Role of Cyclophilin B in Activation of Interferon Regulatory Factor-3*

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IRF-3 is a member of the interferon regulatory factors (IRFs) and plays a principal role in the induction of interferon-β (IFN-β) by virus infection. Virus infection results in the phosphorylation of IRF-3 by IκB kinase ε and TANK-binding kinase 1, leading to its dimerization and association with the coactivators CREB-binding protein/p300. The IRF-3 holocomplex translocates to the nucleus, where it induces IFN-β. In the present study, we examined the molecular mechanism of IRF-3 activation. Using bacterial two-hybrid screening, we isolated molecules that interact with IRF-3. One of these was cyclophilin B, a member of the immunophilins with a cis-trans peptidyl-prolyl isomerase activity. A GST pull-down assay suggested that one of the autoinhibition domains of IRF-3 and the peptidyl-prolyl isomerase domain of cyclophilin B are required for the binding. A knockdown of cyclophilin B expression by RNA interference resulted in the suppression of virus-induced IRF-3 phosphorylation, leading to the inhibition of the subsequent dimerization, association with CREB-binding protein, binding to the target DNA element, and induction of IFN-β. These findings indicate that cyclophilin B plays a critical role in IRF-3 activation.

Interferon regulatory factors (IRFs) are a family of transcription factors that regulate a variety of biological events, including innate immunity. Once activated by the invasion of a pathogen, such as viruses and bacteria, IRFs regulate the expression of various genes encoding immunomodulatory cytokines and chemokines and limit the spread of infection. Among these factors, interferons (IFNs) play important roles in host defense, cell growth regulation, and immune activation (1, 2). IFNs include the type I IFN-α and IFN-β and the type II IFN-γ. Type I IFNs are immediately induced in response to various viral infections, and IRF-3 and IRF-7 play an important role in their induction (3, 4). The mode of IRF-3 involvement against virus infection has been analyzed by using Newcastle disease virus (NDV) and Sendai virus (5–9). IRF-3 is expressed in the cytoplasm as a latent, inactive form, and its C-terminal serine/threonine residues are phosphorylated by IκB kinase ε and TANK-binding kinase 1 (10, 11). Virus-induced C-terminal phosphorylation of IRF-3 represents an important posttranslational modification, leading to dimerization (6, 7), translocation from the cytoplasm to the nucleus, association with CBP/p300 coactivators (6, 9), stimulation of DNA binding to the IFN-stimulated response elements (ISREs), and activation of the corresponding genes (5, 8, 9).

IRF-3 consists of an N-terminal DNA-binding domain that specifically binds to an ISRE motif, and a C-terminal IRF association domain (IAD) that mediates protein-protein interactions. IRF-3 uses the IAD for both intramolecular autoinhibition interactions and intermolecular dimerizations (6, 12). Furthermore, IRF-3 possesses a transactivation domain (amino acids 134–394) and two autoinhibition domains found within the proline-rich sequence (amino acids 134–197) and at the C-terminal end (amino acids 407–414). The two autoinhibition domains are thought to interact with each other to generate a closed conformation that masks the C-terminal IAD, the DNA-binding domain, and the nuclear localization signal of IRF-3, which prevents homodimerization and DNA binding in uninfected cells. The C-terminal phosphorylation of IRF-3 might open the conformation, leading to dimer formation and exposure of the nuclear localization signal and the DNA-binding domain (6, 13, 14). However, the molecular events associated with such a drastic conformational change remain unknown. In the present study, we demonstrate the interaction of IRF-3 with cyclophilin B (CypB), an immunophilin with cis-trans peptidyl-prolyl isomerase and chaperone-like activities (15). The knockdown of CypB by RNA interference prevented the NDV-induced IRF-3 phosphorylation, dimerization, association with CBP, binding to the ISRE, and induction of IFN-β.

