Select Paramyxoviral V Proteins Inhibit IRF3 Activation by Acting as Alternative Substrates for Inhibitor of κB Kinase ε (IKKe)/TBK1*

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Lenette L. Lu†§, Mamta Puri§, Curt M. Horvath§, and Ganes C. Sen†§

From the †Department of Molecular Genetics, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44195, the ‡Graduate Program in Molecular Virology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106, and the †Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208

V accessory proteins from Paramyxoviruses are important in viral evasion of the innate immune response. Here, using a cell survival assay that identifies both inhibitors and activators of interferon regulatory factor 3 (IRF3)-mediated gene induction, we identified select paramyxoviral V proteins that inhibited double-stranded RNA-mediated signaling; these are encoded by mumps virus (MuV), human parainfluenza virus 2 (hPIV2), and parainfluenza virus 5 (PIV5), all members of the genus Rubulavirus. We showed that interaction between V and the IRF3/7 kinases, TRAF family member-associated NFκB activator (TANK)-binding kinase 1 (TBK1)/inhibitor of κB kinase ε (IKKe), was essential for this inhibition. Indeed, V proteins were phosphorylated directly by TBK1/IKKe, and this, intriguingly, resulted in lowering of the cellular level of V. Thus, it appears that V mimics IRF3 in both its phosphorylation by TBK1/IKKe and its subsequent degradation. Finally, a PIV5 mutant encoding a V protein that could not inhibit IKKe was much more susceptible to the antiviral effects of double-stranded RNA than the wild-type virus. Because many innate immune response signaling pathways, including those initiated by TLR3, TLR4, RIG-I, MDA5, and DNA-dependent activator of IRFs (DAI), use TBK1/IKKe as the terminal kinases to activate IRFs, rubulaviral V proteins have the potential to inhibit all of them.

Innate immunity is stimulated by viruses in part via RNA. dsRNA2 specifically is present in several forms: viral genomes, single-stranded RNA virus replication intermediates, DNA virus transcription products, defective viral particles, and debris from lysed cells (1). Although extracellular dsRNA is sensed by Toll-like receptor 3 (TLR3), intracellular dsRNA is detected in part by the RNA helicases retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (Mda-5). These receptors signal through Toll-IL-1R (TIR) domain containing adaptor-inducing IFNβ (TRIF) and IFNβ promoter stimulator 1 (IPS1), respectively, to activate the kinases TANK-binding kinase 1 (TBK1) and inhibitor of κB kinase ε (IKKe). They, in turn, phosphorylate IFN regulatory factor 3 (IRF3), promoting its nuclear translocation and subsequent IFN-stimulated regulatory element-mediated transcription of IFN-stimulated genes (ISGs), such as ISG56, as well as IFN and other cytokines. IFN then, through Janus kinase (JAK)/STAT activation of IRF9, modulates microRNAs in addition to up-regulating itself and more ISGs to heighten the antiviral state and also to initiate the adaptive immune response by promoting dendritic cell maturation, memory T cell proliferation, and B cell differentiation (2–4).

To control this immune response, pathogens and hosts have developed methods of down-regulation and evasion at a variety of different points (5–7). At the level of sensing infection, NS1 from influenza virus binds to and sequesters dsRNA and also interacts with RIG-I (5, 6, 8). At the level of signal transduction, the NS3/4A protease from hepatitis C virus cleaves TRIF and IPS1 (9–11), and vaccinia virus A46R, which contains a TIR domain, inhibits TRIF-dependent activation of IRF3 (12, 13). At the level of IRF-mediated transcription, P proteins from Ebola (5, 6) and borna disease (14) viruses and human papilloma virus E16 (15) interfere with IRF3 activation. For a more global effect, M protein from vesicular stomatitis virus inhibits host machinery to prevent gene transcription (5, 6). Finally, at the level of IFN and cytokines binding to their receptors, human cytomegalovirus encodes a decoy for the chemokine RANTES (regulated on activation normal T cell expressed and secreted) to prevent further signaling (16).

