AKT1-Dependent Activation of NF- κ B by the L Protein of Parainfluenza Virus 5[▽]

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Innate immunity plays a critical role in the control of viral infections. The induction of innate immune responses requires activation of transcription factors. In particular, NF- κ B plays an essential role in activating the expression of cytokines involved in innate immunity such as beta interferon (IFN- β) and interleukin-6 (IL-6). However, the mechanisms by which viruses activate NF- κ B are poorly defined. Infection by parainfluenza virus 5 (PIV5), a prototypical member of the *Paramyxoviridae* family of *Mononegavirales*, has been shown to activate the expression of IFN- β and IL-6. To examine how PIV5 induces this expression, we have examined the activation of NF- κ B by PIV5 proteins. We have found that expression of PIV5 L protein alone is sufficient to activate NF- κ B. The L protein of PIV5, the catalytic component of the viral RNA-dependent RNA polymerase, contains six domains that are conserved among all negative-stranded nonsegmented RNA viruses. We have mapped the region that activates NF- κ B to the second domain, which is thought to be involved in RNA synthesis. The activation of NF- κ B by L requires AKT1, a serine/threonine kinase, since AKT1 small interfering RNA, an AKT inhibitor as well as a dominant-negative mutant of AKT1, blocks this activation. Furthermore, we have found that L interacts with AKT1 and enhances its phosphorylation. We speculate that L may encode AKT1 kinase activity.

Viruses in the *Paramyxoviridae* family of *Mononegavirales* include many important human and animal pathogens such as the human parainfluenza viruses (PIVs), Sendai virus, mumps virus, Newcastle disease virus, measles virus, rinderpest virus, and human respiratory syncytial virus as well as emerging viruses such as Nipah virus and Hendra virus. PIV5, formerly known as simian virus 5 (9), is a prototypical member of the *Rubulavirus* genus of the family *Paramyxoviridae* (22). Although PIV5 was originally isolated from cultured primary monkey cells, its natural host is the dog, in which it causes kennel cough (31). PIV5 can infect humans (10), but no known symptoms or diseases in humans have been associated with exposure to PIV5 (19).

The single-stranded RNA genomes of members of the *Mononegavirales* family range from approximately 11,000 to 19,000 nucleotides in length and encode a linear array of genes separated by nontranscribed sequences (22, 24). The viral RNA-dependent RNA polymerase (vRdRp) that is responsible for both transcription and replication of the nucleocapsid protein (NP or N)-encapsidated RNA genome minimally consists of two proteins, the phosphoprotein (P) and the large polymerase (L) protein (13). The 220- to 250-kDa L proteins of negative nonsegmented RNA viruses (NNSV) encode a number of functions in addition to RNA transcription and replication, including methyltransferase and guanylttransferase transcription, polyadenylation, and RNA editing activities. Sequence comparisons of the L proteins and other RNA poly-

merases indicate that the L proteins have six conserved domains (35, 41).

Innate immunity plays a critical role in control of virus infection. Among the essential elements for the induction of innate immune responses is the activation of nuclear factor κ B (NF- κ B), which regulates the expression of antiviral cytokines such as beta interferon (IFN- β) and of major proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6). The NF- κ B family of transcription factors includes NF- κ B1 (p105), NF- κ B2 (p100), Rel A (p65), Rel B, and c-Rel; this family can be divided into two classes on the basis of transactivation properties and mode of synthesis. p65, Rel B, and c-Rel are translated as mature proteins and contain an N-terminal Rel-homology domain (RHD), essential for dimerization and DNA binding, and a C-terminal transactivation domain. p105 and p100 contain RHDs at their N termini and ankyrin repeats at their C termini and are precursors for p50 and p52, respectively. The precursors undergo ubiquitin-dependent proteolysis to remove the C-terminal domains to generate p50 and p52, which have only RHDs, enabling them to dimerize and bind DNA but not transactivate transcription. p65 has the strongest transactivation domain and is responsible for most NF- κ B transcriptional activities. Pathways leading to activation of NF- κ B family members have been well documented. In the classical pathway, NF- κ B proteins form homodimers or heterodimers and are sequestered in the cytoplasm in association with inhibitor of κ B (I κ B) (5). Activation of NF- κ B is dependent on the activity of the I κ B kinase (IKK) complex, which consists of the IKK α , - β , and - γ subunits. Phosphorylation of I κ B results in its ubiquitin-dependent degradation, thus exposing nuclear localization signals in NF- κ B and inducing translocation of the NF- κ B dimer to the nucleus, where it is further modulated by phosphorylation (51). Acti-

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vation of the IKK complex can be triggered by a number of different signal transduction pathways. Alternatively, in the noncanonical pathway, catalytic subunits of IKK, IKK α , and another kinase, NIK, can be activated to remove the C-terminal domain of p100 to generate p52, allowing p52 dimers to translocate into the nucleus. In this study, we have investigated the mechanism of activation of NF- κ B by PIV5 proteins.

