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**THE ROLES OF LARGE (L) GENE OF PARAINFLUENZA VIRUS 5 IN THE HOST
SIGNALING PATHWAYS**

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by

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ABSTRACT

The innate immune responses play critical roles in combating viral infection. A key feature of this response is production of type I interferons (IFNs) which induce an antiviral and anti-proliferative state in the cells. This process requires activation of transcription factors, such as nuclear factor- κ B (NF- κ B) and interferon regulatory factor-3 (IRF-3). It has been reported that paramyxovirus infection can activate expression of cytokines such as IFN- β , a type I IFN. However, the mechanism of the activation by virus is not well understood. The cytoplasmic viral sensors, the RNA helicases, RIG-I or MDA5, play critical roles in induction of interferon signaling through detection of specific viral components in the infected cells. However, the origin and molecular identity of these viral RNA ligands is not well characterized. Intriguingly, viruses have strategies to evade this host defense response. This interplay between innate immunity and virus infections, is not only crucial to understand virus-host relationships, but also may lead to development of novel antiviral therapeutics.

In this work, we have studied the activation and modulation of innate immune responses by viruses, focusing on the NF- κ B and the interferon signaling pathways using parainfluenza virus 5 (PIV5), a prototypical paramyxovirus as our model system. Paramyxoviruses are enveloped nonsegmented negative stranded (NNS) RNA viruses that include many important human and animal pathogens. PIV5 is a poor inducer of the antiviral responses. However, a recombinant PIV5 lacking the conserved region of the V

protein (rPIV5VΔC) activates expression of cytokines IFN- β and IL-6 through a NF- κ B dependent pathway.

We investigated the role of viral proteins in this activation and determined that the large (L) polymerase protein of PIV5 can activate NF- κ B leading to activation of IFN- β and IL-6. The L protein of PIV5 is the main component of viral RNA-dependent RNA polymerase and is about 250 kDa. It contains six domains that are conserved among all NNS RNA viruses. We analyzed the mechanism of the activation of NF- κ B by the L protein and found that AKT1, a serine/threonine kinase plays a critical role in activation of NF- κ B by L. We found that the L protein interacts with AKT1 and enhances its phosphorylation. We further mapped the region within the L that is sufficient to activate NF- κ B to domain II and discovered its role in activation of IFN- β . Surprisingly, we found that the RNA transcribed from domain II of the L gene is sufficient to activate NF- κ B in an AKT independent pathway and can also induce interferon expression. We have further evaluated the mechanism of this activation and have determined that this viral RNA activates IFN- β through MDA5 and endoribonuclease, RNase L dependent pathway.

Our study has revealed novel roles of the L gene in the host signaling pathways. Our work is the first report identifying a natural ligand for MDA5 that can induce interferon expression and can serve as a possible prototype for the development of broad antiviral therapeutics.

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Chapter 1

Literature Review

1.1 Paramyxovirus Classification

Paramyxoviruses belongs to *Mononegavirales* and are pleomorphic, enveloped nonsegmented negative stranded RNA viruses (NNSV), which are divided into two subfamilies: *Paramyxovirinae* and *Pneumovirinae* (173). Each subfamily is further classified into different genera based on their genomic organization, morphology, sequence similarity and protein functions. *Paramyxovirinae* is divided into five genera namely *Respirovirus*, *Rubulavirus*, *Morbillivirus*, *Avulavirus* and *Henipavirus* (216). The subfamily *Pneumovirinae* consists of two genera, *Pneumovirus* and *Metapneumovirus*. The current taxonomic classification is summarized in **Figure 1.1**. Due to the genetic diversity of paramyxoviruses, some viruses remain unclassified such as Mossman virus, Menangle virus, Salem virus, Ferde-lance virus, Tupaia paramyxovirus, J paramyxovirus (JPV, 18,954 nt) which was isolated from moribund mice (*Mus musculus*) in Queensland, Australia, during the early 1970s (144) and Beilong virus which was isolated from the human mesangial cell line as a contaminant and is identified to possess the largest genome of 19,212 nts (189). The family of paramyxoviruses consists of many human and animal pathogens that can cause severe diseases such as measles, mumps, respiratory

syncytial virus (RSV), Newcastle disease virus (NDV) as well as emerging viruses such as Nipah and Hendra viruses (103, 173).

Parainfluenza virus 5 (PIV5), formerly known as simian virus 5 (SV5) is a member of *Rubulavirus* genus of the family *Paramyxoviridae*. Although PIV5 was originally isolated from cultured primary monkey cells, its natural host is dog in which it causes kennel cough (219). PIV5 can infect humans (62) and its isolates have been obtained from human sources, but no known symptoms or diseases in humans have been associated with PIV5 (53). It is speculated that PIV5 may cause non-symptomatic persistent infection in humans (136).

1.2 PIV5: Virion

The *Paramyxoviridae* viruses generally have a spherical structure of about 150-350 nm in diameter and consists of lipid bilayer envelope containing two glycoproteins, which mediate the entry and exit of the virus (58). The viral envelope is derived from the host cell membrane during the budding process. The glycoproteins present in PIV5 virion are the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins (303). Their spikes extend about 8-12 nm from the surface and can be visualized by electron microscopy (289). The PIV5 envelope also contains the small hydrophobic (SH) protein (124). Inside the viral envelope, there is the helical ribonucleoprotein complex containing the RNA genome of negative polarity that is encapsidated by viral nucleocapsid (NP) protein, phospho (P) protein and the large (L) protein (176). Between the envelope and

nucleocapsid structure, there is a layer of matrix (M) protein, which is important for the formation of virus particle (306). The V protein is a structural component of the virion and interacts with soluble NP (270). It also binds to the RNA through a basic region in its N-terminal domain (194).

The PIV5 has a genome of 15,246 nucleotides in length and contains a series of tandemly linked genes separated by non-transcribed sequences (**Figure 1.2**) (176). The NP-encapsidated RNA genome serves as a template for synthesis of mRNA and antigenome (+) strand (176). *Paramyxoviridae* encodes its own viral RNA-dependent RNA polymerase (vRdRP) minimally consisting of P and L protein which transcribes the nucleocapsid protein (NP or N)-encapsidated genome into 5' capped and 3' polyadenylated mRNAs (93). It is thought that the vRdRP binds to the genomic RNA at 3' entry site and transcribes the genome in a sequential and polar process (1). The vRdRP is also responsible for replication of the viral RNA genome (1, 17, 93, 142)

1.3 PIV5: Viral Proteins

The PIV5 genome consists of seven genes in the order of NP-V/P-M-F-SH-HN-L but encode for eight viral proteins (**Figure 1.2**) (173). The V/P gene is transcribed into two mRNAs, V mRNA and P mRNA through a process of pseudo-templated addition of nucleotides commonly called “RNA editing” (344). The V mRNA is the faithful transcription of the V/P gene by the vRdRP. The vRdRP recognizes a specific RNA sequence in the V/P gene and inserts two non-templated G residues at the site to generate

P mRNA. This process of RNA editing occurs 50% of the time and hence results in equal abundance of V and P mRNAs, which are translated into two proteins, V and P which share identical N-termini but have different C-termini. The RNA editing at a specific site is a common feature among all members of *Paramyxovirinae* subfamily (173). However, the faithful transcription of the V mRNA is characteristic of *Rubulaviruses* while in *Respiroviruses* and *Morbilliviruses*, the P mRNA is faithfully transcribed from the RNA genome and the V mRNA is a result of addition of pseudo-templated G nucleotide(s) (145). Even though the V proteins are produced differently in *Paramyxovirinae*, the sequences of the C-terminal domain of the V proteins are highly conserved among the paramyxoviruses.

1.3.1 The Large Protein

The Large (L) protein, phosphoprotein (P) and nucleocapsid (NP) protein are important for transcription and replication of viral RNA genome (42). The L protein encodes for the polymerase activity and is responsible for transcribing (NP)-encapsidated RNA into 5' capped and 3' polyadenylated mRNA (142) and for replicating the viral RNA genome (1, 93). The L protein of paramyxoviruses is about 220-250 kDa in mass (254). Sequence comparison of L of paramyxoviruses and rhabdoviruses indicates that it consists of six highly conserved regions (“domains”) (**Figure 1.3**) whose functions are not clearly defined (266). Each of these conserved regions are postulated to be involved in different enzymatic functions that are associated with the polymerase complex including transcription initiation, elongation, termination, viral RNA synthesis and

replication, polyadenylation, 5' mRNA capping and methylation (214). It has been suggested that these conserved regions are involved in insertion of non-templated G residues for transcription of the V/P gene. It was also suggested that domain II and III contain the polymerase function. The domain II has a highly conserved region (aa 532-574), which is proposed to be involved in template recognition. Domain III contains a highly conserved peptide sequence GDN which is involved in RNA synthesis (266). A specific mutation at residue 1488 between the region of domain V and VI of VSV L protein causes polyadenylation defect (128). The L protein also has methyltransferase activities (112). Studies have shown that interactions between PIV5 L and P are domain-based, suggesting PIV5 L has structural domains (254). It has been reported that Sendai virus L protein forms oligomers and mutations in different regions of the L are trans-complementary (48). Till date, no structural information is available about the full length L or any of its conserved regions. However, the multifunctional role of the L protein has been supported by mutational and computational analysis studies, which have revealed that by performing targeted amino acids substitution in the different conserved regions of the L protein results in inactivation of the respective polymerase function (182, 183, 242-244).

It is not known whether L is phosphorylated, but there are reports indicating that the L protein of some viruses may have intrinsic kinase activity (20, 21, 92) while other reports indicate that the L-associated kinase activity may come from a host kinase that is associated with L (104, 213). In VSV, protein kinase activity associated with the L protein (295) has been reported and two putative ATP binding sites at amino acid

residues 754-778 and 1332-1351, which are similar to those recognized in certain protein kinases, Gly-X-Gly-X-X-Gly-(X)₁₀₋₂₂ Lys, have been identified (114). But it is not clear whether this protein kinase activity associated with the L protein is of cellular or viral origin (21, 213). In Sendai virus, it has been reported that the L protein possesses protein kinase activity and this putative kinase activity is involved in phosphorylation of viral nucleocapsid proteins, P and NP *in vitro* (92).

1.3.2 The P Protein

The P protein is thought to be the regulatory protein of P-L complex without its own enzymatic activity. The P protein forms a tetramer and binds to the L protein through its C-terminus. A region in the C-terminus is required to interact with the NP in N:RNA template complex (50). The P protein of paramyxoviruses is heavily phosphorylated, hence it is named as phosphoprotein. The exact role of the P phosphorylation is still elusive. However, it has been proposed that the phosphorylation of the P protein might be important for RNA synthesis of nonsegmented negative-stranded RNA viruses but there is lack of conclusive data (173). The two most well studied host kinases related to the P phosphorylation of paramyxoviruses are the serine/threonine kinase, CKII and PKC- ζ . The role of CKII has been identified in respiratory syncytial virus (RSV) (218, 358) and measles virus (72) while for PKC- ζ in human parainfluenza virus 3 (HPIV3) (76) and Sendai virus (SeV) (137). Using minigenome system, the role of putative phosphorylation sites of CKII in the RSV P protein was identified in the viral RNA synthesis, however mutating these sites in the

viral genome had no effect on the virus replication, suggesting phosphorylation of P protein by CKII is not critical for viral RNA synthesis (217). Recently, role of another host kinase, AKT has reported in the replication of paramyxoviruses, where it was demonstrated that AKT interacts and phosphorylate the P protein of PIV5 *in vitro* (329). A recombinant PIV5 virus, CPI- (Canine Parainfluenza virus 5, rPIV5-CPI-) containing six mutations in the shared P/V region elevates viral gene expression, induces apoptosis in infected cells and activates the expression of antiviral responsive genes (IL-6 and IFN- β) (362). These phenotypes have been reported to be associated with the P protein not the V protein as Pcp- protein elevates viral gene expression and Vcp- protein maintains its ability to inhibit viral RNA synthesis in the minigenome system (346). The critical residue in the P protein responsible for this elevated gene expression has been mapped to a single serine position 157 (S157), which consists of SSP motif (346). A serine/threonine kinase, Polo like kinase (PLK1) which plays important role in the cell-cycle progression has been reported to interact with the P protein at residue S157 via SSP motif and phosphorylates it at residue S308 (328). The phosphorylation of S308 residue by PLK1 was demonstrated both *in vitro* and in infected cells. This phosphorylation of P protein by PLK1 has been shown to downregulate the viral RNA synthesis (328). These recent reports demonstrate that phosphorylation of the P protein plays important role in viral gene expression.

1.3.3 The Nucleocapsid Protein

It has been shown that the NP protein is the most abundant protein of

paramyxoviruses-infected cells and forms a RNase resistant nucleocapsid (254). The NP protein plays a critical role in encapsidation of the viral genome and the nascent antigenome RNA. This forms a helical structure that is used as a template for RNA dependent RNA polymerase. The NP protein is important for the formation of virus like particle (VLP) along with the HN and the F protein for PIV5 (306). Studies have defined the functional domains of nucleocapsid proteins which comprise of highly structured N-terminal core region and a C-terminal tail (19, 39, 40). The N-terminal is shown to be important for RNA binding and the encapsidation process while the C-terminal mediates interaction with viral and host proteins. The nucleocapsid protein forms a complex with the P protein. The nucleocapsid protein of measles virus interacts with the P protein (167) and host protein Hsp72 (385, 386) through its C-terminal tail domain. For human RSV (324), henipaviruses (50) and SeV (39, 40, 71), the C-terminal domain is critical for mediating interaction with the P protein. However, in some other viruses including Newcastle disease virus (NDV) and mumps, the N-terminal core domain of nucleocapsid protein is involved in direct interaction with the P protein. The nucleocapsid protein of paramyxoviruses also interacts with the viral matrix (M) protein, which allows efficient genome assembly for virus release (68). For measles (143) and SeV, the C-terminal domain has been reported to be critical for this NP-M interaction. A region at the end of the C-terminal of PIV5 NP protein has been defined which is critical for the VLP production and interaction with the M protein (307).

1.3.4 The V Protein

The V protein of PIV5 consists of about 222 amino acids residues and is a component of PIV5 virion. It is a multifunctional protein and plays important roles in the viral pathogenesis (125, 330). Its N-terminal domain is about 164-residues which is shared with the P protein (392 amino acids residues) and a distinct cysteine rich C-terminal domain (containing seven conserved cysteine residues) (125, 155). The V protein interacts with a cellular protein (DDB1), the 127 kDa subunit of the damage-specific DNA-binding protein (192). The V protein of PIV5 interacts with the soluble NP (270) and the N-terminal domain of V binds RNA through a basic region (193). The V protein also inhibits the interferon- β (IFN- β) production by sequestering IRF-3 in the cytoplasm (125, 268) through its interaction with MDA5, an IFN inducible RNA helicase (10). It has been shown that the V protein plays an important role in inhibition of IL-6 expression (197) and in blocking the apoptosis in PIV5 infected cells (330). The V protein regulates viral RNA synthesis (191) and has been reported to inhibit viral RNA synthesis in minigenome system (196). The V protein is thought to exert this effect likely through its interaction with host kinase, AKT1 (serine/threonine kinase) (329).

1.3.5 The Fusion Protein

The fusion (F) protein in paramyxoviruses mediates virus to cell and cell to cell fusion in a pH independent manner (173). It has also been shown that the F protein is also involved in virus budding (304). The F protein is a class I viral membrane glycoprotein

which exists in trimeric form and is synthesized as an inactive precursor (F_0) that is cleaved by cellular protease (289). This cleavage results in a biologically active form of the F protein, consisting of disulfide-linked peptides of membrane-bound subunit- F_1 and membrane-distal subunit- F_2 (54). In most of the class 1 membrane fusion proteins, the distal subunit consists of the receptor-binding domains (67, 318) but in the case of paramyxoviruses, they take assistance from other viral proteins that are involved with receptor-binding for the fusion activation process (174). The membrane proximal subunit consists of two hydrophobic domains, the fusion peptide and the transmembrane domain (67). The fusion peptide is highly conserved domain within a virus family (133). The role of conserved glycine residues in the F protein of paramyxoviruses has been identified in regulation of fusion activation (286). The fusion peptide is present on the N-terminal of membrane-proximal subunit and once fusion is triggered either by receptor binding or the low pH, the fusion peptide is inserted into the cell membrane (67, 318). The transmembrane domain supports the binding of ectodomain to viral membrane and helps in fusion pore formation (13). The ectodomain consists of two heptad repeats, one present proximal to the fusion peptide (heptad repeat A, HRA) and the other proximal to the transmembrane domain (heptad repeat B, HRB) (67, 174, 287). Once fusion is activated, the HRA mediates insertion of the active fusion peptide into the cellular membrane and forms a prehairpin intermediate, while HRB initiates binding with HRA grooves which forms a hairpin structure to bring the fusion peptide and transmembrane domain close together (67, 174, 287). This protein folding allows fusion of the viral and the target cell membrane and entry of the viral RNP inside the cytoplasm of the host cell. The F protein of paramyxoviruses has about 250 residues long linker region between the two HR

regions which is proposed to act as scaffold to prevent the refolding and release of the fusion peptide and the HR regions (54). The cytoplasmic tails of viral fusion proteins are suggested to regulate virus assembly and activation of the ectodomain (361). For paramyxoviruses, the fusion occurs at neutral pH and requires additional support from hemagglutinin-neuraminidase protein for this process (287, 288).

1.3.6 The Hemagglutinin-Neuraminidase Protein

The hemagglutinin-neuraminidase (HN) is involved in virus entry and release of the virus (361). The HN is a type II membrane integral protein, consisting of a short N-terminal cytoplasmic tail (17 residues), a single N-terminal transmembrane (TM) domain (19 residues) and an ectodomain comprising of a stalk region (82 residues) and a globular head (447 residues) (345). This ectodomain confers receptor binding and neuraminidase activities of the protein and its hydrophobic transmembrane domain is responsible for targeting the HN to ER membrane. The HN exists as a tetramer on the surface of virus-infected cells, consisting of two disulfide-linked dimers that are associated with noncovalent interactions (234). The HN protein serves three important functions: the attachment to cells by binding to sialic acid residues on cell surface, encoding sialidase activity, and activation of the F protein leading to efficient membrane fusion process (174). The sialidase activity is essential in separating the newly formed virions from the infected cells, thus allowing budding and release of virus. The mechanism by which HN is involved in the membrane fusion is poorly understood. There are reports that suggest that the binding of the HN protein to sialic acid receptors may result in alterations of HN

and F interactions that can activate conformational changes in F, thus initiating membrane fusion (176, 339, 383).

1.3.7 The Matrix Protein

The matrix (M) protein is highly abundant and plays an important role in virus assembly and budding (303, 306). It forms a link between viral envelope and the ribonucleoprotein core. The M protein for most of the paramyxoviruses are capable of forming virus like particles (VLPs) when expressed alone. However, the M protein of PIV5 lacks this ability and requires other viral proteins such as nucleocapsid protein for efficient VLP production (118, 306). The late domain near N-terminus of the M protein is critical for the budding process (305). The M protein has also been shown to interact with host proteins, such as Angiomotin like 1 (258), caveolin-1 (273) and 14-3-3 (259).

1.3.8 The Small Hydrophobic Protein

The small hydrophobic (SH) protein is a 44 amino acid type II integral membrane protein and is oriented in membranes with its N-terminus in the cytoplasm (127). It consists of a predicted C-terminal ectodomain of 5 residues, a transmembrane domain of 23 residues and a N-terminal cytoplasmic tail of 16 residues. The SH gene is situated between the HN and the F genes. The SH gene is not commonly found among paramyxoviruses, only a close member of *Rubulavirus* genus, mumps virus (MuV) and recently identified J paramyxovirus (JPV) consisting of one of the largest genome in

Paramyxoviridae family, has been shown to have this SH gene (188). In *Pneumovirus* RSV, an integral protein SH has been identified but it is larger than PIV5. A recombinant PIV5 virus with deletion of the SH gene (rPIV5 Δ SH) grows similar to wild type virus *in vitro* suggesting the SH protein is dispensable for virus viability in tissue culture (123). However, rPIV5 Δ SH virus has been reported to cause cytopathic effect and induces apoptosis in tissue culture cells through the tumor necrosis factor (TNF)- α mediated extrinsic apoptotic pathway (370). The MuV SH protein is found in all the strains of mumps and is type I membrane protein consisting of 57 residues (337). The SH protein of MuV was not found to be essential for virus growth in tissue culture cells (336). The recombinant MuV with deletion of the open reading frame of SH gene has been shown to interfere with TNF- α signaling and play a role in the viral pathogenesis (373). The recombinant RSV lacking the SH gene also grows similar to the wild type virus, causes syncytium formation and causes more apoptosis in L929 and A549 cells (103). The JPV SH protein is a type I membrane protein consisting of 69 amino acid residues with predicted N-terminal ectodomain (5 residues), a transmembrane domain (23 residues) and a C-terminal cytoplasmic tail (41 residues) (144). The SH protein of JPV has been reported to inhibit TNF- α pathway and apoptosis of the cell that might be important for efficient virus spread (188). There is no sequence similarity among PIV5 SH, MuV SH, RSV SH or JPV SH but these SH proteins of paramyxoviruses appear to exhibit similar functions. A common role of SH proteins of paramyxoviruses have been identified in blocking the TNF- α mediated signaling pathway (124, 195, 370).

1.4 Virus Entry

Infection of the host cells by paramyxoviruses requires adsorption of the viruses to the cellular surface followed by fusion of viral and cellular membranes. The attachment protein HN binds to the sialosides on the cell surface (383). This interaction triggers a conformational change in the F protein and activates membrane fusion at the cell surface in a pH independent manner and releases the viral helical nucleocapsid core into the cellular cytoplasm (177). It is unclear how the HN induces activation of the fusion protein but the mechanism is proposed to involve either a conformational change or oligomerization of the HN protein (89). The process of membrane fusion is not a spontaneous process but occurs in discrete steps that involve rearrangement of the lipid bilayer (376). The F protein is a class I viral fusion protein which initiates fusion by refolding into α -helical coiled-coil hairpin structures (375). These class I proteins consist of a hydrophobic fusion peptide, two 4-3 heptad repeat regions (HRA and HRB) and a transmembrane domain (287). The F protein is synthesized as an inactive precursor protein, F_0 and through a proteolytic cleavage, it forms a stable fusion capable complex F_1+F_2 (287-289). Once activated, the F protein inserts its fusion peptide into the host membrane and is triggered to refold HRA and HRB into a hairpin loop structure through binding of HN to its receptor (257). Since the fusion peptide is contiguous to HRA and the transmembrane domain is adjacent to HRB, formation of the hairpin structure brings the viral and host membrane together and allows entry of the RNP into the cell (289).