The family Paramyxoviridae encompasses two subfamilies: Paramyxovirinae and Pneumovirinae. Viruses representing different genuses within the paramyxoviral subfamily are important pathogens for humans such as measles virus (MeV), a Morbillivirus, and mumps virus (MuV) and human parainfluenza virus 2 (hPIV2), both Rubulaviruses. For animals, examples include Hendra virus (HeV), a Henipavirus that infects flying
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foxes, Sendai virus, a Respirovirus that infects rodents, and parainfluenza virus 5 (PIV5), a Rubulavirus that infects dogs (17). Paramyxoviral V accessory proteins have been shown to be important in viral evasion of innate immunity. At the level of IFN signaling, V proteins act as ubiquitin-protein isopeptide ligases (E3) that target STATs for degradation or sequester them, preventing their nuclear translocation and subsequent transcriptional functions (18). At the level of sensing infection, V proteins bind to mda-5 and also to dsRNA itself to inhibit intracellular dsRNA signaling and protein kinase R activation, respectively (19–21). These functions highlight the importance of immune signaling in the viral life cycle. Indeed, VDC PIV5, a mutant PIV5 virus lacking the unique C terminus of V, is no longer able to inhibit IRF3 activation and causes a greater cytotoxic effect in a variety of different cells (22, 23). Taken together, these lines of evidence indicate that V may inhibit dsRNA activation of IRF3 via TLR3.

Here we show that V proteins from hPIV2 (Vp), MuV (Vm), and PIV5 (Vp), but not HeV and MeV, inhibit TLR3 signaling. Analysis of the underlying mechanism revealed that the inhibitory V proteins interacted with the signaling kinases TBK1/IKKe and served as their substrates, thus preventing IRF3 phosphorylation. Our results indicated that the above interaction led to modifications of both partners and their degradation. Therefore, the V proteins from Rubulaviruses and the IRF3-activating kinases TBK1/IKKe are connected by a negative feedback loop.

**EXPERIMENTAL PROCEDURES**

**Viruses, Cells, and Reagents—**W3A strain WT and VDC PIV5 virus, gifts of B. He, were propagated in Vero (22, 24). HT1080-derived 2TGH wild-type V protein-expressing cells, 293 and TLR3 293 have been described (25, 26). To make HT1080-derived 2TGH cell lines permanently expressing mutant Vm, 2TGH cells were co-transfected with respective peFV protein expression vectors and a puromycin-expressing vector for selection. To make TLR3 293 561-TK cells, TLR3 293 was transfected with 561-TK plasmid. This contains the ISG56 promoter (−654 + 3) (27) driving the herpesvirus thymidine kinase (Tk) (28), which was cloned via StsI/Sall into pGL3B (Promega) to replace luciferase. Wild-type peFV protein expression vectors have been described (29, 30). MuV V mutants were constructed with QuikChange® II XL (Stratagene). PCR products from primers targeting residues were cloned with DpnI. PIV5 VDC mutant was constructed with the Expand Hi Fidelity PCR system (Roche Applied Science) using wild-type peFV protein as template and BamHI/BglII and NotI for cloning. peF-Trif-FLAG (31) (gift of K. Fitzgerald), wild-type, and kinase inactive (K38A) IKKe (pCDNA3.1-Myc) (32) (gifts of U. Siebenlist), FLAG-IRF3 and IRF3 5D (33) (gifts of J. Hiscott), and HA-ubiquitin (gift of S. Fuchs) (34) vectors have been described. All transfections were carried out with FuGENE 6 (Roche Applied Science), Ganciclovir (GCV) (Invivogen), leptomycin B (LMB) (Sigma), MG132 (Sigma) in dimethyl sulfoxide (DMSO) (Sigma), and dsRNA (Amersham Biosciences) (27) were used in treatments at 10 μg/ml, 10 ng/ml, 10 μM, and 100 μg/ml, respectively, unless otherwise specified.

**Cell Survival Assay—**TLR3 293 561-TK cells were transfected with pEF-V and treated with dsRNA and GCV. Cells were maintained under selection 4 days to assay survival and create pools expressing V proteins.

**Quantitative PCR—**HT1080 V protein-expressing cells were treated with dsRNA for 6 h. Total RNA was isolated with RNA-Bee (Tel-Test). cDNA was synthesized with SuperScript III (Invitrogen). The quantitative-PCR reaction amplified bp 6–354 of ISG56 and control RPL32 (ribosomal protein L32) with SYBR Green (Applied Biosystems). ISG56 primers are as follows: sense, 5′-TCT CAG AGG AGC CTG GCT AAG-3′, and antisense, 5′-GTC ACC AGA CTC CTC ACA TTT GC-3′. RPL32 primers are as follows: sense, 5′-GCC AGA TCT TAT GCC CCA AC-3′, and antisense, 5′-CGT GCA CAT GAG CTG GCT AAG-3′.

**Immunoblotting and Immunoprecipitation—**Immunoblotting was performed with antibodies HA Y-11 (Santa Cruz Biotechnology), actin (Sigma), p56 (35), c-Myc 9E10 (Santa Cruz Biotechnology), P396 IRF3 (Cell Signaling), total IRF3 (gift of M. David) (36), histone H1 AE4 (Santa Cruz Biotechnology), STAT2 C-20 (Santa Cruz Biotechnology), FLAG M2 (Sigma), and V5, a polyclonal antibody raised against a full-length PIV5 V-GST fusion protein at Cocalico Biologicals Inc.