AKT, also known as protein kinase B (PKB), was first discovered in the AKT8 retrovirus as a viral proto-oncogene capable of transforming certain cells (reviewed in reference 6). Identification and cloning of the AKT gene showed that it has high homology to genes encoding protein kinases A and C; hence the name PKB. Three mammalian AKT genes (AKT1, -2, and -3, also known as PKB α , - β , and - γ , respectively) have been identified, and all have serine/threonine kinase activity. AKT proteins contain a pleckstrin homology domain, a catalytic domain, and a regulatory domain and are activated by phosphorylation. There are two major phosphorylation sites within AKT, amino acid residues Thr308 and Ser473, which are phosphorylated by PDK1 (PI3K-dependent kinase 1) and the rictor-mTOR complex, respectively (8, 40). AKT is a key regulator in the PI3K signaling pathway and plays an important role in many cellular processes such as cell survival, metabolism, growth, proliferation, and mobility. AKT has many downstream targets, including IKK α (IKK α), whose phosphorylation by AKT1 can lead to activation of NF- κ B (11). Recently, we reported that AKT plays a critical role in replication of NNSV, possibly through phosphorylation of the P protein (45). In this study, we found that viral L protein activates NF- κ B through an AKT1-dependent pathway.

MATERIALS AND METHODS

Cells and plasmids. BSR T7 cells (7), a murine cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen)–10% fetal bovine serum–10% tryptose phosphate broth–100 IU of penicillin/ml–100 μ g of streptomycin/ml–400 μ g of G418/ml at 37°C with 5% CO₂.

The plasmids containing LAC (consisting of domains I to IV), Δ LN (consisting of domains IV to VI), LI (consisting of domain I), LI-LII (consisting of domains I and II), or LI-III (consisting of domains I to III) with an antigenic tag (hemagglutinin [HA]) in expression vector pCAGGS (33) were generated by using standard molecular cloning techniques. Plasmids containing PIV5 NP, V, P, M, F, SH, HN, L, and AKT1 with a Flag tag in pCAGGS were described before (25, 27, 28, 49, 50). pRL-TK containing a modified renilla luciferase gene under the control of a thymidine kinase (TK) promoter of herpes simplex virus was from Promega (Madison, WI). pNF- κ B-TATA-F-Luc containing a firefly luciferase (F-luc) gene under the control of NF- κ B binding sites was described before (46). Plasmids containing an F-Luc under the control of various IL-6 promoter mutants (pIL-6wt-F-Luc, pIL-6-TATA-F-Luc, and pIL-6-NF- κ Bmut-F-Luc) were described before (28, 47, 48). Plasmid containing an F-Luc under the control of an IFN- β promoter has been described by Poole et al. (36). The dominant-negative (DN) mutant of AKT, pMT2-AH-AKT1, which contains 1 to 147 residues of AKT with a Myc antigen tag, was described by Khwaja et al. (21). AKT DN, which contains three mutations at phosphorylation sites and an ATP binding site (AAA-AKT1; K179A/T308A/S473A) with an HA tag was described previously by Srinivas et al. (44).

EMSA. BSR T7 cells were transfected with empty vector, pCAGGS L, pCAGGS V, pCAGGS LI, pCAGGS LI-II, or pCAGGS LI-III, and nuclear extracts were prepared using a nuclear extraction kit (Marligen Biosciences). Nuclear extract from TNF- α -treated BSR T7 cells was used as a positive control. The cells were treated with 20 ng of TNF- α /ml for 2 h. The protein concentration was determined using a bicinchoninic acid protein estimation kit (Thermo Scientific). An electrophoretic mobility gel shift assay (EMSA) was carried out as described by Wilson et al. (50). Briefly, double-stranded oligonucleotide containing consensus sequences for NF- κ B binding sites (5'-AGCTCCTGGAAG TCCCAGCGGAAAGTCCCTT-3' and 5'-AGCTAAGGGACTTCCGCTG

GGGACTTTCAGG-3') were end-labeled with [³²P]dCTP by use of Klenow fragments. To examine the NF- κ B binding, 5 μ g of nuclear extracts from transfected cells was used in a volume of 20 μ l of reaction mixture containing 2 μ l of 10 $\times$ EMSA buffer (25 mM HEPES [pH 7.5], 60 mM NaCl, 9% glycerol, 1 mM EDTA, 7.5 mM dithiothreitol, 50 mM MgCl₂)–poly(dI-dC) and incubated at room temperature for 30 min. For controls, unlabeled NF- κ B oligomers were used as a cold competitor at a 20-fold molar excess. Mutant NF- κ B oligomers (5'-AGCTAACTCACTTCCGCTGCTCACTTTCAGG-3' and 5-AGCTCC TGGAAAGTGAGCAGCGGAAAGTGAGTT-3', where the underlined sequences represent changed sequences within the NF- κ B binding site) were used as a specificity control. The nuclear extract and oligomer mixture were resolved on a 6% polyacrylamide gel. The gels were analyzed using a PhosphorImager Storm system (Molecular Dynamics).