EXPERIMENTAL PROCEDURES

Bacterial Two-hybrid Screening—A BacterioMatch™ two-hybrid SystemXR Plasmid cDNA library (Stratagene, La Jolla, CA) was used to screen IRF-3 interacting proteins according to the manufacturer’s protocol. For the construction of the bait plasmid, the GST fusion expression plasmid pGEX-IRF-3 was digested at the Neol site, which overlapped with the initiation codon of IRF-3, filled in with the Klenow fragment of DNA polymerase I, and then digested by Xhol. The plasmid pBT was digested by BamHI, filled in with the Klenow fragment of DNA polymerase I, and then digested by Xhol. The Neol/Klenow fragment containing the IRF-3 coding region was ligated with the pBT fragment. The junction of the cloning site of the resultant plasmid pBT-IRF-3 was verified by sequencing. Competent cells of the BacterioMatch two-hybrid system Escherichia coli reporter strain were transformed with pBT-IRF-3, together with the pTRG-cDNA library (Human HeLa cell plasmid cDNA library, number 982208, Stratagene). As every

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¶ The abbreviations used are: IRF, interferon regulatory factor; IFN, interferon; CBP, cAMP-response element-binding protein-binding protein; ISRE, interferon-stimulated response element; NDV, Newcastle disease virus; ISG, interferon-stimulated gene; GST, glutathione S-transferase; CypB, cyclophilin B; PPIase, peptidyl-prolyl isomerase; siRNA, small interfering RNA.
transformation produced 4.0–5.0 × 10^6 colonies, we finally screened 4.30 × 10^9 colonies after 10 transformations. The pCDA plasmids were recovered from the anti-recombinant and lacZ-positive colonies, and the sequences of the DNA inserts were verified, using the pTRG forward primer (5′-CAGGCTGGAAGTGAAGAGA-3′) and the pTRG reverse primer (5′-ATTCTGTGCCCGCCCATATA-3′), by a PRISM 310 or 3100 sequencer. For the construction of the N-terminal Myc-tag fusion protein expression vector, a synthetic oligonucleotide containing the Myc-tag sequence, followed by HindIII, NcoI, BamHI, and EcoRI sites as multicloning sites, was ligated into pCDNA3 digested with HindIII and EcoRI to yield the plasmid pCDNA/Myc. The cDNAs of the candidate positive clones were amplified by PCR with the pTRG forward and reverse primers, digested with BamHI, NotI, or EcoRI for the 5′-junction and XhoI for the 3′-junction, and then subcloned into the corresponding sites of pCDNA3/Myc.

**GST Pull-down Assay**—The Myc-tagged protein encoded by the cloned cDNA was expressed by the TNT Quick Coupled Transcription/Translation Systems (Promega) with unlabelled methionine. To construct the plasmids pGEX-hIRF-3, pGEX-hIRF-3 was digested with ScaI, SacI, or BglII, blunt-ended with the Klenow enzyme, and then digested with SacII. The 938-bp ScaI-SacI fragment, the 608-bp SacI-BglII fragment, and the 350-bp BglII-SacI fragment were excised and subcloned into the blunt-ended Neol site and the SacI site of the pGEX-3x vector, respectively. To construct pGEX-hIRF-3, the 600-bp BglII-NotI fragment of pGEX-hIRF-3 was blunt-ended with the Klenow enzyme, and then self-ligated. To construct pGEX-hIRF-3ΔC2 and ΔC3, the 724-bp NotI-SacI fragment and the 394-bp SacI-SacI fragment were excised from pGEX-hIRF-3 and then subcloned into the blunt-ended NotI site and the NcoI site of the pGEX-hIRF-3 plasmid, respectively. To create a PFFase-defective mutant of CypB, both the wild-type and position specific mutations were generated in the GST pull-down experiment described above. The antibodies used were the goat polyclonal anti-IRF-3 antibody (C-20), the mouse monoclonal anti-IRF-3 antibody (2F3, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C. After washing twice with complete medium (Dulbecco's modified Eagle's medium containing 10% bovine serum albumin) and incubated with NDV by the addition of 100 μl of allantoic fluid from NDV-infected chicken eggs (1000 hemagglutinin units/ml) for 1.5 h. After washing twice with complete medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 50 μg/ml streptomycin), the cells were further incubated in complete medium for 10.5 h before harvesting for extract preparation.

