Identification of Distinct Signaling Pathways Leading to the Phosphorylation of Interferon Regulatory Factor 3*

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Infection of host cells by viruses leads to the activation of multiple signaling pathways, resulting in the expression of host genes involved in the establishment of the antiviral state. Among the transcription factors mediating the immediate response to virus is interferon regulatory factor-3 (IRF-3) which is post-translationally modified as a result of virus infection. Phosphorylation of latent cytoplasmic IRF-3 on serine and threonine residues in the C-terminal region leads to dimerization, cytoplasmic to nuclear translocation, association with the p300/CBP coactivator, and stimulation of DNA binding and transcriptional activities. We now demonstrate that IRF-3 is a phosphoprotein that is uniquely activated via virus-dependent C-terminal phosphorylation. Paramyxoviridae including measles virus and rhabdoviridae, vesicular stomatitis virus, are potent inducers of a unique virus-activated kinase activity. In contrast, stress inducers, growth factors, DNA-damaging agents, and cytokines do not induce C-terminal IRF-3 phosphorylation, translocation or transactivation, but rather activate a MAPKKK-related signaling pathway that results in N-terminal IRF-3 phosphorylation. The failure of numerous well characterized pharmacological inhibitors to abrogate virus-induced IRF-3 phosphorylation suggests the involvement of a novel kinase activity in IRF-3 regulation by viruses.

Virus infection of mammalian cells triggers multiple signal transduction cascades involved in the activation of a diverse set of immunoregulatory genes and proteins that together create the antiviral state, an intracellular environment that antagonizes virus replication. The type I interferon (IFN) family is essential to the development of the antiviral state and the IFN gene family represents one of the best characterized models of virus inducible gene activation (1). Once produced, these secreted proteins induce gene expression in neighboring cells through cell surface cytokine receptors and the JAK-STAT signaling pathways. STAT1/2 heterodimers, in conjunction with interferon-stimulated gene factor 3 (ISGF3) bind to interferon-stimulated response elements found in numerous IFN-induced genes such as 2’-5’ oligoadenylate synthase and the double stranded RNA (dsRNA) activated kinase (PKR), resulting in the induction of proteins which impair viral gene expression and replication (1). Molecular regulation of IFN gene transcription is tightly regulated by extra- and intracellular signals induced at the site of infection. One of the best characterized models of such regulation is the virus-inducible promoter/enhancer of the IFN-β gene (2–4). This promoter includes an overlapping set of regulatory domains designated positive regulatory sequences (PRDs) 1 to IV, which interact with several signal-responsive transcription factors including NF-κB (p50/p55), ATF-2/c-Jun heterodimers, and interferon regulatory factors (IRF) that bind to PRD II, PRD IV, and PRD I-III, respectively. Together with the chromatin-associated HMG I(Y) proteins, these transcription factors form a stereospecific transcriptional enhancer complex, termed the enhancerome (2–4) that stimulates the high level, transient activation of IFN-β transcription.

The pathways involved in NF-κB and ATF-2/c-Jun activation have been well characterized. Following viral infection, treatment with proinflammatory stimuli like tumor necrosis factor (TNF)-α, interleukin-1, (IL-1), or exposure to dsRNA, these transcription factors are activated through stimulation of distinct kinase cascades. In unstimulated cells, the NF-κB factors are retained in the cytoplasm in association with inhibitory subunits, IκBs; virus-induced phosphorylation at conserved N-terminal residues is accomplished by the IκB kinase (IKK) complex. Phosphorylation triggers a signal that leads to ubiquitination, dependent degradation of IκB, and subsequent nuclear translocation of the NF-κB dimers (reviewed in Ref. 5). The rate-limiting step in this process is the activation of IKK which is composed of two catalytic subunits IKKα and β and one regulatory subunit IKKγ/NEMO. Numerous studies now suggest that the IKKβ catalytic subunit is required for IKK and NF-κB phosphorylation.
activation by TNF-α, interleukin-1, lipopolysaccharide (LPS), dsRNA, and viral infection (6–10). Unlike NF-κB, the heterodimers ATF-2/c-Jun are expressed as nuclear proteins that are activated by phosphorylation of their activation domains by c-Jun amino-terminal kinases (JNKs) which are downstream of a well defined stress-activated kinase cascade (11).

The pathway(s) regulating IRF-3 phosphorylation and activation are also the focus of considerable investigation. IRF-3 belongs to the family of IRFs which include IRF-1 to IRF-7, interferon consensus sequence-binding protein (IRF-8), and interferon-stimulated gene factor 3γ (IRF-9) (12). IRF-3 is expressed constitutively in a variety of tissues, and the relative levels of IRF-3 mRNA do not change in virus-infected or IFN-treated cells. IRF-3 demonstrates a unique response to viral infection. Phosphorylation of latent cytoplasm IRF-3 on serine and threonine residues in the C-terminal region leads to a conformational change, dimerization, cytoplasmic to nuclear translocation, association with the p300/CREB coactivator, stimulation of DNA binding and transcriptional activities (3, 13–17). Activated IRF-3 can in turn induce a specific subset of type 1 IFN genes in response to viral infection including IFN-β and human IFN-α1 (murine α4), as well as the CC-chemokine RANTES and the interleukin-15 (15, 18–22). As with NF-κB activation, the rate-limiting step in this process is C-terminal phosphorylation of IRF-3 by an uncharacterized virus activated kinase (VAK) activity.