Dual Luciferase assay for NF- κ B activation. BSR T7 cells were seeded in a 24-well tissue culture plate at about a 1:10 dilution. Cells at about 80 to 90% confluence were transfected. For each well, DNA was diluted in Opti-MEM to which Plus reagent (Invitrogen) (4 μ l) was added to obtain a final volume of 25 μ l. The mixture was incubated for 15 min at room temperature. Meanwhile, Lipofectamine (Invitrogen) (2 μ l) was diluted in Opti-MEM (23 μ l) and incubated for 15 min at room temperature. Two mixtures were combined and added to each well of a 24-well plate. pCAGGS was used to maintain a constant total amount of DNA in each well. The amounts of plasmids used were as follows: 2.5 ng of pRL-TK and 60 ng of pNF- κ B-TATA-F-Luc, pCAGGS NP, pCAGGS V, pCAGGS P, pCAGGS M, pCAGGS F, pCAGGS SH, pCAGGS HN, and pCAGGS L were used in a concentration range of between 0 and 1,000 ng/ml. All L mutants were used in a range of between 0 and 1,500 ng/ml. Amounts consisting of 120 ng of pIFN β -F-Luc, 60 ng of pIL6wt-F-Luc, pIL-6-TATA-F-Luc, or pIL-6- κ Bmut-F-Luc, and AKT DN pMT2-AH-AKT and AAA-AKT in a range of between 0 and 800 ng/ml were used. At 18 to 24 h after transfection, cells were lysed in 100 μ l of passive lysis buffer (Promega) by using a shaker for 30 to 45 min. Lysate (20 μ l) from each well was then used for a dual luciferase assay according to the protocol of the manufacturer (Promega). To examine the effect of AKT inhibitor on L-activated NF- κ B, 0.5 μ M of AKT inhibitor (IV) was added to BSR T7 cells 4 h after transfection. A dual luciferase assay was performed at 18 to 20 h posttransfection as described before.

ELISA for detecting NF- κ B and phosphorylation of Thr308 of AKT1. To detect NF- κ B activation, an enzyme-linked immunosorbent assay (ELISA)-based experiment was performed according to the recommendations of the manufacturer (Active Motif, Carlsbad, CA). Nuclear extracts were prepared from empty vector (pCAGGS)-, pCAGGS L-, or pCAGGS V-transfected cells as described before. A 2.5- μ g volume of protein was used for the assay.

To detect phosphorylation of AKT1 at Thr308, BSR T7 cells were transfected with empty vector, pCAGGS LI, or pCAGGS LI-II. Cells subjected to platelet-derived growth factor (PDGF) (50 ng/ml) treatment were used as positive control. Cells without PDGF treatment were used as a negative control. The cells were serum starved for 3 h and were treated with PDGF for 10 min in DMEM without serum at 37°C before lysing of the cells. The cells were also left untreated and were maintained in Opti-MEM. The cells were lysed 18 to 21 h posttransfection by the use of cell lysis buffer (Cell Signaling Technology), and the protein concentration was estimated using a bicinchoninic acid protein estimation kit (Thermo Scientific). The cell lysate (4 mg/ml) was used for the ELISA according to the manufacturer's instructions by using a Pathscan Phospho-Akt (Thr308) sandwich ELISA kit (Cell Signaling Technology).

siRNA knockdown of AKT1. Small interfering RNA (siRNA) experiments were performed as described before (45). Briefly, HeLa cells in 24-well plates at about 30 to 50% confluence were transfected with 100 nM of siRNA purchased from Dharmacon (AKT1 siRNA and control siRNA; ATF3) by the use of Oligofectamine (Invitrogen). The cells were washed with Opti-MEM and incubated with 400 μ l of Opti-MEM at 37°C. For each well, 5 μ l of either AKT1 siRNA or ATF3 siRNA (10 μ M stock) was mixed with 95 μ l of Opti-MEM for 5 min at room temperature and 2 μ l of Oligofectamine was mixed with 10 μ l of Opti-MEM. The two diluted mixtures of siRNA and Oligofectamine were combined and incubated for 15 min at room temperature. After the incubation, the siRNA-Oligofectamine mixture was added to the cells. A 250- μ l volume of DMEM–30% fetal bovine serum was added to the cells after 6 h of incubation. After 48 h posttransfection, the cells were transfected with empty vector or L along with pRL-TK and pNF- κ B-TATA-F-Luc as described before. At 1 day posttransfection, the dual luciferase assay and immunoblotting experiments were performed.

