# IRF3 and IRF7 Phosphorylation in Virus-infected Cells Does Not Require Double-stranded RNA-dependent Protein Kinase R or IκB Kinase but Is Blocked by Vaccinia Virus E3L Protein*

## Eric J. Smith‡, Isabelle Marié§, Arun Prakash‡, Adolfo García-Sastre¶, and David E. Levy‡∥

*From the ‡Department of Pathology and Kaplan Comprehensive Cancer Center, Molecular Oncology and Immunology Program, New York University School of Medicine, New York, New York 10016 and the ¶Mount Sinai School of Medicine of New York University, New York, New York 10029*

**Induction of interferon-α (IFNα) gene expression in virus-infected cells requires phosphorylation-induced activation of the transcription factors IRF3 and IRF7. However, the kinase(s) that targets these proteins has not been identified. Using a combined pharmacological and genetic approach, we found that none of the kinases tested was responsible for IRF phosphorylation in cells infected with Newcastle disease virus (NDV). Although the broad-spectrum kinase inhibitor staurosporine potently blocked IRF3 and -7 phosphorylation, inhibitors for protein kinase C, protein kinase A, MEK, SAPK, IKK, and protein kinase R (PKR) were without effect. Both IκB kinase and PKR have been implicated in IFN induction, but cells genetically deficient in IκB kinase, PKR, or the *PKR*-related genes *PERK, IRE1*, or *GCN2* retained the ability to phosphorylate IRF7 and induce IFNα. Interestingly, *PKR* mutant cells were defective for response to double-stranded (ds) RNA but not to virus infection, suggesting that dsRNA is not the only activating viral component. Consistent with this notion, protein synthesis was required for IRF7 phosphorylation in virus-infected cells, and the kinetics of phosphorylation and viral protein production were similar. Despite evidence for a lack of involvement of dsRNA and PKR, vaccinia virus E3L protein, a dsRNA-binding protein capable of inhibiting PKR, was an effective IRF3 and -7 phosphorylation inhibitor. These results suggest that a novel cellular protein that is activated by viral products in addition to dsRNA and is sensitive to E3L inhibition is responsible for IRF activation and reveal a novel mechanism for the anti-IFN effect of E3L distinct from its inhibition of PKR.**

Type I interferon (IFN),[1] consisting of the single *IFNβ* gene

§ Present address: Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris, Cedex 15, France.

∥ To whom correspondence should be addressed: Dept. of Pathology, MSB 556, New York University School of Medicine, 550 First Ave., New York, NY 10016. Tel.: 212-263-8192; Fax: 212-263-8211; E-mail: levyd01@med.nyu.edu.

[1] The abbreviations used are: IFN, interferon; IKK, IκB kinase; dsRNA, double-stranded RNA, PKR, protein kinase R; NDV, Newcastle disease virus; RT-PCR, reverse transcriptase-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; JNK, c-Jun NH₂ kinase; GST, glutathione S-transferase.

and a family of IFNα genes, is rapidly induced by infection of mammalian cells by a broad spectrum of viruses (1, 2). Gene induction is rapid, and can be divided into two distinct phases initiated by induction of *IFNβ* and *IFNα4* expression followed by induction of other members of the *IFNα* gene family (3, 4). Differences in the gene expression profiles of the distinct type I IFN genes can be accounted for by unique properties of their promoter/enhancer structures. *IFNβ* expression is controlled by an enhanceosome that binds three distinct transcription factor complexes in the context of chromatin-organizing proteins (5). Each of these complexes, c-jun/ATF2 (AP1), IRF, and NFκB, become active following protein phosphorylation events induced in response to virus infection. *IFNα* promoters are also activated by IRF proteins, and the distinct DNA binding characteristics and patterns of induction and activation of IRF3 and IRF7 confer the differential expression of the IFNα gene family (3, 6–9).

While several transcription factors required for *IFN* gene induction have been identified, delineation of the signaling pathways stimulated by virus infection that lead to phosphorylation-dependent activation remains incomplete. Activation of NFκB requires phosphorylation-induced degradation of its inhibitor, IκB, through the action of the IκB kinase (IKK) complex composed of IKKα, IKKβ, and IKKγ/NEMO (10). The catalytic activity of IKK is activated by a wide variety of inducers, including viral infection and double-stranded RNA (dsRNA), a common by product of viral infection (11). Activation of IKK by dsRNA and viral infection may depend on a second enzyme, protein kinase R (PKR); however, in this role PKR may function noncatalytically and serve as an adaptor protein (12), although even this requirement has been recently called into question (13). The AP1 transcription factor, also required for *IFNβ* gene induction, is likewise activated by phosphorylation through the action of c-Jun kinase (JNK), which is also activated in virus-infected cells (12). The third transcription factor complex required for IFN gene induction, composed of IRF3 and/or IRF7, is also activated by phosphorylation specifically in virus-infected cells (3, 6, 14–22). However, the kinase responsible for its activation remains to be identified.

Not only is the identity of the IRF3/IRF7 kinase unclear, the nature of the activating component produced during virus infection that leads to kinase activation remains unknown. One candidate has been dsRNA, a common intermediate or by product of many viral infections that directly activates PKR (23). Moreover, many viruses target inactivation of PKR as a strategy to overcome host antiviral responses, by binding dsRNA, interfering with the activation of PKR, blocking its recognition of substrates, or inducing its degradation (24). However, recent

evidence suggests that dsRNA may not be the essential or at least not the only viral component leading to *IFN* gene expression. For instance, disruption of the gene for PKR abrogated the induction of IFN in response to dsRNA, but did not prevent responsiveness to viral infection (25–27). Moreover, cytomegalovirus, which is capable of activating IRF-dependent gene expression (20, 28), can do so from the cell surface without entering cells or replicating (29).

We have investigated the mechanisms of phosphorylation of IRF7 and its close relative, IRF3, during activation of IFN gene expression following Newcastle disease virus (NDV) infection. By all parameters tested, IRF3 and IRF7 appeared to be activated by the same mechanism. Their phosphorylation occurred with similar kinetics which required ongoing protein synthesis during viral infection, was blocked by the broad-spectrum kinase inhibitor staurosporine but not by inhibitors specific for individual kinases, and could be inhibited by the vaccinia virus protein, E3L. However, IRF3 and -7 phosphorylation was unaffected by the inactivation of the *PKR* gene, and *IFNα* gene expression was still induced in virus-infected *PKR*(−/−) and *IKKγ*−/−) cells, as well as in cells deficient for several *PKR*-related genes. These data suggest that IRF7 activation can be stimulated by a viral component other than or in addition to dsRNA, that a novel cellular kinase distinct from PKR is responsible for its phosphorylation, and that the E3L protein exerts its anti-IFN effect by inhibiting not only PKR but also the distinct IRF3/7 kinase(s).