1.5 Virus Transcription

The vRdRP transcribes viral RNA using the negative sense genomic RNA in the RNP complex as a template. The vRdRP begins RNA synthesis at the 3'-end of the leader sequence and transcribes the viral RNA genome into mRNAs consisting of a 5'-cap and a 3'-poly(A) tail in a sequential manner (177). This process of mRNA synthesis follows a “stop-start” model involving termination and reinitiation at each gene junction, resulting in a transcription gradient. Failure to reinitiate transcription at a downstream gene start site results in gradient of mRNA production. The genes closer to the 3'-end of the genome are transcribed more abundantly than the genes closer to the 5'-end (368). Thus, the mRNA production is inversely proportional to the distance of the gene from the 3'-end. For example, the NP gene being closest to the 3'-end leader sequence is the most abundantly transcribed while the L gene which is the farthest from the leader sequence is the least transcribed. The vRdRP is also responsible for capping the viral mRNA.

Each gene junction consists of three nucleotide sequence elements: gene end (GE), intergenic sequence (IGS) and gene start (GS) (368). At the GE sequence, polyadenylation occurs through stuttering on a copying of four to seven- uracyl (U) residues tract and transcription terminates with release of the polyadenylated RNA. The vRdRP either leaves the template, resulting in attenuation or passes over the IGS region which is not present in mRNA and reinitiates the RNA synthesis at the downstream GS sequence (176). The **Figure 1.4** illustrates the mechanism of the RNA synthesis by PIV5.

1.6 Virus Replication

The vRdRP starts viral genome replication of the virus at the 3'-end of the leader sequence generating a complementary copy of the negative strand RNA genome called antigenome (cRNA or antigenomic RNA, positive sense) and subsequently produces negative sense RNA genome from the antigenome starting from the trailer sequence (168). The antigenome is an intermediate for the replication process. The mechanism of the switch from transcription to replication is not clear. This process is thought to start after transcription and translation of primary transcripts. The encapsidation of the genome and antigenome occurs at the same time of the replication. The efficiency of RNA synthesis for paramyxoviruses is affected by many factors: the number of nucleotides in viral RNA template, the leader sequences and the trailer sequences (229). The replication has found to be most effective when the total number of nucleotides is a multiple of six ("rule of six") (43, 168). This rule suggests that the optimal replication of the viral genome requires the 3' end nucleotides of the template to be completely encapsidated by NP or alternatively it is important for recognition of *cis*-acting sequences (230). This "rule of six" requirement varies among different paramyxoviruses. For SeV, this rule is an absolute requirement for efficient RNA replication (43) while for RSV this rule has no obvious advantage (293). PIV5 and HPIV3 have an intermediate requirement, complying "the rule of six" does results in an optimal replication for these viruses but is not absolutely required (88, 230).

The leader and the trailer sequences affect the efficiency of the RNA synthesis by

paramyxoviruses. In PIV5, two important regions have been identified: conserved region I (CRI) which consists of 19 nucleotides at the 3' terminal and conserved region II (CRII) which is present between 72 and 90 nucleotides from the 3' end of the genome and the antigenomic RNA within the coding region of the L gene (229). The sequences of CRII consists of three copies of the conserved 5'-CGNNNN-3' motif (231).

The space between the discontinuous regions of the viral antigenomic promoter also has a role in the viral RNA synthesis (163). In the case of PIV5, the spacing between CRI and CRII is critical for RNA replication irrespective of the “rule of six” (229). This region has been reported to consists of *cis*-acting signals between bases 51 and 66 from the 3' end of the antigenome which is important for replication of the virus (163).

1.7 Virus Assembly and Egress

Viral proteins and RNP assemble together at the selected sites of the infected cell plasma membrane and form particles that are mainly spherical in shape. After the assembly of the viral structural components, the infectious virus particles bud out. The parameters that influence budding are not well defined. The matrix (M) protein plays a critical role in the budding process (267). The M protein acts as a scaffold to bring the viral components together during the assembly process (143). Studies with the recombinant viruses lacking the M proteins show that these viruses lose their ability to bud (306). In some paramyxoviruses such as Nipah (255), Newcastle disease virus (NDV) (250), measles (267) and SeV (338), the M protein expressed in absence of any

other viral protein is sufficient to release virus like particles (VLP) and this production is enhanced in the presence of other viral proteins such as glycoproteins and nucleocapsid protein. While for PIV5 and mumps (184), the M protein lacks the ability to form VLP by itself and requires other viral proteins for VLP formation. In the case of PIV5, the glycoproteins, HN and F assemble with the M protein at the viral plasma membrane to form a platform for the efficient budding of the virus (306). The recombinant viruses with truncations in cytoplasmic tails of the F or the HN proteins bud poorly, indicating that the tails of glycoproteins play an important role in virus budding. A schematic of virus life cycle is shown in **Figure 1.5**.

1.8 Viruses and Innate Immune Responses

The innate immune response is the hallmark of the cellular response that is triggered by invasion of pathogens including viruses. This involves initiation of multiple signaling cascades leading to blockade of the virus replication to clear the infection and also to help with establishment of an effective adaptive immunity against the virus infection. The virus infection can trigger these signaling pathways by activation of transcription factors, such as IRF-3 and NF- κ B (120). The activation of these transcription factors is a critical step for innate immune signaling pathways, resulting in production of interferons and establishing an antiviral state (171). The activation of NF- κ B pathways and interferons can be initiated by different mechanisms by viruses which involve the expression of viral protein, and/or recognition of viral molecular patterns (PAMPs, viral nucleic acids) by the pathogen recognition receptors (PRRs) including

TLR-like receptors and RIG-I-like receptors (5, 25, 171).

1.9 NF- κ B Signaling Pathway

Nuclear factor kappa B (NF- κ B) plays an important role in regulation of immune response pathways by controlling the expression of antiviral cytokine (such as, interferon- β), and major pro-inflammatory cytokines such as TNF- α and IL-6 (121). NF- κ B is one of the critical transcription factors for establishment of an antiviral innate immune response (121, 122). The NF- κ B family of transcription factors consists of five members, p65 (RelA), RelB, c-Rel, p50/p105 (NF- κ B 1) and p52/p100 (NF- κ B 2) (122). They consist of a N-terminal DNA binding/dimerization domain called Rel homology domain (RHD) (120). Through this domain, NF- κ B family members exist as homo or heterodimer and bind to DNA sequences. The RelA, cRel and RelB consist of a C-terminal transcription activation domain that is involved in activating target gene expression. The p50 and p52 lack this domain and form heterodimers with either of the other three family members (RelA, cRel and RelB) (120).

In an uninfected cell, NF- κ B family members exist in an inactive form in the cytoplasm due to their association with the I κ B family of proteins which also prevent the translocation of NF- κ B to the nucleus (122). The activation of NF- κ B can be triggered through different stimulatory signals including virus infections. This process requires the activation of specific protein kinase, “inhibitor of I κ B kinase (IKK) complex”, which

subsequently phosphorylates I κ B and culminates in degradation of I κ B through ubiquitin-proteasomal pathway (120).

IKK complex consists of two catalytically active kinases, IKK α , IKK β scaffold protein NEMO. NF- κ B signaling is generally considered to be mediated by either a classical (canonical) or an alternative (non-canonical) pathway. The classical pathway is simulated by pro-inflammatory cytokines and requires action of IKK β and NEMO to activate RelA and cRel complexes. While in the alternative pathway, IKK α alone is sufficient to phosphorylate p100. Once I κ B is phosphorylated, it is polyubiquitinated and subsequently degraded. The active NF- κ B dimers translocate in the nucleus and activate the expression of target genes (**Figure 1.6**) [reviewed in (120-122, 131)].

Both these pathways are triggered in response to a variety of stimuli, including viral and bacterial infections, stress inducing agents and cytokines (248). NF- κ B is activated by different families of viruses such as HIV-1, HTLV-1, hepatitis B virus, influenza virus, Sendai virus and respiratory syncytial virus (129-131). Viruses have developed strategies to modulate NF- κ B activation to facilitate their own replication, to inhibit virus-induced apoptosis and to evade immune responses (130). It is known that viruses can regulate this innate immune signaling pathway by utilizing their viral components, proteins or nucleic acids (129). The molecular mechanisms of virus regulation of innate immune responses are unclear.

1.10 Host Protein kinase - AKT

AKT, a serine/threonine kinase, also known as protein kinase B (PKB) is a cellular homolog of viral oncogene, AKT8 oncovirus (v-Akt). It is ubiquitously expressed in most cells and has three isoforms PKB α /AKT1, PKB β /AKT2 and PKB γ /AKT3 [reviewed in (38)]. AKT1 and AKT2 are constitutively expressed in many organs and cell types (374) but AKT3 is mostly but not exclusively expressed in neuronal cells. All the three isoforms of AKT are highly conserved (117) and share some redundant functions but also have some distinct roles that are discovered through studies using knockout mice and siRNA (323).

AKT contains a pleckstrin homology (PH) domain, a catalytic domain, and a regulatory domain and is activated by phosphorylation (**Figure 1.7A**). The pleckstrin domain is involved in interaction with membrane lipids such as phosphorylated phosphatidylinositides (PI-3,4-P₂, phosphatidylinositol-3,4-bisphosphate and PI-3,4,5-P₃, phosphatidylinositol-3,4,5-trisphosphate). The catalytic or kinase domain consists of two major phosphorylation sites within AKT, amino acid residue Thr308 and Ser473, which are phosphorylated by PDK1 (phosphoinositide-dependent kinase 1) and the rictor-mTOR complex respectively (49, 296). PDK1 is a 63 kDa serine/threonine kinase and it's a regulator of AKT. The Ser473 residue of AKT is phosphorylated by the rictor-mTOR complex which facilitates phosphorylation of the Thr308 site by PDK1 (296). Mutating these residues to alanine results in loss of kinase activation while mutating these residues to acidic residues renders constitutive activation of the kinase. The C-terminal regulatory

domain of AKT consists of a proline rich region and a hydrophobic motif with a conserved sequence where the phosphorylation of serine or threonine residues is important for activation of AKT.

AKT is activated by different stimuli such as growth factors, phosphatase inhibitors and cellular stress via the phosphoinositide 3-kinase (PI3K) signaling pathway (49). PI3K is activated after binding to phosphorylated receptor tyrosine kinases which leads to a conformational change in the catalytic domain of PI3K and consequent kinase activation. Once activated, PI3K generates PI-3,4-P₂ and PI-3,4,5-P₃ in the cell membrane that bind to the amino-terminal pleckstrin homology (PH) domain of AKT. PI-3,4-P₂ and PI-3,4,5-P₃ also activate PDK1 which then phosphorylates Thr308 of membrane-bound AKT (**Figure 1.7B**) [reviewed in (38, 208)].

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. Many viruses have also shown to utilize this pathway to facilitate their replication and growth. AKT has many downstream targets, including BAD, CREB, GSK-3, p21 CIP1 and I κ B kinase α (IKK α) (87). AKT is reported to be involved in phosphorylation of IKK α and thus can lead to activation of NF- κ B (87). In the case of RSV, it has been reported that it induces NF- κ B activation through the PI3K-AKT pathway in A549 cells to inhibit apoptosis during initial infection, thus resulting in cell survival and creating a favorable environment for RSV growth (343). It has been reported that AKT plays a critical role in replication of nonsegmented negative single stranded

RNA viruses (NNSV), possibly through its role in phosphorylation of the P protein (329). Activated AKT has also been found in many cancers, and targeting AKT using small molecules inhibitors has resulted in reduction of tumor growth in some cases (276, 377).

1.11 Interferon

Interferons (IFN) are a family of cytokines that play an important role in establishing an antiviral state in the host cells to prevent virus infections (210, 297) and therefore, controlling the virus spread. They are grouped in three classes comprising of type I, II and III according to their amino acid sequences. Type I interferon, discovered in 1957 by work of Isaacs and Lindenmann (140, 141), comprise of a large group of cytokines encoded by IFN- α and IFN- β genes which are induced directly in response to viral infection. Type II IFN consists of a single member called IFN- γ , which is induced in response to mitogenically activated T lymphocytes and natural killer cells (15, 16). Type III IFN the most recently identified group of cytokines which consists of IFN λ 1, - λ 2 and - λ 3 also referred as IL-29, IL-28A and IL28B, respectively and are also induced in response to virus infection (11, 12). The IFNs play an important role in modulation of the immune responses that are exerted through binding of the IFN molecules to distinct cellular receptors. This binding triggers signal transduction pathways leading to activation of interferon stimulated genes (ISG) or interferon-inducible genes and hence establishment of an effective antiviral state (272).

The type I IFNs bind to common heterodimeric receptors which are ubiquitously expressed in human cells and are composed of two subunits, IFNAR1 and IFNAR2. These subunits are associated with the Janus activated kinases (JAKs) tyrosine kinase 2 (TYK2) and JAK1, respectively [reviewed in (241, 354)]. The type II IFN, IFN- γ binds a distinct cell-surface receptor known as the type II IFN receptor which is also composed of two subunits, IFNGR1 and IFNGR2 and is associated with JAK1 and JAK2, respectively (15, 264). Type III IFNs are also secreted and bind to receptors such as IL-28 receptor which comprise of a heterodimer of IL10R2 and IFNLR1 and are reported to elicit an antiviral response through a similar mechanism as type I IFN (221, 245, 353), but unlike type I IFN receptors, type III receptor show limited tissue distribution (392). The precise role of type III IFN for the host protection in response to virus infection still needs to be established.

1.12 Viral Inducers

The process of interferon induction is initiated by recognition of specific pathogen-associated molecular pattern (PAMPs) motifs, generated during the virus infection by pathogen recognition receptors (PRRs). These PAMPs are viral components that are produced during virus replication, comprising of viral nucleoproteins or single (uncapped with 5' triphosphate) or double stranded RNA (5). The dsRNA from bacteria, reovirus, vaccinia virus and synthetic polyinosinic:polycytidylic acid [poly(I):poly(C)], have all been shown as potent activators of interferon (63-66, 96, 178, 347) but till now the biochemical basis of the induction by dsRNA remains unclear. The role of dsRNA as

a viral trigger of interferon induction is widely accepted despite the fact that paramyxoviruses restrict the exposure of their RNA during infection. Also the studies using dsRNA-specific antibodies have shown that negative sense RNA viruses do not produce detectible amounts of dsRNA (365). However, this conundrum has been resolved by explanation that the dsRNA can be exposed as a defect or error by virus during its replication process. The detection by dsRNA antibodies can be limited by the amount of dsRNA available in the cells or length of dsRNA molecule.

The other potent inducer of the IFN response is RNA with a 5'-triphosphate group. The RNA polymerase synthesizes RNA with a triphosphate moiety at its 5'-end (132, 260). The cellular RNA are generally processed either through capping, methylation and RNA folding, thus the likelihood of a 5'-triphosphate containing cellular RNA in the cytoplasm is highly uncommon. This makes 5'-triphosphate RNA a distinct viral detection motif. The RNA viruses are known to synthesize this 5'-triphosphate RNA but the question is how are these molecules being exposed in the cells considering the genome of paramyxoviruses is highly encapsidated.

In addition to viral RNA, the roles of viral ribonucleoprotein (RNP) and viral proteins have been identified in the IFN induction. The purified RNP complexes have been shown to induce the IFN response in the cells but it remains unclear if intact RNP is introduced into the cells (341, 342). The NS5A protein of hepatitis C virus has been shown to induce activation of NF- κ B (363). The F protein of RSV has been reported to trigger activation of pro-inflammatory cytokines through TLR4 (172).

1.13 Cellular Receptors

The two distinct virus sensors that induce interferon include surface/endosomal Toll-like receptors (TLRs) and the cytoplasmic Retinoic acid inducible gene I (RIG-I)-like receptors (RLRs). The secreted interferons establish immunity by alerting bystander cells through triggering signaling cascades that express IFN-stimulated genes.

1.13.1 TLR Signaling Pathway

The TLRs are type I transmembrane glycoproteins that consist of leucine rich repeats and a cytoplasmic Toll/IL-1 receptor homology (TIR) domain (3). These receptors encounter the pathogens either on the cell surface or on lysosomes/endosomes as a result of endocytosis or autophagy of cytoplasmic material (335). Different receptors recognize distinct viral nucleic acids. At present, 13 mammalian TLRs have been identified, of which TLR3, TLR7 and TLR8, located intracellularly in the endosomal compartments have been reported to be viral RNA sensors while the other members are involved in detection of bacteria, fungi and DNA viruses which are commonly located on cell surfaces (4, 335). TLR3 is involved in detection of double stranded (ds) RNA present on the cellular membrane or endosomal compartments of macrophages, fibroblasts, conventional dendritic cells (cDCs) and epithelial cells (7). In plasmacytoid dendritic cells (pDCs), TLR3 can also respond to dsRNA presented in the phagocytosed apoptotic cells. TLR7 and TLR8 are involved in recognition of single stranded (ss) RNA that are

rich in G/U residues (126) while TLR9 recognizes unmethylated DNA with CpG motifs (27). TLR7 is widely found in endosomal compartments of pDCs while TLR8 in myeloid dendritic cells (mDCs) (82). After the recognition of the ligand, the TLR mediated signaling pathways are activated by Toll-interleukin (IL)-1-resistance (TIR) domain containing adaptor molecules, TRIF (recruited by TLR3) or MyD88 (recruited by TLR7/8 or TLR9). This stimulates activation of kinases further leading to activation of the transcription factors, IRF-3 and NF- κ B (161) which further leads to activation of IFN- β gene. The optimal induction of IFN- β also requires binding of ATF-2/c-jun on the promoter leading to recruitment of co-factors such as CREB-binding protein (CBP)/p300 and RNA polymerase II (223).

In NF- κ B mediated activation of IFN, TRIF acts as a scaffold to recruit tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and receptors-interacting protein 1 (RIP1). TRAF6 initiates polyubiquitination of itself and RIP1 which is recognized by TAK-1 binding proteins and recruits transforming growth factor β -activated kinase 1 (TAK1). This complex then recruits IKK complex (IKK α , β , γ) which results in phosphorylation of IKK β by TAK-1 and leads to activation of NF- κ B by phosphorylation and degradation of I κ B.

In the interferon regulatory factor 3 (IRF-3) mediated signaling pathway, TRIF recruits TRAF3 which interacts with the TRAF family member-associated NF- κ B activator (TANK) which is associated with TANK-binding kinase 1 (TBK-1). The

activated TBK1/IKK ϵ phosphorylates IRF-3 leading to its dimerization and translocation of IRF-3 into the nucleus. In pDCs, TLR7 and TLR8 are activated in response to ssRNA and recruit myeloid differentiation factor 88 (MyD88), that activate a complex of kinases consisting of interleukin-1 receptor-associated kinase 4 (IRAK4)/IRAK1/TRAF6 and leads to IRF-7 activation (349). TRAF6 activation can also stimulate NF- κ B signaling pathway through IKK. TLR9 are localized on the endosomes that can activate IFN production through MyD88 and TRAF6 mediated pathways in response to unmethylated DNA. These signaling cascades results in production of type I interferons and pro-inflammatory cytokines (**Figure 1.8**) [reviewed in (272, 350)]. However, the roles of TLRs involved in recognition of extracellular viral dsRNA are dispensable for IFN response in cell types except plasmacytoid dendritic cells.

1.13.2 RLR Signaling Pathway

Retinoic acid-inducible gene-I (RIG-I), melanoma differentiation associated gene-5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) belongs to the RLR family of RNA helicases involved in cytoplasmic sensing of some RNA viruses (5). These cytoplasmic sensors play an essential role in activation of IFN expression in most cell types, including epithelial, fibroblastic and conventional dendritic cells. Both RIG-I and MDA5 act in parallel to activate signaling cascade for IFN production after being triggered by the viral PAMPs (378). Following the binding of RIG-I and MDA5 with their respective RNA ligands, they homo-oligomerize to expose their CARD domains (157) and recruit a CARD domain containing adaptor protein which is located on the

outer mitochondrial membrane called as interferon- β promoter stimulator protein-1 (IPS-1) [also known as mitochondrial antiviral signaling protein (MAVS), virus-induced signaling adaptor (VISA) or CARD adaptor inducing IFN- β (Cardiff)]. As a consequence of this association, IPS-1 initiates a downstream signaling cascade which involves activation of serine kinases [I κ B kinase (IKK)–related kinases, IKK-I and TBK1], resulting in activation of the transcription factors, NF- κ B and IRF-3 and subsequently, leading to production of IFN- β and pro-inflammatory cytokines (**Figure 1.8**) (5, 158, 379). IFN- β binds to its receptors on the uninfected cells and induces the expression of interferon stimulated genes which establishes an antiviral state.

A. RIG-I

RIG-I was identified as a key sensor of viral RNA and as an inducer of interferon production through a cDNA screen for its ability to induce IRF reporter upon poly(I):poly(C) treatment (379). Studies have demonstrated the role of RIG-I as a key receptor for different RNA viruses including negative stranded RNA viruses such as Sendai virus, influenza A and B, Newcastle disease virus, rabies virus, measles virus, respiratory syncytial virus (RSV), vesicular stomatitis virus (VSV) and some positive stranded RNA viruses such as hepatitis C virus (157, 201, 327).

RIG-I consists of three basic domains, the N-terminal terminal caspase activation and recruitment domains (CARD), a central DExD/H-Box RNA helicase domain and C-terminal regulatory domain (378) (**Figure 1.9A**). RIG-I contains two copies of CARD,

which are involved in its interaction with the IPS-1 CARD domain and thus transducing signals for IFN production (160). The CARD1 of RIG-I is required for its interaction with IPS-1, and CARD2 undergoes ubiquitination which is important for RIG-I activation. The RIG-I constructs lacking either or both of the CARD domains have been reported to act as dominant negative (291). Interestingly, the CARD domain itself is sufficient to induce IFN induction without any virus stimulus for its activation. But this function of CARD domain is only observed when expressed in cells with wild type RIG-I cells but not in RIG-I deficient cells (291). The native conformation of RIG-I is an inactive state and requires an appropriate signal for its activation (378).