**Western Blotting**—The procedures were the same as those used in the GST pull-down experiment described above. The antibodies used were the goat polyclonal anti-IRF-3 antibody (C-20), the mouse monoclonal anti-IRF-3 antibody (2F3, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C. After washing twice with complete medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 50 μg/ml streptomycin), the cells were further incubated in complete medium for 10.5 h before harvesting for extract preparation.

**Preparation of Whole Cell Extracts**—The cells were washed with ice-cold phosphate-buffered saline (−) and then scraped and suspended in 400 μl of cell lysis buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 2 mM Na_3VO_4, 10 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride). The cells were lysed by vortexing briefly, and the lysates were incubated on ice for 10 min. The whole cell extracts were recovered after centrifugation at 15,000 rpm for 10 min at 4°C.

**Affinity Purification**—The proteins bound to DNA. The cross-linked proteins were purified and immunoprecipitated with the anti-IRF-3 antibody (C-20). After reversal of the cross-linking, the DNA was amplified using primers for the ISG15 promoter (5′-TTTCCCTGTCCCTTCGGTAGCAGTCC-3′, 5′-TTATAAGCGTGGACACGCAAA-3′) and for the control (5′-ACAGAAGACGGAGAGGGGTTT-3′, 5′-CCACCCUGUGGUCCUGUUGTTT-3′) were annealed according to the provider's instructions, respectively (JBlue, San Jose, CA). Cells were transfected with siRNAs using Lipofectamine 2000 (Invitrogen). In brief, 1 × 10^6 cells/well were seeded in a 6-well tissue culture plate. After 24 h of incubation, the medium was replaced by culture medium without antibiotics. To allow the siRNA-liposome complexes to form, 5 μl of siRNAs (20 μM) and 5 μl of Lipofectamine 2000 reagent were combined in 500 μl of Opti-MEM medium and incubated at room temperature for 20 min. This mixture was added to the cells and incubated for 24 h. The cells were then washed twice with dilution medium (Dulbecco's modified Eagle's medium containing 10% bovine serum albumin) and infected with NDV by the addition of 100 μl of allantoic fluid from NDV-infected chicken eggs (1000 hemagglutinin units/ml) for 1.5 h. After washing twice with complete medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 50 μg/ml streptomycin), the cells were further incubated in complete medium for 10.5 h before harvesting for extract preparation.
RESULTS
Isolation of Putative IRF-3-binding Proteins by the Bacterial Two-hybrid Screening—To isolate the protein(s) that associate with IRF-3, we performed a screening by a bacterial two-hybrid system (16, 17). The bait was fused to the full-length bacteriophage λ repressor protein (λI), whereas the corresponding target protein was fused to the N-terminal domain of the α-subunit of RNA polymerase. When the bait and target interact, they recruit and stabilize the binding of RNA polymerase at the promoter and activate the transcription of reporter genes, the carbenicillin-resistance and the β-galactosidase genes. In addition, this system offers the ability to detect an interaction between a pair of protein domains with an equilibrium dissociation constant in the high nanomolar range. In the present study, we used IRF-3 as the bait and a human HeLa cell cDNA library as the target. We obtained 272 antibiotic-resistant and lacZ-positive clones from 4.30 × 10⁶ screened clones. To identify the protein encoded by the candidate positive clones, the nucleotide sequence of each clone was determined. Among the clones obtained, only 25 clones encoded in-frame fusion proteins to the λI repressor. These clones were used in a search for homology in the NCBI data base. The cDNA fragments of the positive clones were subcloned into an expression vector to produce N-terminally Myc-tagged fusion proteins. However, the positive clones were subcloned into an expression vector to produce N-terminally Myc-tagged fusion proteins. However, we found that cyclophilin A (CypA), the most abundant cyclophilin