Previous studies have demonstrated that treatment with dsRNA was sufficient to trigger the nuclear accumulation of IRF-3 (17) and the formation of an IRF-3 containing DNA binding complex (3, 16). Recent studies also suggest that phosphorylation and activation of IRF-3 is not restricted to viral infection, since LPS, DNA-damaging and stress-inducing agents all stimulate nuclear accumulation of IRF-3, DNA binding activity, and transactivation (23–25). Using a variety of pharmacological and molecular approaches, we now demonstrate that IRF-3 is uniquely activated via C-terminal virus-dependent phosphorylation. In addition to Sendai virus and Newcastle disease virus (NDV), measles virus (MeV) and vesicular stomatitis virus (VSV) are also identified as potent inducers of VAK activity. In contrast, exposure of cells to stress inducers, growth factors, DNA-damaging agents, and cytokines including doxorubicin and TNF-α, resulted in N-terminal phosphorylation but not C-terminal IRF-3 phosphorylation by an uncharacterized virus activated kinase (VAK) activity.

Materials and Methods

Reagents—PDT, sorbitol, LPS, and ribavirin were purchased from Sigma and dissolved in distilled water or phosphate-buffered saline. All other pharmacological inhibitors were from Calbiochem or Biomol and resuspended in dimethyl sulfoxide or ethanol. Reconstitute macrophage inflammatory protein 1α, macrophage inflammatory protein 1β, and RANTES were from R&D Systems. Pertussis Toxin, epidermal growth factor, platelet-derived growth factor-βB, insulin, and thrombin were kind gifts from Dr. Sylvain Meloche.

Plasmid Constructions and Mutagenesis—CNDV-IRF-3, -IRF-3 5A, -IRF-3 5D; pFlag-IRF-3 1–240, the reporter plasmids containing the IRF-3 reporter plasmids (4). The bacterial plasmids containing two PRD II sites, pgL3-P2/Ptk-LUC and the IFNβ promoter, pgL3-IFN-β-LUC were described previously (13, 19, 26). The Kb-mutated RANTES promoter, pgL3-Bm-RANTES-LUC, was prepared by cloning the BgIII/SaIII fragment (~397 to +5, filled in with the Klenow enzyme) from the Bm-RANTES-CAT reporter plasmid (19) into the Nhel site (filled in with the Klenow enzyme) of the pgL3-basic vector. The expression constructs encoding different C-terminal IRF-3 truncations, pFlag-IRF-3-3 (1–198), (1–186), (1–174), and (1–150) were generated by overlap polymerase chain reaction mutagenesis using Vent DNA polymerase. Constructs encoding for MAPKKKs, PCDNA3-MEK1-1HA, and pIKS-MYC-Cot were kind gifts from Drs. Richard Gerszten and Warner Greene, respectively.

Cell Culture—The rTA-Jurkat, rTA-Jurkat IRF-3wt, and rTA-Jurkat IRF-3-5D were described previously (27). Human embryonic kidney (HEK) 293 cells and HeLa cells were grown in α-minimal essential medium and Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% fetal bovine serum, glutamine, and antibiotics. The monocytic cell line U937 was cultured in RPMI supplemented with 10% fetal bovine serum. The human bronchial lung carcinoma cell line A549 was purchased from ATCC (CCL-185) and cultured in F12K supplemented with 10% fetal bovine serum. Extracts of primary monocytes uninfected or infected with NDV were a kind gift of Dr. Sandra Gessani, ISS, Rome.

Transfections and Luciferase Assays—All transfections were carried out on subconfluent HEK 293 cells grown in 60-mm Petri dishes or 24-well plates (luciferase assay). 5 μg of DNA constructs (per 60-mm dish) or 10 ng of pRLTK reporter (Renilla luciferase for internal control), 100 ng of pGL3 reporter (firefly luciferase, experimental reporter) and 250–500 ng of expression plasmids (24-well plate) were introduced into target cells by calcium phosphate coprecipitation method. At 24 h post-transfection, cells were infected with Sendai virus for 12 h (80 hemagglutinating units (HAU)/ml) or treated with the induc-ers the indicated times. At 36 h, cells were collected, washed in ice-cold phosphate-buffered saline and assayed for reporter gene activities (Promega); whole cell extracts (WCE) were prepared in Nonidet P-40 lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 30 mM NaF, 5 mM EDTA, 10% glycerol, 1,0 mM Na3VO4, 40 mM β-glycerophosphate, 10−4 M phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml of each leupeptin, pepstatin, and aprotinin, and 1% Nonidet P-40) and stored at −80°C.

Immunoblot Analysis—To verify the state of phosphorylation of IRF-3 and to confirm the expression of the transgenes, WCE (30–60 μg) were subjected to electrophoresis on 7.5, 10, or 12% acrylamide gels. Proteins were electrothermally transferred to Hybond-C nitrocellulose membranes (Amersham Pharmacia Biotech, Inc.) in 25 mM Tris, 192 mM glycine, and 20% methanol. The membranes were blocked in TBS containing 5% nonfat dry milk and 0.1% Tween 20 at 1 h at 25°C before incubation for 1.5 h at 25°C with anti-IRF-3 (a kind gift from Dr. Paula Pitha), anti-α-actin (Sigma), or anti-Flag M2 (Sigma) (1:500 to 1:1000) in blocking solution. After washing four times in TBS, 0.1% Tween 20, the membranes were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:10000) in blocking solution. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Inc.).