The helicase domain consists of six conserved DExD/H helicase motifs and exhibits two important enzymatic functions, ATPase and helicase activity (379). Like other DExD/H-Box proteins, RIG-I also requires ATP hydrolysis function for translocating and unwinding the RNA (18). The *in vitro* biochemical studies have also shown that while ATP hydrolysis is critical for RIG-I signaling but it is not sufficient for inducing IFN production. It has been demonstrated that many *in vitro* transcribed RNA or synthetic double stranded RNA molecules exhibiting ATPase activity *in vitro*, were failed to induce IFN production when transfected into the cells (301, 302, 334). Some studies have suggested that the regulatory domain of RIG-I can also be important for ATP hydrolysis function thus complementing the ATPase activity by helicase domain (70). The helicase domain is also involved in RNA binding. The *in vitro* binding assays and competition experiments have suggested that the helicase domain has a preference for

binding to dsRNA with 5'-OH groups than any RNA with 5' triphosphate (ss or ds), which are recognized by regulatory domain of RIG-I (18, 302, 334).

The carboxy-terminal domain (CTD) also known as the repressor domain (RD) is responsible for self inhibition of RIG-I signaling. This repression is likely initiated through the association of RD with both CARD and helicase domain. Upon RNA binding, a conformational change occurs in RIG-I that allows it to unfold and thus exposes the CARD to initiate downstream signaling. The role of RD has also been identified as the RNA recognition domain of RIG-I (70, 334). The structural studies of RD have identified a basic groove on one side and acidic region located on the opposite side. It has been suggested that the basic groove can serve as a site for 5'-triphosphate RNA recognition (334) while the acidic surface provide suitable region for interaction with the CARD domain of RIG-I. The RD has also been reported to be important for RIG-I dimerization. *In vitro* studies have revealed that like full length RIG-I, the RD alone is sufficient to form dimer in presence of 5'ppp-RNA (70, 291, 379).

The role of RIG-I as a viral sensor is well established in the innate antiviral immunity. The RNA detection by RIG-I is dependent on substrate specificity and many studies have highlighted the key features of RNA recognized by RIG-I. The studies with *in vitro* transcribed (IVT) RNA have demonstrated that RNA, single or double stranded with an uncapped terminal 5'-triphosphate (5'-ppp) of at least 19 nucleotides in length, act as potent inducers of the RIG-I mediated IFN pathway (132, 260). The importance of the 5'-ppp was discovered by the studies using treatment of the IVT RNA with calf

intestinal alkaline phosphatase (CIP), which rendered the RNA refractory to detection by RIG-I. Interestingly, a synthetic ssRNA of the same sequence as IVT RNA with a 5'-OH group was not able to induce any IFN production (157). The physiological relevance of 5'-ppp RNA as a RIG-I PAMP was established using viral genomic RNA. The RNA isolated from influenza A or rabies virus, did not stimulate IFN activation upon treatment with CIP and also no IFN production was detected upon transfection of viral RNA in RIG-I deficient MEFs (157). The single or double stranded 5'-ppp RNA is widely accepted as a RIG-I ligand. This concept is interesting as in the case of most paramyxoviruses the 5'-ppp is present on the genome and the antigenome which makes them an inherent part of the virus life cycle and less likely to be mutated. However, viruses have evolved strategies to hide these immune response stimulants through tightly encapsidating the viral genome. The 5'-ppp also serves as a distinguishing factor between viral and cellular RNA, as in the case of the cellular RNAs 5'-end are processed either by capping, methylation, cleavage and base modifications [(233), reviewed in (28, 300)].

Interestingly, it has been reported that the 5'-ppp end also requires a dsRNA complementarity region of at least 10-18 nucleotides for induction of RIG-I mediated IFN activation (302). It was shown that a chemically-synthesized 5'-ppp ssRNAs were unable to activate RIG-I-mediated IFN production but if these RNAs were first hybridized to complementary RNA oligos, they could activate RIG-I (301, 302). Furthermore, these studies analyzed the true nature of 5'-ppp *in vitro* transcribed T7 ssRNA transcripts by separating them on polyacrylamide gel and found that the RNA transcripts consist of a mixture of dsRNA and ssRNA products and purifying the RNA

fragment corresponding to ssRNA did not induce any IFN production upon transfection (301, 302). It still remains unclear whether short dsRNAs require a 5'ppp or if a monophosphate is sufficient to induce RIG-I activation (300). The more insightful information about dsRNA as RIG-I ligand was provided by the study that utilized short poly(I):poly(C) about 30-1000 bp, where the IFN induction was found to be solely dependent on RIG-I (156). Another feature of the RIG-I ligand has been demonstrated is the sequence composition of RNA, if it is rich in homopolyuridine or homopolyriboadenosine motifs, it induces more efficient IFN response than without it (260, 290, 292).

B. MDA5

Similar to RIG-I, MDA5 is also DExD/H helicase family member consisting of two N-terminal CARD domains, a central helicase domain with dsRNA dependent ATPase motif and a C-terminal regulatory domain (**Figure 1.9A**) (153). MDA5 was first identified in a screening of genes that were upregulated with IFN treatment and involved in growth suppression of melanoma cells (153). The role of MDA5 as an antiviral protein was implicated in studies with PIV5, where it was reported that MDA5 is involved in interaction with interferon antagonist, the V protein (10). This interaction of MDA5 has been demonstrated with the V proteins of all paramyxoviruses which prevent the binding of dsRNA and self-association of MDA5, thus inhibiting the downstream signaling for IFN activation (57). Similar to RIG-I, overexpression of MDA5 stimulates IFN production in the presence of synthetic double stranded RNA (dsRNA), poly(I):poly(C)

(10). Like in RIG-I, the CARD domain of MDA5 alone is sufficient to induce IFN expression while the helicase domain exhibits a dominant negative phenotype when expressed alone (10). However, unlike RIG-I, the RD of MDA5 does not have an autoinhibitory activity as the MDA5 construct lacking the RD is capable of IFN induction (291). The binding efficiency of RD is very poor to dsRNA or *in vitro* transcribed RNA (70, 333, 334). The dsRNA is known as a potent inducer of MDA5 stimulated IFN production but considering that MDA5 has a weak binding efficiency for dsRNA, it remains unclear how MDA5 recognizes dsRNA and activates the IFN production in response to dsRNA .

The studies with knockout MDA5 mice show that MDA5 is an important receptor for *in vivo* recognition of poly(I):poly(C) and picornaviruses (encephalomyocarditis virus (EMCV), mengo virus) (108, 157, 201, 327), *Caliciviridae* (murine Noravirus-1 in dendritic cells) (220) and coronavirus, Mouse Hepatitis Virus (MHV) in brain macrophages (280). MDA5 has been reported to recognize stable, long, double stranded RNA structures greater than 2 kilobase pairs (kb) generated during RNA virus infection which are not typical of cellular RNA) (156). Long synthetic dsRNA polymers of poly(I):poly(C) are often used as a surrogate for the putative activator of MDA5 (108). A natural ssRNA trigger for MDA5 has not been identified.

The picornaviruses do not contain 5'-triphosphate RNA which make them less likely to be recognized by RIG-I, however a recent study has identified role of picornavirus proteinase 3C(pro) which specifically cleaves RIG-I, thus suggesting RIG-I

can also be important for detection of picornavirus infections (24). While some RNA virus infections such as West Nile virus, Dengue virus type 2 (DENV2) and type 3 Dearing (T3D) reovirus, type1 Lang (TIL) reovirus can be recognized by both RIG-I and MDA5 (101, 201), suggesting these two receptors have overlapping functions. The variable recognition by these two receptors is attributed to the specificity of the viral nucleic acid generated during infection and also to the cell type. For instance, in the case of Sendai virus infection which expresses inhibitors for both RIG-I and MDA5 (326), it is shown to be sensed by RIG-I in MEFs (157) but in dendritic cells, it is primarily recognized by MDA5 (382). This suggests that the differential recognition by RIG-I and MDA5 can be due to differences in the presence of distinct viral PAMPs. It remains unclear what is responsible for this cell type specificity of the virus whether it is the differential expression of the viral sensors or differences in the virus replication.

C. LGP2

The third member of the RLR family is LGP2 which possesses a DexD/H-Box helicase domain and a C-terminal regulatory domain, similar to RIG-I and MDA5, however it lacks the CARD domain hence, it is incapable of signaling through IPS-1 for IFN induction (**Figure 1.9A**) (378). The RD of LGP2 is structurally and functionally very similar to RIG-I (262). It is involved in RNA binding and dimer formation *in vitro* (228). The RD expression by itself is sufficient to inhibit RIG-I-mediated signaling, however this is not as efficient as full length LGP2 (228). The RD of all three receptors, RIG-I, MDA5 and LGP2, have a very similar structure that consists of conserved cysteine

residues which play roles in RD function (70, 185, 228, 333, 334). The RNA binding feature is exhibited by the basic surface present on RD for all the receptors (70, 262, 334). LGP2 can also bind to any dsRNA without any requirement of a 5'-triphosphate (228, 262).

LGP2 activity has been reported to be specific to the RLR signaling as its expression does not affect TLR induced IFN production (378). It is known to be a negative regulator of RIG-I and a positive regulator of MDA5 (298, 378). Its role as negative regulator of the RIG-I pathway has been identified in Lgp2 deficient mice infected with VSV (357). The mechanism of LGP2 interference of the RIG-I pathway is not clear but some studies have suggested few possibilities for this inhibition. It has been suggested that LGP2 can inhibit RIG-I-mediated viral RNA recognition likely by sequestering the viral RNA due to the fact that LGP2 possesses a strong binding affinity to dsRNA (378). The LGP2 can likely interfere with RIG-I and IPS-I binding through its regulatory domain as LGP2 and RIG-I forms a complex in the infected cells (281, 291). LGP2 can also possibly exert RIG-I inhibition by competing with the kinases recruited by IPS-1 to suppress the RLR signaling (169). This probable mechanism is supported by the study which has shown that LGP2 can compete with IKK ϵ for IPS-1 binding (169).

Apart from its negative role, LGP2 also acts as a positive regulator of MDA5-mediated IFN induction. This role has been identified in studies with picornavirus infections. LGP2 interacts with MDA5 in the infected cells. Its role as a regulator of MDA5 signaling was recognized in studies utilizing *in vivo* models of Lgp2 deficient

mice and mice with an inactive ATPase activity in the DExD/H-box RNA helicase domain. In these studies, the IFN response to EMCV infections was found to be severely impaired (291). The same animals were found to be resistant to RIG-I specific virus such as SeV or NDV, which supported the differential role of LGP2 in regulation of RIG-I and MDA5 mediated signaling. In accordance with the *in vivo* model, the LGP2 knockout MEFs have also been reported to have poor IFN production after poly(I):poly(C) transfection (357). It has also been demonstrated that MDA5 dependent activation of IFN in response to poly(I):poly(C) reached to its maximal level in the presence of an equal amount of LGP2 (262). Interestingly, it has been shown that the paramyxovirus V protein interact with both LGP2 and MDA5, but not with RIG-I, demonstrating a unique link between these two proteins (251). The helicase domain of LGP2 and MDA5 mediates this interaction with the V protein which dampens the ATPase activity of both receptors (251). The role of LGP2 as a negative or positive regulator of RIG-I and MDA5 mediated signaling still remains perplexing, as the two receptors act in parallel for IFN induction. However, the relative abundance and differences in the kinetics profiles of these three receptors in various cell types may account for their differential activities.

D. RNase L and the Antiviral Response

The endoribonuclease RNase L is important for activating the antiviral immune response to RNA virus infection or dsRNA stimulation (389). RNase L is one the major components of the IFN regulated pathway known as the 2-5A system. The 2-5A pathway is an antiviral pathway, other than dsRNA dependent protein kinase (PKR) which is

stimulated by IFNs and dsRNA (224). The human RNase L is ubiquitously expressed in almost every mammalian cell type. It is an unusual nuclease enzyme which requires binding with a small oligonucleotide, 2-5A for its activation (59). The 2-5A are 5'-triphosphorylated oligoadenylates with 2'-5' phosphodiester bonds (60). The activation pathway of RNase L is stimulated by IFN. The IFN can stimulate transcription of several 2-5A synthetase (59, 275). The 2'-5' oligoadenylate synthetase (OAS) is activated through direct recognition of viral dsRNA (viral replication intermediates or viral mRNA) and produces 5'-triphosphorylated 2'-5' oligoadenylate (2-5A) species from ATP (235, 275). Several viral RNAs that can activate 2-5A synthetase have been identified including, TAR RNA of HIV-1 (204), EBER-1 RNA of Epstein-Barr virus (310) and the adenoviral VAI RNA (79). These 2-5A species specifically bind to RNase L and activate it. Once RNase L is activated, it leads to cleavage of single stranded regions of cellular and viral RNA at 3'UpUp and UpAp sequences, resulting in duplex RNA (99, 116, 205, 236). The viral mRNA cleavage by RNase L has been shown in treatment of VSV infected cells with 2-5A (134), HIV-1 and EMCV infected cells (186, 211). These duplex RNA structures can also be recognized by the helicases and thus stimulate the signaling pathways to activate IFN gene expression.

The 2-5A species are very unstable and are generally dephosphorylated by phosphatases at their 5'-end and can also be degraded by 2'-phosphodiesterase making it incapable to activate RNase L (170). The specific features of 2-5A species have been characterized which are essential for RNase L activation. These oligomers require the presence of at least two adenylates, 5'-monophosphate, 2'-5'-phosphodiester bonds and

3'-hydroxyl groups of the second adenosine (from the 5' terminus) to activate RNase L (313, 316). The 5'-terminal adenine base is important for binding with RNase L while the adenine of 2'-terminal adenosine is essential for the nuclease function of RNase L (86).

The RNase L was first identified in a 185kDa complex by gel filtration (319) and then as a 78-80kDa protein under denaturing gel conditions (100). RNase L is found to exist in two forms that can bind to 2-5A, depending on the cell type (29, 34). In the case of mouse spleen and liver and in Ehrlich ascites tumor (EAT) cells, a small proteolytic cleavage product of RNase L, about 40-46kD was identified that can bind to 2-5A and also performs 2-5A dependent nuclease activity (29, 100). The human RNase L is a 741-amino acid protein with a molecular mass of 83.539 kDa (389). It consists of three domains: the N-terminal ankyrin repeat domain which serves as a regulatory domain facilitating its interaction with 2-5A, a protein kinase homology domain and a C-terminal ribonuclease domain (**Figure 1.9B**) (340). The N-terminal domain consists of eight complete and one partial ankyrin motifs (R1-R9) with two walker A motifs (ATP or GTP fixation) within R7 and R8 (119, 226). The ankyrin motifs are involved in mediating protein-protein interactions and also interact with 2-5A (226). The C-terminal half of the protein is responsible for the ribonuclease activity of RNase L (84, 86). The interaction of 2-5A with the ankyrin domain of RNase L mediates a conformational change which exposes the C-terminal domain and leads to homodimerization and nuclease activation of RNase L (83-86, 232).

RNase L is a very fascinating enzyme which is reported to cleave not only viral mRNA and rRNA but also the cellular mRNA, thus playing an important role in regulation of various biological activities (186, 205, 317, 371). The role of RNase L as an antiviral protein has been clearly demonstrated in cells infected with EMCV (369), vaccinia virus (278), reovirus (237), West Nile virus and herpes simplex virus 1. It has been shown that transfection of 2-5A confers resistance to EMCV (364) and *in vivo* the antiviral role of the 2-5A pathway was demonstrated using RNase L knockout mice which were unable to stimulate IFN activation (32, 390). These knockout mice were found to be highly susceptible to virus infections including, West Nile virus, EMCV, herpes simplex virus and also showed reduced IFN production (97, 294, 299, 387).

The viruses have also evolved strategies to counteract this antiviral activity of 2-5A/RNase L pathway. In some virus infections, despite presence of the 2-5A species, the activation of RNase L is not detected. In case of herpes simplex virus 1 and 2 infection, 2-5A analogues are synthesized which compete with authentic 2-5A for RNase L binding and preventing activation of RNase L (47). The NS1A protein of influenza virus A can sequester dsRNA and thus inhibits 2-5A activation (225). The EMCV and HIV-1 can recruit a host protein, ribonuclease L inhibitor (RL1) to inhibit RNase L activity (33, 211, 212). Interestingly, HCV can also escape RNase L activity by introducing mutation at RNase L recognition sites (115, 116). The mRNA of IFN resistant genotypes of HCV (HCV 1a and 1b) consist of less UA and UU dinucleotides which are the cleavage sites of RNase L than IFN sensitive genotypes of HCV (HCV 2a, 2b, 3a and 3b) (115). It is speculated that the interferon therapy is not effective for the patients infected with HCV

1b viruses than to HCV genotype 2, as HCV 1b virus is able to escape the RNase L activity by introducing silent mutations in mRNA at UA and UU dinucleotides.

RNase L is also implicated in regulation of translation and stability of cellular mRNA, thus it is important for maintaining cellular processes (181). For instance, MyoD mRNA stability is found to be regulated by RNase L which is important for muscle cell differentiation (35). It also regulates mitochondrial mRNA stability which can contribute to anti-proliferative activity of IFNs (180). RNase L is also implicated to be important for PKR mRNA stability and interferon stimulated genes: ISG43 and ISG15 (164, 187). The role of RNase L has also been identified in apoptosis in response to virus infections (45, 46). RNase L expression can degrade mitochondrial mRNA and induce IFN α mediated apoptosis which requires caspase-3 activity and results in elimination of virus infected cells (51, 285). RNase L has also been shown to have tumor suppressive activities and has been linked to prostate cancer (200). The RNase L gene was reported to be one of the genes linked to Hereditary prostate cancer (HPC) (44, 372). Several germline mutations or variants in *HPC1/RNASEL* have been identified and the cases with mutations in one or more copies on RNase L gene have found to possess higher risk of prostate cancer (314). RNase L is a unique nuclease enzyme with diverse functions involved in regulation of innate immunity and cellular processes but its exact mechanism of action still remains to be defined.

1.14 Significance of the study

The early innate response to intrusion of pathogens is characterized by expression of pro-inflammatory chemokines and antiviral cytokines. This host defense mechanism is responsible for detecting and clearing the virus infections. The most critical antiviral response is the interferon system which involves a series of signaling events for its activation. The innate immune responses help in combating virus infection and also aid in shaping up an effective adaptive immune response. The proteins responsible for recognition of virus infections to initiate these immune responses have been identified and their roles in this intricate signaling are now starting to be characterized. The TLRs and RLRs, along with Protein Kinase R (PKR) and RNase L form a wide group of viral sensors (205, 352). They are involved in initiating antiviral responses which are marked by activation of transcription factors such as NF- κ B and IRF-3 and ultimately leads to production of interferons. It is possible that these viral sensors are working in accordance with each other in the infected cells to combat virus infection but depending on the cell type, virus and infection conditions, one sensor is more active than the other. The studies using the knockout mice and cells have clearly established the roles of these viral sensors in the innate immunity but it also insinuates the possibility of the existence of other unidentified sensors. The other piece of the puzzle is formed by the viral recognition motifs that can stimulate an antiviral response through recognition by these sensors. Even though the dsRNA and 5'-ppp RNA are now accepted as paradigm molecules for virus detection, still the actual molecular nature of the viral signature molecules and their physiological roles in triggering interferon activation need to be characterized.

Our study has been focused on understanding the molecular mechanisms and identifying viral components that can lead to the activation of these innate immune responses. In this study, we discuss a novel role of the L protein in activation of NF- κ B through a host kinase protein, AKT and also identified the RNA sequence of the L gene that can induce innate immune responses through RNase L and MDA5 suggesting a possible natural ligand for MDA5.

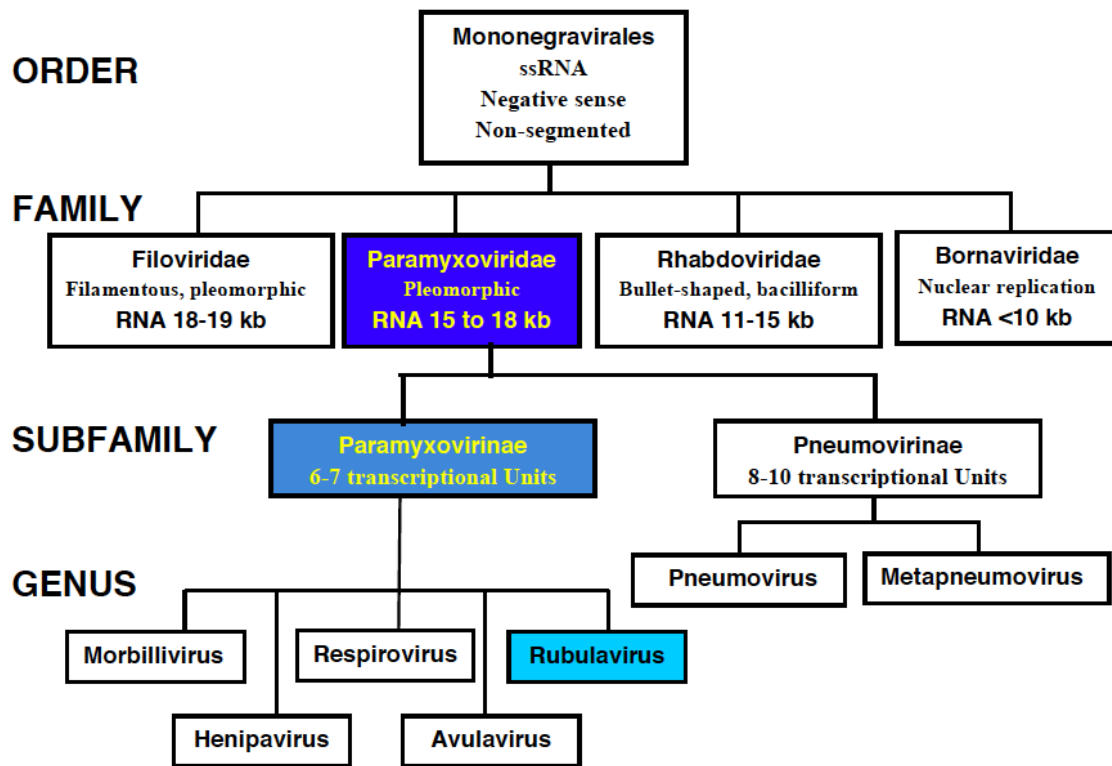


Figure 1-1. Classification of Nonsegmented Negative Stranded RNA Viruses. This chart represents the current classification of *Mononegavirales* with the new genera *Henipavirus* and *Avulavirus*. The highlighted boxes represent the Family, Subfamily and the Genus of PIV5. Modified from Wang *et. al.* (360).