## MATERIALS AND METHODS

*Cell Culture*—293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. *PKR*(−/−) and wild type control fibroblasts were immortalized by the 3T3 process (30) from mouse embryo fibroblasts obtained from *PKR* gene-targeted animals (26), the kind gift of Joan Durbin (Ohio State University) and John Bell (University of Ottawa, Canada), and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. *Perk*(−/−) (31), *IRE1α/β*(−/−) (32), *GCN2*(−/−) embryonic stem cells (33) and wild type control cells were the kind gift of David Ron (New York University, NY). *GCN2*(−/−) teratoma cells were derived from *GCN2*(−/−) embryonic stem cells by passage in nude mice and selection in culture in the presence of G418 (400 μg/ml). NEMO (IKKγ)-deficient 1.3E2 and control 70Z/3 cells (11) were the kind gift of Gilles Courtois (Pasteur Institute, Paris, France) and were maintained in RPMI medium supplemented with 10% fetal bovine serum and 50 μM β-mercaptoethanol.

For kinase inhibition studies, inhibitors were added to growth media 5 h post-infection, unless otherwise indicated, at the following final concentrations: staurosporine, 500 nM; genistein, 300 μM; H7, 45 μM; H8, 45 μM; acetylsalicylic acid, 5 mM; sodium salicylate, 5 mM. Dimethyl sulfoxide treatment was performed by adding 0.1% dimethyl sulfoxide by volume to growth media. Cycloheximide (75 μg/ml) was added to cells at the indicated time points post-infection. Additional inhibitors were used at the concentrations listed in Table I.

*Plasmid Constructs*—Mouse IRF3 and IRF7 (3), and E3L and K3L expression vectors (34), the kind gift of Robert Schneider (New York University, NY), were driven by the cytomegalovirus immediate-early promoter. The K167A point mutation in E3L was created by PCR-mediated site-directed mutagenesis using the following primers: sense, 5′-CGATAAGGCAGATGGAAAATCTGCTCGAGATGCTAAAAATAAT-GC-3′; antisense, 5′-GCATTATTTTTAGCATCTCGAGCAGATTTTCC-ATCTGCCTTATCG-3′. DRBP76 (35) and Staufen expression vectors (36) were the kind gifts of Ganes Sen (Cleveland Clinic, OH) and Juan Ortín (Centro Nacional de Biotecnologia, Madrid, Spain), respectively. The adenovirus VA gene was a kind gift of Robert Schneider (New York University, NY) and the MKK7(D) construct was a kind gift of Jan Vilcek (New York University).

*Transfections and Viral Infections*—293T cells were transfected by the calcium phosphate method (37). 12 h after transfection, plates of similarly transfected cells were pooled and distributed onto 60-mm plates. Cells were infected 12 h later with NDV, Manhattan strain (3), or influenza virus (38), as described. Unless otherwise noted, cells were harvested 7 h post-infection, and nuclear and cytoplasmic fractions were prepared, as described (39, 40).

*Orthophosphate Labeling*—*PKR*(−/−) fibroblasts were transfected with epitope-tagged IRF7 or IRF3 using LipofectAMINE 2000 (Life Technologies, MD). After 24 h, cells were infected with NDV, and 2 h post-infection were washed twice with phosphate-buffered saline, incubated in phosphate-free medium for 3 h, and then incubated for 2 h with 2.5 mCi/ml [$^{32}$P]orthophosphate. Cells were washed twice and lysed in whole cell lysis buffer (300 mM NaCl, 50 mM HEPES, pH 7.6, 1.5 mM MgCl$_2$, 10% glycerol, 1% Triton X-100, 10 mM Na$_4$P$_2$O$_7$, 20 mM NaF, 1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM Na$_4$VO$_3$, and protease inhibitors). Following cell lysis, the transfected protein was isolated by immunoprecipitation overnight at 4 °C using 1 μg of M2 anti-Flag antibody (Sigma), and the immunoprecipitated protein was analyzed by 8% SDS-PAGE.

*RNA Analysis*—Total RNA was prepared using 3 ml of Trizol reagent (Life Technologies, MD) per 100-mm plate. Semi-quantitative reverse transcriptase-PCR analysis was performed as previously described (3).

*Protein Analysis*—Electromobility shift assays (EMSA) were performed using nuclear extracts, as described (6, 40). Western blots were performed by standard techniques (41), except that E3L proteins were transferred to polyvinylidene difluoride with a 0.2-μm pore size. Rabbit antisera to IRF3 and IRF7 (Zymed Laboratories Inc.) were used at 0.5 μg/ml. Chicken anti-NDV (SPAFAS, North Franklin, CT) was used at a dilution of 1:1000. Monoclonal anti-E3L antibody Tw2.3 (42) was an ammonium sulfate fraction from supernatants of hybridoma cultures, the kind gift of Jonathan Yewdell (National Institutes of Health, Bethesda, MD). DRBP76 and Staufen proteins were detected using antibodies to epitope tags. *In vitro* kinase assays for IKK were performed by immunoprecipitating IKK from mouse fibroblasts, and incubating the recovered protein with 1 μg of GST fusion protein substrates in 15 μl of kinase buffer (20 mM Hepes, pH 7.6, 20 mM magnesium chloride, 20 mM β−glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 1 mM Na$_4$VO$_3$, 1 mM EDTA, plus protease inhibitors) in the presence of 10 μCi of [γ-$^{32}$P]ATP at 30 °C for 30 min, followed by analysis by 10% SDS-PAGE and autoradiography. Antibody against IKK was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). GST-IRF7 was expressed in bacteria from the pGEX-2T vector (43). GST-IκB and mutant IκB were kind gifts of Jan Vilcek (New York University).

## RESULTS

*IRF3 and IRF7 Can Be Phosphorylated with Equivalent Kinetics during NDV Infection*—IRF3 is constitutively expressed in cells while IRF7 is induced in response to an early wave of IFN production following viral infection (44). This differential expression complicates the question of whether these two proteins can be activated by the same kinase. To alleviate this technical shortcoming, we ectopically expressed IRF3 or IRF7 by transient transfection so that expression would be normalized under the control of an artificial promoter and phosphorylation could be measured independent from protein induction. Infection of transfected cells with NDV led to phosphorylation, as judged by a mobility shift following SDS-PAGE that is dependent on phosphorylation (3, 6, 14, 16), of both IRF7 (Fig. 1*A*) and IRF3 (Fig. 1*B*). Moreover, both proteins were activated with equivalent kinetics, becoming phosphorylated at 5 h post-infection and showing maximal phosphorylation 7–8 h post-infection that remained high for several hours (data not shown). Acquisition of DNA binding activity, as judged by EMSA, correlated precisely with phosphorylation for both proteins (data not shown). We validated this transfection system by examining the kinetics of phosphorylation of endogenous IRF3 (Fig. 1*D*) which was phosphorylated with the same kinetics as the ectopically expressed protein. Therefore, the sequential activation of IRF3- and IRF7-target genes normally observed in untransfected cells following NDV infection can be accounted for by the requirement for induction of the IRF7 protein rather than by utilization of distinct kinases.