To assay V interaction with IKKe, TLR3 293 cells were transfected with relevant expression plasmids. Immunoprecipitations were performed as described previously (30) with agarose-conjugated HA F7 (Santa Cruz Biotechnology), FLAG M2 (Sigma), or protein A/G PLUS-agarose (Santa Cruz Biotechnology) with c-Myc 9E10 at 4 °C. To assay for in vitro V phosphorylation, the same procedure was used to isolate V. The resulting samples were then treated with 100 units of calf intestine phosphatase (USB Corp.) 37 °C 3 h where indicated.

**IRF3 Activation Assays—**To assess Ser-396 phosphorylation and IRF3 nuclear localization, HT1080 Vm cells were treated with dsRNA for 3 h before collection of whole cell or nuclear lysates, respectively, described previously (27). To look at localization by immunofluorescence, the same cells were plated on coverslips and treated with dsRNA for 1 h and then LMB for 2 h. Cells were stained for IRF3 and 4′,6-diamidino-2-phenylindole (27). To determine IRF3 transcriptional activity, the same cells were treated with dsRNA for 1 h and then with LMB for 6 h. Whole cell extracts were prepared for immunoblotting.

**In Vitro Kinase Assay—**To purify FLAG-IRF3 and Vm, the respective vectors were transfected for 24 h into TLR3 293 cells, and lysates were prepared as described (37). FLAG-tagged proteins were immunoprecipitated with FLAG M2 agarose (Sigma), eluted with FLAG peptide (Sigma), and concentrated with Microcon filter devices YM-3 (Millipore). Myc-TBK1 was purified similarly with c-Myc 9E10 and Trueblot immunoprecipitation beads (Ebioscience). For in vitro kinase assays, purified FLAG-IRF3 or Vm were incubated with GST-IKKe (Cell Signaling) or Myc-TBK1 in [y-32P]ATP (37). Quantitation for autoradiograph was performed by GE Healthcare PhosphorImager and for immunoblotting by Odyssey (Licor).

**Vm degradation—**To follow Vm degradation by IKKe, control empty or IKKe-expressing vectors were co-transfected with V-expressing vectors into TLR3 293 cells for 8 h, media were changed, and whole cell lysates were prepared 48 h later. To follow Vm degradation by dsRNA, the same cells were
simultaneously transfected with pEF-V and dsRNA-treated with either MG132 or control DMSO for 16 h.

**RESULTS**

Some, but Not All, Paramyxoviral V Proteins Inhibit TLR3 Signaling—We used a cell survival assay to examine the potentials of V proteins from different paramyxoviral subfamilies to inhibit TLR3 signaling. This assay uses a TLR3-expressing 293 cell line (TLR3 293) in which a selection gene has been introduced; this gene is driven by the promoter of ISG56 (27) and encodes the herpesvirus TK protein (28). Any signaling that activates transcription factors containing IRF proteins, such as TLR3 signaling or type I IFN signaling, induces TK production and causes cell death in the presence of GCV. If an inhibitor of signaling blocks TK production in a cell, that cell survives and proliferates even in the presence of GCV. Using this assay, we determined that V proteins from the Rubulaviruses hPIV2 (VH), MuV (VM), and PIV5 (VP) inhibited TLR3 signaling (Fig. 1A, top panel, lanes 4–6). In contrast, V proteins from HeV and MeV were ineffective (Fig. 1A, top panel, lanes 2 and 3), although the different V proteins were expressed at similar levels in the transfected cells (Fig. 1B). Expression of the V proteins themselves, without any dsRNA treatment, did not affect cell survival at all (Fig. 1A, bottom panel). The above observations suggested that rubulaviral V proteins could block induction of cellular dsRNA-inducible genes as well, a conclusion that was confirmed by measuring the levels of ISG56 mRNA, using a quantitative reverse transcription-PCR assay in HT1080 cells permanently expressing different V proteins. The mRNA was induced strongly upon dsRNA treatment of cells (Fig. 1C, lane 5) and the induction was almost completely blocked by VH, VM, and VP (Fig. 1C, lanes 6–8). Similar inhibitions of the induction of p56 protein were observed (Fig. 1D).