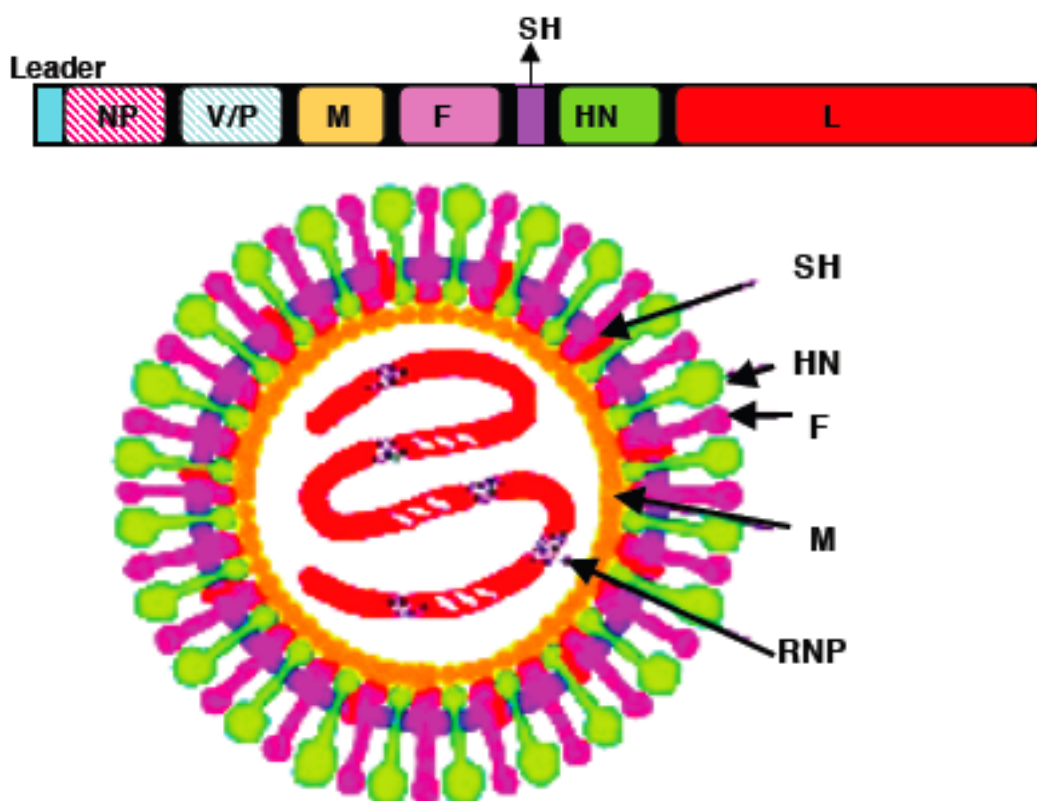


Figure 1-2. Schematic representation of Parainfluenza Virus 5 genome and particle structure. PIV5 (genome size 15,246 nucleotides) is an enveloped nonsegmented negative stranded RNA virus which has seven genes but encodes for eight proteins. The RNA genome of the virus is bound by the nucleocapsid (NP) protein to form helical structures called ribonucleoprotein (RNP) complex. The L (Large) polymerase protein and the P protein are important part of RNA dependent RNA polymerase which is responsible for transcribing the NP (nucleoprotein)-encapsidated RNA genome into 5'-capped and 3'-polyadenylated mRNA and replicates the virus in the cytoplasm. The F (fusion) is responsible for mediating virus entry by promoting cell-cell and virus-cell fusion in a pH independent manner. The HN (hemagglutinin–neuraminidase) protein is involved in virus entry by attachment of virus-cell and release from the host cells by removing sialic acid. The M (matrix) protein is important in virus assembly and budding. The SH (small hydrophobic) protein is a 44 amino acid integral membrane protein with its N-terminal in the cytoplasm which plays role in blocking the TNF- α mediated extrinsic apoptotic pathway. The V/P is transcribed into V and P mRNA though RNA editing. The V is a multifunctional protein, important for viral pathogenesis and virus replication. The leader and the trailer sequences are important for viral RNA synthesis (transcription and replication). Modified from Lamb and Kolakofsky (173)

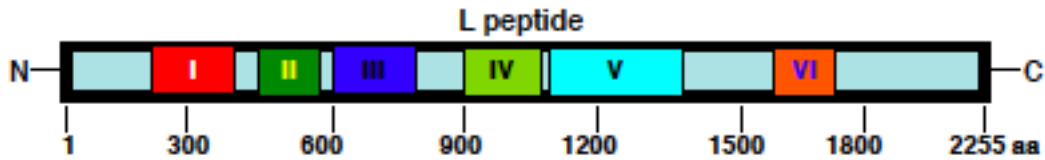


Figure 1-3. Schematic illustration of L protein. L is 250 kDa protein consisting of six domains (marked I-VI) that are conserved among paramyxoviruses. Modified from Higuchi *et. al.* and Poch *et. al.* (128, 266).

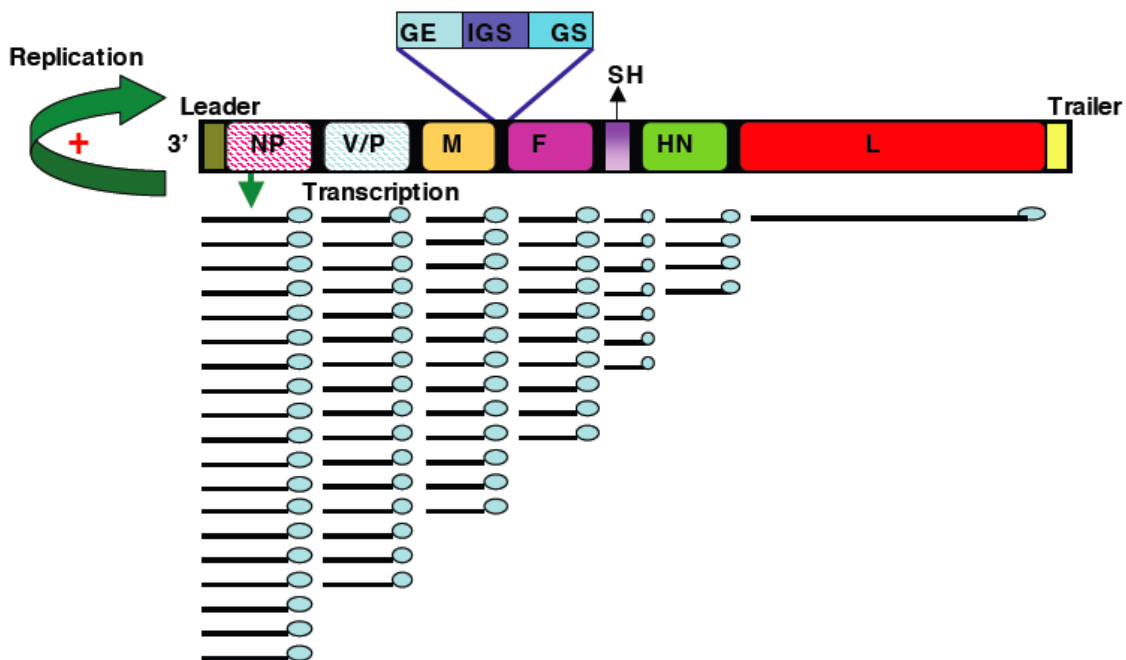


Figure 1-4. RNA synthesis of PIV5. Synthesis of all viral mRNAs initiates at the 3'-end leader sequence and vRdRp (P and L complex) transcribes viral RNA genome (vRNA) into mRNAs containing a 5'-cap structure and a 3'-poly(A) tail in a sequential manner. The junctions between each gene consist of three-nucleotide sequence elements. At the gene-end (GE) sequence, polyadenylation occurs and transcription terminates releasing a polyadenylated RNA. At this juncture, the vRdRp either leaves the template (attenuation) or passes over an intergene sequence (IGS) not found in mRNAs, and then reinitiates mRNA synthesis at a downstream transcriptional gene-start (GS) sequence. A failure to reinitiate transcription at a downstream site results in a gradient of mRNA production inversely proportional to the distance from the 3' end of the genome. Modified from Lamb and Kolakofsky (173).

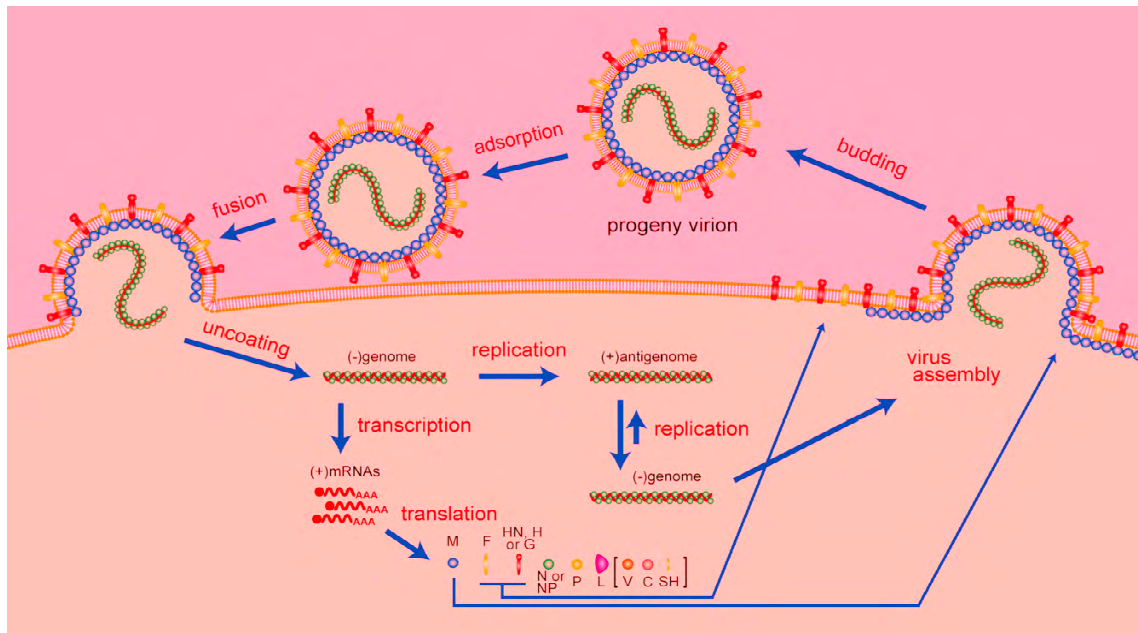


Figure 1-5. Schematic of paramyxovirus life cycle. Paramyxovirus infections are initiated with the binding of the virus to the host cell surface receptors (Adsorption). This process involves the attachment protein, HN which binds to sialic acid receptors in the case of SeV, PIV5 or MuV while the H protein of measles virus and the G protein of *Henipaviruses* bind to the protein receptors. Following attachment, fusion proteins initiate fusion of viral membrane with the host cell membrane which allows the viral genetic material (RNP) to enter the cytoplasm. RNA dependent RNA polymerase starts the transcription process with negative sense RNA genome serving as the template for more mRNA production. RNA polymerase then switches to replication mode which allows production of positive sense antigenome and simultaneous production of negative sense genomes. The newly synthesized viral proteins and RNP complex are assembled together with help of M protein at the plasma membrane and buds from the cell surface which completes the virus life cycle. The V protein and other accessory proteins such as SH or C are involved in inhibiting host antiviral innate immune responses, allowing virus to replicate. Modified from Harrison, M.S. *et al.* (118)

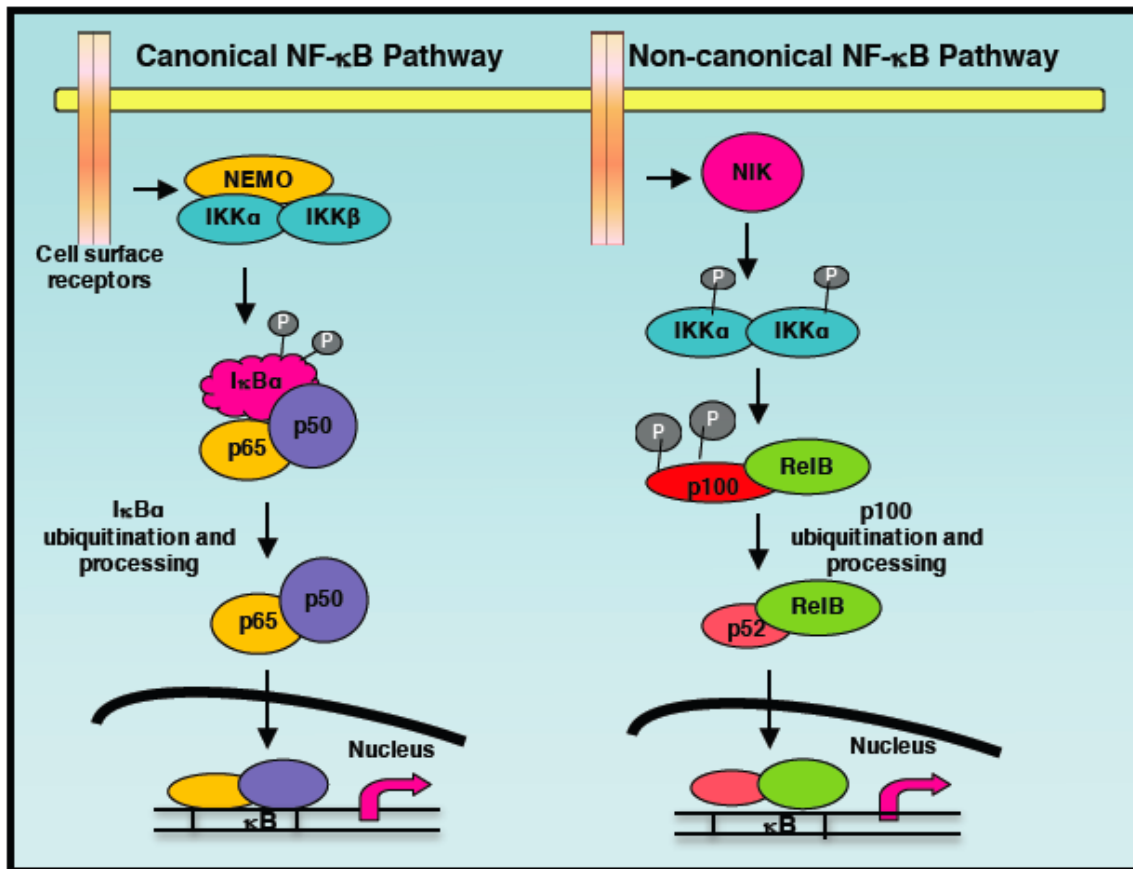


Figure 1-6. A model depicting classical (canonical) and Alternative (non-canonical) pathways for NF-κB activation. The classical pathway is mediated by IKKβ leading to phosphorylation of IκB. The alternative pathway is initiated by NIK activation of IKKα and leads to the phosphorylation and processing of p100, generating p52:RelB heterodimers. Modified from Gilmore, T.D. and Hayden, M.S. and Ghosh, S. (107, 120).

A.



B.

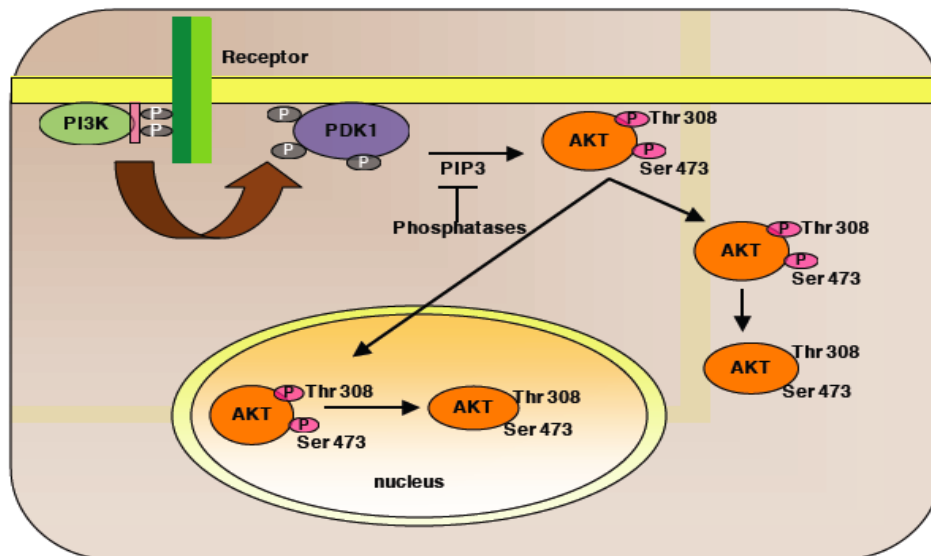


Figure 1-7. AKT signaling pathway (A) A schematic of AKT illustrating its domain architecture. AKT consists of three domains: a pleckstrin homology (PH) domain, a catalytic (kinase) domain, and a C-terminal regulatory domain. It is activated by phosphorylation. The two major phosphorylated sites are Thr308 and Ser473. **(B) PI3K–AKT activation signaling pathway.** Activated PI3K leads to synthesis of phosphoinositide triphosphates (PIP3). AKT translocates to the plasma membrane and binds to PIP3, where it undergoes phosphorylation at two sites Ser473 and Thr308. The activated AKT translocates from plasma membrane to cytoplasm or nucleus where it can phosphorylate the effector proteins. Finally AKT is dephosphorylated by protein phosphatases (inositol polyphosphate phosphatases) which completes this activation cycle. Modified from Du, K. and Tsichlis, P.N. (87).

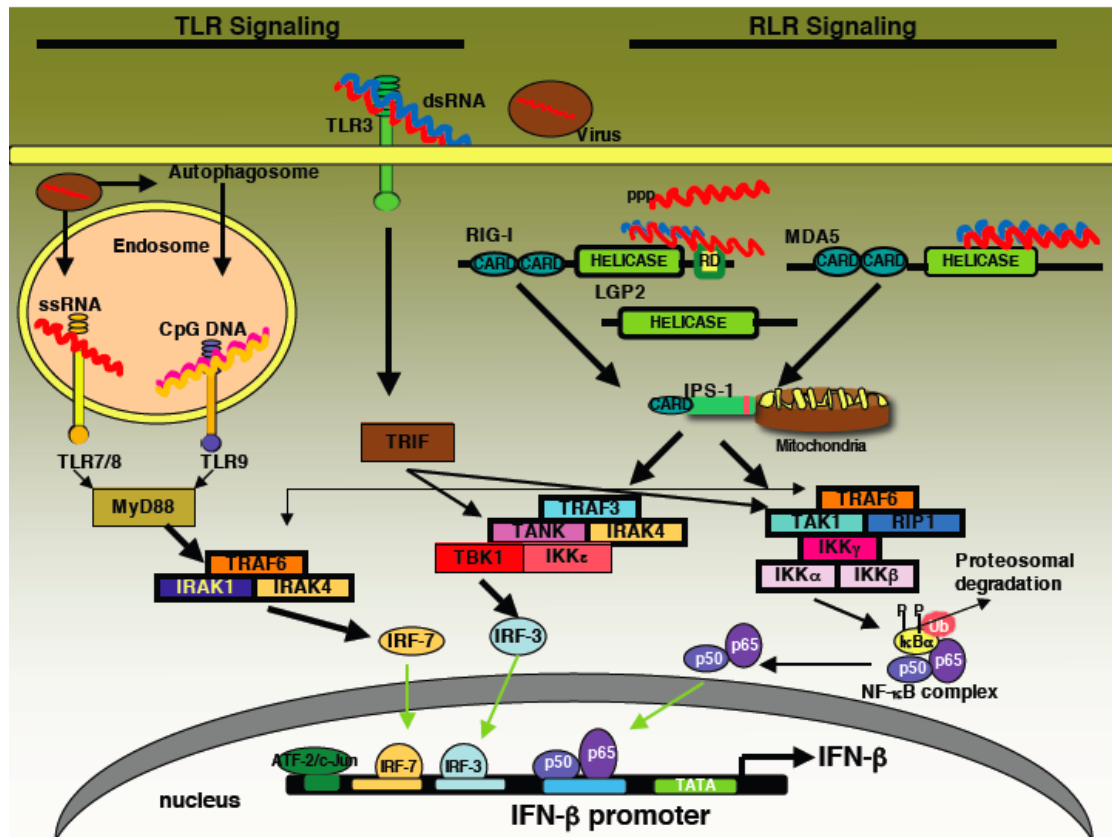
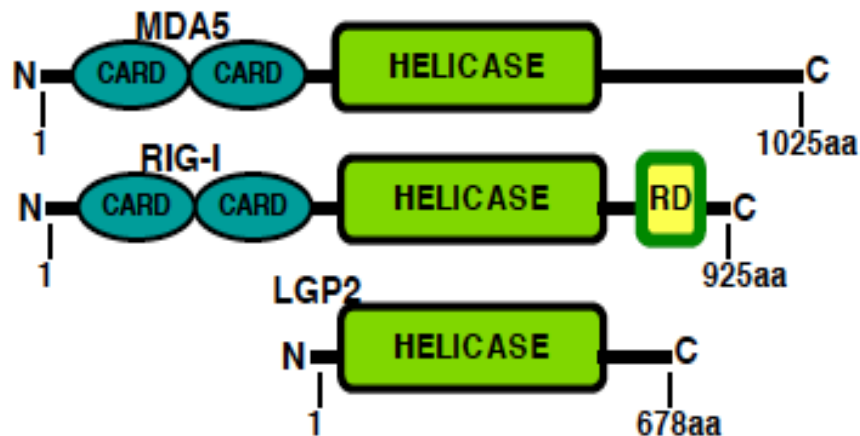


Figure 1-8. Innate Immune signaling pathways activated by virus infections. **TLR Signaling.** The TLR dependent signaling is activated in response to virus presented outside the cell membrane or on endosomes by endocytosis or by autophagocytosis. Activated TLR3 receptor recruits the adaptor TRIF that acts as the scaffold to activate the IRF-3 or NF- κ B pathway. In pDCs, TLR7/8 and TLR9 are present on endosomal compartments, activated in response to ssRNA or DNA respectively. These TLRs engage in MyD88, an adaptor protein which further recruits IRAK4 and IRAK1 and leads to activation of the IRF7 or NF- κ B pathway for promoting IFN expression. **RLR signaling.** RIG-I and MDA5 are the RNA helicases that recognize specific viral RNA motifs and undergo a conformational change for activation. While RIG-1 can be activated in response to 5'-ppp ssRNA or short dsRNA, MDA5 requires long dsRNA molecules for activation of the IFN signaling pathway. These helicases signal through a common adaptor protein called IPS-1 which involves interaction of their CARD domains. This results in activation of the downstream signaling cascade involving either of the two serine/threonine kinases (TBK- or IKK) which phosphorylates transcription factors, IRF-3 or NF- κ B and leads to production of interferons. Modified from Basler, C.F., and Garcia-Sastre, A.; Randall, R. E., and Goodbourn, S. (26, 272).