Considerable viral protein synthesis occurred within the first 7 h of infection, as judged by immunoblotting for viral proteins using antisera directed against NDV virions (Fig. 1*C*). This observation prompted us to consider whether protein synthesis, including viral protein synthesis, might be involved in IRF activation. To test this notion, protein synthesis was blocked by
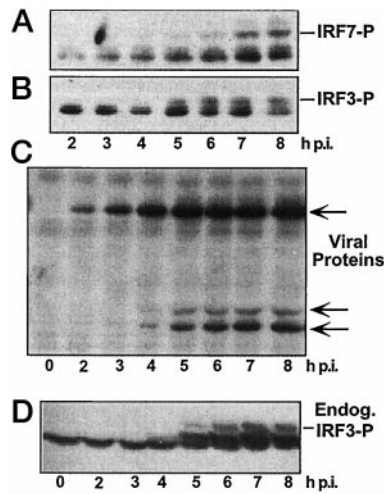
FIG. 1. **Kinetics of IRF phosphorylation.** *A,* kinetics of IRF7 phosphorylation. 293T cells transfected with 10 μg of an IRF7 expression construct were infected with NDV 12 h later, and nuclear extracts harvested at the indicated times post-infection were analyzed for IRF7 phosphorylation by SDS-PAGE and immunoblotting. *B,* kinetics of IRF3 phosphorylation. Cells were transfected with 10 μg of IRF3 and were analyzed as described in *A. C,* kinetics of NDV protein accumulation. Nuclear extracts from NDV-infected 293T cells were analyzed by immunoblotting for virion proteins. The 0-h time point represents uninfected cell extract. *D,* kinetics of phosphorylation of endogenous IRF3 parallel those for ectopically expressed protein. Whole cell extracts from mouse 3T3 cells infected with NDV for various times, as indicated, were analyzed by SDS-PAGE and immunoblotting. *h.p.i.*, hours post-infection.

addition of cycloheximide at various times after infection. Inhibition of protein synthesis at any point up until ~3 h post-infection prevented phosphorylation of IRF7 (Fig. 2, *lanes 3–5*). However, addition of cycloheximide after that point was without effect (Fig. 2, *lanes 6–9*). Consistent with the requirement of phosphorylation of IRF3 (16) and IRF7 for acquisition of DNA binding (6), cycloheximide added early in infection also blocked IRF7 activity as measured by EMSA (data not shown). These data suggest that proteins accumulating during the first 3 h of NDV infection are required for activation of the IRF7 kinase. Since no serine kinases are encoded by NDV (45), it is likely that viral proteins are involved either directly or indirectly in activation of a cellular kinase that targets IRF7. Alternatively, cycloheximide might block synthesis of a short-lived cellular protein.

*No Specific Kinase Inhibitor Blocks IRF7 Phosphorylation—* Cellular kinases potentially involved in IRF phosphorylation were investigated initially by use of pharmacological inhibitors. To distinguish a direct effect of inhibition on an IRF kinase from a possible indirect effect caused by, for instance, inhibition of necessary viral protein synthesis, pharmacological inhibitors were added late in infection (5 h post-infection), just prior to the observed appearance of phosphorylated protein and at a time point at which ongoing protein synthesis was no longer required. IRF7 phosphorylation was blocked by staurosporine (Fig. 3*A, lane 4*), a broad-spectrum kinase inhibitor previously shown to block phosphorylation of IRF1 in virus-infected cells (46). IRF7 phosphorylation was also reduced by the related inhibitor, K252a (Table I). However, none of the more specific kinase inhibitors tested were capable of preventing IRF phosphorylation (Fig. 3*A* and Table I). These included inhibitors of PKR (2-amino purine (47)) and of IKK (acetylsalacylic acid and salacylic acid (48)), kinases known to be activated in response to virus infection and involved in activation of NFκB. Other agents ineffective in blocking IRF phosphorylation included inhibitors of PKC, PKA, p38, extracellular kinase, phosphatidylinositol 3-kinase, and tyrosine kinases. The
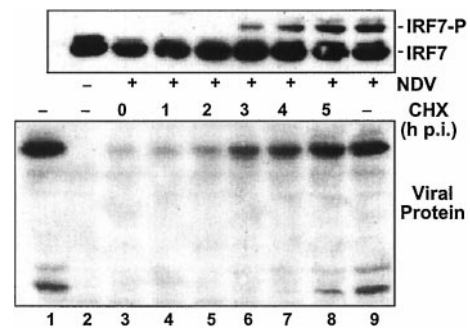


FIG. 2. **Cycloheximide (*CHX*) inhibition of IRF7 phosphorylation.** 293T cells were transfected with 10 μg of an IRF7 expression construct, infected with NDV as indicated, and cycloheximide (75 μg/ml) was added to growth media at the indicated times (h) post-infection. *Lanes 1, 2,* and *9* did not receive cycloheximide treatment. Cells were harvested at 7 h post-infection and nuclear extracts were analyzed for IRF7 phosphorylation (*upper panel*) and viral protein accumulation (*lower panel*). *Lane 1* (*lower panel*) represents mock-transfected cells infected with NDV for 7 h.
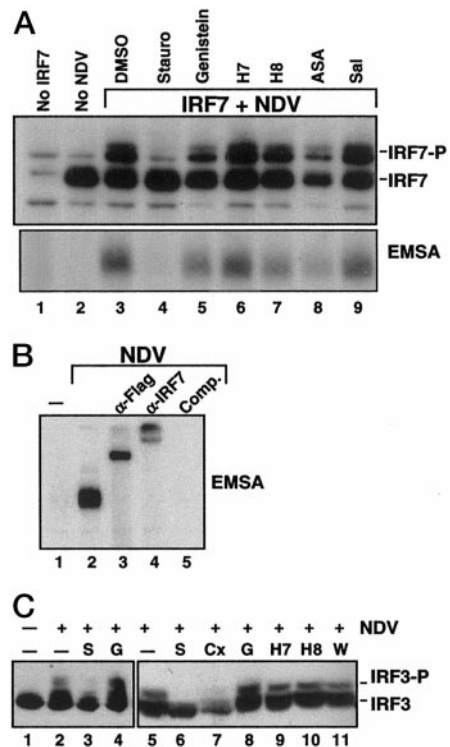