For co-preparation studies, WCE (200–1000 μg) were incubated with 1 μg of anti-CBP antibody A-22 (Santa Cruz) cross-linked to 30 μl of protein A-Sepharose beads for 3 h at 4°C (Amersham Pharmacia Biotech). The beads were washed five times with Nonidet P-40 lysis buffer, resuspended in denaturing sample buffer, and the eluted IRF-3 protein associated with CBP were analyzed by immunoblotting.

Cytoplasmic and Nuclear Extracts Preparations—To examine subcellular localization of the IRF-3 protein, nuclear and cytoplasmic extracts were prepared from HeLa cells after treatment with different inducers for 8 h. The cells were washed in buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM PMSF) and were resuspended in buffer A containing 0.1% Nonidet P-40. The cells were then chilled on ice for 10 min before centrifugation at 10,000 × g. This procedure was performed twice to remove cytoplasmic contaminants in the nuclear extracts. After centrifugation, supernatants were kept as cytoplasmic extracts. The pellet were then resuspended in buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 5 μg/ml of each leupeptin, pepstatin, aprotinin, and spermidine). Samples were incubated on ice for 15 min before being centrifuged at 10,000 × g. Nuclear extract supernatants were diluted with buffer C (20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 50 mM KCl, 0.5 mM dithiothreitol, 0.5 mM PMSF). Equivalent amounts of nuclear and cytoplasmic extracts (20 μg) were subjected to SDS-PAGE in a 10% polyacrylamide gel. The membranes were electrothermally transferred to Hybond-C nitrocellulose membranes which were probed with IRF-3 antibody as described earlier.

Phosphatase Treatment—HEK 293 cells were left untransfected or transfected with expression plasmids encoding wild-type or mutated forms of IRF-3. At 36 h post-transfection, cells were stimulated and WCE were prepared. Endogenous IRF-3 (400 μg) or overexpressed IRF-3 (150 μg) proteins were immunoprecipitated with anti-IRF-3 an
tibody (Santa Cruz) or anti-Flag antibody (Sigma) cross-linked to 30 μl of protein G-Sepharose beads for 4 h at 4 °C. Precipitates were washed two times in Nonidet P-40 lysis buffer followed by two washes in of protein G-Sepharose beads for 4 h at 4 °C. Precipitates were washed anti-Flag antibodies.

The reactions were incubated at 37 °C for 2 h and stopped by washing the beads once with Nonidet P-40 lysis buffer and addition of 50 μl of 2 × denaturating sample buffer. The samples were resolved by SDS-gel electrophoresis and analyzed by immunoblotting using anti-IRF-3 and anti-Flag antibodies.

**RESULTS**

**Multiple Forms of IRF-3 Phosphoprotein—C-terminal phosphorylation of IRF-3 following paramyxovirus infection is a prerequisite for its nuclear translocation, association with CBP/p300 co-activators, and transcriptional activation (13, 16, 17, 28).** VAK activity is relatively easy to detect in extracts from virus-infected cells, since phosphorylated IRF-3 migrates slower in SDS-PAGE than nonphosphorylated IRF-3 (13, 16, 17), a phenomenon observed with many phosphoproteins. To characterize the different forms of phosphorylated IRF-3 in virus-infected cells, IRF-3-specific immunoblotting was used to reveal two forms of IRF-3 (designated forms I and II) in uninfected HEK 293, U937 and Jurkat cells (Fig. 1B, lanes 1, 3, and 7). These forms were also present in human epithelial HeLa cells, human bronchial epithelial A549 cells, primary human monocytes (see Figs. 3 and 6) and freshly isolated primary B cells (data not shown). Sendai virus infection resulted in the appearance of two slowly migrating forms of IRF-3 (forms III and IV) in HEK 293, U937, and IRF-3-expressing Jurkat cells (Fig. 1B, lanes 2, 4, 5, 6, and 8). Forms III and IV represent IRF-3 phosphorylated at a cluster of serines near the C-terminal end of the protein (Ref. 13 and see Fig. 4C). In addition, a net decrease in the amount of IRF-3 was observed between 4 and 12 h after virus infection of U937 cells, supporting the idea that C-terminal phosphorylated IRF-3 is subject to proteasome-mediated degradation (13). Overexpression of the constitutively active form of IRF-3-(5A/D) (13, 19, 26, 27) in Jurkat cells demonstrated that the phosphomimetic form migrated slower in SDS-PAGE than endogenous IRF-3 protein, at a position similar to form IV observed in cells infected with Sendai virus (Fig. 1B, lane 9). This initial experiment, while largely confirming previous observations, nevertheless clearly demonstrates that multiple forms of IRF-3 phosphoprotein exist in unstimulated and virus-infected cells.