A.



B.

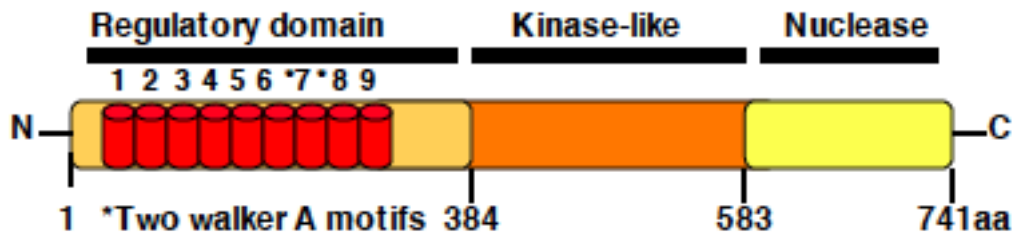


Figure 1-9. Schematic representation of RIG-I like receptors and RNase L. (A) Schematic of RIG-I like receptors. The figure illustrates the primary structure and functional domains of MDA5, RIG-I and LGP2. CARD: Caspase activating recruitment domain, RD: Repressor domain. Modified from Barral, P.M. et.al. (25) **(B) Schematic representation of RNase L structure.** RNase L consists of a N-terminal domain which is considered to be regulatory domain, a kinase domain and a C-terminal ribonuclease domain. The N-terminal domain consists of eight complete and one partial ankyrin motifs (R1-R9) and two walker A motifs which are located within R7 and R8. Binding of 2-5 A to ankyrin repeats on the N-terminal domain is proposed to induce conformational change, unmasking the C-terminal domain and thus activating the nuclease activity of RNase L. Modified from Bisbal, C. and Silverman. R.H. (36)

Chapter 2

Materials and Methods

2.1 Cells

BSR T7 cells (41), 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 µg of streptomycin/ml–400 µg of G418/ml at 37°C with 5% CO₂. HeLa, Vero, and 293T cells were maintained at 37°C with 5% CO₂ in DMEM supplemented with 10 % FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. For Huh 7.0 and Huh 7.5 cells, additional 1% nonessential amino acids were added. Mouse embryonic fibroblasts (MEF) cells, both wild-type (T antigen transformed) and RNase L–deficient (Tag transformed) cells were grown in RPMI 1640 media (Invitrogen) containing 10 % FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES and L-glutamine (205).

2.2 PLASMIDS

The plasmids containing LAC (consisting of domains I to IV), LAN (consisting of I and II), LI-III (consisting of domains I to III), L-II (consisting of domain II) and L-II mut (consisting of STOP codon instead of START codon in LII background) with an antigenic tag (hemagglutinin [HA]) in an expression vector pCAGGS (238) 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 (194, 196, 197, 361, 370). phRL-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).

Plasmids containing a firefly luciferase (F-luc) gene under the control of NF- κ B binding sites (pNF- κ B-TATA-F-Luc) (331), a F-Luc gene under the control of various IL-6 promoter mutants (pIL-6hwt-F-Luc, pIL-6-TATA-F-Luc, and pIL-6-NF- κ Bmut-F-Luc) (197, 355, 356), and a F-Luc gene under the control of an IFN- β promoter (268) were described before. The dominant negative (DN) mutant of AKT, pMT2-AH-AKT1, which contains 1 to 147 residues of AKT with a myc antigen tag (165) and AKT DN, which contains three mutations at phosphorylation sites and an ATP binding site (AAA-AKT1; K179A/T308A/S473A) with a HA tag, were described previously (322).

Plasmids encoding wild-type RNase L, RNase L mutant (R667A), the RIG-I DN (Flag-tagged RIG-I consisting of residues 218-925), and the IPS-1 DN (with the deletion of the CARD domain) were previously described (198, 205). The plasmids were prepared using a maxi prep kit (Qiagen). The endotoxin concentration was measured using a LAL endotoxin assay kit (GenScript). The endotoxin concentrations of all plasmids were lower than 0.1 EU/ μ g of DNA.

2.3 Dual Luciferase Assay

Cells in 24-well tissue culture plates at about 80-90% confluency were transfected. For BSR T7 cells, the transfection was performed using Plus and Lipofectamine (Invitrogen), and for 293T or HeLa cells, transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The vector 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, pIL6wt-F-Luc, pIL-6-TATA-F-Luc, or pIL-6- κ Bmut-F-Luc, 120-240 ng of pIFN β -F-Luc. The plasmids encoding NP, V, P, M, F, SH, HN and L were used in a concentration range of between 0 and 1,000 ng/ml. A range of concentrations up to 1,500 ng of plasmids encoding all L mutants, AKT DN and pMT2-AH-AKT were used at 800 ng and plasmid c-RIG (RIG-I DN) and IPS-1 DN were used at 500 ng. At 18-24 h after transfection, cells were lysed in 100 μ l of passive lysis buffer (Promega) for 30-45 minutes. 20 μ l of lysate from each well was then used for a dual luciferase assay following manufacturer's protocol (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 hrs after transfection and a dual luciferase assay was performed at 18 to 20h posttransfection as described before.

2.4 Electrophoretic Mobility Shift Assay (EMSA) or Gel Shift Assay

BSR T7 cells were transfected with plasmid expressing empty vector, L, V, L-I, L-I-II, L-II, L-II mut or LI-III. The nuclear extracts were prepared 18-20h after transfection using a nuclear extraction kit (Marligen Biosciences) following manufacturer's instructions or alternatively, by using buffers A and C. The media was removed from the cell culture plate and cells were trypsinized and washed in cold PBS and were pelleted in an Eppendorf tube. The cells were resuspended in 500 μ l of cold buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.1% NP-40, 0.1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride and 1X of protease inhibitor cocktail) for 15 min on ice. The tubes were centrifuged at 10,000 g (Microfuge 18; Beckman Coulter) for 10 minutes. The supernatant (cytoplasmic extract) was discarded, and the cells were resuspended again in 500 μ l of the same buffer and centrifuged for additional 10 minutes. The pellet was resuspended in 50 μ l of cold buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol (v/v), 0.1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail) was added. The suspension was sonicated twice at 60% power on ice with a three seconds pulse and a 10 seconds pause in between. The cell extract was centrifuged for 10 minutes at 10,000 g. The supernatant (nuclear extract) was collected and stored at $-70^{\circ}C$. TNF- α -treated BSR T7 cells (20 ng of TNF- α /ml for 2h) were used as a positive control. The protein concentration was determined using a bicinchoninic acid (BCA) protein estimation kit (Thermo Scientific). An electrophoretic mobility gel shift assay (EMSA) was carried out as described by Wilson et al. (370). Briefly, double-stranded oligonucleotide containing

consensus sequences for NF- κ B binding sites,

(5'-AGCTCCTGGAAAGTCCCCAGCGGAAAGTCCCTT-3') and

(5'-AGCTAAGGGACTTTCCGCTGGGGACTTTCCAGG-3')

were end-labeled with [32 P]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 10X EMSA buffer (25 mM HEPES [pH 7.5], 60 mM NaCl, 9% glycerol, 1 mM EDTA, 7.5 mM dithiothreitol, 50 mM MgCl₂)– polyd(I):polyd(C) 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'-AGCTAACTCACTTTCCGCTGCTCACTTTCCAGG-3') and

(5'-AGCTCCTGGAAAGTGAGCAGCGGAAAGTGAGTT-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).

2.5 Small Interfering RNA (siRNA) experiments

siRNA experiments were performed as previously described (329). Cells in 24-well plates at about 30 to 50% confluency were transfected with 100 nM of siRNA purchased from Dharmacon [non-target siRNA pool (NT), AKT1 siRNA and control

siRNA; ATF3 or MDA5 siRNA] and Santa Cruz (RNaseL, RIG-I siRNA) with 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 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.

At 48h after siRNA transfection, the cells were transfected with empty vector, plasmids expressing L, L-II, or L-II mutant (1 μ g/ μ l), or poly(I):poly(C) (500 ng/ml) using lipofectamine 2000 along with phRL-TK and pNF- κ B-TATA-F-Luc or pIFN-Luc as previously described. At one day post-transfection, the dual luciferase assay, IFN- β ELISA and immunoblotting experiments were performed.

2.6 Enzyme Linked Immunosorbent Assay (ELISA) for detecting NF- κ B, phosphorylation of T308 of AKT1 and IFN- β

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 cells transfected with plasmid encoding empty vector (pCAGGS), L-, or V- as described before. A 2.5 μ g

volume of protein was used for the assay.

To detect phosphorylation of AKT1 at T308, BSR T7 cells were transfected with plasmids: empty vector, LI, or 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 3h 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 21h 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 (T308) sandwich ELISA kit (Cell Signaling Technology).

For detection of IFN- β , 293T or HeLa cells were treated with siRNA targeting MDA5, RIG-I, RNase L or non-target as mentioned in section 2.5. After 48h, the cells were transfected with empty vector, plasmids expressing L, L-II, L-II mut (1 μ g/ μ l) or poly(I):poly(C). Alternatively, the cells without siRNA treatment were transfected with the respective plasmids. The medium was collected 24h after transfection and centrifuged to remove cell debris. 50 μ l of the cleared medium or the IFN- β standard were used in duplicate for detection of IFN- β using a human IFN- β ELISA kit (PBL Interferon Source, NJ) following manufacturer's instructions.

2.7 Co-immunoprecipitation

A. Radiolabel Immunoprecipitation

BSR T7 cells were seeded in a 6-cm-diameter tissue culture plate and transfected with plasmid encoding AKT1. At 16 to 18h after transfection, cells were lysed with whole-cell extraction buffer (WCEB; 1 M Tris [pH8.0], 280 mM NaCl, 0.5% NP-40, 2mM EGTA, 0.2 mM EDTA, 10% glycerol, 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 3h at 4°C. The cell lysate were spun down and washed twice with WCEB. The precipitate was used for further immunoprecipitation with LI, LII or LI-II. The LI, LII or LI-II with a HA tag were synthesized *in vitro* using TNT-coupled transcription-translation systems (Promega). Templates for LI, LII or 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, LII or LI-II were incubated with precipitated AKT1 with beads and antibody for 2 to 3h at 4°C. To examine the expression levels of *in vitro*-synthesized LI and LI-II, they were immunoprecipitated with anti-HA antibody. The samples were resolved on a 10% polyacrylamide gel and visualized using a PhosphorImager Storm system (Molecular Dynamics).

Cells infected (wt-PIV5) or transfected with plasmids encoding LI, LII, LI-II, or LII mutant with a HA tag, vector or plasmids encoding for the PIV5 viral proteins were metabolically labeled with [35 S]Met and [35 S]Cys for 3h. After 24h post-transfection or infection, the cells were lysed with WCEB. The lysates were then precipitated with anti-HA antibody or with the respective antibody against the PIV5 viral proteins. The precipitated proteins were resolved by 15% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using a Storm PhosphorImager (Molecular Dynamics).

B. Non-radiolabeled Immunoprecipitation

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), AKT1-Flag (1 μ g), L-I (5 μ g), LI-II (5 μ g), and AKT-Flag (1 μ g) plus LI-II (5 μ g). A sample in which 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 21h 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 3h 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 5X 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 T308 antibody diluted in 1.5% BSA–TBST (Cell Signaling technology), or rabbit antiphospho-AKT-S473 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).

2.8 Immunoblotting

The lysates from the luciferase assays were diluted (1: 1) with protein lysis buffer (2% sodium dodecyl sulfate, 62.5 mM Tris-HCl pH 6.8, 2% dithiothreitol) and sonicated. 100 µl of the lysate was resolved in 10% SDS-PAGE and immunoblotting was performed using respective antibodies as described previously.

2.9 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.

2.10 RNA purification and transfection

293T or Vero cells were transfected with empty vector, or a plasmid encoding L, LI, LII, LI-II or LII mut. Cells (HeLa or Vero) were infected with wild type PIV5 or rPIV5VAC, or mock-infected. 18-20h after transfection or infection, total RNA was isolated using a Qiagen RNeasy kit, or mRNA was isolated using Qiagen Oligotex direct mRNA purification kit, following manufacturer's instructions. The purified total RNA (1

μg/μl per well of 24-well plate) or mRNA (200 ng/μl per well of 24-well plate) was transfected into 293T cells using lipofectamine 2000. Poly(I):poly(C) (500 ng/μl per well of 24-well plate) was used as a positive control. At one day post-transfection, IFN-β production was determined using human IFN-β ELISA kit (PBL Interferon Source, NJ), following manufacturer's instructions.

2.11 Northern Blot Analysis

The RNA samples purified from BSR T7 cells that were transfected with empty vector (pCAGGS), or a plasmid encoding LI, LII, or LII mut at 18h post-transfection were electrophoresed on a 1.2% agarose gel in the presence of 0.44 M formaldehyde, transferred to a positively charged nylon membrane (Roche Diagnostics), fixed by UV crosslinking, and analyzed by hybridization with DIG-labeled RNA probes that were generated by *in vitro* transcription using the DIG Northern Starter kit (Roche Applied Sciences). The hybridized probes were detected with anti-digoxigenin-AP Fab fragments and were visualized using chemiluminescence substrate CDP-Star (Roche Applied Sciences) on X-ray films. The DNA templates for generating DIG-RNA probes were prepared using PCR with gene-specific sense oligomer and antisense oligomer with T7 RNA polymerase promoter sequence. The amplified PCR fragments were purified using a PCR purification column and gel purification kit (GenScript). A digoxigenin-labeled RNA molecular weight marker (Roche) was used to indicate the size of RNA.

2.12 RNase H treatment

The purified RNA from L-II-transfected cells or infected cells was used for a reverse transcription reaction using L-II specific primer or NP-specific primer. The RT products were treated with RNase H and purified using a RNeasy column. The treated or untreated RT product or L-II RNA were transfected into 293T cells and at one day post-transfection, the concentration of IFN- β was measured in the medium using ELISA.

2.13 *In vitro* RNA transcription

DNA containing the region I or II of the L gene were amplified by PCR with sequence-specific sense primer containing T7 polymerase promoter sequence and the antisense primer, using plasmid containing LI or LII as template. The LI or LII RNA fragments were *in vitro* synthesized using Riboprobe *in vitro* transcription systems (Promega). The synthesized fragments were treated with DNase I to remove the DNA template and were then purified using a RNeasy column (Qiagen). The *in vitro* synthesized RNA fragments were treated with calf intestinal phosphate (CIP) for two hours and were purified. The purified CIP-treated *in vitro* RNA transcripts (200 ng) were transfected into 293T cells using Lipofectamine 2000. At one day post-transfection, IFN- β production was measured using a human IFN- β ELISA kit.

2.14 Real Time PCR

The 293T cells in 6-well plates were transfected with 1 μ g of purified RNA (vector, LI or LII mut) or poly(I):poly(C) (250 ng) in OPTI-MEM using lipofectamine 2000. Four hours after transfection, the media was changed to complete media with DMSO or cyclohexamide (CHX-20 μ g/ml). After 16 hours incubation, the total RNA was isolated using RNeasy Mini kit (Qiagen). For each samples, 11 μ l of total RNA was used for reverse transcription using Superscript III reverse transcriptase (invitrogen) with oligo (dT)₁₅ according to manufacturer's protocol. The cDNA (4 μ l of 1:20 diluted cDNA) from each sample was used for a real time PCR reaction on Step one Plus Real Time PCR System (Applied Biosystems) using Taqman Universal PCR Master Mix (Applied Biosystems) and Taqman Gene Expression 1 Assays (Applied Biosystems) for IFN- β gene with FAM dye and β -actin gene with VIC dye. Results were analyzed to obtain Ct values. Relative levels of IFN mRNA and β -actin mRNA were determined by calculating ΔC_T (comparative threshold) values. The expression of IFN- β gene was normalized to levels of β -actin mRNA. The normalized IFN- β expression levels of vector transfected cells was set to 1 to which expression levels of samples were compared and presented as fold changes. Each sample was run in three replicates.

Chapter 3

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

This Chapter is partly a reprint of a paper originally published in The Journal of Virology. The co-authors are: Dengyun Sun, Matthew Wolfgang and Biao He. The Copyright Permission for the use of material has been approved by the American Society of Microbiology.

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0022-538X/08/\$08.00_0 doi:10.1128/JVI.00806-08.**

3.1 ABSTRACT

Innate immunity plays a critical role in controlling 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.

3.2 Background

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 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. The sequence comparison of the L proteins and the other RNA polymerase indicate that the L proteins have six conserved domains (266, 312).

Innate immunity plays a critical role in controlling 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 anti-viral cytokines such as interferon- β , and major proinflammatory cytokines such as TNF- α and 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, which can be divided into two classes dependent on their 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, interaction with I κ Bs and DNA binding, and a C-terminal transactivation domain. p105 and p100 contain RHD domains 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 only have RHD domains 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) (30). 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 (NEMO, NF- κ B essential modifier) subunits. Phosphorylation of I κ B at two N-terminal serine residues by IKK β complex results in its ubiquitin-dependent degradation, thus exposing nuclear localization signals in NF- κ B and translocation of the NF- κ B dimer to the nucleus, where it is further

modulated by phosphorylation (388). Activation of the IKK complex can be triggered by a number of different signal transduction pathways. Alternatively, in the non-canonical 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 and bind to specific sequences of promoter or the enhancer regions of the target genes. The molecular mechanisms of the activation of many of these cytokines by virus are not clear. Previously, it has been shown that NF- κ B factors and IL-6 are activated by infection with a mutant parainfluenza virus 5 lacking a cysteine rich C-terminus of the V protein (rPIV5V Δ C) (197). 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 (38)). 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 (AKT 1, -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 T308 and S473, which are phosphorylated by PDK1 (PI3K-dependent kinase 1) and the rictor-mTOR complex, respectively (279, 296). 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 alpha ($IKK\alpha$), whose phosphorylation by AKT1 can lead to activation of NF- κ B (87). Recently, it was reported that AKT plays a critical role in replication of NNSV, possibly through phosphorylation of the P protein (329). In this study, we found that the viral L protein activates NF- κ B through an AKT1-dependent pathway.

3.3 RESULTS

3.3.1 Activation of NF- κ B by PIV5 proteins

Previously, it has been shown that a recombinant PIV5 lacking the conserved region 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 (125, 197). 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 transfection control plasmid, phRL-TK which has renilla luciferase (R-Luc, or RL) under the control of a HSV TK promoter were transfected with a plasmid encoding PIV5 viral protein NP, V, P, M, F, SH, HN or L. The luciferase activities were determined one day post-transfection. Among the known PIV5 proteins, the L protein demonstrated the highest level of activation of NF- κ B-dependent reporter gene expression (**Figure 3.1A**), indicating that L is capable of activating NF- κ B. The expression levels of HN and L necessary for NF- κ B activation was 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 (**Figure 3.1B**). To further confirm the activation of NF- κ B by L, an electrophoretic mobility gel shift assay (EMSA) was performed. As expected, nuclear

extracts prepared from L-transfected cells at 1 day post transfection shifted the mobility of a ^{32}P -labeled NF- κB probe (**Figure 3.2A**), whereas nuclear extract from mock or V-transfected cells had no effect. This binding was competed away by excess “cold” competitor, but not a “cold” mutant NF- κB probe, confirming that the L protein specifically activates NF- κB .

NF- κB consists of five subunits. To investigate which NF- κB factor is activated by L, an ELISA-based assay was carried as described before (103, 197). As shown in **Figure 3.2B**, L expression activates the p50, p52 and p65 subunits of NF- κB , the same set of NF- κB factors that are activated in rPIV5V ΔC -infected cells and to a lesser extent activated c-Rel and RelB (**Figure 3.2C**) (197). Taken together these results, we suggest that L is capable of activating NF- κB and is the major player in NF- κB activation during infection.

Previously it has been reported that the V protein expression can block the NF- κB activation in rPIV5V ΔC -infected cells (197). We investigated the effect of the V protein expression on NF- κB activation by the L protein. A dual luciferase experiment was performed and we observed that the co-expression of the V protein with the L protein, results in significant reduction in NF- κB promoter activation (**Figure 3.2D**). This suggested that the V protein plays a role in blocking activation of NF- κB by L, as observed in the case of rPIV5V ΔC -infected cells (197).

3.3.2 Activation of IFN- β and IL-6 Promoters by L

It is known that NF- κ B activation plays an important role in expression of cytokines, chemokines and growth receptors, thus playing 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 reporter gene assay as above. As shown in **Figure 3.3A**, transfection of increasing amounts of L plasmid results in higher luciferase transcription from the IFN- β promoter, indicating that L plays 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 (**Figure 3.3B**). Mutating the NF- κ B binding 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- κ B binding.

3.3.3 Mapping of the region within L essential for NF- κ B activation

The PIV5 L protein is ~250kD and consists of six domains that are conserved among all NNSVs. To investigate which domain(s) is involved in NF- κ B activation, a series of L mutants was generated (Fig. 3.4A) and tested using the NF- κ B-dependent reporter gene assay as described above. The L mutant plasmid consisting of N-terminal four domains of L (L Δ C) was capable of activating NF- κ B but L Δ N did not (**Figure 3.4B**). Further analysis of the L Δ C indicated that domains I and II are sufficient to

activate NF- κ B. Since the domain I alone does not activate NF- κ B, domain II is likely the essential region of L for NF- κ B activation. However, it is not clear whether the domain II is sufficient for activating NF- κ B.