Coimmunoprecipitation. BSR T7 cells were seeded in a 6-cm-diameter tissue culture plate and transfected with pCAGGS AKT1. At 16 to 18 h after transfection, cells were lysed with whole-cell extraction buffer (WCEB; 1 M Tris [pH 8.0], 280 mM NaCl, 0.5% NP-40, 2 mM EGTA, 0.2 mM EDTA, 10% glycerol,

1 $\times$ protease inhibitor, 0.1 mM phenylmethylsulfonyl fluoride). Cell lysates were precleared with 40 μ l of protein A-labeled Sepharose beads. The lysates were then immunoprecipitated with 2 μ l of anti-AKT1 antibody (Cell Signaling) and Sepharose A beads for 2 to 3 h at 4°C. The cell lysate were spun down and washed twice with WCEB. The precipitate was used for further immunoprecipitation with LI and LI-II. LI and LI-II with an HA tag were synthesized in vitro using TNT-coupled transcription-translation systems (Promega). Templates for LI and LI-II were generated using oligomers containing a T7 promoter and were purified with phenol-chloroform extraction. Manufacturer's instructions were followed for in vitro transcription-translation. In vitro-synthesized LI and LI-II were incubated with precipitated AKT1 with beads and antibody for 2 to 3 h at 4°C. To examine the expression levels of in vitro-synthesized LI and LI-II, they were immunoprecipitated with HA antibody. Samples were resolved on a 10% polyacrylamide gel and visualized using a PhosphorImager Storm system (Molecular Dynamics).

Immunoprecipitation and immunoblotting. To examine phosphorylation of AKT1 by LI-II, 6-cm-diameter plates seeded with BSR T7 cells were transfected with empty vector (6 μ g), pCAGGS AKT1-Flag (1 μ g), pCAGGS LI (5 μ g), pCAGGS LI-II (5 μ g), and pCAGGS AKT1-Flag (1 μ g) plus pCAGGS LI-II (5 μ g). A sample in which pCAGGS AKT1-Flag-transfected cells were treated with PDGF (50 ng/ml) for 15 min in DMEM without serum before lysing of the cells was used as a positive control after serum starving them for 40 min. At 18 to 21 h after transfection, cells were lysed with WCEB and lysates were washed once with WCEB. The cell lysates were incubated with Flag antibody (Red anti-Flag M2 Affinity Gel antibody, Sigma) (10 μ l) and 40 μ l of Sepharose A beads slurry for 2 to 3 h at 4°C. The beads were washed with WCEB once. The samples were resuspended in sodium dodecyl sulfate loading buffer without dithiothreitol and were resolved on a 10% polyacrylamide gel. The immunoprecipitated proteins were transferred onto an Immobilon P polyvinylidene difluoride transfer membrane (Millipore Corporation) in a buffer consisting of 800 ml of 5 $\times$ transfer buffer (60 g of Tris [Trizma base], 285 g of glycine), 800 ml of methyl alcohol, and 2.4 liters of distilled water by use of a wet-gel transfer apparatus. The membranes were blocked in 5% milk-phosphate-buffered saline containing 0.3% (vol/vol) Tween 20 (PBST) or in 3% bovine serum albumin (BSA)-Tris-buffered saline containing 0.3% (vol/vol) Tween 20 (TBST) for phosphorylated AKT analysis. The membranes were incubated with mouse anti-AKT1 antibody diluted in 5% milk-PBST (Cell Signaling Technology), rabbit anti-phospho-AKT Thr308 antibody diluted in 1.5% BSA-TBST (Cell Signaling technology), or rabbit anti-phospho-AKT-Ser473 antibody diluted in 1.5% BSA-TBST (Cell Signaling Technology) overnight at 4°C. The excess antibody was removed by three 10-min washes with the corresponding buffers. To analyze the bound antigen, mouse (in 5% milk-PBST) or rabbit (in 1.5% BSA-TBST) secondary antibodies conjugated with horseradish peroxidase were used. The bound secondary antibody was detected using ECL Plus Western blotting reagents (Amersham) and visualized using a PhosphorImager Storm system (Molecular Dynamics).

To examine the expression levels of L mutants, 6-cm-diameter plates of BSR T7 cells were transfected with 6 μ g of plasmids containing LI, LI-II, or LI-III. The next day, cells were lysed in 500 μ l of protein lysis buffer (2% sodium dodecyl sulfate, 62.5 mM Tris-HCl [pH 6.8], 2% dithiothreitol) and subjected to sonication to shear DNA. Up to 100 μ l of the lysate was resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to immunoblotting with anti-HA and anti-actin antibodies as described above.

Interaction of LI-III and AKT1 in a yeast two-hybrid system. To examine the interaction between L and AKT1 by use of a yeast two-hybrid system, domains LI-III were placed at the N terminus of the LexA DNA binding domain in pHybLex/Zeo (Invitrogen, Carlsbad, CA) and AKT1 was placed at the C terminus of the B42 activation domain in pYESTrp2 (AKT1-AD) (Invitrogen, Carlsbad, CA). Positive controls containing plasmids containing LI-III-BD plus pYESTrp2, AKT1-AD plus pHybLex/Zeo, or LI-III-BD plus AKT1-BD as well as positive controls Fos-AD plus Jun-BD were transformed into strain L40 of *Saccharomyces cerevisiae*. Transformants were plated onto yeast minimal medium lacking tryptophan and uracil but containing Zeocin (YC-WU Z300) (300 mg/ml). Samples were then plated onto yeast minimal medium lacking histidine, uracil, and lysine but containing Zeocin (YC-HUK Z300) (300 mg/ml) to examine whether LI-III-AD or AKT1-BD alone activated reporter gene expression and whether LI-III-AD and AKT1-BD together activated reporter gene expression.