FIG. 3. **IRF phosphorylation is inhibited by staurosporine.** *A,* 293T cells were transfected with 10 μg of an IRF7 expression construct, infected with NDV, and the indicated treatment was added to growth media at 5 h post-infection. Cells were harvested at 7 h post-infection and nuclear extracts were analyzed for IRF7 by SDS-PAGE (*upper panel*) and EMSA (*lower panel*). *B,* IRF7-DNA complex formed in response to NDV infection of transfected 293T cells (*lane 2*) was specifically competed by antibody against an epitope tag (anti-Flag, *lane 3*) or against native IRF7 (*lane 4*) and by excess binding site competitor (*lane 5*). *C,* 293T cells (*lanes 1–4*) were transfected with 10 μg of an IRF3 expression construct and treated as described in *A.* Inhibition by staurosporine (*S*) and not by genistein (*G*) is shown. 3T3 cells (*lanes 5–11*) were infected with NDV without (*lane 5*) or in the presence of staurosporine (*lane 6*), cycloheximide (*lane 7*), genistein (*lane 8*), H7 (*lane 9*), H8 (*lane 10*), or wortmanin (*lane 11*), and then analyzed for endogenous IRF3 phosphorylation. All inhibitors were added 5 h post-infection, with the exception of cycloheximide which was added simultaneously with virus.

phosphorylation status of IRF7 correlated with its ability to bind DNA, as detected by EMSA (Fig. 3*A, lower panel*). Specificity of DNA binding was confirmed by antibody and DNA competition reactions (Fig. 3*B*) using anti-Flag (*lane 3*), anti-

TABLE I
*Inhibitors tested*

| Treatment (primary target/effect) | Concentration[a] | Inhibition[b] |
|---|---|---|
| Kinase inhibitors | | |
| Staurosporine (kinases) | 500 nM | + |
| K252a (kinases) | 1 μM | +/− |
| KT5823 (kinases) | 1 μM | − |
| Genistein (tyrosine kinases) | 300 μM | − |
| Herbimycin (tyrosine kinases) | 2 μM | − |
| HA1004 (cAMP/cGMP kinases) | 45 μM | − |
| H7 (PKC) | 45 μM | − |
| H8 (PKA) | 45 μM | − |
| 2-Aminopurine (PKR) | 10 mM | − |
| Acetylsalicylic acid (IKK) | 5 mM | − |
| Sodium salicylate (IKK) | 5 mM | − |
| Wortmanin (PI3K) | 100 nM | − |
| LY294002 (PI3K) | 25 μM | − |
| PD98059 (MEK1) | 50 μM | − |
| SB203580 (p38) | 10 μM | − |
| Other pharmacological agents | | |
| Tunicamycin (glycosylation) | 2.5 μg/ml | − |
| MG132 (26 S proteasome) | 7.5 μM | − |
| DRB[c] (RNA polymerase) | 100 μM | − |
| Actinomycin D (DNA polymerase) | 5 μg/ml | − |
| Methyl methanesulfonate (DNA damage) | 100 μg/ml | − |
| Cycloheximide (protein synthesis) | 75 μg/ml | + |
| BAPTA-AM[d] (calcium ions) | 40 μg/ml | − |
| Okadaic acid (PP2A) | 1 μM | − |
| Other[e] | | |
| MKK7(D) (activated MKK) | | − |
| E3L (PKR) | | + |
| K3L (PKR) | | − |
| VA (PKR) | | − |
| NS1[f] (PKR) | | + |

[a] Maximum concentration tested.

[b] Inhibition of IRF7 phosphorylation in response to NDV infection is indicated by (+). K252a treatment (+/−) resulted in partial inhibition at the highest concentration tested. None of these agents caused IRF7 phosphorylation in the absence of NDV infection.

[c] 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole.

[d] 1,2-Bis(2-aminophenoxy)ethane-$N,N,N',N'$-tetraacetic acid tetrakis(acetooxymethyl ester).

[e] Agents tested by transfection or infection.

[f] NS1 inhibition was deduced from the higher level of IRF7 phosphorylation induced after infection by NS1-deficient influenza virus relative to wild type virus.

IRF7 (*lane 4*), or homologous DNA competitor (*lane 5*). IRF3 phosphorylation was also blocked by staurosporine treatment (Fig. 3C, *lane 3*) but not by any other inhibitors tested, and none of the pharmacologic agents tested led to IRF phosphorylation in the absence of infection (data not shown). Phosphorylation of ectopically expressed IRF3 paralleled that of the endogenous protein whose phosphorylation was blocked by staurosporine and cycloheximide (Fig. 3C, *lanes 6* and *7*) but not by genistein, H7, H8, or wortmanin (*lanes 8–11*).

Another technique used to probe the nature of the IRF kinase involved a search for agents that might block a virally activated cellular signaling step, or, alternatively, that might activate IRF7 phosphorylation in the absence of viral infection. We treated cells with DNA damaging agents, calcium chelators, proteosome inhibitors, phosphatase inhibitors, and activators of the unfolded protein response. We also overexpressed a constitutively active form of MKK7, an activator of the JNK pathway (49, 50). None of these treatments caused IRF7 phosphorylation in the absence of viral infection nor prevented IRF7 activation when added to virus-infected cells, allowing us to rule out involvement of the known stress- and virus-activated signaling pathways in stimulating IRF7 phosphorylation (Table I).
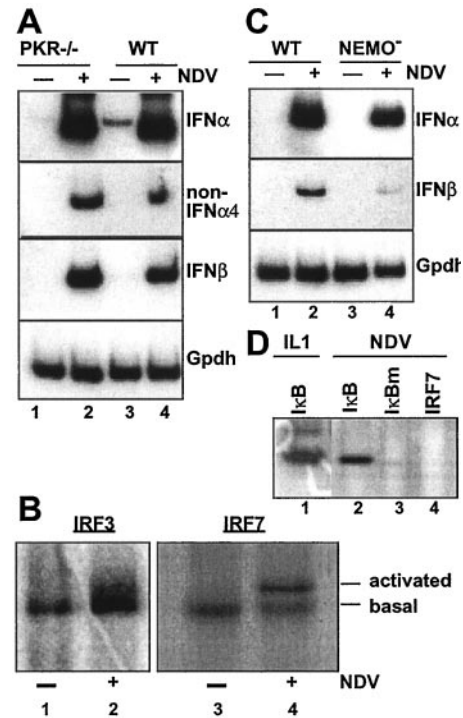


FIG. 4. **PKR and IKK are not responsible for IFNα gene induction or IRF phosphorylation.** *A, PKR*(−/−) and wild type cells were infected with NDV for 9 h, as indicated, and expression of IFN genes was measured by RT-PCR, as indicated. *B, PKR*(−/−) mouse fibroblasts were transfected with 20 μg of IRF3 or IRF7 expression constructs, as indicated, labeled with [³²P]orthophosphate, and IRF proteins were recovered by immunoprecipitation with anti-Flag antibodies and analyzed by SDS-PAGE and autoradiography. Positions of basal and activated IRF isoforms are indicated. *C,* wild type and *NEMO* (IKKγ)-deficient cells were infected with NDV as indicated and assayed for *IFNα* and *IFNβ* gene expression by RT-PCR, as indicated. *D,* activated IKK was recovered from interleukin 1 (*IL1*)-treated (*lane 1*) or NDV-infected mouse fibroblasts (*lanes 2–4*) by immunoprecipitation and incubated with GST-IκB (*lanes 1* and *2*), mutant IκB (*lane 3*), or GST-IRF7 (*lane 4*) in the presence of [³²P]ATP, followed by analysis by SDS-PAGE and autoradiography. Gpdh, glyceraldehyde-3-phosphate dehydrogenase.