To confirm this observation, a gel shift assay was conducted as described before with nuclear extracts prepared from L-, LI-, LI-II-, or LI-III- transfected cells. The NF- κ B-protein complex was observed in nuclear extracts from L-, LI-II-, or LI-III- transfected cells, but not from that of vector or LI- transfected cells, confirming activation of NF- κ B by L-I-II (**Figure 3.4C**). The expression levels of LI and LI-II in this experiment were comparable as determined by immunoblotting (**Figure 3.4D**).

3.3.4 AKT1 plays an essential role in NF- κ B activation by L

To further elucidate the mechanism of NF- κ B activation by PIV5 L protein, we 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- κ B through phosphorylation of IKK α (152, 247, 279). It has been reported that AKT1 plays a critical role in replication of PIV5, a process for which L is also important (329). 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 **Figure 3.5A**, 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 dominant negative mutant of AKT1 (AH-AKT1) were co-transfected with L and NF- κ B-dependent transcription was assayed as above. Co-expression of AH-AKT1 inhibited L-activated NF- κ B reporter activity in a dosage dependent manner (**Figure 3.5B** and **Figure 3.5C**), confirming that AKT1 plays a critical role in the activation of NF- κ B by L. Similar results were obtained using a different AKT1 dominant negative (AAA-AKT1) that contains mutations at the phosphorylation sites (Thr308 and Ser473) and the ATP binding site (**Figure 3.5D** and **Figure 3.5E**). To test whether, AKT1 plays a critical role in activation of NF- κ B, AKT1 expression level was reduced by transfecting cells with siRNA (**Figure 3.5F**). As predicted, reducing AKT1 expression level results in the reduction of NF- κ B-dependent reporter gene expression by L (**Figure 3.5G**), indicating that AKT1 plays an essential role in activation of NF- κ B by L.

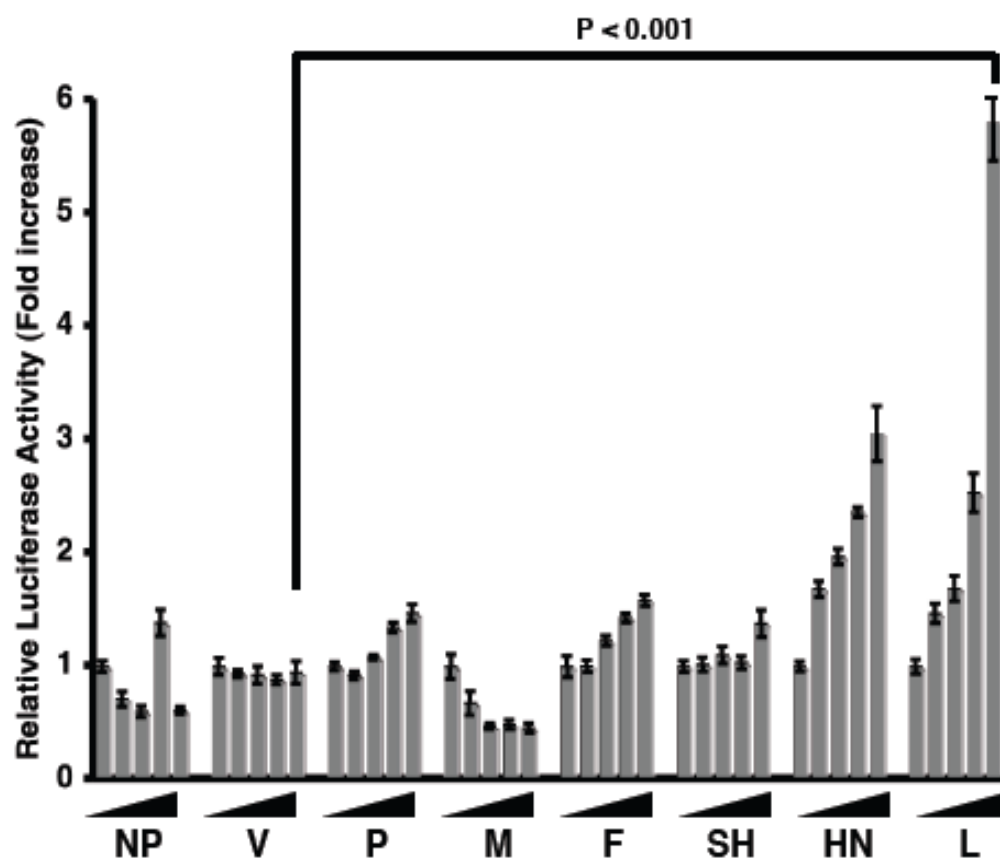
3.3.5 Phosphorylation of AKT1 by PIV5 L protein

AKT1 is activated through phosphorylation at specific sites: T308 and S473. The role of phosphoinositide 3-kinase (PI3K) signaling pathway has been identified in activation of AKT. To examine if PI3K signaling is critical for L-mediated activation of NF- κ B, we used inhibitor against PI3K, wortamannin. Interestingly, we found that the activation of NF- κ B by L was not affected in presence of the inhibitor suggesting that AKT is activated independent of PI3K (**Figure 3.6A** and **Figure 3.6B**). 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 plasmids expressing AKT1, L-I or LI-II. The cells were lysed and the amount of phosphorylated AKT1 at residue T308 were measured using an ELISA kit. As shown in **Figure 3.6C**, the cells transfected with LI-II gives raise higher level of T308 phosphorylation than the cells transfected with other plasmids. However, the level of T308 in LI-II-transfected cells is low compare with positive control, PDGF-treated cells. This is likely due to the fact that transfection only introduces a portion of cells with plasmids while AKT1 in all cells is activated with PDGF treatment. To further investigate phosphorylation of AKT1, a plasmid encoding Flag-tagged-AKT1 was co-transfected with 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 **Figure 3.6D**, expression of LI-II enhanced phosphorylation of AKT1 at both T308 and S473. The level of the enhancement is comparable to the positive control, PDGF-treatment (**Figure 3.6E**).

We next investigated whether L and AKT1 can interact using an *in vitro* transcription/translation system. As shown in **Figure 3.7A**, AKT1 co-precipitated with both LI and LI-II, indicating that the domain I of PIV5 L can interact with AKT1. In addition, we confirmed this interaction by a yeast two-hybrid system (**Figure 3.7B**), using LI-III, which is sufficient for NF- κ B activation (**Figure 3.4B**), as the bait and AKT1 as the prey. We found yeast grew on selective plates only when transformed with both LI-III and AKT1 plasmids, indicating that AKT1 interacts with the N-terminal three domains of L.

A.



B.

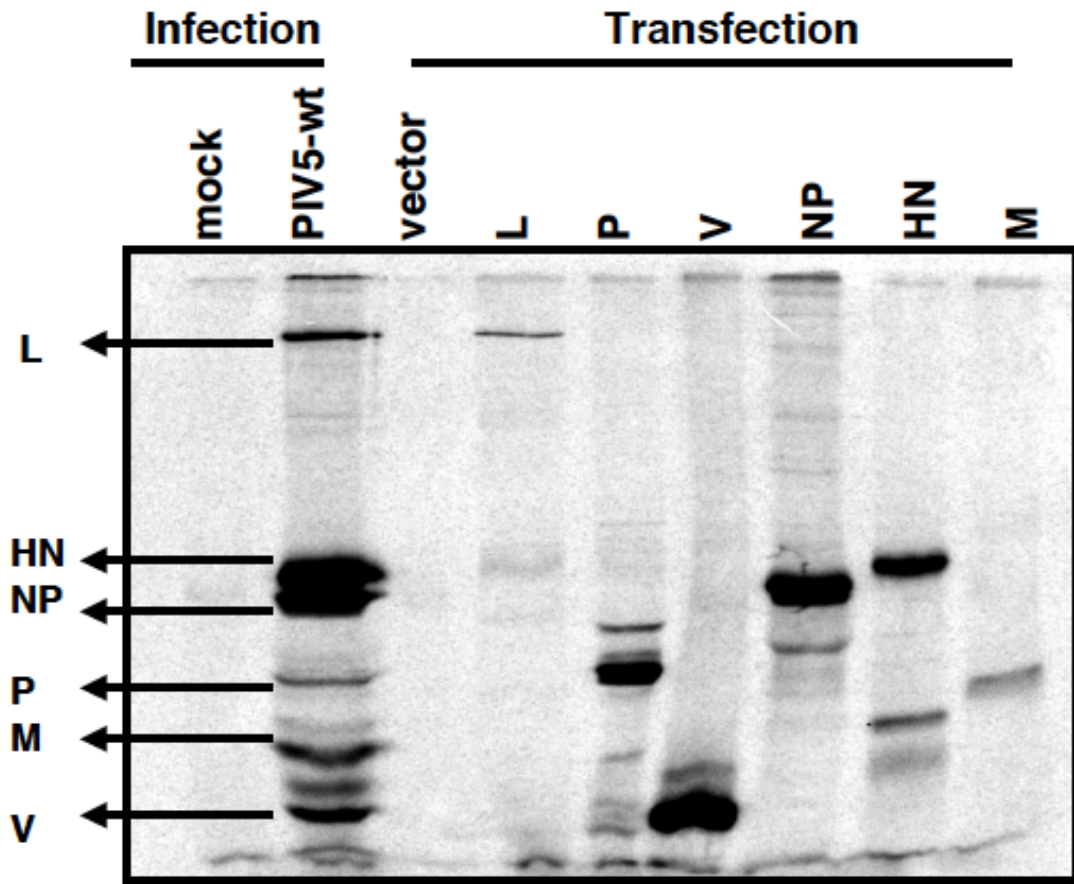
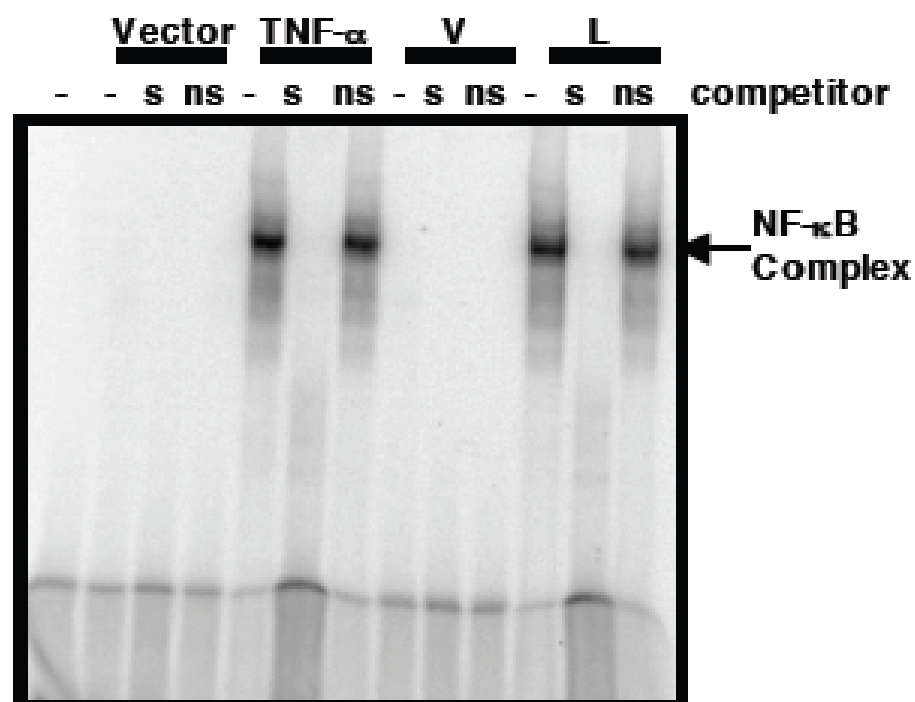
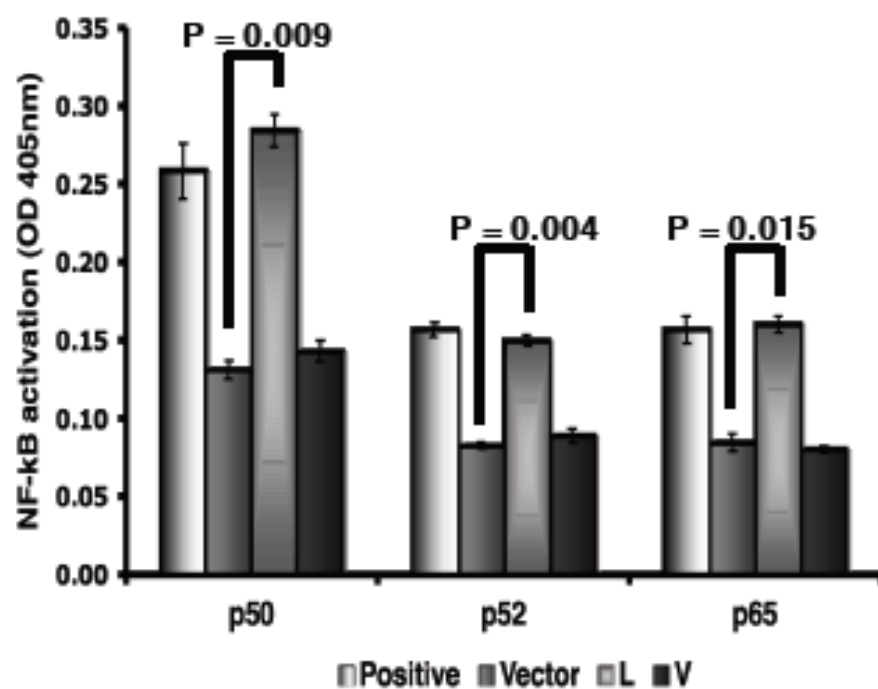


Figure 3-1. NF- κ B activation by PIV5 proteins. (A) L activates NF- κ B promoter activation. A plasmid encoding a F-Luc under the control of NF- κ B responsive elements was co-transfected into cells with increasing amounts (0, 250, 500, 750, and 1,000 ng) of a plasmid encoding each PIV5 viral protein along with a plasmid encoding 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 post-transfection 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. **(B) Expression of viral proteins.** The BSR T7 cells were infected (wt-PIV5) or transfected (with plasmids encoding PIV5 viral proteins) and [35 S]Met and [35 S]Cys metabolic labeled immunoprecipitation experiment was performed. The precipitated proteins were resolved by SDS-PAGE and visualized using a Storm PhosphorImager (Molecular Dynamics).

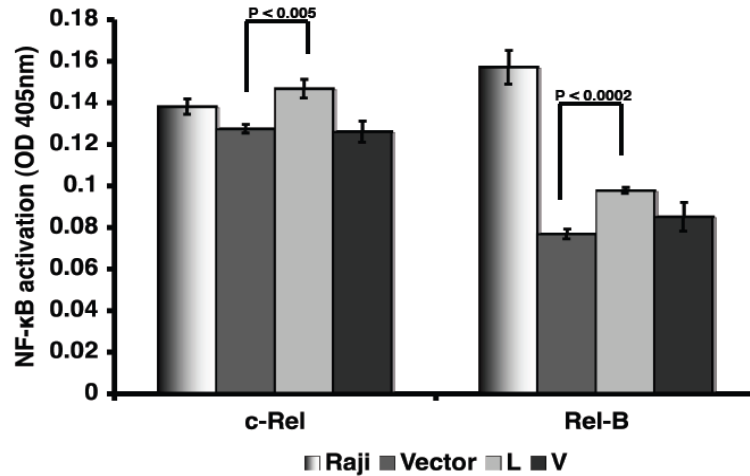
A.



B.



C.



D.

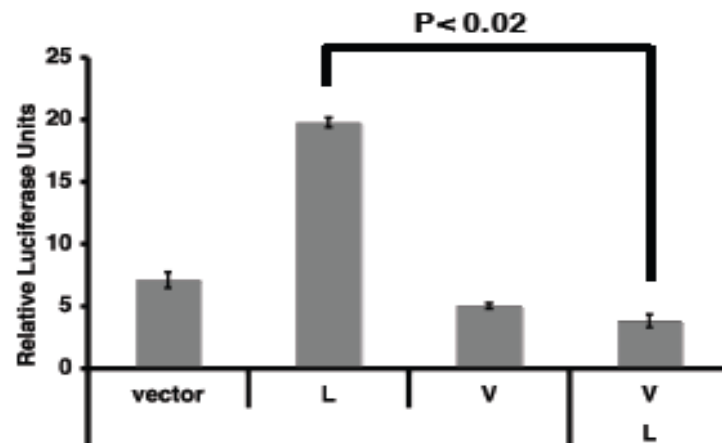
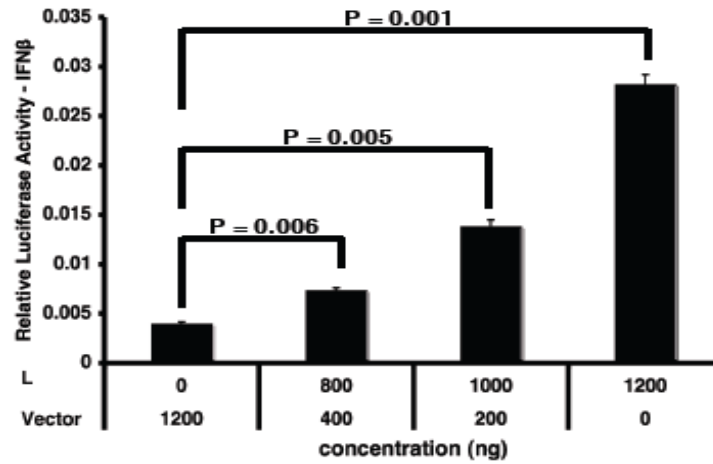


Figure 3-2. Activation of NF-κB by L. (A) EMSA. Nuclear extracts were obtained from the transfected cells with appropriate plasmids for EMSA and incubated with 32 P-labeled NF-κB probe and appropriate competitors. TNF- α , nuclear extracts from cells were treated with 20 ng of TNF- α /ml for 2h. “Probe (-)”- NF-κB DNA primers labeled with 32 P; “S (specific competitor)”- unlabeled NF-κB probe (20-fold excess); “NS (Non-specific competitor)”- unlabeled mutant NF-κB probe (20-fold excess). **(B and C) ELISA.** The nuclear extracts from the samples 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. **(D) V inhibits activation of NF-κB by L.** A dual luciferase experiment was performed for NF-κB activation. Plasmids encoding for V (800 ng) and L (800 ng) along with luciferase plasmids were transfected into cells and the reporter assay was performed 20h after transfection as described before Fig 3.1A.

A.



B

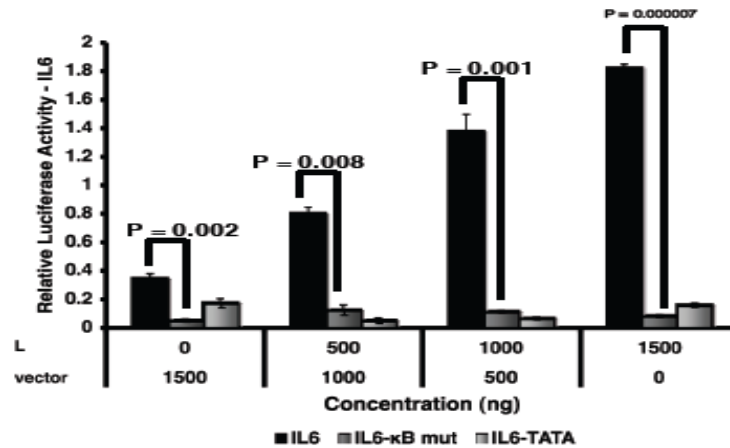
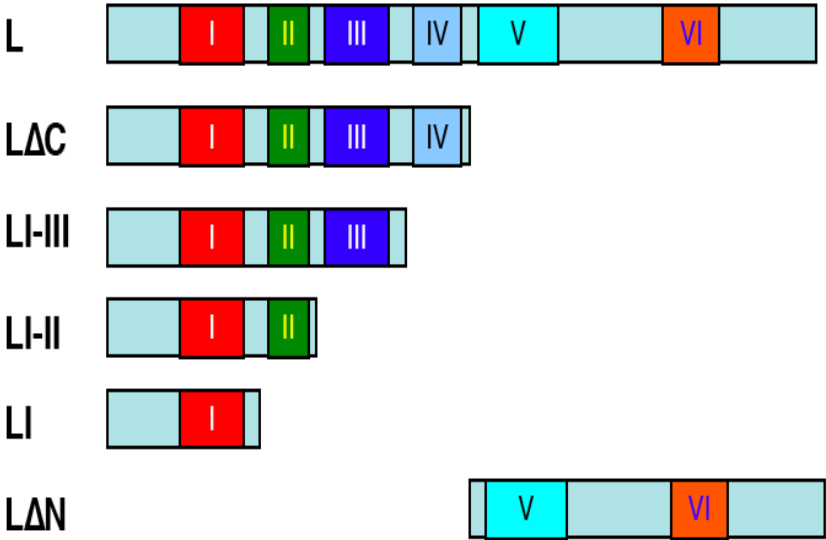
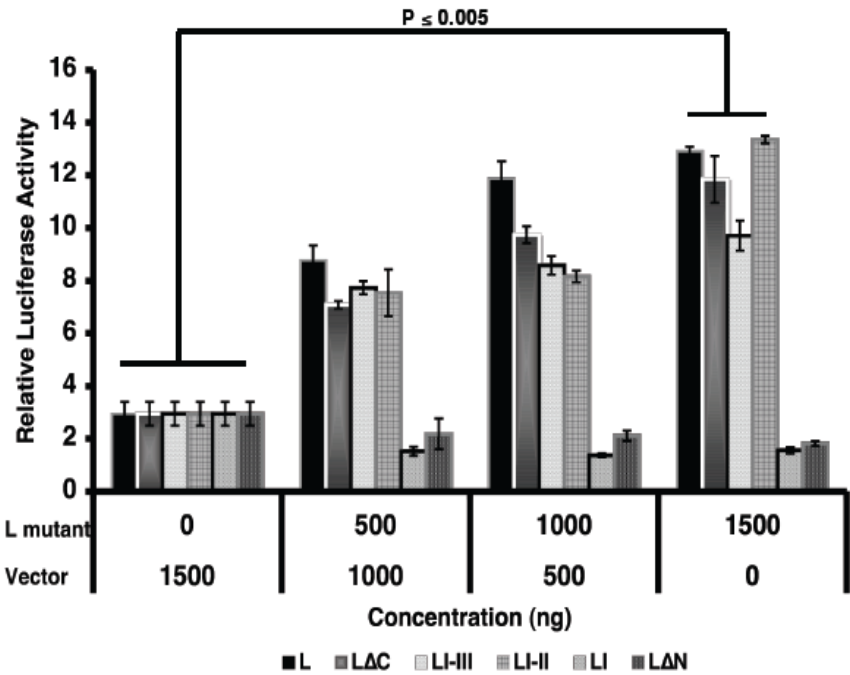


Figure. 3-3. Activation of IFN- β and IL-6 promoters by L. (A) IFN- β promoter activation. Dual luciferase reporter gene assays were performed similar to in Fig. 3.1A with firefly luciferase under the control of the IFN- β promoter instead of a NF- κ B-responsive element. **(B) IL-6 promoter activation.** A luciferase assay was performed as described in 3.3A using plasmid constructs containing a firefly luciferase gene under the control of the wild-type IL-6 promoter (pIL6wt-F-Luc), an NF- κ B binding site mutant of the IL-6 promoter (pIL6- κ B mut), 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.