FIG. 1. Mapping the interaction domains of IRF-3 and CypB. A, schematic representation of IRF-3 deletion mutants. Structures of wild type (top) and deletion mutants (ΔN1–3 and ΔC1–3) of IRF-3 are shown. Locations of functional domains are indicated. DBD, DNA-binding domain; NES, nuclear export signal; Pro, proline-rich region; IAD, interferon-associated domain. B, GST pull-down assay between IRF-3 mutants and CypB. Input, 5% of in vitro translated Myc-tagged CypB was included in the binding reactions. GST, GST protein only (not fused with IRF-3); FL, GST fusion of wild type IRF-3 protein; ΔN1, GST fusion of IRF-3 truncated 131 residues from the N terminus; ΔN2, GST fusion of IRF-3 truncated 241 residues from the N terminus; ΔN3, GST fusion of IRF-3 truncated 328 residues from the N terminus; ΔC1, GST fusion of IRF-3 truncated 99 residues from the C terminus; ΔC2, GST fusion of IRF-3 truncated 186 residues from the C terminus; ΔC3, GST fusion of IRF-3 truncated 296 residues from the C terminus. C, schematic representation of cyclophilin B PPIase mutants. The wild type CypB structure (top) is shown. The amino acid sequence of the wild type CypB and the mutated residues are shown. CsA, Cyclosporin A; D, GST pull-down assay between CypB, CypB PPIase mutant, or CypA and wild type IRF-3. Input, 5% of in vitro translated Myc-tagged CypB, Myc-tagged CypB PPIase mutant, or Myc-tagged CypA was included in the binding reactions. D, GST, GST protein only (not fused with IRF-3); GST, GST fusion of wild type IRF-3 protein. The solid and empty arrowheads indicate CypB and CypA, respectively. E, co-immunoprecipitation assay to detect an endogenous association between IRF-3 and CypB in vivo. Whole cell lysates prepared from cells, either mock-infected or NDV-infected for 10 min, were used as the input. IgG, lysates incubated with goat IgG (lanes 1 and 3); IP, lysates incubated with goat polyclonal anti-IRF-3 antibody (C-20) (lanes 2 and 4); IB, immunoblot. The data are representative of three independent experiments.

Then, we examined the interaction between IRF-3 and CypB in vivo. The association of endogenous IRF-3 and CypB was detected when an anti-IRF-3 antibody was used for the precipitation of an extract prepared 10 min after NDV infection (Fig. 1E, lane 4) but not 30 min after the infection (data not shown). On the other hand, the available anti-CypB antibodies could
Role of CypB in IRF-3 Activation

Not precipitate the endogenous IRF-3 with CypB, even 10 min after the infection. We also tried to characterize the binding domains of IRF-3 with CypB by using mutant IRF-3 expression plasmids. Unfortunately, the IRF-3 expression was inhibited when the CypB plasmid was introduced simultaneously. This is consistent with previous reports that CypB may participate in inducing the degradation of exogenously introduced DNA (18, 19).

Cyclophilin B Is Required for Virus-induced Phosphorylation of IRF-3—To examine the physiological roles of CypB in the IRF-3 function, we performed RNA interference experiments to knock down CypB in vivo and then examined the biochemical activities of IRF-3 after NDV infection. As shown in Fig. 2, the expression of CypB was almost completely suppressed by the specific siRNA interference (lanes 3 and 6, third panel). NDV infection in the mock- or control siRNA-transfected cells resulted in a mobility shift of IRF-3, which reflected the C-terminal phosphorylation of IRF-3 (Fig. 2, lanes 4 and 5, first panel). However, IRF-3 showed an intermediate mobility in CypB siRNA-transfected/NDV-infected cells (Fig. 2, lane 6, first panel). When the same blot was reprobed with an antibody that specifically recognized a phosphorylated form of IRF-3 (21), we found that the amounts of phosphorylated IRF-3 (phosphorylated Ser-396) were reduced in the CypB siRNA-transfected cells, as compared with the mock- or control siRNA-transfected cells (Fig. 2, compare lanes 6 with lanes 4 and 5, second panel). These results strongly suggest that the knockdown of CypB resulted in the defect in IRF-3 phosphorylation by virus infection.