Phosphatase treatment of immunoprecipitated IRF-3 isolated from cells overexpressing IRF-3wt revealed that form II was also a phosphoprotein (Fig. 1C, lanes 4–6). Phosphatase treatment resulted in the disappearance of form II from the extract and an increase in IRF-3 form I (Fig. 1C, compare lanes 4 and 5), an effect that was blocked by addition of phosphatase inhibitors (Fig. 1C, lanes 6). Interestingly, IRF-3-(5A) and IRF-3-(5D), in which the five phosphoacceptor sites in the C terminus were mutated to alanine (5A) and aspartic acid (5D) (see Fig. 1A), were still expressed as two forms, form II (5A lane 7) and form IV (5A lane 10) (see also Fig. 1B, lane 9). These forms remained sensitive to phosphatase treatment (Fig. 1C, lanes 8 and 11) but were present when phosphatase inhibitors were used (lanes 9 and 12). Also, when endogenous IRF-3 was immunoprecipitated from Sendai virus-infected HEK 293, forms III and IV were readily detected (Fig. 1C, lane 13); CIP treatment resulted in the conversion of forms III and IV to forms I and II (Fig. 1C, lane 14), an effect that was also blocked by phosphatase inhibitors (Fig. 1C, lane 15). Based on these preliminary observations, it appeared that multiple forms of IRF-3 phosphoprotein could be detected and basal IRF-3 phosphorylation, represented by forms II and V (for IRF-3-(5D)), did not occur at the C-terminal phosphoacceptor sites implicated in IRF-3 activation.

**Pharmacological Inhibitors Fail to Block VAK Activity**—In the effort to identify the pathway(s) activated by viral infection and implicated in IRF-3 phosphorylation, the effect of well

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**Figure 1. Multiple forms of IRF-3 phosphoprotein.** A, schematic representation of IRF-3. The DNA-binding domain, the NES element, the proline-rich region, and the C-terminal IRF association domain are indicated. The region between aa 382 and 414 are expanded below the schematic. The amino acids targeted for alanine or aspartic acid substitutions are shown in large letters. The point mutations are indicated below the sequence: 5A, S396A, S398A, S402A, T404A, S405A; 5D, S396D, S398D, S402D, T404D, S405D. cDNAs encoding for IRF-3 lacking the C-terminal region (IRF-3-(1–240), -(1–198), -(1–186), -(1–174), -(1–163), -(1–153), -(1–143), -(1–133), -(1–123)) are also shown. B, phosphorylation of IRF-3 in HEK 293, U937, and rTAT-Jurkat, rTAT-Jurkat IRF-3wt, and rTAT-Jurkat IRF-3(5D) cells. Jurkat cells were induced with Dox (1 μg/ml) for 16 h. Then HEK 293, U937, and rTAT-Jurkat IRF3wt were infected withSendai virus (80 HAU/ml) for 4, 8, or 12 h or left uninfected (−). Endogenous IRF-3 proteins were detected in whole cell extracts (55 μg) by immunoblotting using anti-IRF-3 antibody (from Dr. Paula Pitha). C, forms II, III, and IV are sensitive to phosphatase treatment. HEK 293 cells were transfected with vector alone pBSCMV (pBS) or constructs encoding for IRF-3(wt), IRF-3(5A), and IRF-3(5D) or left untransfected (Sendai virus). At 36 h post-transfection or 8 h after infection withSendai virus (80 HAU/ml), whole cell extracts were prepared and subjected to immunoprecipitation using IRF-3 antibody covalently linked to protein A-Sepharose beads. Immunoprecipitated IRF-3 was then used in a phosphatase assay as described under “Materials and Methods.” The resulting immunoprecipitated proteins were resolved by 7.5% SDS-PAGE. IRF-3 phosphorylated forms were analyzed by immunoblotting using anti-IRF-3 antibody (from Dr. Paula Pitha).
Distinct Pathways Leading to IRF-3 Phosphorylation

**Activation of IRF-3 Is Restricted to Viral Infection**—The antagonizing effect of ribavirin and UV treatment on virus-dependent IRF-3 activation (Fig. 2, B and C) indicated that C-terminal phosphorylation may be specific to virus infection. Viruses from different families were tested for their capacity to induce IRF-3 phosphorylation and activation. Two paramyxoviridae family members, MeV and NDV, and one rhabdoviridae family member, VSV, were also able to induce the generation of forms III and IV in HEK 293 cells, primary monocytes and human bronchial epithelial A549 cells, respectively (Fig. 3A, lanes 2, 4, and 7). These viruses resulted in a phosphorylation-dependent degradation of IRF-3 which was no longer detected in primary monocytes after infection with NDV for 18 h (Fig. 3A, lane 5, and data not shown). Induction of IRF-3 forms III and IV by MeV infection also resulted in transactivation of human bronchial epithelial A549 cells, respectively (Fig. 3A, lanes 2, 4, and 7).

**Stress Inducers, DNA Damaging Agents, Growth Factors, and NF-xB Inducers Stimulate N-terminal IRF-3 Phosphorylation**—Recent studies showed that DNA-damaging agents and stress inducers activated IRF-3 in HeLa cells (23, 25). To determine which forms of IRF-3 were activated by this diverse array of agents, HEK 293 cells were induced with Sendai virus, stress inducing stimuli sorbitol and anisomycin, DNA-damaging agent doxorubicin, and the growth factor/NF-kB, and also epidermal growth factor (data not shown) resulted in the accumulation of form II without the generation of forms III and IV (Fig. 4A, lanes 2, 4, and 7). The conversion of forms I to II using growth factor and also epidermal growth factor (data not shown) resulted in the accumulation of form II without the generation of forms III and IV (Fig. 4A, lanes 2, 4, and 7). The conversion of forms I to II using growth factor and also epidermal growth factor (data not shown) resulted in the accumulation of form II without the generation of forms III and IV (Fig. 4A, lanes 2, 4, and 7).