V Proteins Block IRF3 Activation—Once we established that the three V proteins blocked TLR3 signaling, we investigated the underlying mechanism. Exogenous expression of signaling proteins downstream of TLR3 is known to activate IRF3 and induce synthesis of p56, as shown in Fig. 2A. Expression of TRIF, IKKe, and TBK1 induced p56 (lane 1), and the induction was blocked by all three V proteins. In contrast, p56 induction from expression of a constitutively active IRF3 5D mutant was not blocked (Fig. 2A, lanes 2–4). These results indicated that the V proteins blocked a step in signaling downstream of TLR3, TRIF, and the IRF3 kinases and upstream of events following activation of IRF3. Inactive IRF3 shuttles in and out of the nucleus, whereas activated IRF3 translocates to the nucleus and induces transcription of target genes, such as ISG56 (38). IRF3 was not localized to the nucleus in cells expressing VM (Fig. 2B, lane 4). VM and VP (data not shown). By using LMB, a drug that blocks nuclear export of proteins (39), we examined whether VM blocked nuclear import of IRF3 or enhanced its export. As expected, in untreated cells, IRF3 was in the cytoplasm of both control and V-expressing cells (Fig. 2C, row 1). dsRNA treatment alone caused nuclear accumulation of IRF3 only in the absence of V protein (Fig. 2C, row 2). In both untreated and dsRNA-treated cells, LMB caused similar nuclear accumulation of IRF3 in the absence and the presence of V protein (Fig. 2C, row 3). These results suggested that the V proteins could block nuclear accumulation of IRF3 by inhibiting nuclear import of IRF3.

**FIGURE 1. Select paramyxoviral V proteins inhibit TLR3-mediated gene induction.** A, paramyxoviral V proteins were transiently expressed in TLR3 293 561-TK cells, which were then treated with dsRNA and GCV for 4 days (top panel) or GCV alone (bottom panel). Estimated cell survival is shown. Error bars represent standard error from two experiments. Lane 1, no V protein; lane 2, HeV protein; lane 3, MeV protein; lane 4, HPIV2 V protein/VM; lane 5, MuV V protein/VM; lane 6, PIV5 V protein/VP; lane 7, PIV5 V protein/VP; lane 8, PIV5 V protein/VP. B, p56 mRNA levels were measured by quantitative reverse transcription-PCR in untreated (lanes 1–4) or dsRNA-treated (lanes 5–8) HT1080 cells stably expressing V proteins. Error bars represent standard error from triplicate samples. C, p56 mRNA levels were measured by quantitative reverse transcription-PCR in untreated (lanes 1–4) or dsRNA-treated (lanes 5–8) cells described in C. Immunoblotting for actin and FLAG-V served as controls.
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**Figure 2.** V proteins from hPIV2, MuV, and PIV5 inhibit IRF3 activation. A, pools of TLR3 293 561-TK cells expressing V proteins were transfected with expression vectors for different components of the TLR3 pathway; the specific signaling protein expressed is shown on the left. Induced p56 levels were determined by immunoblotting. Actin served as a loading control. Lane 1, no V; lane 2, Vp (labeled); lane 3, VM (M); lane 4, VM (H). B, nuclear extracts from control cells (lanes 1 and 2) or cells stably expressing VM (lanes 1 and 3) or treated with dsRNA (lanes 2 and 4) were immunoblotted for IRF3, with histone as loading control. C, control and VM-expressing HT1080 cells were treated with dsRNA (rows 2 and 4) and LMB (rows 3 and 4). Subcellular location of IRF3 was determined by immunofluorescence. D, cell extracts from samples, as described for C, rows 2 and 4, were used for immunoblotting to detect p56, FLAG-V, and actin. E, IRF3 phosphorylation at serine 396 (P396) was detected by immunoblotting cell extracts with a phospho-IRF3-specific antiserum, with total IRF3 as a control. Whole cell extracts were prepared from control cells (lanes 1 and 3) or cells stably expressing VM (lanes 2 and 4) that were untreated (lanes 1 and 2) or treated with dsRNA (lanes 3 and 4).

2C, rows 3 and 4). These results suggested that either VM inhibited nuclear import of only activated IRF3 or VM enhanced export of activated IRF3 through exportin 1, which is blocked by LMB (39). To address these possibilities, we examined whether the IRF3 sequestered within the nucleus by LMB was transcriptionally active in the presence of dsRNA. Nuclear IRF3 in LMB-treated cells could not induce p56 in control cells unless they were dsRNA-treated as well (Fig. 2D, lanes 1 and 3). In dsRNA-treated V-expressing cells, although LMB treatment caused nuclear translocation of IRF3, p56 was not induced (Fig. 2D, lanes 2 and 4). These results indicated that V protein inhibited import of activated IRF3 to the nucleus and did not enhance its nuclear export. Furthermore, these results indicated that nuclear localization, although necessary, was insufficient for IRF3 to induce genes; additional activation was required by post-translational modifications of the protein. We then asked whether the observed block of IRF3 activation was due to a block in its phosphorylation, which is known to activate it. Indeed, phosphorylation of Ser-396, a hallmark of IRF3 activation, was inhibited in the presence of VM (Fig. 2E, lane 4). These results indicated that the major block caused by VM was at the level of IRF3 phosphorylation.

