the N-terminal and the C-terminal regions (Fig. 1C). As shown in Fig. 1D and 1E, the C-terminal truncations (aa 1–382, 1–357, and 1–201) in fusion with GFP all retained the ability to translocate into the nucleus, whereas the N-terminal truncations (GFP-IRF3131-427) lost the ability to accumulate in the nucleus. However, the N-terminal truncation GFP-IRF364-427 retained the ability to translocate into the nucleus. These results indicate that the NLS domain of IRF3 resides within the N-terminal region of aa 64–131.

Mutational analysis of IRF3 NLS
Because most of the known NLS domains contain clusters of basic residues, we examined the IRF3 sequence to search for clusters of basic amino acids within the region of aa 64–131 and then performed site-directed mutagenesis to evaluate their contribution to IRF3 nuclear accumulation. As shown in Fig. 2A, seven clusters of basic amino acids could be recognized and were replaced by uncharged amino acids. The resulting IRF3 mutants were designated RK68/70SQ, KR77/78NG, R81L, RK86/87LQ, R91L, R98L, R101L, R104L, RK115/116LQ, R119L, R126L, R127L, and RK130/131LQ. As shown in Fig. 2B, all seven mutants failed to translocate into the nucleus, indicating that the NES of IRF3 is necessary for nuclear accumulation.

Figure 1. Mapping of the IRF3 NLS domain. (A) HEK 293 cells were infected with SeV (50 HA/ml) at the indicated time before nuclear–cytoplasm extraction assays were performed; 5% of cytoplasmic extracts and 10% of nuclear extracts were subjected to SDS-PAGE analysis and immunoblotting with the indicated Abs. (B) MEFs were left uninfected or were infected with SeV for 12 h, immunofluorescently stained with IRF3 Ab (green) and DAPI (blue), and examined using confocal microscopy. Scale bars, 10 μm. (C) Schematic presentation of IRF3-GFP truncation mutants. (D) HEK 293 cells were transfected with the indicated IRF3-GFP truncation mutant plasmids for 24 h before confocal microscopy. Scale bars, 10 μm. (E) The intensities of nuclear/cytoplasmic GFP were quantitated using the ImageJ processing program and normalized to that of total GFP.
FIGURE 2. Screening of the IRF3 NLS domain for key basic amino acids. (A) Schematic presentation of IRF3 and mutation of the basic amino acids to neutral amino acids in the region spanning aa 64–130. (B) IRF3−/− Irf7−/− MEFs were transfected with the indicated IRF3 site-directed mutant plasmids for 24 h and then left uninfected or infected with SeV for 8 h before luciferase assays and immunoblotting. The immunoblots show the protein expression of IRF3 variants and endogenous GAPDH in the SeV-infected group. Assays were performed in triplicate. RK96/87SQ, and K105Q. Upon virus infection, IRF3 undergoes dimerization and nuclear accumulation and then activates the IFN-β promoter. The site mutation that results in a deficiency in IRF3 nuclear transportation fails to activate IFN-β. We tested whether the above IRF3 mutants could activate IFN-β by transfecting these site mutations into IRF3−/− Irf7−/− MEFs together with IFN-β promoter reporter plasmids. IRF3−/− Irf7−/− MEFs were used to avoid the interference of endogenous IRF3 and Irf7. As shown in Fig. 2B, the mutants RK68/70SQ, R91L, RK96/87SQ, and K105Q retained the ability to activate IFN-β, indicating that these are not the amino acids that specify IRF3 nuclear accumulation. In contrast, the mutants KR77/78NG, R81L, and RK86/87LQ failed to activate IFN-β, indicating these basic amino acids may be responsible for IRF3 nuclear import and act as part of the NLS.