RESULTS

Activation of NF- κ B by PIV5 proteins. Previously, we showed that a recombinant PIV5 lacking the conserved region

FIG. 1. NF- κ B activation by PIV5 proteins. A plasmid encoding a firefly luciferase gene (F-Luc) under the control of NF- κ B responsive elements was cotransfected into cells with increasing amounts (0, 250, 500, 750, and 1,000 ng) of a plasmid encoding each PIV5 viral protein along with a plasmid encoding a renilla luciferase (R-Luc) as a transfection efficiency control. Empty vector was used to maintain a constant amount of DNA. Luciferase activities were measured at 1 day posttransfection and are expressed as the F-Luc/R-Luc ratio values normalized to vector-transfected cell results. All transfections were carried out in replicates of four; error bars represent standard errors of the means.

of the V protein (rPIV5V Δ C) activates expression of IFN- β and IL-6 and that NF- κ B is activated in rPIV5V Δ C-infected cells (18, 28). To investigate the mechanism of NF- κ B activation, we examined the ability of viral protein expression to activate NF- κ B-dependent reporter gene expression. A reporter gene construct, pNF- κ B-TATA-F-Luc, containing a firefly luciferase (F-Luc or FL) gene under the control of a promoter containing three NF- κ B binding sites along with a transfection control plasmid, pRL-TK, which has renilla luciferase (R-Luc, or RL) under the control of a herpes simplex virus TK promoter, was transfected with a plasmid encoding PIV5 viral protein NP, V, P, M, F, SH, HN, or L. The luciferase activities were determined at 1 day posttransfection. Among the known PIV5 proteins, the L protein demonstrated the highest level of activation of NF- κ B-dependent reporter gene expression (Fig. 1), indicating that L is capable of activating NF- κ B. The expression levels of HN and L necessary for NF- κ B activation were lower than in PIV5-infected cells, while expression levels of the other proteins greater than those seen in infected cells were insufficient to induce NF- κ B-dependent transcription (data not shown). To further confirm the activation of NF- κ B by L, an EMSA was performed. As expected, nuclear extracts prepared from L-transfected cells at 1 day posttransfection shifted the mobility of a 32 P-labeled NF- κ B probe (Fig. 2A), whereas nuclear extract from mock- or V-transfected cells had no effect. This binding was competed away by excess "cold" competitor but not by a "cold" mutant NF- κ B probe, confirming that the L protein specifically activates NF- κ B.

NF- κ B consists of five subunits. To investigate which NF- κ B factor is activated by L, an ELISA-based experiment was carried as described before (14, 28). As shown in Fig. 2B, L expression activates the p50, p52, and p65 subunits of NF- κ B, the same set of NF- κ B factors that are activated in rPIV5V Δ C-infected cells (28). Taking these results together, we conclude that L is capable of activating NF- κ B and is the major player in NF- κ B activation during infection.

FIG. 2. Activation of NF-κB by L. (A) EMSA. Cells were transfected with empty vector or plasmids encoding either L or V protein. Nuclear extracts were obtained for EMSA as described before (26). TNF-α, nuclear extracts from cells were treated with 20 ng of TNF-α/ml for 2 h. Probe (-), NF-κB DNA primers labeled with 32 P; s, unlabeled NF-κB probe (20-fold excess); ns, unlabeled mutant NF-κB probe (20-fold excess). (B) ELISA. The nuclear extracts from the samples described above were analyzed for NF-κB factor activation by using an NF-κB transcription factor ELISA from Active Motif and following the manufacturer's instructions. The positive control was nuclear extract from Raji cells provided by the manufacturer. *P* values are shown in the graph. OD, optical density.

Activation of IFN-β and IL-6 by L. It is known that NF-κB activation plays an important role in expression of cytokines, chemokines, and growth receptors and thus plays an essential role in immune responses. To examine whether PIV5 L has a role in cytokine activation, its effect on IL-6 and IFN-β activation was studied by the use of a promoter assay as described above. As shown in Fig. 3A, transfection of increasing amounts of L plasmid results in higher luciferase transcription from the IFN-β promoter, indicating that L can play a role in IFN-β activation. As expected, L activates IL-6-dependent reporter gene expression in a dosage-dependent manner, indicating that L can also activate the IL-6 promoter (Fig. 3B). Mutating the NF-κB site within the IL-6 promoter as well as removing the regulatory sequences of the IL-6 promoter abolished activation of the IL-6 promoter by L, indicating that IL-6 promoter activation by L requires NF-κB binding.