**List of pharmacological inhibitors, their cellular targets, and effects on Sendai virus-induced IRF-3 phosphorylation in HEK 293 cells**

| Pharmacological inhibitors | Target | Inhibition of IRF-3 phosphorylation |
|---------------------------|--------|-----------------------------------|
| Ser/Thr kinases           |        |                                   |
| Go6976 (0.1–10 μM)        | cPKCs  | --                                |
| GF199205X (0.01–10 μM)    | c,-nPKCs| --                                |
| SB203580 (10–50 μM)       | Jp2    | --                                |
| KN93 (10 μM)              | CAM kinase II | --                                |
| NaSal (20 mM)             | IKKβ   | --                                |
| K-252a (200 nM)           | Ser/Thr kinases | --                                |
| H-7 (1 μM)                | Ser/Thr kinases | --                                |
| Rapamycin (10–25 ng/ml)   | mTOR/FRAP | --                                |
| Dual specificity kinase   |        |                                   |
| PD 98059 (50–100 μM)      | MEK1,2 | --                                |
| Tyrosine kinases          |        |                                   |
| Genistein (25–100 μM)     | Py3     | --                                |
| AG490 (50 μM)             | JAK-2   | --                                |
| Phosphatidylinositol kinase|        |                                   |
| LY294002 (50 μM)          | PI 3-kinase| --                                |
| Wortmannin (100 nM)       | PI 3-kinase| --                                |
| mRNA and protein synthesis|        |                                   |
| Actinomycin D (5 μg/ml)   | DNA    | +                                 |
| Ribavirin (0.25–1 mg/ml)  | RNA polymerase of paramyxoviruses| +                                 |
| Miscellaneous             |        |                                   |
| Okadaic acid (25–100 nM)  | PP-1/PP-2A phosphatases | --                                |
| PDTC (100 μM)             | Reactive oxygen species | +                                 |
| Pertussis toxin (100 ng/ml)| G/G pathways | --                                |
| Cytochalasin D (1 μM)     | Actin filaments| +                                 |
| BAPTA-AM (15 μM)          | Calcium chelator| +                                 |

characterized pharmacological inhibitors on IRF-3 phosphorylation following Sendai virus infection was examined (Fig. 2 and Table I). Use of specific pharmacological inhibitors that targeted MEK1/2 (PD98059), p38α and β2 (SB203580), phosphatidylinositol 3-kinase (wortmannin and LY294002), and mTOR/FRAP (rapamycin) (29–35) did not affect the generation of the two hyperphosphorylated forms of IRF-3 (III and IV) by Sendai virus (Fig. 2A, lanes 3–7). Pretreatment of cells with the intracellular calcium chelating agent BAPTA-AM (Fig. 2A, lane 10) did, however, induce a shift from forms I to II. It also completely blocked virus-induced IRF-3 phosphorylation (Fig. 2A, lane 11, and Table I), suggesting that a calcium-dependent phosphatase might be involved in the generation of form I and more importantly, that a calcium-dependent pathway may be upstream of IRF-3 activation. Many other pharmacological inhibitors also failed to block IRF-3 phosphorylation (Table I). Interestingly, ribavirin, a selective inhibitor of the RNA polymerase of paramyxoviruses (36) had a dose-dependent inhibitory effect on IRF-3 phosphorylation (Fig. 2B, lanes 3–8, and Table I), possibly due to its ability to inhibit the replication of Sendai virus (data not shown). Furthermore, UV-treated Sendai virus was unable to induce C-terminal IRF-3 phosphorylation (Fig. 2C), suggesting that complete IRF-3 activation through C-terminal phosphorylation requires replication competent virus.
Distinct Pathways Leading to IRF-3 Phosphorylation

Fig. 3. Activation of IRF-3 is restricted to virus infections. A, phosphorylation of IRF-3. Whole cell extracts (75 μg) were prepared from HEK 293 cells, freshly isolated primary monocytes, and A549 cells infected (−) or infected with MeV (multiplicity of infection of 1.0), NDV (100 HAU/ml), and VSV (multiplicity of infection of 10) for different time points, were resolved by 7.5% SDS-PAGE and transferred to nitrocellulose. IRF-3 was analyzed by immunoblotting for the presence of phosphorylated IRF-3 forms (II to IV) with anti-IRF-3 antibody. B, transactivation of PRD I–III- and interferon-stimulated response elements containing promoters. HEK 293 cells were transiently transfected with reporter constructs containing IFN-β promoters. HEK 293 cells were transiently transfected with reporter constructs containing IFN-β enhancer (IFN-β-LUC) and the eB-mutated RANTES promoter (eBm-RANTES-LUC). At 24 h post-transfection, cells were treated as indicated in the figure and LUC activity was analyzed 12 h later. Relative LUC activity was measured as fold activation as described under “Materials and Methods.” The resulting immunoprecipitated proteins were then used in a phosphatase assay, as described under “Materials and Methods.” Each value represents the mean ± S.E. of triplicate determinations. The data are representative of at least two different experiments with similar results. The concentration of viruses used was: Sendai virus (50), 80 HAU/ml; measles virus (MeV), multiplicity of infection of 1.0.

also that dsRNA treatment was sufficient to trigger the nuclear accumulation of IRF-3 and the formation of a functionally active IRF-3-containing DRAF complex (3, 16, 17). LPS treatment of U373 astrocytoma cells was also shown to induce IRF-3 nuclear translocation and DNA binding activity (24). Surprisingly, dsRNA treatment of HEK 293 cells and LPS treatment of U937 and HeLa cells did not induce any phosphorylation of IRF-3, as detected by immunoblot analysis (data not shown). Other cytokines and growth factors such as CC-chemokines (macrophage inflammatory protein 1-α, macrophage inflammatory protein 1-β, and RANTES) thrombin, insulin, platelet-derived growth factor-BB also had also no effect on IRF-3 phosphorylation (data not shown).