*Genetic Evidence for Lack of Involvement of PKR and IKK*— Because both PKR and IKK are known to be activated in response to viral infection, we sought additional evidence for their apparent lack of involvement in IRF phosphorylation by examining mutant cell lines lacking these enzyme activities. Embryo fibroblasts were prepared from mice devoid of the *PKR* gene due to gene targeting (26). Induction of *type I IFN* genes following NDV infection was assessed as a measure of endogenous IRF phosphorylation. *IFNα*4 and *IFNβ* expression, dependent on IRF3 phosphorylation, and expression of additional members of the *IFNα* gene family (non-*IFNα*4 genes), dependent on IRF7 phosphorylation, were detected in approximately equal abundance in both wild type and *PKR*-null cells (Fig. 4A). Similar results were obtained using *PKR*-null cells derived from a different mouse strain that sustained a distinct mutation (27), corroborating the results obtained with inhibitors (data not shown). However, *IFNα* induction in response to dsRNA as opposed to virus infection was impaired in *PKR*-null cells (data not shown), as previously reported (25).

Phosphorylation of IRF3 and IRF7 was directly tested in virus-infected, *PKR*-null cells. Flag epitope-tagged IRF3 or IRF7 were transfected into *PKR*-null cells which were subsequently infected with NDV and labeled with [³²P]inorganic phosphate. Protein extracts were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. Phosphorylated IRF3 or IRF7 was recovered from *PKR*-mutant cells (Fig. 4B),
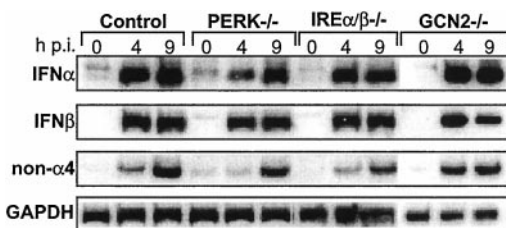
FIG. 5. **PKR related kinases are not required for *IFN* gene induction by NDV infection.** Cells from the indicated mouse cell lines were infected with NDV, and total cellular RNA prepared at the indicated hours post-infection (*h.p.i.*) was analyzed for *IFN* gene induction by RT-PCR. *0 h* represents uninfected cells. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

demonstrating the PKR independence of this process. It should be noted that both IRF3 and IRF7 are basally phosphorylated in the absence of viral infection and become additionally phosphorylated in response to infection, as indicated by their change in electrophoretic mobility. Neither basal nor induced phosphorylation was inhibited by the absence of the *PKR* gene.

Another enzyme known to be activated in response to virus infection and required for activation of NF$\kappa$B is IKK (12). To confirm the inhibitor studies that suggested the lack of its involvement in IRF phosphorylation, we took advantage of a mutant cell line lacking the IKK$\gamma$/NEMO subunit that is therefore completely defective for IKK activity (11). Consistent with a lack of requirement for IKK activity for IRF phosphorylation and no requirement of NF$\kappa$B function for *IFN$\alpha$* gene transcription, *IFN$\alpha$* gene expression was induced normally in NDV-infected mutant cells (Fig. 4*C*). As expected, *IFN$\beta$* induction was impaired (Fig. 4*C, lane 4*), due to its demonstrated requirement for NF$\kappa$B (51). The modest reduction in *IFN$\alpha$* gene induction is likely due to the absence of IFN$\beta$-dependent positive feedback (3, 52). Finally, we asked whether IKK was capable of phosphorylating IRF7, even though it was not required. Activated IKK was immunoprecipitated from extracts of virus-infected cells and tested for its ability to phosphorylate IRF7 *in vitro*. Although phosphorylation of I$\kappa$B was readily detected with virus-activated IKK (Fig. 4*D, lane 2*) to levels similar to that achieved with IKK isolated from interleukin 1$\beta$-treated cells (Fig. 4*D, lane 1*), no phosphorylation of IRF7 was observed using the virus-activated kinase (Fig. 4*D, lane 4*). A mutant form of I$\kappa$B in which serine residues 23 and 25 were changed to alanine (I$\kappa$Bm) served as specificity control for activation of IKK (Fig. 4*D, lane 3*).

Several genes have been isolated recently that bear homology to *PKR*. One possibility raised by the ability of *PKR*-null cells to phosphorylate IRF proteins in response to virus infection while being deficient in response to dsRNA might be the existence of *PKR*-related enzymes that are activated by a viral component other than dsRNA. To test this notion, we examined *IFN* gene expression in response to virus infection of cells deficient for individual *PKR*-related genes due to gene targeting. Cells deficient for *PERK*, an eIF2$\alpha$ kinase (31), for *IRE1$\alpha$* and -$\beta$, enzymes involved in activation of JNK (32), or for mouse *GCN2*, another eIF2$\alpha$ kinase (33, 53, 54), were all capable of producing *IFN$\alpha$*, *IFN$\beta$*, and the *non-IFN$\alpha$4* subset of *IFN$\alpha$* genes in response to viral infection (Fig. 5). Therefore, none of these PKR-related kinases is individually required for *IFN* gene induction and is unlikely to be a required IRF kinase.

*IRF7 Phosphorylation Is Blocked by Vaccinia Virus E3L Protein*—Viruses maintain numerous strategies to evade host innate immune defenses, including inhibition of PKR (55). Three such viral inhibitors that target inhibition of PKR are vaccinia virus E3L and K3L proteins and adenovirus VA RNA (56–62). To test the potential of these genes as inhibitors of

IRF phosphorylation, cells were co-transfected with E3L, K3L, or VA expression constructs along with IRF3 or IRF7, infected with NDV, and assayed for IRF phosphorylation by SDS-PAGE. Expression of K3L or VA were without effect (Fig. 6*A,* and data not shown); in contrast, expression of E3L blocked IRF7 phosphorylation in a dose-dependent manner (Fig. 6*A, top panel*). Coexpression of E3L with IRF7 also blocked its ability to bind DNA following isolation from virus-infected cells (Fig. 6*A, lower panel*), likely a reflection of the lack of phosphorylation. Coexpression of K3L was without effect in either assay (Fig. 6*A*).