Mapping of the region within L essential for NF-κB activation. The PIV5 L protein is ~250 kDa and consists of six domains that are conserved among all NNSV. To investigate which domain(s) is involved in NF-κB activation, a series of L mutants was generated (Fig. 4A) and tested using the NF-κB-

dependent reporter gene assay as described above. The N-terminal four domains of L are capable of activating NF-κB as LΔC-activated NF-κB, but LΔN is not (Fig. 4B). Further analysis of the LΔC indicates that domains I and II are sufficient to activate NF-κB. Since domain I alone does not activate NF-κB, domain II is likely the essential region of L for NF-κB activation. However, it is not clear whether domain II is sufficient for activation of NF-κB.

To confirm this observation, EMSA was conducted as described before with nuclear extracts prepared from L-, LI-, LI-II-, or LI-III-transfected cells. The DNA-protein complex was observed in nuclear extracts from L-, LI-II-, or LI-III-transfected cells but not in those from vector- or LI-transfected cells, confirming activation of NF-κB by LI-II (Fig. 4C). The expression levels of LI and LI-II in this experiment as determined by immunoblotting were comparable (Fig. 4D).

AKT1 plays an essential role in NF-κB activation by L. To further elucidate the mechanism of NF-κB activation by PIV5 L protein, we have examined the role of AKT1 in this process. We have focused on the role of AKT1 for two reasons. AKT1 is known to activate NF-κB through phosphorylation of IKKα

FIG. 3. Activation of IFN-β and IL-6 promoters by L. (A) IFN-β promoter activation. Dual luciferase reporter gene assays similar to the ones represented in Fig. 1A were performed with firefly luciferase under the control of the IFN-β promoter instead of a NF-κB-responsive element. (B) IL-6 promoter activation. A luciferase assay was performed as described above using plasmid constructs containing a firefly luciferase gene under the control of the wild-type IL-6 promoter (pIL6wt-F-Luc), an NF-κB binding site mutant of the IL-6 promoter (pIL6-κB mut), or IL-6-TATA, which consists only of the TATA box of IL-6 promoter. Relative luciferase activity values represent ratios of F-Luc to R-Luc (transfection control). Graphs represent data from replicates of four experiments $\pm$ standard errors of the means.

FIG. 4. Mapping of L sequences that are required for activation of NF- κ B. (A) Schematic of L mutants. All the mutants with an antigenic tag (HA) in pCAGGS expression vector were generated using standard molecular cloning techniques. (B) NF- κ B activation. A dual luciferase assay was performed as described before by using the indicated amounts of expression plasmids. Relative luciferase activity values represent ratios of F-Luc to R-Luc (transfection control). All transfections were carried out in replicates of four experiments; error bars represent standard errors of the means. (C) EMSA. Cells were transfected with empty vector and plasmids encoding L, LI, LI-II, or LI-III. Nuclear extracts were prepared and incubated with 32 P-labeled NF- κ B probe and appropriate competitors and were resolved on 6% polyacrylamide gel as described in the text. TNF- α , nuclear extracts from cells were treated with 20 ng/ml of TNF- α for 2 h. Probe (-), NF- κ B DNA primers labeled with 32 P; s, unlabeled NF- κ B probe (20-fold excess); ns, unlabeled mutant NF- κ B probe (20-fold excess). (D) Expression levels of L mutants. Immunoblotting with anti-HA antibody was used to analyze the expression of L mutants in transfected cell extracts.

(20, 34, 38). Also, we recently reported that AKT1 plays a critical role in replication of PIV5, likely through phosphorylation of the P protein (45). Therefore, we examined the ability of L to activate NF- κ B-dependent reporter gene expression in the presence of AKT inhibitor IV, a chemical compound which can inhibit all three isoforms of AKT. As shown in Fig. 5A, AKT inhibitor treatment resulted in reduced luciferase activities in the presence of L, indicating that AKT is important in activation of NF- κ B by L. To further confirm these results, increasing amounts of a plasmid encoding a DN mutant of AKT1 (AH-AKT1) cotransfected with L- and NF- κ B-dependent transcription were assayed as described above. Coexpression of AH-AKT1 inhibited L-activated NF- κ B reporter activity in a dosage-dependent manner (Fig. 5B and C), confirming that AKT1 plays a critical role in the activation of NF- κ B by L. Similar results were obtained using a different DN AKT1 that contains mutations at the phosphorylation sites (Thr308 and Ser473) and the ATP binding site (data not shown). To test whether AKT1 plays a critical role in activation of NF- κ B, AKT-1 siRNA was transfected to cells to reduce the AKT1 expression (Fig. 5E). As predicted, reducing AKT1 expression levels results in the reduction of NF- κ B-dependent reporter gene expression by the L protein (Fig. 5D), confirming that AKT1 plays an essential role in activation of NF- κ B by L.