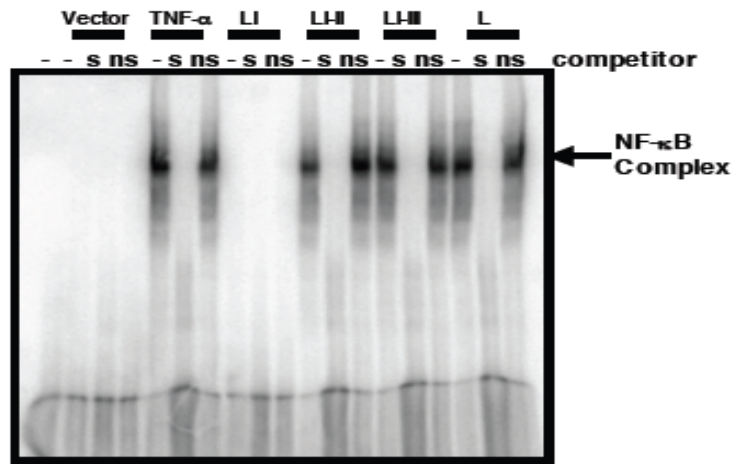
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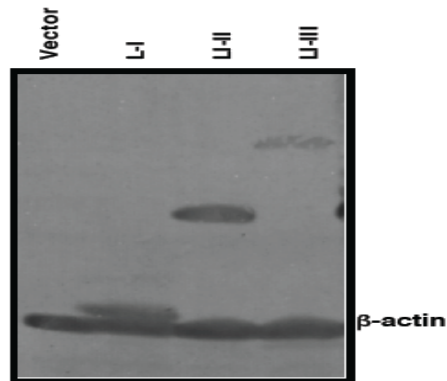
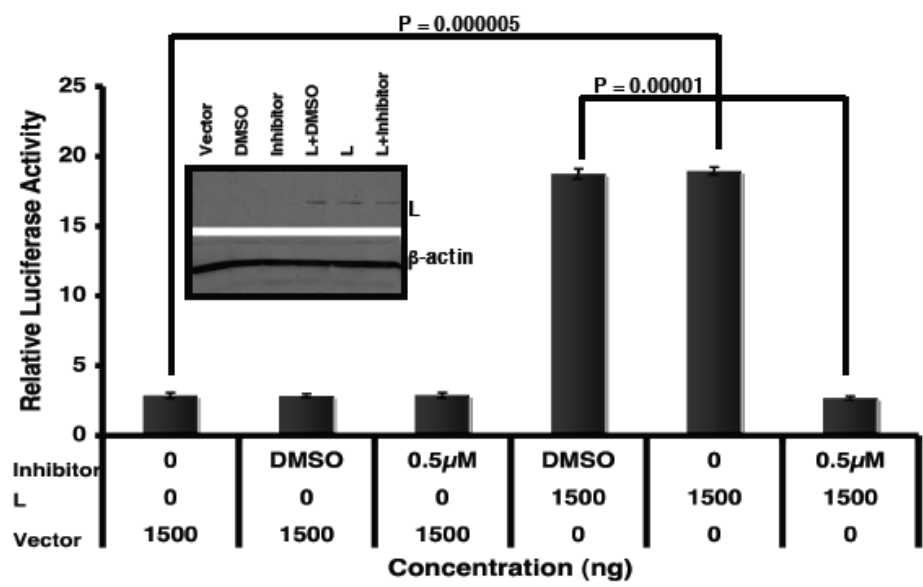
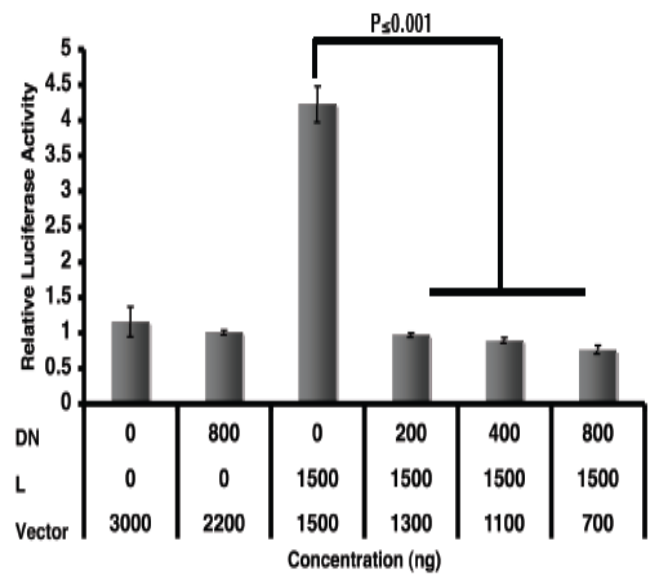


Figure 3-4. Activation of NF-κB by L mutants. (A) Mapping of L sequences that are required for activation of NF-κB. Schematic of L mutants. All the mutants with an antigenic tag (HA) in pCAGGS expression vector were generated using standard molecular cloning techniques. **(B) NF-κB activation.** A dual 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. 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 using Marligen Kit and were incubated with ^{32}P -labeled NF-κB probe and appropriate competitors and were resolved on 6% polyacrylamide gel. TNF-α, nuclear extracts from cells were treated with 20 ng/ml of TNF-α for 2h. “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). **(D) Expression levels of L mutants.** Immunoblotting with anti-HA antibody was used to analyze the expression of L mutants in transfected cell extracts.

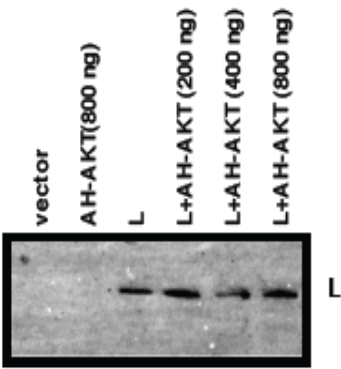
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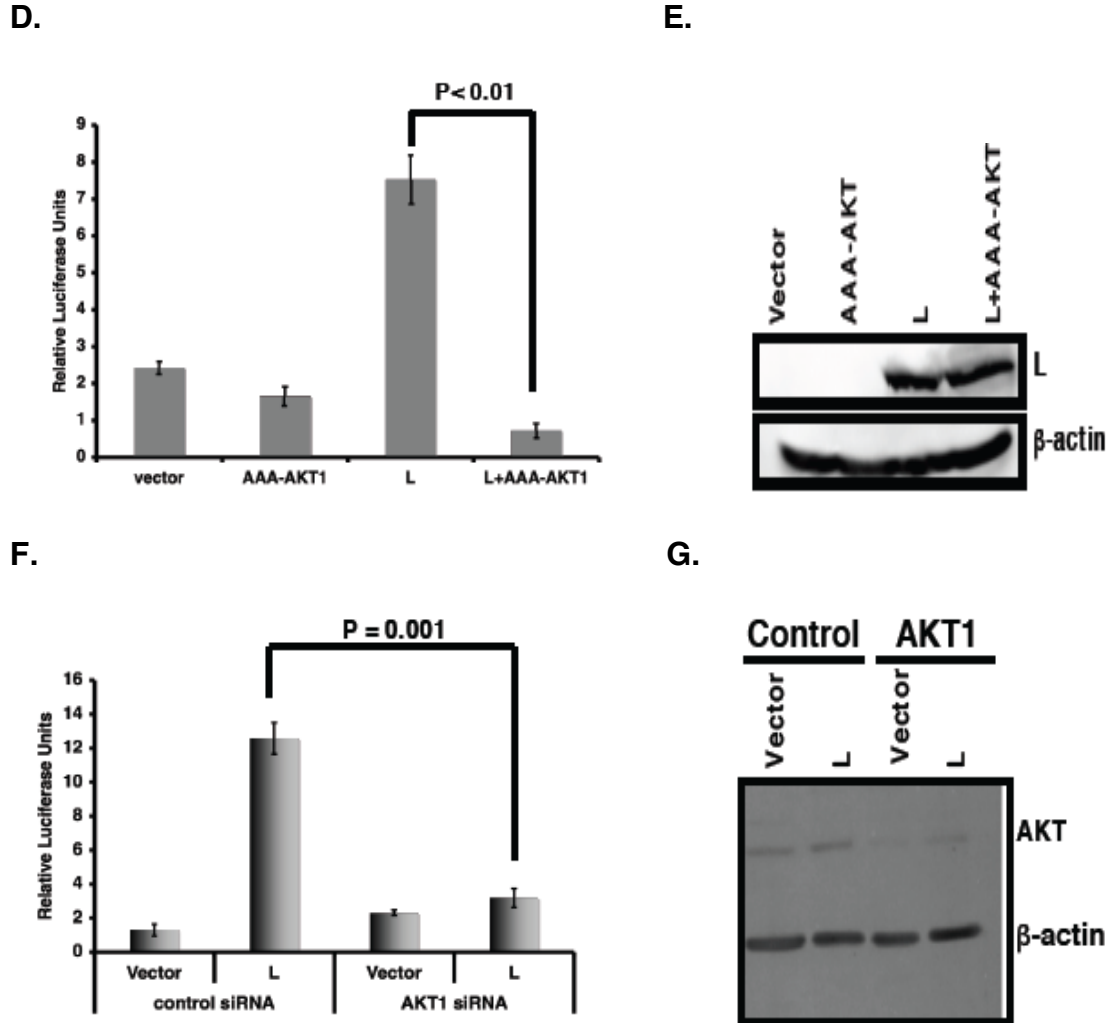
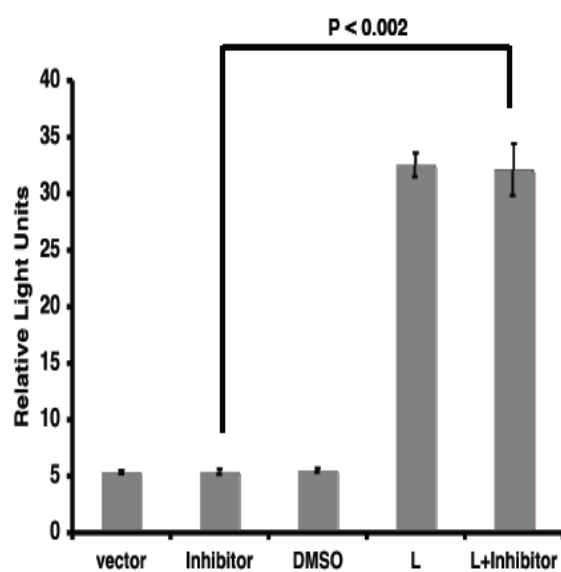
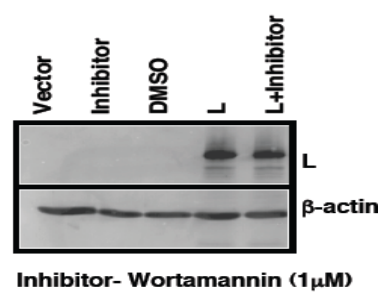


Figure 3-5. AKT plays a critical role in activation of NF- κ B by L. (A) Effect of AKT inhibitor on L-activated NF- κ B. A dual luciferase assay was performed as described for Fig.3.1A in the presence of AKT IV inhibitor (0.5 μ M) (Calbiochem) or vehicle (dimethyl sulfoxide [DMSO]). **(B and D) Inhibition of L-activated NF- κ B by DN AKT1.** Reporter gene assays were carried out as described before in presence of dominant negative of AKT: AH-AKT or AKT1-AAA (800 ng/ μ l). **(C and E) 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 examine the expression of L in the presence of AH-AKT or AAA-AKT. **(F) Reduction of NF- κ B activation after AKT1 knockdown with siRNA.** The cells were transfected with siRNA targeting AKT1 or with control siRNA. After 48h of siRNA transfection, the cells were transfected with plasmids encoding L along with reporter luciferase genes as described before. Luciferase activities were measured at 24h after plasmid transfection. **(G) Expression levels of AKT1 in siRNA-transfected cells.** The amounts of AKT1 and β -actin were examined using the lysates from luciferase for immunoblotting with anti-AKT1 and anti- β -actin antibodies.

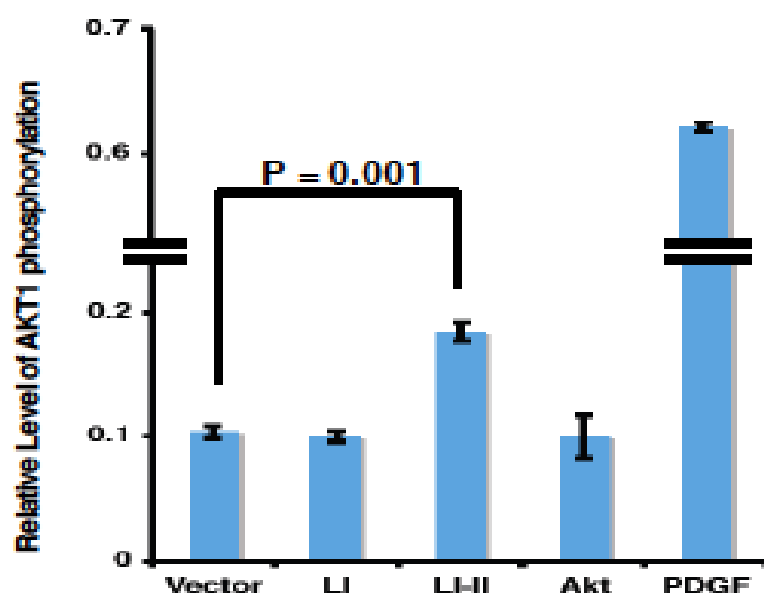
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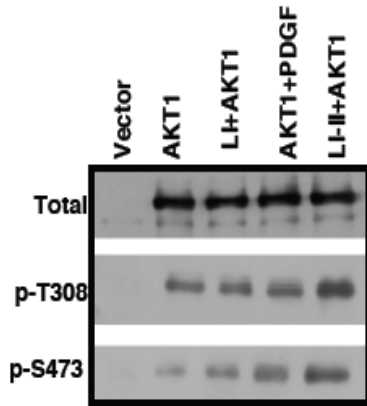
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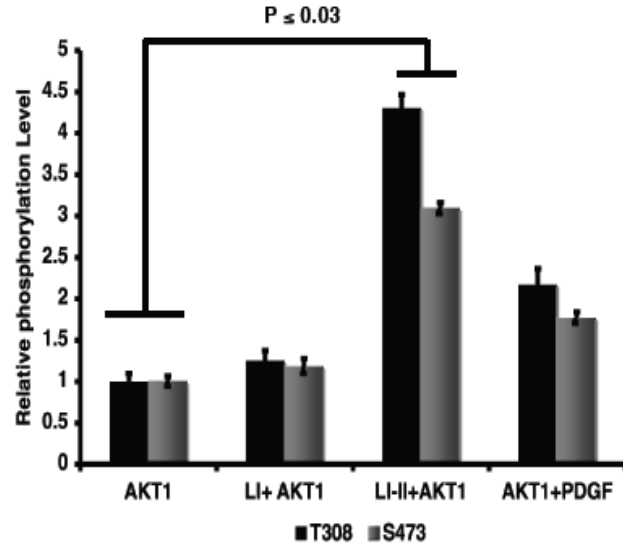
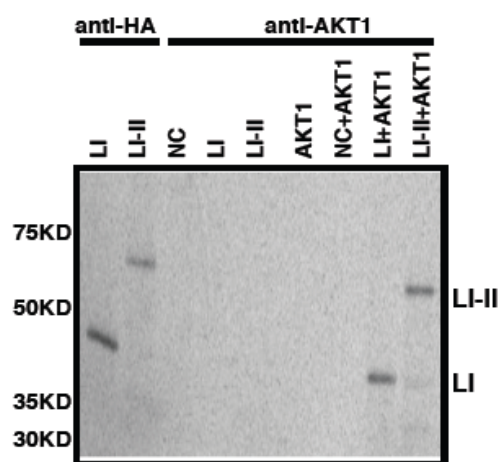


Figure 3-6. L enhances phosphorylation of AKT1. (A) **AKT activation is independent of PI3K.** A dual luciferase experiment was performed as described in Fig. 3.5A, in presence of PI3K inhibitor -Wortmannin (1 μ M) or vehicle DMSO. (B) **Expression of L in presence of PI3K inhibitor.** The lysates from luciferase assay were collected and were used for western blotting with anti-Flag antibody to examine the expression of L in the presence of PI3K inhibitor. (C) **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 T308 were examined using an ELISA kit (Cell Signaling) with T308 phosphorylation-specific AKT1 antibody. PDGF, cells were treated with 50 ng/ml PDGF for 10 min in media without serum after serum starvation of cells for 3h. NC, negative control, untransfected cells without PDGF treatment. (D) **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. Cells transfected with 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. (E) **Quantification of phosphorylation of AKT1 by L.** The average values for phosphorylation of AKT1 from the results of three independent experiments were graphed. The ratio of phosphorylated AKT (at T308 or S473) to total AKT from cells transfected by AKT1 alone was set at 1. Error bars represent standard errors of the means.

A.



B.

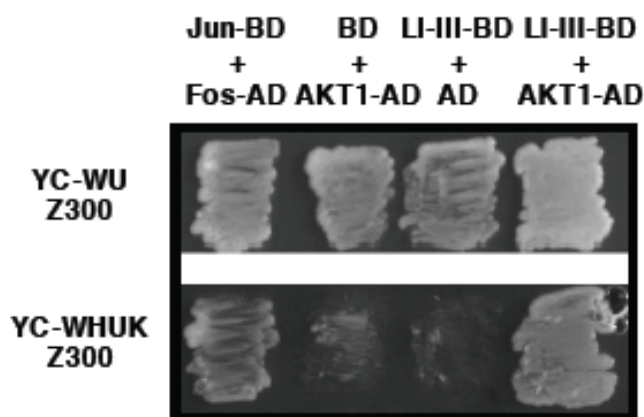


Figure 3-7. L interacts with AKT1. (A) Co-immunoprecipitation of L and AKT1. ³⁵S-labeled LI and LI-II were synthesized by *in vitro* transcription and translation kit. AKT1 was obtained from cells transfected with AKT1 expression plasmid. ³⁵S-labeled LI or LI-II was mixed with cell lysate containing 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.

3.4 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 pro-inflammatory (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 IRF-3 and NF- κ B. Recently, a great deal of progress has been made in elucidating the pathways that lead to IRF-3 activation after viral infection (reviewed in (272)). However, stimulation of NF- κ B by viruses has been less well characterized. Previously, it has been reported that a mutant PIV5 (a recombinant PIV5 lacking the conserved C-terminus of the V protein, rPIV5V Δ C) can activate expression of IFN- β and IL-6 (125, 197, 268). Further studies indicated that IFN- β induction was the result of activation of IRF-3 by the RIG-like helicase pathway, specifically through MDA5 (9). While IL-6 expression by rPIV5V Δ C infection requires NF- κ B activation (197), 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 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 position 308 (Threonine, T308) and 473 (Serine, S473). It is well established

that PDK1 and the rictor-mTOR complex can phosphorylate T308 and S473 respectively. Interestingly, the expression of the L protein alone caused enhanced phosphorylation at both sites of AKT1 and PI3K was not found to be important for this activation. In the case of PDGF activation of AKT1, it is thought that phosphorylation of the residue 308 facilitates the phosphorylation of residue 473. How L stimulates AKT phosphorylation still remains to be determined.

Previously, it has been reported that AKT1 plays a critical role in the replication of NNSVs (329). We speculate that AKT is important for NNSV replication due to its ability to phosphorylate the viral P protein, an essential co-factor of the 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*. Thus, L would not only provide the enzymatic functions for RNA synthesis, but would also stimulate transcription/replication by inducing the phosphorylation of P. Activation of NF- κ B via the AKT1 pathway would therefore be a by-product 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 the L protein. A proposed mechanism of L mediated activation of AKT and NF- κ B is shown in **Figure 3.8**.

The structure of the L protein is well conserved among all NNSVs. There are six conserved domains within L, though the functions of these domains are not well defined. Domain III contains a conserved region resembling catalytic center of polymerase and is

thus considered the catalytic domain (206, 321). Domain VI contains a methylase domain since mutations in the domain affect methylation of the 5' viral mRNA (112, 182). 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 (227, 320). In this study, we found that domain II is essential for stimulating the phosphorylation of AKT1, suggesting that the 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 VSV (New Jersey serotype) and Sendai virus may have intrinsic kinase activity (20, 21, 91, 114, 295). However, other reports suggest that the L-associated kinase activity may come from a host kinase that interacts with L (105, 213). In RSV, it has been reported that L does not have intrinsic kinase activity (22). Our results indicate that L interacts with AKT1 and enhances its phosphorylation. It is possible that L and AKT1 form a complex with a cellular kinase, which in turn, phosphorylates and activates AKT1.

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.