E3L binds dsRNA and has been thought of as a competitive inhibitor of PKR that sequesters dsRNA (56, 60) and directly inhibits PKR through protein-protein interaction following binding to dsRNA (63, 64). We tested the requirement of dsRNA binding for the inhibition of IRF7 phosphorylation by E3L using a point mutant version of E3L, in which lysine 167 was converted to alanine (K167A), and is incapable of binding dsRNA (65). Loss of RNA binding by E3L disrupted its ability to inhibit IRF7 phosphorylation (Fig. 6*B, lane 6*). To evaluate whether direct sequestering of dsRNA might be the sole mechanism of the inhibitory function of E3L, we tested two additional dsRNA-binding proteins. Expression of neither DRBP76 (35) nor mammalian Staufen (66) prevented IRF7 phosphorylation, nor did they block the inhibitory action of E3L (Fig. 6*C* and data not shown), despite these proteins being implicated in regulation of PKR and viral infection (35, 36, 66). Additionally, E3L but not K3L effectively inhibited IRF3 phosphorylation following NDV infection (Fig. 6*D*). Therefore, it is likely that E3L directly inhibits a cellular kinase that targets both IRF3 and IRF7 rather than functioning merely to sequester dsRNA, although its ability to bind dsRNA is necessary for this inhibitory function, as it is for inhibition of PKR.

## DISCUSSION

Activation of *IFN* gene expression is a cellular response resulting from innate immune recognition of virus infection, but how cells sense viral replication is only poorly characterized. While production of viral products such as dsRNA play a role in activation of IKK and possibly of JNK (12), the data reported here suggest that additional signals stimulate phosphorylation of IRF3 and IRF7, essential events in induction of the protective IFN response. This evidence is severalfold. First, the known target of dsRNA in mammalian cells, PKR, was not required for stimulation of IRF3 or -7 phosphorylation. Neither pharmacological inhibitors of PKR nor ablation of the *PKR* gene resulted in impaired IRF phosphorylation. Second, while *PKR*-mutant cells were defective in responses to dsRNA, they retained responsiveness to viral infection, demonstrating that infection provided additional signals beyond simply dsRNA. Finally, protein synthesis during viral infection was required for IRF phosphorylation, and the kinetics of phosphorylation and of the required protein synthesis correlated well with the major synthesis of viral proteins. Therefore, it is likely that newly synthesized viral proteins play a necessary role in activation of the cellular IRF kinase. A model for regulation of IRF phosphorylation is shown in Fig. 7. Viral infection leads to production of dsRNA and activation of PKR. Infection also leads to an independent activation event in a protein synthesis-dependent manner, targeting a novel cellular kinase (X) and leading to IRF phosphorylation. dsRNA-activated PKR might directly phosphorylate IRF or might activate the same kinase X, leading to IRF phosphorylation. We cannot rule out the possibility that the required viral protein synthesis induces a secondary product (possibly an RNA) that is the ultimate activator of the IRF kinase. It is also possible that synthesis of a cellular protein is necessary during viral infection. In either
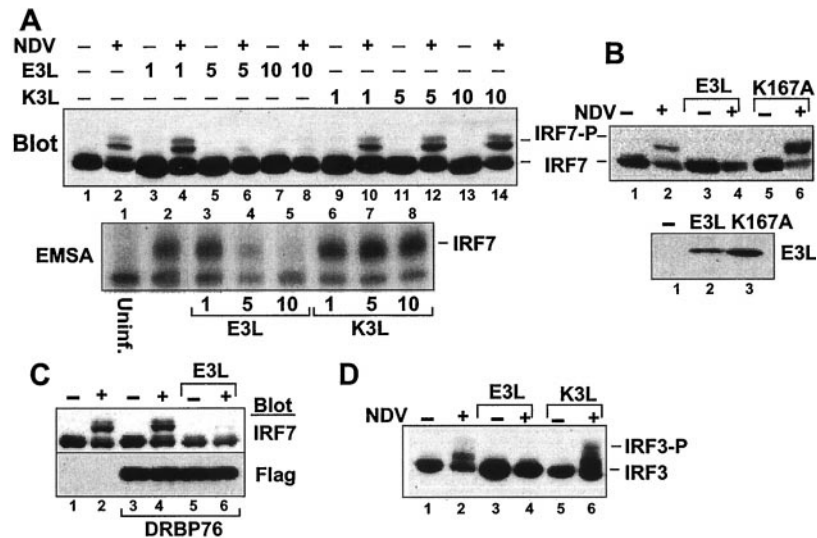
FIG. 6. **IRF7 phosphorylation is inhibited by E3L.** *A,* dose-dependent inhibition of IRF7 phosphorylation by E3L. 293T cells were transfected with an IRF7 expression vector and the indicated amount (*μg*) of E3L or K3L expression constructs and infected with NDV, as indicated. Nuclear extracts were analyzed for activation of IRF7 by SDS-PAGE (*upper panel*). Nuclear extracts from uninfected (*lane 1*) or NDV-infected cells (*lanes 2–8*) that had been co-transfected with IRF7 and the indicated amounts of E3L or K3L were analyzed by EMSA (*lower panel*). *B,* dsRNA binding is necessary but not sufficient to inhibit IRF7 phosphorylation. 293T cells were transfected with 10 μg of the following expression constructs: IRF7 (all lanes), E3L (*lanes 3 and 4* and *11 and 12*) and E3L-K167A (*lanes 5 and 6*), infected with NDV, as indicated, and nuclear extracts harvested 7 h post-infection were analyzed for IRF7 (*upper panel*), E3L (*lower panel*). *Lanes 1–3* of the *lower left panel* correspond to the NDV infected lanes in the *upper panel* (*lanes, 2, 4,* and *6*). *C,* DRBP76 does not inhibit IRF7 phosphorylation. Cells were analyzed as described for *panel B* following transfection with Flag-tagged DRBP76, as indicated. *D,* IRF3 phosphorylation is inhibited by E3L. 293T cells were transfected with 10 μg of an IRF3 expression vector and 10 μg of E3L (*lanes 3 and 4*) or K3L (*lanes 5 and 6*), infected with NDV, as indicated, and nuclear extracts were analyzed for IRF3 by immunoblotting.
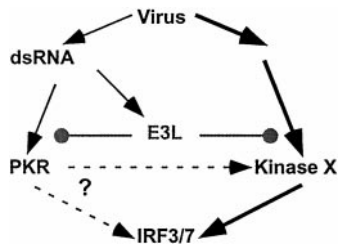


FIG. 7. **Proposed model of IRF3 and -7 phosphorylation.** Virus-induced dsRNA activates PKR while a second, protein synthesis-dependent step activates an unknown kinase X which phosphorylates IRF3 and -7. Activated PKR either directly or indirectly through kinase X also leads to IRF3 and IRF7 phosphorylation. E3L protein blocks both PKR and kinase X in a dsRNA-dependent fashion.

case, these events are independent of PKR and its close homologues PERK, IRE1α/β, and GCN2.