**Interaction of V with IKKe Is Essential for Inhibiting TLR3 Signaling**—To further analyze the mechanism of V-mediated blocking of TLR3 signaling, we examined possible interactions of the viral proteins with known components of the signaling pathways. VM, Vp, and VP did not interact with TLR3, TRIF, IRF3, Src, phosphatidylinositol 3-kinase, and IKK α, β, and γ as revealed by co-immunoprecipitation assays, although the same assay demonstrated their known interactions with STAT2 (Fig. 3A and B). In contrast, all three viral proteins interacted with both IKKe (Fig. 3C) and TBK1 (data not shown). IKKe co-immunoprecipitated with Vp (lane 3), VM (lane 5), and VP (lane 7) (Fig. 3C, top panel). Note that the mobility of co-immunoprecipitated IKKe was slower than that of the protein in cell extracts. This was also the case for co-immunoprecipitated STAT2 (Fig. 3C, middle panel, lanes 3, 5, and 7). Since the V proteins have E3 ubiquitin ligase activity (18), the observed mobility differences could be due to V-mediated polyubiquitination of the co-immunoprecipitated proteins. Indeed, exogenously introduced ubiquitin was covalently bound to IKKe purified by immunoprecipitation, and this ubiquitination was VM-dependent (Fig. 3D).

The functional significance of the V-IKKe interaction was determined by testing mutant VM. Among six mutant proteins tested (Fig. 3E), only the VM mutant W174A/W178A/W188A (VM-AAA) failed to bind to IKKe (lane 3, left top panel). This mutant protein could not block TLR3 signaling as revealed by the cell survival assay (data not shown) and in HT1080 cells permanently expressing mutant VM-AAA (Fig. 3G, lane 8). In contrast, WT VM and mutant VC189A could inhibit signaling (Fig. 3G, lanes 4 and 6). Both bound to IKKe (Fig. 3E, lanes 7 and 8) and, as shown when electrophoresed longer, both shifted the mobility of co-immunoprecipitated IKKe, indicating its ubiquitination (Fig. 3F, lanes 2 and 4, compared with lanes 1 and 3). These results strongly indicated that the V proteins blocked TLR3 signaling by blocking the action of the signaling kinases.

**V Proteins Are Substrates for IKKe and TBK1**—Because the V proteins bound to the kinases, in the next experiments, we tested whether they were phosphorylated as a consequence. Indeed, both VM (Fig. 4A) and VP (Fig. 4B) were phosphorylated by IKKe in vivo. As expected, the phosphorylated V proteins migrated more slowly (Fig. 4, A and B, lane 2), and upon phosphatase treatment, they co-migrated with the unphosphorylated proteins (Fig. 4, A and B, lane 3). Similar results were seen for TBK1 (data not shown). Expression of enzymatically inactive IKKe did not cause phosphorylation of the V proteins (Fig. 4, A and B, lane 4), suggesting that IKKe directly phosphorylated V. To test this suggestion, purified VM and IKKe were added to an in vitro protein kinase reaction in the presence of [γ-32P]ATP, and radiolabeling of VM was measured. As a positive control, purified IRF3, a known substrate of IKKe, was used. Both IRF3 and VM were phosphorylated by IKKe (Fig. 4C, lanes 1 and 2). Control reactions showed that the addition of both IKKe and VM was needed to observe the radiolabeled protein (Fig. 4C, lanes 3 and 4). Quantification of the incorporated phosphates demonstrated that, on a molar basis, VM was a slightly better substrate than IRF3 (114 versus 100). Similar in vitro reactions demonstrated the ability of purified TBK1 to
phosphorylate $V_M$ (130 versus 100) (Fig. 4D, lane 3). These results established the V proteins as authentic substrates for IKKe/TBK1.

As a consequence of phosphorylation, V appeared to be destined for faster degradation (Fig. 5A). Co-expression of V with kinase active (lane 2), but not kinase inactive (lane 3) IKKe, caused major diminution of the cellular level of V, as compared with control (lane 1). Furthermore, this was not caused just by overexpression of IKKe because dsRNA-mediated activation of the endogenous kinase caused a dramatic lowering of the level of V as well (Fig. 5B, lane 2), and this diminution could be inhibited by the proteasome inhibitor MG132 (Fig. 5B, lane 3). Finally, interaction between V and IKKe was required for degradation; $V_{M,AAA}$, a mutant that did not co-immunoprecipitate with IKKe (Fig. 3E, lane 3), was not degraded (Fig. 5C).