DNA damaging agents activate the classical stress pathway MKK4/SEK1 and JNK (37–39); furthermore, the catalytic activity of MKK4/SEK1 is regulated by MAPKK family members of which MEKK1 is the best described member (40). Anisomycin, epidermal growth factor, and hyperosmolarity are also good inducers of MEKK1 activity (data not shown and Refs. 41–43). Therefore, the effect of overexpressing MEKK1 on IRF-3 phosphorylation was examined. Fig. 4B demonstrates that Flag-tagged IRF-3 was expressed as nonphosphorylated form I and phosphorylated form II (Fig. 4B, lane 1). Importantly, coexpression of MAPKKs MEKK1 and Cot, a member of the MAPK family recently implicated in NF-κB activation following T cell receptor engagement (44), induced the accumulation of form II in transfected cells (Fig. 4B, lanes 2–5). This form represented the phosphatase-sensitive form of IRF-3 (Fig. 4B, lanes 5, 6, 8, and 9) as observed above with stress inducers and DNA-damaging agents (Fig. 4A). In contrast to Sendai virus infection (see Fig. 1B, lanes 5 and 6), no degradation of IRF-3 was observed in cells overexpressing MEKK1/Cot or treated with stress inducers and DNA-damaging agents after 16 to 24 h of treatment (data not shown).

Partial mapping of the region of IRF-3 phosphorylated by these agents or by MAPKKs overexpression revealed that phosphorylation did not occur in the C-terminal region (Fig. 4C). Overexpression of IRF3wt showed the accumulation of
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Form II following sorbitol treatment or when MEKK1 and Cot were co-transfected (Fig. 4C, lanes 8, 12, and 16). However, when the Ser-Thr cluster at aa 396–405 was mutated to Ala (5A), the shift from form I to form II still occurred under the same conditions (Fig. 4C, lanes 9, 14, and 17). Overexpression of IRF-3wt and 5A showed that the cluster of serine residues in the C-terminal region was essential for Sendai virus-induced generation of forms III and IV (Fig. 4C, compare lanes 4 and 6). In addition, no accumulation of form II was observed in Sendai virus-infected cells overexpressing IRF-3 (5A) (Fig. 4C, lane 6). Therefore an independent pathway leading to IRF-3 phosphorylation, distinct from the virus inducible C-terminal specific pathway, appears to be stimulated by stress inducers, DNA-damaging agents, and growth factors.

To further pinpoint the region of IRF-3 targeted for phosphorylation by stress inducers and DNA damaging agents, a series of IRF-3 deletion mutants were evaluated. As illustrated in Fig. 5A, truncation of full-length IRF-3 to a protein of 240 or 198 aa did not alter the generation of forms I and II (Fig. 5A, lanes 4–6); however, truncation to a protein of 186 aa resulted in a single form of IRF-3 (Fig. 5A, lane 3), indicating that the modification occurred between aa 186–198. With IRF-3(1–198), anisomycin resulted in the conversion of form I to form II (Fig. 5B, compare lanes 1 and 13); CIP treatment reverted form II to form I in a manner that was sensitive to phosphatase inhibitors (Fig. 5B, lanes 1 and 15). The 150, 174, and 186 aa IRF-3 truncations were expressed as a single form in both control and anisomycin-treated cells and were insensitive to phosphatase (Fig. 5B, lanes 4–12 and 16–24). As shown above for full-length IRF-3, stress inducers, DNA damaging agents, and growth factors such as doxorubicin and PMA as well as MEKK1 and Cot1 overexpression resulted in the complete or partial conversion of Flag-tagged IRF-3(1–198) from form I to form II (Fig. 5C), thus indicating that the phosphorylation site was located between aa 186 and 198. Analysis of this region of IRF-3 revealed a single potential site of Ser phosphorylation located within the sequence 186GPSENPLKRLLVP198. In addition, Sendai virus infection did not induce accumulation of form II (Fig. 5C, lane 2, and Fig. 4C, lanes 5 and 6), suggesting that the modification of IRF-3 by stress inducers and DNA-damaging agent was not used by virus to induce the activated forms of IRF-3 (form III and IV).

N-terminal Phosphorylation Does Not Alter IRF-3 Function—To examine the functional consequences of N-terminal phosphorylation on IRF-3 activity, cells were stimulated with stress inducers and evaluated for IRF-3 functions such as CBP association, nuclear accumulation of IRF-3, DNA binding, and transactivation activity. Fig. 6A shows that PMA, doxorubicin, stress inducers, such as anisomycin, sorbitol, and NaCl, and TNF-α induced a shift from form I to form II (Fig. 6A, lanes 3–8) without inducing the slowly migrating forms of IRF-3 observed when cells were infected by Sendai virus (Fig. 6A, lane 2). The effect of TNF-α on the conversion of form I to form II was transient, with maximal conversion to form II occurring after 30 min and returning to equal proportions of form I and II after 2 h (data not shown).2