Our data are consistent with a single kinase targeting both IRF3 and IRF7. By studying transfected cells, we avoided the requirement for IRF7 induction that normally limits its action to a secondary wave of IFN production (3). Under these experimental conditions, both IRF3 and IRF7 were phosphorylated with the same kinetics, and endogenous IRF3 was phosphorylated with similar kinetics, validating the transfection system. Moreover, IRF3 and IRF7 phosphorylation showed the same sensitivity and resistance to a panel of pharmacological agents. Indeed, only the broad-spectrum kinase inhibitors staurosporine and to a lesser extent K252a were effective IRF kinase inhibitors. Although staurosporine was first characterized as an inhibitor of PKC, other inhibitors of PKC or down-regulation of PKC protein by prolonged exposure to phorbol ester were ineffective in preventing IRF phosphorylation. Other cellular stress responses, such as induction of the unfolded protein response or stimulation of MAP kinase cascades, did not appear to play a role in the IRF response to virus, suggesting that a novel cellular signaling pathway allows the innate immune system to recognize viral infection. Hiscott and colleagues have recently reached a similar conclusion (67).

Despite the lack of evidence for dsRNA and PKR involvement in IRF phosphorylation by virus, we found that the vaccinia virus PKR inhibitor E3L, but not K3L, was an effective IRF phosphorylation inhibitor (Fig. 6). Moreover, the ability of E3L to bind RNA was necessary for its anti-IRF action. These data suggest that dsRNA is one and possibly an essential aspect of the recognition of viral infection. However, while the action of dsRNA alone is dependent on the activity of PKR, the action of dsRNA in the context of other components of viral replication acted in a PKR-independent manner, although it remained sensitive to E3L inhibition. These findings suggest that an enzyme related to PKR could be a likely candidate for the virus-activated IRF kinase; however, none of the known *PKR*-related genes tested was individually required, including *PERK, IRE1α, IRE1β,* and *GCN2.* It remains a possibility that another PKR-related enzyme, for instance, HRI (68) or an as yet to be identified related kinase, is the IRF kinase. Alternatively, a combination of these genes operating in a redundant fashion could have obscured the requirement for any one individual enzyme.

Whether IRF phosphorylation depends on a novel gene or a combination of PKR-related enzymes, it is likely that E3L protein directly inhibits the catalytic function of such an enzyme, as it does PKR. Inhibition by E3L likely required the presence of dsRNA, possibly serving to activate the inhibitory function of E3L. However, sequestering of dsRNA alone is probably insufficient to inhibit IRF phosphorylation since other RNA-binding proteins were unable to mimic this action of E3L. Interestingly, the non-RNA binding amino terminus of E3L is required for full inhibition of PKR (63, 64) and must interact with PKR, not simply sequester its activator dsRNA to block catalytic activity. The amino terminus of E3L also induces multimerization (69) that may be necessary for its function. In contrast, the K3L protein inhibits PKR catalysis by acting as a psuedo-substrate, mimicking the PKR substrate eIF2α and blocking the catalytic site of the enzyme. The finding that K3L was not an effective inhibitor of the IRF3/7 kinase suggests a

substrate specificity distinct from PKR. The ability of these two viral proteins to target independent aspects of the IFN pathway may explain why both genes have been maintained in the vaccinia virus genome.

The action of E3L to inhibit IRF3 and IRF7 phosphorylation in response to NDV infection is reminiscent of the recently reported action of the influenza viral protein NS1 to inhibit IRF3 nuclear translocation (70). Indeed, we also observed inhibition of IRF7 phosphorylation in the presence of NS1 protein (Table I). We would speculate that both E3L and NS1, and possibly additional, virally-encoded inhibitors of IFN induction, function by binding and inhibiting a cellular IRF protein kinase which is activated in response to dsRNA plus additional viral components.