$V$ Protein Determines the Efficacy of Virus Replication in TLR3-activated Cells—To evaluate the biological significance of our observations, we chose to use the PIV5 mutant virus VDC, which encodes a C-terminally truncated V protein (22). VDC was not phosphorylated in vivo by IKKe as seen in WT PIV5 V protein (Fig. 6A). Furthermore, we determined that the truncated V protein could not block signaling induced by exogenous expression of IKKe (data not shown). Results showed that WT virus replicated better as compared with the mutant virus in both TLR3-expressing and non-expressing cells (Fig. 6B). Since the mda-5/RIG-I pathway is still intact in these cells, this difference may be due to the lack of ability of the truncated V protein to inhibit that signaling pathway (19). In cells not expressing TLR3, treatment with dsRNA did not have any effect on the replication of either virus. In contrast, in TLR3-expressing cells, dsRNA treatment strongly inhibited the replication of the mutant virus (5 logs), whereas the effect on WT virus was minor. These results demonstrated that inhibition of TLR3 signaling by V protein was relevant for effective virus replication.

**DISCUSSION**

The results presented above revealed several new features of the equilibrium established in an infected cell between the virus and the host (Fig. 7). Induction of viral stress-inducible genes, including IFNs, is blocked by rubulaviral V proteins by inhibiting IRF3 phosphorylation, which is required for its activation because the V proteins can themselves be phosphorylated by TBK1/IKKe. A similar observation has been made by Unterstab et al. (14) for borna disease virus P protein. It is not yet clear exactly how V proteins can
inhibit IRF3 phosphorylation by TBK1/IKKe. Because they themselves are substrates of the same kinases, one possibility is that V simply competes out IRF3 as a substrate. In the in vitro kinase assays, quantitation of phosphorylation showed that IRF3 and VM were equally phosphorylated by IKKe, when present in equimolar amounts either singly or in combination (data not shown), indicating that the two proteins are equally competent substrates of the kinases. Hence, to compete out IRF3, V has to be present at a high molar excess, a possibility not unlikely because V proteins are produced in large quantities in Paramyxovirus-infected cells (17). Alternatively, V proteins can have stronger affinity for TBK1/IKKe, thus not allowing access to IRF3. The fact that V and IKKe were co-immunoprecipitated efficiently indicated that the two proteins could interact strongly, thus providing credence to the second scenario. Although our study was designed to determine whether different paramyxoviral V proteins could inhibit TLR3 signaling, our observations are equally relevant for other signaling pathways that converge on TBK1/IKKe. Thus, we can expect rubulaviral V proteins to block signaling initiated by the cytoplasmic receptors RIG-I, mda-5, and DNA-dependent activator of IRFs (DAI) or the membrane receptor TLR4, which are known to be triggered by RNA, DNA, or lipopolysaccharide (3). Indeed, we observed that RIG-I/mda-5 signaling to IRF3 triggered by transfected dsRNA was inhibited by V_M, V_M, and V_p (data not shown).

An additional consequence of the action of V proteins as alternative substrates of IKKe could be inhibition of phosphorylation of STAT1. Because STAT1 phosphorylation by IKKe is essential for its ability to induce a subset of ISGs (40), in addition to causing STAT1 degradation (18), rubulaviral V proteins may block actions of IFN using this mechanism.

The interaction with V caused ubiquitination of IKKe as indicated by the observed slower mobility of co-immunoprecipitated IKKe and its V-dependent ubiquitination when co-transfected with tagged ubiquitin. A similar change in the mobility of IKKe was observed when C189A mutant of VM was used; this was unexpected because the mutant cannot bind DDB1 (data not shown), a component of the ubiquitin ligase complex (18). This indicates the existence of alternative pathways for the
the degradation of \( V_M \) observed over the course of a viral infection (41). Because \( V \) was needed for the optimum replication of viruses, even in the absence of dsRNA signaling (Fig. 6), its destruction should be a potent antiviral mechanism in that context as well. Phosphorylation and degradation of \( V \) provide an opportunity for temporal regulation of IRF3 activation; when the level of \( V \) is high, IRF3 phosphorylation is blocked by competition. As more and more \( V \) is phosphorylated and degraded, the relative concentration of IRF3 will increase, leading to its phosphorylation, activation, and degradation. Thus, two negative feedback loops, one between \( V \) and IRF3 and the other between IKKe/TBK1 and \( V \), regulate the equilibrium between the virus and the cell.