Determination of specific basic amino acids that contribute to IRF3 nuclear import

We then determined whether the failure of the IRF3 mutants in IFN-β activation lies in their nuclear inaccessibility. Because it is well known that dimerization and nuclear import are two prerequisites for IRF3 activation (31), we first determined at which step these mutants lost their activity. As shown in Fig. 3A, all of these mutants retained the ability to dimerize, but dimer formation of mutant KR77/78NG was reduced. As shown in Fig. 3B, dimerized KR77/78NG failed to be imported into the nucleus after virus infection, indicating that basic residues KR77/78 are required for IRF3 nuclear import. The mutant RK86/87LQ also failed to be imported into the nucleus following SeV infection, but R81L retained the ability to translocate to the nucleus (Fig. 3B). The expression level of RK86/87 mutant was reduced ∼35% compared with other IRF3 variants in HEK 293 cells (Fig. 3A). In contrast, there was no significant difference in expression between RK86/87LQ and other IRF3 variants in IRF3−/− Irf7−/− MEFs (Fig. 2B) or other cell lines, such as HeLa cells and HEC-1B cells (Supplemental Fig. 1). Because RK86/87LQ mutation totally abolished IRF3 nuclear import and IRF3-mediated IFN-β induction in different cell lines, irrespective of its expression level, all of the data clearly support the role of RK86/87 in nuclear localization. We observed the same results when we transfected the synthetic viral RNA analog polyinosinic-polycytidylic acid into the cells in place of SeV infection (data not shown). The decrease in cytoplasmic IRF3 could not be detected (Fig. 3B, lower panel), probably because IRF3 expression was driven by the strong CMV promoter, and the persistent synthesis of IRF3 may obscure the turnover of cytoplasmic IRF3. These results indicate that both clusters of basic residues (KR77/78 and RK86/87) are essential for nuclear import of IRF3.

To determine the specific contribution of these clusters of basic residues to IRF3 nuclear import, we generated NES-deficient IRF3 mutants (IL139/140MM), resulting in the mutants IRF3-NES, KR77/78NG-NES, R81L-NES, and RK86/87LQ-NES. As shown in Fig. 4A, prior to virus infection, IRF3-NES, KR77/78NG-NES, and R81L-NES were able to be imported into the nucleus; upon virus infection, the nuclear localization of these mutants was markedly enhanced, and the nuclear IRF3 following virus infection was hyperphosphorylated, which could be readily recognized by the band shift in Fig. 4A. However, RK86/87LQ-NES could
not translocate into the nucleus, with or without virus infection (Fig. 4A). Furthermore, we tested the ability of the mutants to activate IFN-β. As shown in Fig. 4B, IRF3-NES activated IFN-β production following virus infection, but it failed to activate IFN-β before virus infection, even though it was able to translocate into the nucleus. Dimerization analysis showed that nuclear IRF3-NES, KR77/78NG-NES, and R81L-NES were in a monomeric state before virus infection (Fig. 4C). The full-length IRF3 monomer could not bind to the promoter ISRE and, thus, is not able to activate IFN-β without virus infection. KR77/78NG-NES, R81L-NES, and RK86/87LQ-NES failed to activate IFN-β, despite the fact that all three mutants could dimerize when stimulated by virus. RK86/87LQ-NES failed to be imported into the nucleus, even in the absence of NES function, suggesting that amino residues RK86/87 represent an essential part of the IRF3 NLS. In contrast, the mutant KR77/78NG-NES could enter the nucleus, although at low efficiency (Fig. 4A), indicating that the role of KR77/78 in IRF3 nuclear import could be partially offset by NES in intact IRF3. Although KR77/78NG-NES and R81L-NES were able to translocate into the nucleus after virus infection, they were unable to activate IFN-β, implying that these two mutants were deficient in the subsequent transcription activation, which was tested in our subsequent experiments. The same results were observed when we transfected these mutants into HEC-1B cells (Supplemental Fig. 2), indicating that the function of these basic clusters responsible for IRF3 nuclear import is not cell type specific.

**Immunofluorescence analysis of IRF3 nuclear accumulation**

We carried out immunofluorescence assays to confirm the role of the clusters of basic residues in IRF3 nuclear import. We used a tet-off expression system to avoid the consistent overexpression of transfected IRF3. IRF3 wild-type (WT), KR77/78NG, R81L, and RK86/87LQ were reintroduced into Irf3−/− Irf7−/− MEFs, and the x-ray crystallographic analysis of complexes between the DNA enhancer and the recombinant DBD of IRF3 revealed that the IRF3 DBD binds to DNA through positioning the amino residues RK86/87 represent an essential part of the IRF3 NLS. In contrast, the mutant KR77/78NG-NES could enter the nucleus, although at low efficiency (Fig. 4A), indicating that the role of KR77/78 in IRF3 nuclear import could be partially offset by NES in intact IRF3. Although KR77/78NG-NES and R81L-NES were able to translocate into the nucleus after virus infection, they were unable to activate IFN-β, implying that these two mutants were deficient in the subsequent transcription activation, which was tested in our subsequent experiments. The same results were observed when we transfected these mutants into HEC-1B cells (Supplemental Fig. 2), indicating that the function of these basic clusters responsible for IRF3 nuclear import is not cell type specific.