Phosphorylation of AKT1 by PIV5 L protein. AKT1 is activated through phosphorylation at specific sites: Thr308 and Ser473. To investigate the mechanism of AKT1-dependent activation of NF- κ B by L, the effect of L on endogenous AKT1 phosphorylation was examined. BSR T7 cells were transfected with empty vector or with plasmids expressing LI or LI-II. The cells were lysed, and amounts of phosphorylated AKT1 at residue Thr308 were measured using an ELISA kit (Cell Signaling). As shown in Fig. 6A, the cells transfected with LI-II produced higher levels of Thr308 phosphorylation than the cells transfected with others. However, the level of Thr308 in LI-II-transfected cells was low compared with positive-control, PDGF-treated cell results. This is likely because transfection introduces the plasmid into only a portion of cells whereas AKT1 is activated with PDGF treatment in all cells. To further investigate phosphorylation of AKT1, a plasmid encoding Flag-tagged-AKT1 was cotransfected with LI or LI-II plasmid or empty vector into cells. AKT1 was immunoprecipitated using anti-Flag antibody, and total or phosphorylated AKT1 was detected by immunoblotting. As shown in Fig. 6B, expression of LI-II enhanced phosphorylation of AKT1 at both Thr308 and Ser473. The level of the enhancement was comparable to that seen with the positive control, PDGF treatment (Fig. 6C).

We next used an in vitro transcription/translation system to

FIG. 5. AKT plays a critical role in activation of NF- κ B by L. (A) Effect of AKT inhibitor on L-activated NF- κ B. A dual luciferase assay was performed as described for Fig. 1A in the presence of AKT IV inhibitor (0.5 μ M) (Calbiochem) or vehicle (dimethyl sulfoxide [DMSO]). Relative luciferase activity values represent ratios of F-Luc to R-Luc (transfection control). (B) Inhibition of L-activated NF- κ B by DN AKT1. Reporter gene assays were carried out as described in the text. DN, AKT DN (AH-AKT) used in increasing amounts. (C). Expression levels of L in the presence of AKT1 DN. Lysates from the luciferase assay were used for Western blotting with anti-Flag antibody to check the expression of L in the presence of AH-AKT. (D) Reduction of NF- κ B activation after AKT1 knockdown with siRNA. The cells were transfected with siRNA targeting AKT1 or with control siRNA. At 48 h after siRNA transfection, the cells were transfected with plasmids encoding L along with reporter luciferase genes as described in the text. Luciferase activities were measured at 24 h after plasmid transfection. (E) Expression levels of AKT1 in siRNA-transfected cells. The amounts of AKT1 and actin were examined using the lysates from luciferase for immunoblotting with anti-AKT1 and anti- β -actin antibody.

FIG. 6. L enhances phosphorylation of AKT1. (A) Expression of L enhances phosphorylation of endogenous AKT1. Cells were transfected with vector, LI, or LI-II and then lysed at 1 day after transfection. The levels of phosphorylation at Thr308 were examined using an ELISA kit (Cell Signaling) with Thr308 phosphorylation-specific AKT1 antibody as described in Materials and Methods. PDGF, cells were treated with 50 ng/ml PDGF for 10 min in media without serum after serum starvation of cells for 3 h. NC, negative control, untransfected cells without PDGF treatment. (B) Expression of L enhances phosphorylation of AKT1. Immunoprecipitation of cells transfected with Flag-tagged AKT1 along with vector, LI, or LI-II followed by immunoblotting with AKT1 antibody or phosphorylation-specific AKT1 antibodies was performed as described in Materials and Methods. Cells transfected with pCAGGS AKT1-Flag and treated with PDGF (50 ng/ml) for 15 min in DMEM without serum after serum starvation for 40 min were used as a positive control. (C) Quantification of phosphorylation of AKT1 by L. The average values for phosphorylation of AKT1 from the results of three experiments were graphed. The ratio of phosphorylated AKT (at Thr308 or Ser473) to total AKT from cells transfected by AKT1 alone was set at 1. Error bars represent standard errors of the means.

investigate whether L and AKT1 can interact. As shown in Fig. 7A, AKT1 coprecipitated with both LI and LI-II, indicating that domain I of PIV5 L can interact with AKT1. In addition, we confirmed this interaction by use of a yeast two-hybrid system (Fig. 7B), with LI-III, which is sufficient for NF- κ B activation (Fig. 4B), as the bait and AKT1 as the prey. We found that yeast grew on selective plates only when trans-

formed with both LI-III and AKT1 plasmids, indicating that AKT1 interacts with the N-terminal three domains of L.

DISCUSSION

A critical factor in the control of virus infection is the induction of innate immunity, such as induction of the expression of antiviral (e.g., IFN- β) and proinflammatory (e.g., IL-6) cytokines. Cytokine production requires the coordinated activity of a number of signal transduction molecules, leading to the activation of cytokine transcription by specific transcription factors such as IRF3 and NF- κ B. Recently, a great deal of progress has been made in elucidating the pathways that lead to IRF3 activation after viral infection (reviewed in reference 37). However, stimulation of NF- κ B by viruses has been less well characterized. Previously, we reported that a mutant PIV5 (a recombinant PIV5 lacking the conserved C terminus of rPIV5V Δ C, the V protein) can activate expression of IFN- β and IL-6 (18, 28, 36). Further studies indicated that IFN- β induction was the result of activation of IRF3 by the RIG-like helicase pathway, specifically through MDA-5 (1). While IL-6 expression induced by rPIV5V Δ C infection requires NF- κ B activation (28), the mechanisms by which this occurs are not clear. Here, we provide evidence that the PIV5 L protein activates NF- κ B through an AKT1-dependent pathway. To the best of our knowledge, this is the first report showing that a viral polymerase can activate NF- κ B and that AKT1 plays a critical role in this activity.