It is known that IKKe interacts with STAT1 and that STAT1 can interact with \( V \) (30, 40). However, the interaction between IKKe and \( V \) reported here is not mediated by STAT1 because \( V_M \) could block TLR3 signaling in U3B cells (42), which lack functional STAT1 (data not shown). The tryptophan residues of \( V \) that were required for IKKe binding are located in the C-terminal region of the protein (43). Because this region is not shared between viral V and P proteins, whose mRNAs are produced by alternative transcription of the same open reading frame, P proteins are not expected to bind to IKKe. This prediction is consistent with previous findings that \( V \), but not P, blocks dsRNA-mediated IRF3 activation (22).

The experiments with LMB (Fig. 2, C and D) illuminated subtle, but important, aspects concerning IRF3 activation. According to the current paradigm of the activation process, IRF3 is phosphorylated at multiple serine residues, and this leads to its nuclear translocation, a process that is necessary for its action as a transcription factor (38). We found that forcing nuclear localization of IRF3 by LMB treatment of cells was not sufficient for its ability to induce genes. Therefore, there are consequences to IRF3 phosphorylation in addition to nuclear translocation that are necessary for activation.

It is interesting to note that among the \( V \) proteins from paramyxoviral subfamilies tested, all inhibit IFN signaling, but only members of the genus Rubulavirus were able to inhibit dsRNA-TLR3-mediated IRF3 activation (17). These observations seem to correlate with what seems to be a more important role for \( V \) in the life cycle of Rubulaviruses as compared with others. Rubulaviral \( V \) proteins, in contrast to P in other Paramyxoviruses, represent the default open reading frame transcribed at the P/V locus, resulting in higher levels of basal expression. Correspondingly, virions from Rubulaviruses contain a much higher level of \( V \) as compared with others and are conveniently present during the initial stages of infection and signaling (17). This importance of rubulaviral \( V \) proteins may be in part compensation for the lack of W or C accessory proteins, which Respiroviruses and Henipaviruses encode to counteract host defense (5, 17, 44).

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REFERENCES
1. Bowie, A. G., and Fitzgerald, K. A. (2007) Trends Immunol. 28, 147–150
2. Hovanessian, A. G. (2007) Cytokine Growth Factor Rev. 18, 351–361
Paramyxoviral V Proteins Are Substrates for IKKe/TBK1