## REFERENCES

1. Pestka, S., Langer, J. A., Zoon, K. C., and Samuel, C. E. (1987) *Annu. Rev. Biochem.* **56**, 727–777
2. Doly, J., Civas, A., Navarro, S., and Uze, G. (1998) *Cell. Mol. Life Sci.* **54**, 1109–1121
3. Marié, I., Durbin, J. E., and Levy, D. E. (1998) *EMBO J.* **17**, 6660–6669
4. Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T., and Tanaka, N. (1998) *FEBS Lett.* **441**, 106–110
5. Thanos, D., and Maniatis, T. (1995) *Cell* **83**, 1091–1100
6. Marié, I., Smith, E., Prakash, A., and Levy, D. E. (2000) *Mol. Cell. Biol.* **20**, 8803–8814
7. Yeow, W. S., Au, W. C., Juang, Y. T., Fields, C. D., Dent, C. L., Gewert, D. R., and Pitha, P. M. (2000) *J. Biol. Chem.* **275**, 6313–6320
8. Lin, R., Genin, P., Mamane, Y., and Hiscott, J. (2000) *Mol. Cell. Biol.* **20**, 6342–6353
9. Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S., Tanaka, N., and Taniguchi, T. (2000) *Immunity* **13**, 539–548
10. Karin, M. (1999) *J. Biol. Chem.* **274**, 27339–27342
11. Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998) *Cell* **93**, 1231–1240
12. Chu, W. M., Ostertag, D., Li, Z. W., Chang, L., Chen, Y., Hu, Y., Williams, B., Perrault, J., and Karin, M. (1999) *Immunity* **11**, 721–731
13. Iordanov, M. S., Wong, J., Bell, J. C., and Magun, B. E. (2001) *Mol. Cell. Biol.* **21**, 61–72
14. Lin, R., Heylbroeck, C., Pitha, P. M., and Hiscott, J. (1998) *Mol. Cell. Biol.* **18**, 2986–2996
15. Sato, M., Tanaka, N., Hata, N., Oda, E., and Taniguchi, T. (1998) *FEBS Lett.* **425**, 112–116
16. Yoneyama, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E., and Fujita, T. (1998) *EMBO J.* **17**, 1087–1095
17. Weaver, B. K., Kumar, K. P., and Reich, N. C. (1998) *Mol. Cell. Biol.* **18**, 1359–1368
18. Schafer, S. L., Lin, R., Moore, P. A., Hiscott, J., and Pitha, P. M. (1998) *J. Biol. Chem.* **273**, 2714–2720
19. Wathelet, M. G., Lin, C. H., Parekh, B. S., Ronco, L. V., Howley, P. M., and Maniatis, T. (1998) *Mol. Cell* **1**, 507–518
20. Navarro, L., Mowen, K., Rodems, S., Weaver, B., Reich, N., Spector, D., and David, M. (1998) *Mol. Cell. Biol.* **18**, 3796–3802
21. Au, W. C., Moore, P. A., LaFleur, D. W., Tombal, B., and Pitha, P. M. (1998) *J. Biol. Chem.* **273**, 29210–29217
22. Juang, Y., Lowther, W., Kellum, M., Au, W. C., Lin, R., Hiscott, J., and Pitha, P. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9837–9842
23. Jacobs, B. L., and Langland, J. O. (1996) *Virology* **219**, 339–349
24. Alcami, A., and Koszinowski, U. H. (2000) *Immunol. Today* **21**, 447–455
25. Kumar, A., Yang, Y. L., Flati, V., Der, S., Kadereit, S., Deb, A., Haque, J., Reis, L., Weissmann, C., and Williams, B. R. (1997) *EMBO J.* **16**, 406–416
26. Abraham, N., Stojdl, D. F., Duncan, P. I., Methot, N., Ishii, T., Dube, M., Vanderhyden, B. C., Atkins, H. L., Gray, D. A., McBurney, M. W., Koromilas, A. E., Brown, E. G., Sonenberg, N., and Bell, J. C. (1999) *J. Biol. Chem.* **274**, 5953–5962
27. Yang, Y. L., Reis, L. F., Pavlovic, J., Aguzzi, A., Schafer, R., Kumar, A., Williams, B. R., Aguet, M., and Weissmann, C. (1995) *EMBO J.* **14**, 6095–6106
28. Zhu, H., Cong, J. P., and Shenk, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 13985–13990
29. Boyle, K. A., Pietropaolo, R. L., and Compton, T. (1999) *Mol. Cell. Biol.* **19**, 3607–3613
30. Aaronson, S. A., and Todaro, G. J. (1968) *J. Cell. Physiol.* **72**, 141–148
31. Harding, H., Zhang, Y., Bertolotti, A., Zeng, H., and Ron, D. (2000) *Mol. Cell* **5**, 897–904
32. Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H., and Ron, D. (2000) *Science* **287**, 664–666
33. Harding, H., Novoa, I., Zhang, Y., Zeng, H., Schapira, M., and Ron, D. (2000) *Mol. Cell* **6**, 1099–1108
34. Feigenblum, D., Walker, R., and Schneider, R. J. (1998) *J. Virol.* **72**, 9257–9266
35. Patel, R. C., Vestal, D. J., Xu, Z., Bandyopadhyay, S., Guo, W., Erme, S. M., Williams, B. R., and Sen, G. C. (1999) *J. Biol. Chem.* **274**, 20432–20437
36. Marion, R. M., Fortes, P., Beloso, A., Dotti, C., and Ortin, J. (1999) *Mol. Cell. Biol.* **19**, 2212–2219
37. Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745–2752
38. García-Sastre, A., Egorov, A., Matassov, D., Brandt, S., Levy, D. E., Durbin, J. E., Palese, P., and Muster, T. (1998) *Virology* **252**, 324–330
39. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489
40. Levy, D. E. (1998) *Methods* **15**, 167–174
41. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
42. Yuwen, H., Cox, J. H., Yewdell, J. W., Bennink, J. R., and Moss, B. (1993) *Virology* **195**, 732–744
43. Smith, D. B., and Johnson, K. S. (1988) *Gene* (*Amst.*) **67**, 31–40
44. Nguyen, H., Hiscott, J., and Pitha, P. M. (1997) *Cytokine & Growth Factor Rev.* **8**, 293–312
45. de Leeuw, O., and Peeters, B. (1999) *J. Gen. Virol.* **80**, 131–136
46. Watanabe, N., Sakakibara, J., Hovanessian, A. G., Taniguchi, T., and Fujita, T. (1991) *Nucleic Acids Res.* **19**, 4421–4428
47. Hu, Y., and Conway, T. W. (1993) *J. Interferon Res.* **13**, 323–328
48. Yin, M. J., Yamamoto, Y., and Gaynor, R. B. (1998) *Nature* **396**, 77–80
49. Holland, P. M., Suzanne, M., Campbell, J. S., Noselli, S., and Cooper, J. A. (1997) *J. Biol. Chem.* **272**, 24994–24998
50. Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 7337–7342
51. Lenardo, M. J., Fan, C. M., Maniatis, T., and Baltimore, D. (1989) *Cell* **57**, 287–294
52. Erlandsson, L., Blumenthal, R., Eloranta, M. L., Engel, H., Alm, G., Weiss, S., and Leanderson, T. (1998) *Curr. Biol.* **8**, 223–226
53. Sood, R., Porter, A. C., Olsen, D. A., Cavener, D. R., and Wek, R. C. (2000) *Genetics* **154**, 787–801
54. Berlanga, J. J., Santoyo, J., and De Haro, C. (1999) *Eur. J. Biochem.* **265**, 754–762
55. Levy, D. E., and Garcia-Sastre, A. (2000) *Cytokine Growth Factor Rev.,* in press
56. Davies, M. V., Chang, H. W., Jacobs, B. L., and Kaufman, R. J. (1993) *J. Virol.* **67**, 1688–1692
57. Shors, S. T., Beattie, E., Paoletti, E., Tartaglia, J., and Jacobs, B. L. (1998) *J. Interferon Cytokine Res.* **18**, 721–729
58. Shors, T., Kibler, K. V., Perkins, K. B., Seidler-Wulff, R., Banaszak, M. P., and Jacobs, B. L. (1997) *Virology* **239**, 269–276
59. Chang, H. W., Uribe, L. H., and Jacobs, B. L. (1995) *J. Virol.* **69**, 6605–6608
60. Chang, H. W., Watson, J. C., and Jacobs, B. L. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 4825–4829
61. Jagus, R., and Gray, M. M. (1994) *Biochimie* (*Paris*) **76**, 779–791
62. O'Malley, R. P., Mariano, T. M., Siekierka, J., and Mathews, M. B. (1986) *Cell* **44**, 391–400
63. Sharp, T. V., Moonan, F., Romashko, A., Joshi, B., Barber, G. N., and Jagus, R. (1998) *Virology* **250**, 302–315
64. Romano, P. R., Zhang, F., Tan, S. L., Garcia-Barrio, M. T., Katze, M. G., Dever, T. E., and Hinnebusch, A. G. (1998) *Mol. Cell. Biol.* **18**, 7304–7316
65. Ho, C. K., and Shuman, S. (1996) *J. Virol.* **70**, 2611–2614
66. Falcon, A. M., Fortes, P., Marion, R. M., Beloso, A., and Ortin, J. (1999) *Nucleic Acids Res.* **27**, 2241–2247
67. Servant, M. J., ten Oever, B., LePage, C., Conti, L., Gessani, S., Julkunen, I., Lin, R., and Hiscott, J. (2001) *J. Biol. Chem.* **276**, 355–363
68. Berlanga, J. J., Herrero, S., and de Haro, C. (1998) *J. Biol. Chem.* **273**, 32340–32346
69. Ho, C. K., and Shuman, S. (1996) *Virology* **217**, 272–284
70. Talon, J., Horvath, C. M., Polley, R., Basler, C. F., Muster, T., Palese, P., and Garcia-Sastre, A. (2000) *J. Virol.* **74**, 7989–7996

**IRF3 and IRF7 Phosphorylation in Virus-infected Cells Does Not Require Double-stranded RNA-dependent Protein Kinase R or I κB Kinase but Is Blocked by Vaccinia Virus E3L Protein**

Eric J. Smith, Isabelle Marié, Arun Prakash, Adolfo Garci?a-Sastre and David E. Levy

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