AKT1 is a serine/threonine kinase that plays a critical role in many cellular processes. Activation of AKT1 often involves phosphorylation of two critical amino acid residues at positions 308 (Thr) and 473 (Ser). It is well established that PDK1 and the rictor-mTOR complex can phosphorylate Thr308 and Ser473,

FIG. 7. L interacts with AKT1. (A) Coimmunoprecipitation of L and AKT1. 35 S-labeled LI and LI-II were synthesized by *in vitro* transcription and translation; AKT1 was obtained from cells transfected with AKT1 expression plasmid. 35 S-labeled LI or LI-II was mixed with cell lysate containing AKT1 and immunoprecipitated with anti-AKT1 antibody. NC, negative control containing no *in vitro*-synthesized fragment. (B) Interaction between LI-III and AKT1 in yeast two-hybrid system. The plasmids encoding BD or AD alone or hybrid proteins LI-III-BD or AKT1-AD were transformed into the L40 yeast strain that contains His as a reporter gene. The transformed yeast cells were grown on YC-WU Z300, which selects for the two plasmids encoding BD and AD. Interaction of LI-III-BD and AKT1-AD led to activation of His, resulting in growth in YC-WHUK Z300 His-deficient medium. Jun-BD and Fos-AD, which are known to interact with each other, were used as positive controls.

FIG. 8. Model for the activation of AKT in virus-infected cells and the roles of AKT in virus replication. Previously, we showed that AKT plays a critical role in phosphorylation of P, which is an essential component of vRdRp. We propose that L activates AKT by causing phosphorylation of AKT. The activated AKT contributes to the activation of the expression of cytokines such as IFN- β and IL-6 as well as phosphorylation of P. Since V blocks expression of IFN- β and IL-6 and V interacts with AKT, we speculate that V can block the activation of NF- κ B through its interaction with AKT.

respectively. Interestingly, the expression of L protein alone caused enhanced phosphorylation at both sites of AKT1. It is not clear whether L encodes a kinase activity that can phosphorylate AKT1 directly or whether it activates a distinct kinase(s) to perform this function. In the case of PDGF activation of AKT1, it is thought that phosphorylation of residue 308 facilitates the phosphorylation of residue 473. Thus, it is possible that L directly phosphorylates Thr308 primarily, resulting in enhanced phosphorylation of Ser473 by a cellular kinase.

We recently reported that AKT1 plays a critical role in the replication of NNSV (45). We speculate that AKT is important for NNSV replication due to its ability to phosphorylate the viral P protein, an essential cofactor of vRdRp, whose function is regulated by extensive phosphorylation. Since AKT1 is not constitutively active in untransformed cells, we reason that the virus would need to activate AKT1 in order to replicate efficiently *in vivo* (Fig. 8). Thus, L would not only provide the enzymatic functions for RNA synthesis but would also stimulate transcription and replication by inducing the phosphorylation of P. Activation of NF- κ B via the AKT1 pathway would therefore be a byproduct of stimulating the kinase activity of AKT1. Interestingly, the V protein of PIV5, which is known to prevent IFN- β production and signaling, can block the activation of NF- κ B by L (data not shown).

The structure of the L protein is well conserved among all NNSV. There are six conserved domains within L, though the functions of these domains are not well defined. Domain III contains a conserved region resembling the catalytic center of polymerase and is thus considered the catalytic domain (29, 43). Domain VI contains a methylase domain, since mutations in the domain affect methylation of 5' viral mRNA (16, 23). The function of domain II has not been defined. Previous reports indicate that this region may contain a template-binding site and is involved in RNA synthesis (32, 42). In this study, we found that

domain II is essential for phosphorylation of AKT1, suggesting that domain II may have intrinsic kinase activity or may be associated with a host kinase. Whether NNSV L proteins have kinase activity has been controversial. There are reports indicating that L of vesicular stomatitis virus (New Jersey serotype) and Sendai virus may have intrinsic kinase activity (2, 3, 12, 17, 39). However, other reports suggest that the L-associated kinase activity may come from a host kinase that interacts with L (15, 30). It has been reported that L in respiratory syncytial virus does not have intrinsic kinase activity (4). Our results indicate that L directly interacts with AKT1 and enhances its phosphorylation. Thus, we favor the hypothesis that L encodes an intrinsic kinase activity; however, it is possible that L and AKT1 form a complex with a cellular kinase which in turn phosphorylates and activates AKT1. We are currently investigating whether the L protein of PIV5 has intrinsic kinase activity or associates with additional host kinases.

In summary, this work demonstrates that the L protein enhances phosphorylation of AKT1 and induces activation of NF- κ B, suggesting that the L protein may play a role in viral pathogenesis in addition to its well-known roles in viral RNA synthesis.

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