Since association of IRF-3 with CBP coactivator is a critical step in IRF-3 activation (13, 16, 17, 28), the relationship between the conversion of form I to form II and association with CBP coactivator was evaluated. Co-immunoprecipitation analysis demonstrated that in HeLa, HEK 293, and U937 cells, the association between IRF-3 and CBP was only detected in Sendai-infected cells when forms III and IV are present (Fig. 6B, lane 2); similarly when cytoplasmic and nuclear partitioning was evaluated, only virus-induced IRF-3 translocated from the cytoplasm to the nucleus of HeLa cells (Fig. 6C, lanes 2 and 7). Next, the effect of DNA-damaging and stress-inducing agents on the transactivating potential of IRF-3 was measured using a reporter gene assay with the IRF-3 responsive xBm-RANTES-LUC (19). Sendai virus infection resulted in a 25-fold induction of RANTES activity in HEK 293 cells (Fig. 6D). Virus-inducible expression of the RANTES promoter was inhibited by co-transfection with a dominant-negative mutant of IRF-3 (ΔNIRF-3) (19, 27), demonstrating that the inducibility of the RANTES promoter was essentially dependent on IRF-3 activation (Fig. 6D) (19). Under the same conditions, stimula-

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2 R. Lin, unpublished data.
Distinct Pathways Leading to IRF-3 Phosphorylation

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Fig. 6. N-terminal phosphorylation does not alter IRF-3 subcellular localization or function. A, IRF-3 phosphorylation. Whole cell extracts prepared from HEK 293 cells untreated (−) or treated with different agents or infected with Sendai virus (Sv; 80 HAU/ml) for 8 h (except for TNF-α-treated cells where a 30-min stimulation is shown) were prepared. Protein extracts (75 μg) were analyzed by immunoblotting for the presence of phosphorylated IRF-3 (II to IV) with anti-IRF-3 antibody. The concentration of agents used were: sorbitol (S), 0.3 M; NaCl (Na), 0.25 M; anisomycin (A), 1 μg/ml; doxorubicin (D), 1 μg/ml; PMA (P), 100 ng/ml; TNF-α (T), 25 ng/ml; B, interaction between IRF-3 and CBP coactivator. HeLa, HEK 293, and U937 cells were treated as described in A. Whole cell extracts (500 μg) were immunoprecipitated with anti-CBP antibody A22, covalently linked to protein A-Sepharose beads. The immunoprecipitated proteins were resolved by SDS-gel electrophoresis on 7.5% acrylamide gel and transferred to nitrocellulose membrane. The membrane was probed with anti-IRF-3 antibody. As indicated, only forms III and IV were found to bind CBP. The concentration of agents used are described in A. LPS (L), 10 μg/ml. C, cytoplasmic to nuclear translocation of IRF-3. Hela cells were treated as indicated in A and B. Cytoplasmic and nuclear fractions were prepared as described under “Materials and Methods.” Each isolated fraction was subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-IRF-3 antibody. Lower panels, membranes were stripped and reblotted with an α-actin antibody. D, transactivation of interferon-stimulated response elements and FRD II containing promoters. HEK 293 cells were transfected with the α-Bm-mutated RANTES promoter (α-Bm-RAN- TES-LUC) or P2(2)tk-LUC reporter plasmids and the MEKK1 (M, 250 ng) or ANIRF-3 (ΔN, 500 ng) expression plasmids when indicated. At 24 h post-transfection, cells were treated as indicated below the bar graph and LUC activity was analyzed 12 h later. Relative LUC activity was measured as fold activation. Each value represents the mean ± S.E. of triplicate determinations. The data are representative of at least three different experiments with similar results. The concentration of agents used were: Sendai virus (Sv), 80 HAU/ml; PMA (P), 100 ng/ml; LPS (L), 10 or 100 μg/ml; TNF-α (T), 10 or 100 ng/ml; doxorubicin (D), 1 μg/ml; anisomycin (A), 1 μm; and sorbitol (S), 0.20 M.

Discussion

In the present study, we describe a series of pharmacological and molecular experiments designed to further characterize the signaling pathway(s) leading to IRF-3 phosphorylation and activation following virus infection or treatment with a variety of activating agents. IRF-3 phosphoprotein exists as two forms in uninfected cells: form I represents non-phosphorylated IRF-3, while form II represents a basally phosphorylated form of IRF-3 that is sensitive to phosphatase treatment. Mapping studies using IRF-3 deletions and point mutations demonstrate that phosphorylation of form II does not occur within the previously characterized cluster of serine residues at the C terminus of IRF-3 (13, 17). Rather, the phosphoacceptor site involved in the generation of form II appears to map to the N-terminal domain of IRF-3 between aa 186 and 198. Treatment with stress inducers, DNA-damaging agents, cytokines, and growth factors, does not induce C-terminal IRF-3 phosphorylation, translocation, or transactivation but rather activates a MAPKKK-related signaling pathway that increases the proportion of N-terminal phosphorylated IRF-3 resulting in the accumulation of form II. Following viral infection, two additional slowly migrating forms of IRF-3 are detected, designated form III and IV, that are sensitive to phosphatase treatment and represent C-terminal phosphorylation of IRF-3. Only forms III and IV translocate to the nucleus of virus-infected cells, and only C-terminal phosphorylated IRF-3 possesses DNA binding potential, CBP coactivator association, and transcriptional activity. Several well characterized, specific pharmacological inhibitors failed to block virus-induced C-terminal phosphorylation, thus apparently ruling out many known signaling pathways in the virus activation cascade. Furthermore, in vitro kinase assays demonstrated that extracellular-activated kinases (ERK 1/2), JNK, p38, IKKα/β, and PKR were unable to phosphorylate the C-terminal end of IRF-3 (data not shown). Full activation of IRF-3 appears to be restricted to viral infection including paramyxoviruses (MeV, Sendai, and NDV) and rhabdoviruses (SVV) which are potent inducers of VAK activity. Our data thus provide evidence of an uncharacterized virus-regulated kinase pathway involved in C-terminal IRF-3 phosphorylation and activation.