The Cyclophilin B Knockdown Inhibited Virus-induced IRF-3 Dimerization—It is known that the phosphorylation of IRF-3 is prerequisite for the dimerization of IRF-3 in siRNA-transfected cells. To do this, we used the native PAGE assay that sensitively detects the difference between the monomer and dimer forms of IRF-3 (22). IRF-3 existed as a monomer—dimerization of IRF-3 (Fig. 2, third panel). The anti-IRF-3 antibody (second panel), the anti-CypB antibody (third panel), or the anti-actin antibody (fourth panel). The solid and empty arrowheads indicate the phosphorylated IRF-3 and the unphosphorylated IRF-3, respectively. The data are representative of three independent experiments.

FIG. 2. Cyclophilin B knockdown inhibits virus-induced IRF-3 phosphorylation. HT1080 cells were mock-transfected (lanes 1 and 4) or transfected with control siRNA (lanes 2 and 5) or CypB siRNA (lanes 3 and 6), respectively, followed by mock infection (lanes 1–3) or infection with NDV for 12 h (lanes 4–6). Whole cell extracts were prepared and subjected to immunoblotting using the anti-IRF-3 (C-20) antibody (first panel), the anti-IRF-3 Ser(P)-396 antibody (second panel), the anti-CypB antibody (third panel), or the anti-actin antibody (fourth panel). The solid and empty arrowheads indicate the phosphorylated IRF-3 and the unphosphorylated IRF-3, respectively. The data are representative of three independent experiments.

Demonstrated in the phosphorylation of Ser-386, which is the critical residue in IRF-3 activation (23). CypB siRNA-transfected/NDV-infected cells failed to phosphorylate Ser-386 (Fig. 3, panel 2, lane 6).

FIG. 3. Cyclophilin B knockdown inhibits virus-induced IRF-3 dimerization. Whole cell extracts were prepared as described in Fig. 2 and were analyzed by native PAGE followed by immunoblotting with the anti-IRF-3 (C-20) antibody (upper panel) and the anti-IRF-3 Ser(P)-396 antibody (lower panel). The solid and empty arrowheads indicate the monomer and the dimer of IRF-3, respectively. The data are representative of three independent experiments.

The Cyclophilin B Knockdown Inhibited IRF-3 Binding to the ISRE and Association with CBP—Phosphorylated IRF-3 undergoes homodimerization (6, 7) and associates with the coactivators CBP/p300 (6, 9). The holocomplex has the ability to specifically recognize the target DNA sequence, called the ISRE (5, 8, 9). We examined the effects of CypB on IRF-3 in terms of the DNA binding to the ISRE and the association with the CBP/p300 coactivator, using electromobility shift assay. In the absence of NDV infection, no DNA-protein complex was observed in the mock-, control siRNA-, and CypB siRNA-transfected cells (Fig. 4, lanes 1, 6, and 11). NDV infection induced the formation of a DNA-protein complex bound to the ISRE of the ISG15 gene in the mock- and control siRNA-transfected cell extracts (Fig. 4, lanes 2 and 7). The addition of specific antibodies against IRF-3 to the binding reactions reduced the amount of the complex band and the induction of supershifted bands, indicating that the complex contained IRF-3 (Fig. 4, lanes 3 and 8). The addition of specific antibodies against CBP to the binding reactions reduced the amount of the complex but did not induce the formation of supershifted bands, indicating that complex formation was partly blocked by the antibody (Fig. 4, lanes 4 and 9). This is consistent with the previous result that CBP/p300 is involved in the holocomplex of IRF-3 (6–9). However, the knockdown of CypB severely impaired the DNA binding activity of IRF-3 (Fig. 4, lane 12). Thus, the inhibition of CypB also resulted in reduced holocomplex formation, which is required for the target gene activation by IRF-3. A chromatin immunoprecipitation analysis showed the in vivo binding of IRF-3 to the ISG15 promoter in NDV-infected cells (Fig. 4B, lane 8) but not in uninfected cells (Fig. 4B, lane 2). When NDV-infected cells were pretreated with CypB siRNA, the binding was significantly reduced, and no band was detected after 30 PCR cycles (Fig. 4B, lane 12), although a band was visible after 40 cycles (data not shown). The control siRNA could not reduce the binding (Fig. 4B, lane 10).