3. Medzhitov, R. (2007) Nature 449, 819–826
4. Platanias, L. C. (2005) Nat. Rev. Immunol. 5, 375–386
5. Garcia-Sastre, A. (2004) Curr. Top. Microbiol. Immunol. 283, 249–280
6. Haller, O., Kochs, G., and Weber, F. (2006) Virology 344, 119–130
7. Liew, F. Y., Xu, D., Brint, E. K., and O’Neill, L. A. (2005) Nat. Rev. Immunol. 5, 446–458
8. Mibayashi, M., Martinez-Sobrido, L., Loo, Y. M., Cardenas, W. B., Gale, M., Jr., and Garcia-Sastre, A. (2007) J. Virol. 81, 514–524
9. Li, K., Foy, E., Ferroen, J. C., Nakamura, M., Ferroen, A. C., Ikeda, M., Ray, S. C., Gale, M., Jr., and Lemon, S. M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 2992–2997
10. Loo, Y. M., Owen, D. M., Li, K., Erdjument-Bromage, A. K., Johnson, C. L., Fish, P. M., Carney, D. S., Wang, T., Ishida, H., Yoneyama, M., Fujita, T., Saito, T., Lee, W. M., Hagedorn, C. H., Lau, D. T., Weinman, S. A., Lemon, S. M., and Gale, M., Jr. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 6001–6006
11. Cheng, G., Zhong, J., and Chisari, F. V. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 8499–8504
12. Bowie, A., Kiss-Toth, E., Symons, J. A., Smith, G. L., Dower, S. K., and O’Neill, L. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10162–10167
13. Stack, J., Haga, I. R., Schroder, M., Bartlett, N. W., Maloney, G., Reading, P. C., Fitzgerald, K. A., Smith, G. L., and Bowie, A. G. (2005) J. Exp. Med. 201, 1007–1018
14. Unterstab, G., Ludwig, S., Anton, A., Planz, O., Dauber, B., Krappmann, D., Heins, G., Ehret, D., and Wolff, T. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 13640–13645
15. Ronco, L. V., Karpova, A. Y., Vidal, M., and Howley, P. M. (1998) Genes Dev. 12, 2061–2072
16. Wang, D., Bresnahan, W., and Shenk, T. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 16642–16647
17. Lamb, R. A., and Parks, G. D. (2007) in Fields’ Virology (Knipe, D. M., and Howley, P. M., eds) 5th Ed., pp. 1449–1496, Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia
18. Horvath, C. M. (2004) Cytokine Growth Factor Rev. 15, 117–127
19. Andrejeva, J., Childs, K. S., Young, D. F., Carlos, T. S., Stock, N., Goodbourn, S., and Randall, R. E. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 17264–17269
20. Childs, K., Stock, N., Ross, C., Andrejeva, J., Hiltunen, L., Skinner, M., Randall, R., and Goodbourn, S. (2007) Virology 359, 190–200
21. Gainey, M. D., Dillon, P. J., Clark, K. M., Manuse, M. J., and Parks, G. D. (2008) J. Virol. 82, 828–839
22. He, B., Paterson, R. G., Stock, N., Durbin, I. E., Durbin, R. K., Goodbourn, S., Randall, R. E., and Lamb, R. A. (2002) Virology 303, 15–32
23. Poole, E., He, B., Lamb, R. A., Randall, R. E., and Goodbourn, S. (2002) Virology 303, 33–46
24. Paterson, R. G., and Lamb, R. A. (1993) in Molecular Virology: a Practical Approach (Davisson, A. J., and Elliott, R. M., eds) pp. 35–73, IRL Press, Oxford; New York
25. Parisien, J. P., Lau, J. F., Rodriguez, I. J., Ulane, C. M., and Horvath, C. M. (2002) J. Virol. 76, 4190–4198
26. Sarkar, S. N., Smith, H. L., Rowe, T. M., and Sen, G. C. (2003) J. Biol. Chem. 278, 4393–4396
27. Peters, K. L., Smith, H. L., Stark, G. R., and Sen, G. C. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 6322–6327
28. Li, X., Commene, M., Burns, C., Vithalani, K., Cao, Z., and Stark, G. R. (1999) Mol. Cell. Biol. 19, 4643–4652
29. Rodriguez, J., Wang, L. F., and Horvath, C. M. (2003) J. Virol. 77, 11842–11845
30. Ulane, C. M., Kentsis, A., Cruz, C. D., Parisien, J. P., Schneider, K. L., and Horvath, C. M. (2005) J. Virol. 79, 10180–10189
31. Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., Coyle, A. J., Liao, S. M., and Maniatis, T. (2003) Nat Immunol 4, 491–496
32. Chariot, A., Leonard, A., Muller, J., Bonif, M., Brown, K., and Siebenlist, U. (2002) J. Biol. Chem. 277, 37029–37036
33. Lin, R., Heylbroeck, C., Pitha, P. M., and Hiscott, J. (1998) Mol. Cell. Biol. 18, 2986–2996
34. Kumar, K. G., Krolewski, J. J., and Fuchs, S. Y. (2004) J. Biol. Chem. 279, 46614–46620
35. Guo, J., Peters, K. L., and Sen, G. C. (2000) Virology 267, 209–219
36. Navarro, L., and David, M. (1999) J. Biol. Chem. 274, 35535–35538
37. ten Oever, B. R., Sharma, S., Zou, W., Sun, Q., Grandvaux, N., Julkunen, I., Hemmi, H., Yamamoto, M., Akira, S., Yeh, W. C., Lin, R., and Hiscott, J. (2004) J. Virol. 78, 10636–10649
38. Hiscott, J. (2007) J. Biol. Chem. 282, 15325–15329
39. Kumar, K. P., McBride, K. M., Weaver, B. K., Dingwall, C., and Reich, N. C. (2000) Mol. Cell. Biol. 20, 4159–4168
40. Teneoever, B. R., Ng, S. L., Chua, M. A., McWhirter, S. M., Garcia-Sastre, A., and Maniatis, T. (2007) Science 315, 1274–1278
41. Hu, A., Schwartz, S., Utter, G., Orville, C., Kovanen, P., and Norry, E. (1993) Arch. Virol. 133, 201–209
42. Muller, M., Laxton, C., Briscoe, J., Schindler, C., Improtta, T., Darnell, J. E., Jr., Stark, G. R., and Kerr, I. M. (1993) EMBO J. 12, 4211–4228
43. Nishio, M., Garcin, D., Simonet, V., and Kolakofsky, D. (2002) Virology 300, 92–99
44. Shaw, M. L., Cardenas, W. B., Zamarin, D., Palese, P., and Basler, C. F. (2005) J. Virol. 79, 6078–6088