The results of this study contradict a number of recent investigations demonstrating that stress inducers and DNA-damaging agents functionally activate IRF-3. Navarro and David (24) reported that LPS treatment of human U937 astrocytoma cells resulted in IRF-3 activation, via a Toll-receptor and p38-dependent pathway. The authors demonstrated nuclear translocation and DNA binding of IRF-3 but did not examine the phosphorylation state of IRF-3 or the functional activity of the LPS-induced complex. Also the IRF-3-DNA complex that was
identified migrated rapidly in EMSA at a position consistent with a complex that did not include CBP/p300 coactivator. The functionally active complex contains minimally IRF-3, CBP/p300, and DNA, resulting in a high molecular weight virus-induced complex (45) or virus-activated factor (3). In light of the present findings, an interpretation consistent with these observations is that LPS-induced IRF-3 phosphorylation occurs in the N-terminal domain. In U373 cells, LPS appears to be sufficient to induce translocation of IRF-3 into the nucleus followed by enhanced IRF-3 DNA binding (24). However, because of the absence of C-terminal phosphorylation, IRF-3 was unable to engage CBP/p300 coactivator. Furthermore, in our hands with several cell types, LPS was unable to induce functional IRF-3 activation (data not shown).

Kim et al. (23, 46) in a series of recent papers, demonstrated that stress inducers and genotoxic agents such as DNA damaging agents doxorubicin and UV radiation stimulate IRF-3 (and IRF-7) phosphorylation, nuclear translocation, CBP association, and transcriptional activation of an IRF-3 responsive promoter. These experiments raise the exciting possibility that IRF-3 activation may be central to the innate host response to environmental stress. However, the analysis of IRF-3 phosphorylation by Kim et al. (23, 46) was not resolved sufficiently to delineate the different IRF-3-phosphorylated forms. Furthermore, the construct used to measure IRF-3 functional activity consisted of an artificial construct containing five Gal4-binding sites to measure the activity of a Gal4-IRF-3 fusion construct. As detailed in the present manuscript, DNA damaging agents did stimulate IRF-3 phosphorylation at the N-terminal site but failed to induce nuclear accumulation through CBP association or transcriptional activation of a natural IRF-3 responsive promoter-RANTES, even using the identical HeLa cell model. At this stage, we believe that overexpression of IRF-3 coupled with a sensitive but artificial transcriptional readout may lead to IRF-3 activation in response to genotoxic stress.

Interestingly, a small molecule CG18 that stimulates MEKK1 activity was used to activate the stress-mediated signaling pathway and was shown to stimulate the formation of the IFN-β enhanceosome (25). All the enhancer binding activities, ATF, c-Jun, IRF-3, and NF-κB were activated. MEKK1 activated IRF-3 through the JNK pathway but not through p38 or IKK pathways. These experiments imply that MEKK1 can induce IRF-3 and ATF2/c-Jun through the JNK pathway and NF-κB through the IKK pathway, resulting in the integration of multiple signal transduction pathways leading to the proper assembly of the IFN-β enhanceosome. The phosphorylation sites targeted by the MEKK1-related pathway are distinct from the C-terminal sites, since the IRF-3(5A) protein was still phosphorylated in response to CG18 and MEKK1.

The IRF-3 function regulated by N-terminal phosphorylation remains to be elucidated. However, based on the present study, several scenarios are possible. N-terminal phosphorylation by the stress-induced pathway may alter IRF-3 conformation, thus making the C-terminal Ser-Thr cluster more accessible to VAK (Fig. 7, pathway 1). This possibility was also proposed by Kim et al. (25). The two-step mechanism is, however, questionable since viral infection did not induce N-terminal phosphorylation of IRF-3 (Figs. 4C and 5C), indicating that VAK activity does not require this modification to activate IRF-3. Another possibility is that N-terminal phosphorylation may control IRF-3 activity at a step preceding nuclear translocation, such as relief of autoinhibition or dimerization (Fig. 7, pathway 2). Finally, the possibility exists also that N-terminal phosphorylation has no major effect on IRF-3 activity as a transcription factor, but may rather be involved in a distinct function of IRF-3 based on the observation that IRF-3 interacts with regulatory proteins that are not involved in transcription control (data not shown).

Finally, these experiments demonstrate for the first time that replication competent virus is required for full activation of IRF-3 since UV inactivation or ribavirin inhibition of virus replication blocked IRF-3 activity. Furthermore, the paramyxovirus MeV and the rhabdovirus VSV may be added to the growing list of viruses capable of activating IRF-3 function.
Interestingly, influenza virus (as well as other viruses of different classes) was unable to activate IRF-3.\(^3\) Consistent with this observation, a recent study has demonstrated that the influenza virus NS1 protein, a dsRNA-binding protein, specifically inhibited IRF-3 (47), although the mechanism of inhibition remains to be elucidated. These studies demonstrate that, as with many other viruses, the ability to interfere with the IFN antiviral cascade may contribute significantly to the virulence and pathogenicity of viral infection.

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\(^3\) B. ten Oever, unpublished data.