The Cyclophilin B Knockdown Reduced IFN-β Production by Newcastle Disease Virus Infection—Finally, we examined the
Role of CypB in IRF-3 Activation

The present in vitro analysis suggests that autoinhibition of IRF-3 and the catalytic domain bearing the peptidyl-prolyl isomerase activity of CypB are required for the interaction.

CypB, a member of the cyclophilins, possesses a cis-trans peptidyl-prolyl isomerase activity (15). Via their PPIase activity, cyclophilins facilitate protein folding and have been shown to contribute to the maturation and trafficking of several proteins (24). Furthermore, cyclophilins regulate signal transduction cascades, as revealed by their modulation of transforming growth factor-β signaling and the transactivation of c-myc and IRF-4 (25, 26). Among the cyclophilins, CypB is distinguished from the others by the presence of an endoplasmic reticulum-directed signal sequence (15). However, CypB is found not only in the endoplasmic reticulum but also in the extracellular space and the nucleus (15). CypB has been reported to interact with prolactin. The proximal action of prolactin is mediated by its cell surface receptor. PRL activity, however, is also associated with the internalization and translocation of this hormone into the nucleus. To retrotransport it to the nucleus, and to potentiate prolactin-induced proliferation, the interaction with CypB with its PPIase activity is essential (27, 28). Similar to the interaction with prolactin, the PPIase domain of CypB is required for IRF-3 binding in a GST pull-down assay. We also found that retaining either one of the autoinhibition domains of IRF-3 is required for the binding. Previously, Mamane et al. (29) reported the interaction between IRF-4, a member of the IRFs, and FKBPS2, another member of the immunophilins. They demonstrated that IRF-4 would not co-immunoprecipitate with FKBPS2 unless the C-terminal autoinhibition domain of IRF-4 was removed. This observation raises the possibility that the interactions of immunophilins with IRF family proteins are sensitive to the conformations or the tertiary structures of IRFs.

As demonstrated by our RNA interference analysis, the specific knockdown of CypB in vivo resulted in the inhibition of virus-induced IRF-3 activation. This was confirmed at multiple steps, including phosphorylation, dimerization, DNA binding, coactivator binding, and IFN-β induction. If the phosphorylation of IRF-3 is a prerequisite for the following events after the virus-induced activation of IRF-3, then it is likely that CypB is involved in the phosphorylation reaction of IRF-3. The early involvement of CypB is supported by the notion that the in vivo association of CypB with IRF-3 was only detected 10 min after the infection but not after 30 min. At present, it has been reported that the C-terminal phosphorylation of IRF-3 is mediated by IkB kinase-ε and TANK-binding kinase 1 (10, 11). The mutagenesis of IRF-3 revealed key residues for virus-induced activation. Substitutions of the serine residues at 385 or 386 to alanine, glutamic acid, or aspartic acid made the molecule unresponsive to stimuli (7, 21). Substitutions of other serine/threonine residues, present at positions 396, 398, 402, 404, and 405, to aspartic acid made IRF-3 constitutively active (20). Recently, Servant et al. (21) identified Ser-396 and Ser-398 as the minimal phosphorylation sites critical for activation, based on their observation that S396D and S396D/S398D are constitutively active. More recently, Mori et al. (23) identified Ser-386 as the target of IRF-3, using an antibody that specifically detects the phosphorylation of Ser-386. As protein kinases are often associated with, in addition to their regulators, molecular chaperones that sometimes need to exert their specificity for the substrates, it is interesting to speculate that CypB associates with the IRF-3 kinases, IkB kinase-ε and TANK-binding kinase 1, in a similar manner. Although it will be important in the future to determine whether it is the CypB binding, catalytic activity, or both that is responsible for its effect on IRF-3, our results indicate that CypB plays a significant role in modulating IFN-β gene expression via its interaction with IRF-3.
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