**Integration of the DNA-recognition motif and NLS domain**

The x-ray crystallographic analysis of complexes between the DNA sequence that covers the PRD III–I region (nt −96 to −64) of the IFN-β enhancer and the recombinant DBD of IRF3 revealed that the IRF3 DBD binds to DNA through positioning the α3 helix in the major groove and loop L1 near the minor groove (32). As shown in Fig. 6A, K77, R78, R81, R86, and K87 are all positioned in the α3 helix, making direct or water-mediated hydrogen bonds with the nitrogenous bases or phosphate backbone. To determine the contribution of these basic amino acids to DNA binding, we expressed recombinant IRF3 DBD (1–115 aa) and its site-directed mutants (Fig. 6B). First, we performed a dose-dependent EMSA to measure the binding ability of IRF3 DBD to the ISRE sequence. As shown in Fig. 6C, when the molar ratio of IRF3/DNA was 1:1, all of the DNA is bound, and no dissociation of the complex occurs during electrophoresis. In contrast, recombinant IRF3 could not bind with the mutated DNA probe, indicating that the observed band shift resulted from the specific binding of IRF3 and the probe DNA (Fig. 6C). We observed that adding IRF3 Ab to the mixture of
recombinant protein IRF31–115 and the DNA probe severely impaired the interaction between IRF31–115 and the DNA probe compared with the control IgG–treated lane, resulting in more unbound probe DNA in the IRF3 Ab–treated lane (Supplemental Fig. 3) and indicating that the antigenic determinants of IRF3 recognized by these IRF3 Abs may overlap with the region responsible for DNA binding or that DNA binding may have changed the conformation of IRF3. Although super shift was not observed, the specific binding between recombinant IRF31–115 and the DNA probe was confirmed using the mutated probe. Next, we tested the DNA-binding ability of the IRF3 mutants at a 2:1 ratio. As shown in Fig. 6D, both KR77/78NG and R81L completely lost their DNA-binding ability, whereas RK86/87LQ retained its DNA-binding activity but at a reduced efficiency compared with that of WT IRF3. Such data indicated that the basic amino acid clusters responsible for IRF3 nuclear import are also critical for DNA binding. This also may explain why KR77/78NG-NES R81L and R81L-NES could translocate into the nucleus but were unable to activate IFN-β after SeV infection. These results demonstrate that the two functions of IRF3, DNA binding and nuclear import, are integrated in its N-terminal region.

The antiviral function of IRF3 requires intact NLS
To explore the potential effects of these basic amino acids responsible for both nuclear import and DNA binding on the IRF3–mediated antiviral response, we tested the replication capacity of VSV-GFP in IRf3−/− IRf7−/− MEFs stably expressing vector and the indicated IRF3 mutants. As shown in Fig. 7A, IRF3 WT and IRF3-NES expression markedly suppressed VSV-GFP replication. Consistently, VSV-GFP titers were much lower in IRf3−/− IRf7−/− MEFs complemented with IRF3 WT or IRF3-NES than were those in control vector–restored IRf3−/− IRf7−/− MEFs (Fig. 7B).
FIGURE 7. IRF3 NLS mutants are defective in inducing antiviral responses. (A) *Ifir*3^−/− *Irf7*^−/− MEFs were transfected with the indicated IRF3 site mutant plasmids for 24 h and infected with VSV-GFP (multiplicity of infection [MOI] = 0.01) before microscopy. (B) The cell culture supernatant in (A) was collected to measure the VSV-GFP titer by plaque assay. (C) The cell culture supernatant in (A) was collected to measure the secretion of IFN-β cytokine. Assays were performed in triplicate. Bar graphs show mean ± SD (n = 3). ND, not detected.

R81L, RK86/87LQ, or their relative NES mutants behaved as *Ifr*3^−/− *Irf7*^−/− MEFs complemented with vector plasmid control. In line with these results, *Ifr*3^−/− *Irf7*^−/− MEFs restored with IRF3 WT or IRF3-NES exhibited remarkably enhanced IFN-β production following SeV infection, whereas *Ifr*3^−/− *Irf7*^−/− MEFs complemented with vector or other mutants showed no IFN-β secretion under the same conditions (Fig. 7C). Sequence alignment and structural analysis of IRF3 NLS domain revealed that IRF3 NLS is highly reserved among different species but unique among the IRF family proteins (Fig. 8). Collectively, the data indicate that these basic amino acids that are responsible for both nuclear import and DNA binding are highly reserved and are essential in virus-induced IFN-β production and cellular antiviral responses.

**Discussion**

Our results define a bipartite NLS that is integrated within the DNA-recognition region of IRF3. We mapped the NLS of IRF3 to aa 64–130, partially overlapping with the DBD. Basic amino acids KR77/78 and RK86/87 are required for efficient nuclear import of IRF3. Significantly, we demonstrate that the NLS of IRF3 also plays an important role in the DNA-binding activity. The IRF family contains nine mammalian members (IRF1, IRF2, IRF3, IRF4, IRF5, IRF6, IRF7, IRF8, and IRF9), which are most conserved in their DBD. IRF1 and IRF2, which are closely related to each other, contain a conserved NLS located immediately C-terminal to the DBD, involving aa 120–138 (33). IRF4, IRF8, and IRF9 are highly conserved with each other and use the homologous NLS (aa 66–85) to direct their accumulation in the nucleus (34). Interestingly, IRF5 contains two monopartite consensus NLSs, a N-terminal NLS and a C-terminal NLS (35). Our study, together with previous reports, demonstrated that the NLSs of IRFs are generally positioned within or in close proximity to the DBD, and the principal type of NLSs for these IRFs is structurally bipartite. Sequence alignment reveals that the conservation of NLS domain of the IRF family proteins is related to their phylogenetic relationship (Fig. 8A, 8B). The NLSs of IRF family proteins have been identified, with the exception of IRF6 and IRF7. Phylogenetically, IRF6 is most closely related to IRF5, and IRF7 is most closely related to IRF3. Thus, the NLSs of IRF6 and IRF7 may be homologous to IRF5 and IRF3, respectively. Our subsequent research goal is to define the NLS of IRF6 and IRF7. Sequence alignment (Fig. 8B) reveals that IRFs display high overall homology among the DNA-recognition helix, which is critical for the DNA-binding activity of the IRF family proteins. However, only IRF3 uses this region as an NLS domain, and the key basic amino acids corresponding to IRF3 NLS are not conserved in the IRF family. The unique NLS domain on IRF3 is highly conserved from fish to primates (Fig. 8C). Bipartite basic signals typically consist of interdependent basic amino acid clusters separated by a flexible linker of 9–12 aa (29, 36). The linker between the two basic clusters (KR77/78 and RK86/87) on IRF3 NLS domain contains only seven residues, shorter than the length of the conventional linkers. The IRF3 NLS domain contains an α-helical motif, and KR77/78 and RK86/87 are 90° apart (Fig. 8D), which makes a distance of 1–2 aa for the spatial distance between the two basic clusters that interact with the minor binding site and major binding site in importin-α, respectively. A previous report also showed that Ty1 integrase contains a bona fide bipartite NLS with a 29-aa linker (37). Our data, together with these reports, reveal that the traditional definition of NLS is too restrictive; the linker length can vary depending on amino acid composition and conformation. These findings also allow us to expand the definition of a classic bipartite NLS consensus beyond the previous limitations of a 9–12-aa linker.

With regard to the nature of the NLS of IRF3, there is clear difference between our results and those of a previous study (30). We showed in this study that IRF3 NLS is bipartite, consisting of residues KR77/78 and RK86/87, whereas the earlier work claimed that the residues KR77/78 are the sole determinant of IRF3 NLS. Such a disparity could result from the use of different experimental methodologies. Examination of the earlier work by Kumar et al. (30) reveals several likely pitfalls in their studies. First, they used only GFP-IRF3^1–131 fusion for nuclear-localization studies by fluorescence microscopy. Small proteins can pass the nuclear pore in an energy- and receptor-independent manner (38); in fact, the GFP-IRF3^1–131 fragment (∼42 kDa) is able to diffuse between the nucleus and the cytoplasm of cells (Fig. 1D), thus giving rise to indefinite judgment of NLS determinants. Second, they used a cell line that contains endogenous WT IRF3. Because IRF3 can...
form dimers, the intrinsic IRF3 may influence the behaviors of GFP-fused IRF3 or its mutants. In our study, we characterized the IRF3 NLS through two experimental methods: nuclear–cytoplasm extraction assays and fluorescence microscopy. We also used Irf3/ Irf7-null MEFs to avoid the interference of endogenous native IRF3. Furthermore, we also analyzed the role of the key amino acid residues in type I IFN responses and IRF3-mediated antiviral activity. All of these studies point out that the amino acid residues KR77/78 and RK86/87 make up the bipartite NLS of IRF3 and are essential to virus-induced innate immunity.

Based on the crystal structure of IRF3 (39), inactive IRF3 is in “the autoinhibitory state,” in which two regions corresponding to residues 380–427 and 98–240 of IRF3 interact to form a closed structure in the inhibited state (40). Virus-induced multiple phosphorylation of C-terminal serine/threonine residues or phosphorylation-mimetic mutation of IRF3 (IRF3-5D) introduces massive negative charges, leading to unmasking of the NLS domain and realignment of the DBD for transcriptional activity. Interestingly, our study showed that mutation RK96/98SQ in IRF3 generated a strong activator of IFN-β gene expression (Fig. 2B). The RK96/98SQ mutant alone was able to stimulate IFN-β expression as strongly as virus infection. These results suggest that RK96/98 may be directly involved in the interaction of residues 98–240 with the C-terminal phosphor-acceptor region in the closed structure of IRF3, and the dispelling of negative charge at RK96/98 appears to be equal to the C-terminal phosphorylation in unleashing the autoinhibition state of IRF3.

It is widely accepted that dimerization of IRF3 is crucial for nuclear import and transcriptional activity of IRF3. However, analysis using the mutant IRF3-NES demonstrated that the NES-deficient monomeric IRF3 could enter the nucleus but was unable to activate the IFN-β promoter, suggesting that the NLS of IRF3 is not masked in the autoinhibitory state. Monomeric IRF3 in the autoinhibitory state cannot associate with CBP/p300 and is not ready to realign the DBD for transcriptional activity, which may account for the inability of IRF3-NES to activate the IFN-β promoter prior to viral infection. These results also suggest that dimerization is not a prerequisite for IRF3 nuclear import.

It is notable that IRF proteins contain both NLSs and NESs; thus, regulation of subcellular distribution is emerging as a common method to control IRF functions. The predominant cytoplasmic localization of IRF3 before virus infection implies that nuclear export is more dominant than nuclear import prior to virus infection. However, IRF3 resides predominantly in the nucleus after viral infection, indicating that nuclear import is dominant when IRF3 is activated. Because IRF3 is a major transcriptional factor for type I IFN responses, elaborate regulation at the steps of nuclear import and export can be envisaged. Therefore, further characterization of
the molecular processes will help to reveal the mechanisms of IRF3 activation and IRF3-mediated antiviral immunity.

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Disclosures

The authors have no financial conflicts of interest.

References

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Supplementary Figure 1. The expression levels of IRF3 variants in different cells. (A) HeLa cells were transfected with the indicated IRF3 site mutant expression plasmids before immunoblotting with anti-FLAG and GAPDH antibody. (B) Similar to (A), except that HEC-1B cells were used.
Supplementary Figure 2. Determination of the contribution of the key basic clusters. (A) HEC-1B cells were transfected with the indicated IRF3 site mutant plasmids for 24 hr, and then left uninfected or infected with SeV for 8 hr before nuclear-cytoplasm extraction assays were performed. 5% of cytoplasmic extract and 10% of nuclear extract were subjected to SDS-PAGE analysis and immunoblotting with the indicated antibodies. LMNB1 and β-tubulin were used as markers for nuclear and cytoplasmic proteins, respectively.
Supplementary Figure 3. The specific binding between the recombinant IRF3_{1-115} and the DNA probe. EMSA was performed by incubating WT DNA probe (20 µM) with recombinant IRF3_{1-115} (40 µM), IRF3 antibody or rabbit IgG control.