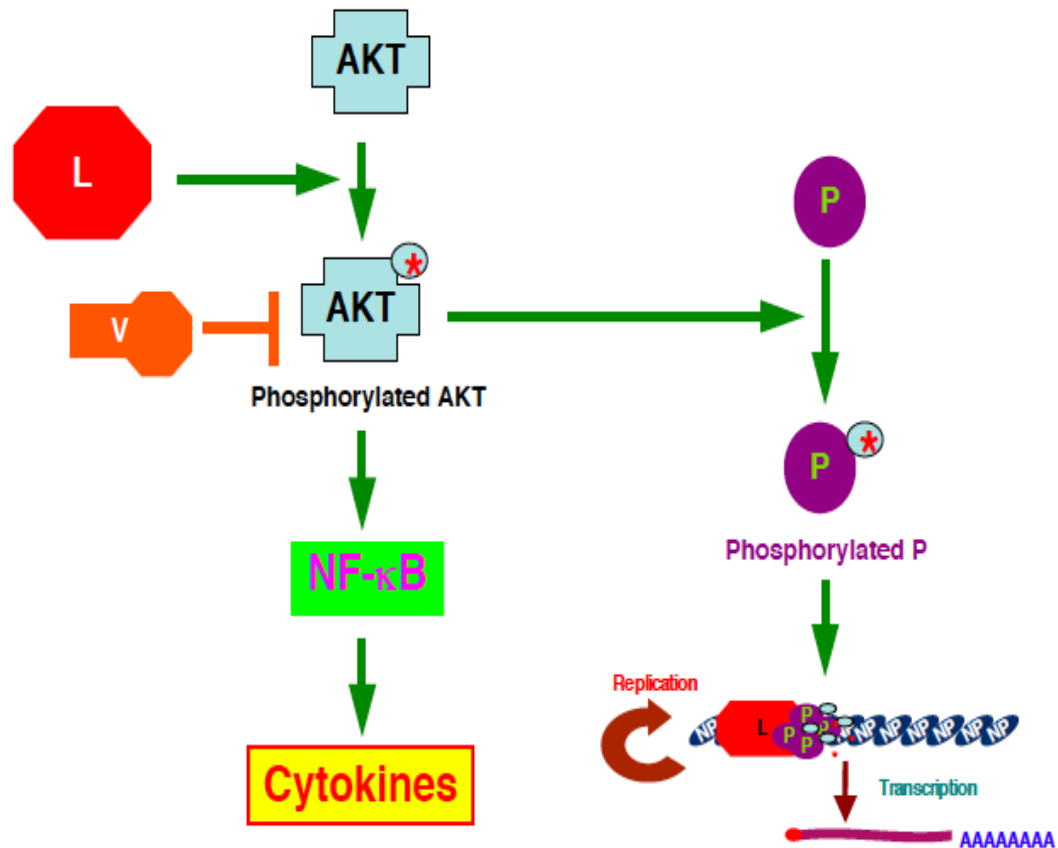


Figure 3-8. Proposed mechanism for the activation of AKT in virus-infected cells and the roles of AKT in virus replication. Previously, it has been shown that AKT plays a critical role in phosphorylation of the P protein, which is an essential component of vRdRp (329). We propose that the L protein activates AKT by stimulating 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 the V protein blocks expression of IFN- β and IL-6 and the V protein interacts with AKT, we speculate that the V protein can block the activation of NF- κ B likely through its interaction with AKT.

Chapter 4

Activation of Interferon- β expression by a viral mRNA through RNase L and MDA5

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4.1 Abstract

The innate immune response mediated by interferons (IFNs) is the frontline defense against viral infections in vertebrate animals. IFNs initiate signal transduction to antiviral genes that limit infections by many types of viruses. MDA5, a RNA helicase, is a key component in activating expression of type I IFNs in response to certain types of viral infections. It has been reported that MDA5 senses non-cellular RNA and triggers the signaling cascade that leads to IFN production. Short synthetic and viral double-stranded RNAs are known activators of MDA5. However, natural single-stranded RNAs have not been reported to activate MDA5. Here, we have serendipitously identified a viral mRNA encoded by the L gene of PIV5 that activates IFN expression through MDA5 and provide evidence that the signaling pathway includes the antiviral enzyme, RNase L. Our results indicate that a viral mRNA, with 5'-cap and 3'-poly (A), can activate IFN expression through an RNase L-MDA5 pathway.

4.2 BACKGROUND

Interferon plays a critical role in the innate immune responses against viral infections. Viruses trigger expression of IFN- β in initially infected cells and IFN- β can lead to activation of IFN- α expression through phosphorylation of IRF-7 (210, 297). IFNs induce an antiviral state in cells that inhibits spreading infection. MDA5 (melanoma differentiation-associated gene 5), a RNA helicase, plays an essential role in the activation of IFN expression (10). MDA5 is involved in the cytoplasmic sensing of infections by some RNA viruses (5). Recognition of RNA molecules generated during viral infections by MDA5 leads to activation of IFN- β promoter stimulator (IPS)-1, NF- κ B and IFN expression (158).

How MDA5 differentiates between self and non-self RNA is not clear but it has been reported that stable, long, double stranded (ds) RNA structures greater than 2 kilobase pairs (kb) in size, presumably with 5'-triphosphates generated during RNA virus infection (not typical of self RNA) may serve as a distinguishing factor for MDA5 specific recognition (156). Long synthetic dsRNA polymers of poly(I):poly(C) are often used as a surrogate for the putative activator of MDA5 (108). However, Pichlmair *et. al.* reported that long dsRNA was not sufficient to activate IFN expression through MDA5-dependent pathway and higher-order RNA structures containing both dsRNA and single stranded (ss) RNA are activators of MDA5-dependent IFN expression (261). The nature

of the higher-order RNA structure remains undefined and the actual molecular identity of the ligands is unclear. A natural RNA trigger for MDA5 has not been identified.

The role of MDA5 in regulating IFN expression was first reported in the studies of parainfluenza virus 5 (PIV5) [formerly known as simian virus 5 (SV5)] (10, 53). PIV5 is a prototypical paramyxovirus in a family of nonsegmented, negative-stranded RNA viruses that includes many important human and animal pathogens, including mumps virus, measles virus, Nipah virus, and respiratory syncytial virus (173). PIV5 was originally isolated from cultured primary monkey cells, its natural host is the dog in which it causes kennel cough (219). PIV5 can infect humans (61), but no known symptoms or diseases in humans have been associated with PIV5 (135). PIV5 has seven genes and encodes eight viral proteins (175). The viral RNA-dependent RNA polymerase, minimally consisting of the L protein and the P protein, transcribes the nucleocapsid protein (NP or N)-encapsidated viral genome RNA into 5' capped and 3' polyadenylated mRNAs (94).

The V protein of PIV5, a component of PIV5 virions (~350 molecules per virion), is a multifunctional protein and plays important roles in viral pathogenesis. The V protein C-terminal domain contains seven cysteine residues, resembling a zinc finger domain, and binds atomic zinc (199, 256), which is conserved in all paramyxoviruses. The V protein of PIV5 interacts with the soluble NP protein (271) and the N-terminal domain of the V protein binds RNA through a basic region (193). The V protein regulates viral RNA synthesis (190) and interacts with a cellular protein (DDB1), the 127 kDa subunit

of the damage-specific DNA-binding protein (DDB) that is involved in damaged DNA repair. The V protein of PIV5 can cause degradation of STAT1 protein, an essential regulator of IFN signaling, through a proteasome-mediated pathway in human cells but not in mouse cells (81) by forming a complex with DDB1, Cul4A, STAT1 and STAT2, and V has an E3 ubiquitin ligase activity (253, 351). A recombinant virus lacking the C-terminus of the V protein of PIV5 (rPIV5VΔC) induces a higher level of IFN expression than wild-type virus, indicating that the V protein plays an essential role in blocking IFN production in virus-infected cells (125, 268). Andrejeva *et. al.* found that the V protein interacts with MDA5, resulting in a blockade of activation of IFN- β expression (10). They also found that knocking down expression of MDA5 reduces IFN expression induced by poly(I):poly(C), indicating that MDA5 plays an essential role in induction of IFN expression by dsRNA. The endoribonuclease RNase L has been reported to be important for activating the antiviral immune response to RNA virus infection or dsRNA stimulation (205). RNase L is activated by 2-5 oligoadenylate and results in production of small RNA cleavage products and hence initiation of interferon production. In this work, the activation of IFN by rPIV5VΔC infection has been investigated and a viral mRNA encoded by L gene of PIV5 with 5'-cap has been identified as an activator of IFN expression through MDA5-dependent pathway that includes RNase L.

4.3 Results

4.3.1 Region II of the L gene activated NF- κ B independent of AKT1

Previously, it has been reported that rPIV5 Δ C induces expression of IL-6, as well as activates NF- κ B (197, 203), furthermore the L gene of PIV5 is sufficient to activate NF- κ B (203). The L gene has six regions that are conserved among all the L proteins of negative stranded, nonsegmented, RNA viruses (265). The portion of the L gene containing the conserved regions I and II (L-I-II) together is sufficient to activate NF- κ B (203). To further investigate the role of the L gene in activation of NF- κ B, we performed deletion mutagenesis of the region within the L gene. We found that region II, which contains 144 amino acid residues, was sufficient for the activation of NF- κ B using a reporter gene assay (**Figure 4.1A**) and the result was confirmed using electrophoretic mobility shift assay (EMSA) (**Figure 4.1B**). This result came as somewhat a surprise since previously we have shown that the activation of NF- κ B by the L gene requires AKT1 and region I (L-I) which binds to AKT1 (203).

We examined the interaction between AKT1 and L-II and confirmed that L-II did not bind to AKT1 (**Figure 4.2A**). Interestingly, this activation by L-II did not require AKT1 because an AKT1 inhibitor, AKTIV or an AKT1 dominant negative (DN) mutant, had no effect on activation of NF- κ B by the L-II (**Figure 4.2B** and **Figure 4.2C**). These results indicate that the L-II region activated NF- κ B through a novel mechanism.

4.3.2 The RNA of region II of the L gene activated NF- κ B

Because RNA can activate NF- κ B (272), we speculated that the RNA sequence within the L-II region, not the amino acid residues encoded by the L-II region, might be responsible for NF- κ B activation. We mutated the start codon of L-II into a stop codon (L-II mut) (**Figure 4.3A**) and found that L-II mut did not express protein although the expression levels of RNAs were similar between L-II and L-II mut (**Figure 4.3B** and **Figure 4.3C**). Interestingly, this mRNA generated from the plasmid pCAGGS, which is under the control of a pol-II promoter (238), activated NF- κ B (**Figure 4.4A**) and the result was confirmed by EMSA (**Figure 4.4B**), suggesting that a mRNA of viral origin can activate NF- κ B. This activation was not inhibited by the AKT1 inhibitor, AKTIV or an AKT1 DN mutant (**Figure 4.4C** and **Figure 4.4D**) consistent with previous observations (**Figure 4.2B** and **Figure 4.2C**).

4.3.3 The RNA of region II of the L gene activated IFN- β expression

To investigate the ability of this RNA to activate IFN expression, a plasmid containing a reporter gene (F-Luc) under control of an IFN- β promoter was co-transfected with a plasmid encoding the L-II or L-II mut. As shown in **Figure 4.5A**, the plasmid expressing the L-II RNA activated IFN- β promoter-driven reporter gene expression, suggesting that the RNA activated the IFN promoter. Further, the amount of IFN- β in the medium of cells transfected with plasmids L-II- or L-II mut-expressing respective mRNAs were measured using ELISA. The putative mRNA from the L-II mut

plasmid induced equivalent IFN- β production to the positive control, poly(I):poly(C) (500 ng/ml) (**Figure 4.5B**), suggesting that the mRNA indeed activated expression of IFN- β . To further confirm that it is the RNA that activated IFN- β expression, the RNAs from transfected cells were purified and transfected into fresh cells, and the levels of IFN- β in the medium of the RNA-transfected cells were measured after one day. As shown in **Figure 4.5C**, the RNA from the L-II mut-transfected cells produced a higher level of IFN- β than the RNAs from vector-transfected cells. The ability of RNA to induce IFN- β from virus-infected cells was also examined. Interestingly, both RNAs from wild-type and rPIV5V Δ C induced expression of IFN- β , indicating that RNAs capable of activating IFN- β expression exists in virus-infected cells as well (**Figure 4.5C**). To further confirm that the activation of IFN- β is due to the mRNA from the plasmid encoding the region II mRNA, mRNAs from the cells transfected with plasmids encoding L-II RNA were purified and transfected into fresh cells. The amounts of IFN- β in the medium of cells transfected with mRNA from cells transfected with a plasmid expressing L-II mRNA were similar to that of those stimulated with poly(I):poly(C). IFN- β was not detected in the medium from cells transfected with control RNA from vector-transfected cells (**Figure 4.5D**), indicating that the mRNA from region II plays a critical role in activating expression of IFN- β .

The L-II RNA activated IFN- β transcription in the presence of a protein synthesis inhibitor cyclohexamide (CHX) (**Figure 4.6A**), indicating that the activation of IFN- β does not require protein synthesis and that the L-II RNA activated IFN- β expression at the RNA level. To validate the role of L-II mRNA in activating IFN- β expression, the

mRNA was removed from the total RNA purified from the L-II plasmid-transfected cells by carrying out a reverse transcription (RT) reaction using an L-II specific primer, followed by treating the RT products with RNase H, which digests RNA in a RNA-DNA hybrid. This L-II mRNA-depleted mRNA did not activate expression of IFN- β , indicating that L-II mRNA is essential for activation of IFN- β expression (**Figure 4.6B**). A similar experiment was carried out using RNA purified from virus-infected cells (**Figure 4.6C**). The reverse transcription using L-specific primer (region II) but not NP-specific primer reduced induction of IFN- β expression, indicating that the L mRNA in virus infection is responsible for activating expression of IFN- β .

To determine whether the L-II RNA is capable of activating IFN- β expression by itself, we generated a L-II RNA by *in vitro* transcription using T7 RNA polymerase, and we analyzed their sizes by using an agarose gel and also on RNA chip using AGILENT bioanalyzer (**Figure 4.6D** and **Figure 4.6E**). Both RNAs from the L-I and L-II region activated expression of IFN- β as expected (**Figure 4.6F**), since T7 RNAP transcripts have 5'-triphosphate, which is a known activator of IFN through RIG-I. Interestingly, while removing 5'-triphosphate with calf intestinal phosphatase (CIP) reduced activation of IFN- β by the L-I RNA, this had minimal impact on the effect of L-II RNA, confirming that the L-II RNA in its own right can activate IFN- β expression (**Figure 4.6F**). Furthermore, a plasmid expressing a mutant L gene with two stop codons placed downstream in-frame of its start codon induced activation of NF- κ B and IFN- β , confirming that the L mRNA is capable of activating expression of IFN- β (**Figure 4.7A**).

and **Figure 4.7B**). We also confirmed that this new L mut did not express any protein by performing immunoblotting experiment (**Figure 4.7C**).

4.3.4 The RNA of region II of the L gene activated IFN- β expression through a MDA5-dependent pathway

There are two known cytoplasmic proteins that sense non-cellular RNA: RIG-I and MDA5 (157). Both of these proteins activate IFN expression through IPS-1 protein (309, 379). Expression of a dominant negative (DN) mutant of IPS-1 blocked the activation of NF- κ B by plasmids expressing L-II RNA (**Figure 4.8A**), implying that RIG-I and/or MDA5 may play a role in L-II-induced NF- κ B activation.

To determine whether these two proteins are involved in NF- κ B activation by L-II RNA, we transfected plasmids encoding the L-II RNA into Huh7 or Huh7.5 cells, which has a defective RIG-I gene (327). We did not observe a difference in NF- κ B activation between the two cell lines, suggesting that RIG-I does not play a role in NF- κ B signalling (**Figure 4.9A**). Furthermore, RIG-I DN had no effect on the activation of NF- κ B (**Figure 4.9B**), confirming that RIG-I does not play a role in L gene-induced activation of NF- κ B.

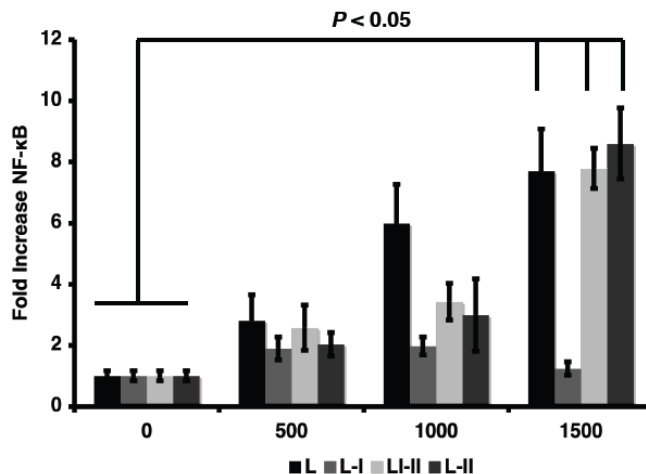
To investigate the role of MDA5, we reduced MDA5 expression using siRNA and found that this resulted in reduced NF- κ B activation (**Figure 4.10A**), indicating that MDA5 plays a critical role in NF- κ B activation. To further examine the roles of RIG-I and MDA5 in activating IFN- β expression, the effects of siRNA targeting RIG-I or MDA5 on IFN- β promoter activation were investigated. It was observed that siRNA

targeting MDA5, but not RIG-I, reduced IFN- β promoter activation by the plasmid expressing L-II mut RNA (**Figure 4.10B**). This was further confirmed by examining IFN- β production by cells transfected with plasmids expressing L-II RNA after treatment with siRNA targeting RIG-I or MDA5 using ELISA. As shown in **Figure 4.10C**, MDA5 siRNA reduced IFN- β production after plasmid transfection, whereas RIG-I siRNA had no effect, indicating that MDA5, not RIG-I, plays a role in activating IFN- β expression by L-II RNA.

4.3.5 The RNA of region II of the L gene activated IFN- β expression through a RNase L-dependent pathway

Previously, it has been reported that RNase L plays an important role in IFN expression (205). To examine the role of RNase L, we have used MEF cells with or without RNase L. We found that plasmids expressing L-II RNA activated NF- κ B and the IFN- β promoter in wild-type MEF, but not in MEF lacking RNase L (**Figure 4.11 A** and **Figure 4.11B**), suggesting that RNase L plays an important role in activation of IFN- β by this viral mRNA. To confirm these results, we transfected wild-type RNase L or a defective RNase L into MEF lacking RNase L. We found that the plasmid expressing the L-II RNA activated IFN- β in the presence of wild-type RNase L, but not in the presence of a defective RNase L lacking ribonuclease activity in MEF (**Figure 4.11C**). To confirm the role of RNase L in activation of IFN- β expression by L-II RNA, effects of siRNA targeting RNaseL were examined. As shown in **Figure 4.11D**, siRNA targeting RNaseL reduced induction of IFN- β production.

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B.

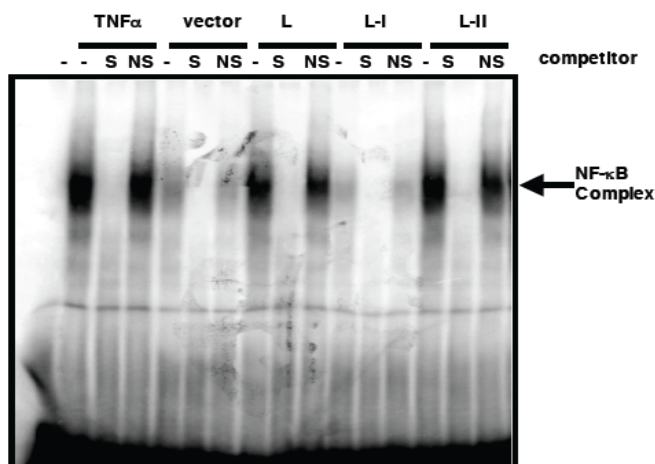
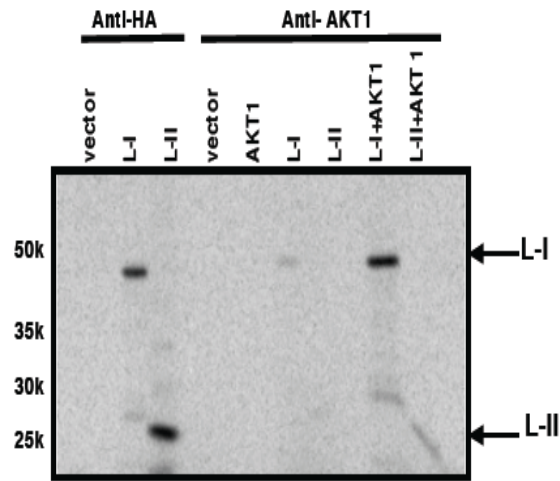
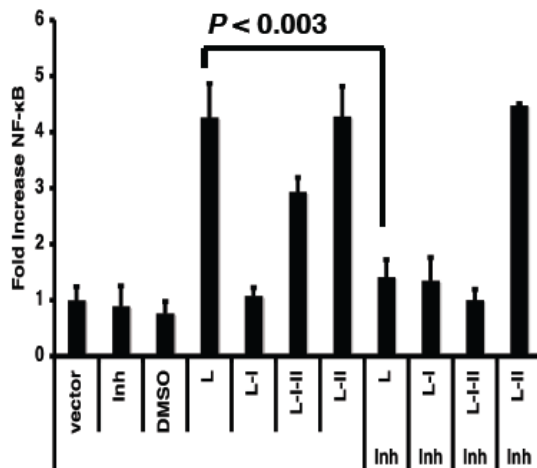


Figure 4-1. Activation of NF-κB by region II of the L gene. (A) A dual luciferase assay. BSR T7 cells were transfected with a plasmid encoding firefly luciferase gene (F-Luc) under the control of NF-κB responsive elements and indicated amounts of mentioned plasmids, along with a plasmid encoding *Renilla* luciferase (R-Luc) as an indicator for transfection efficiency. Empty vector was used to maintain a constant total transfected DNA. Luciferase activities were measured at 1 day post-transfection. Ratios of F-Luc to R-Luc are used as an indicator of reporter gene activity. The activity of vector was set as 1 and the rest samples were standardized to the vector. All transfections were carried out in replicates of four and error bars represent standard deviation (SD). **(B) EMSA.** Cells were transfected with empty vector and plasmids encoding L, L-I, or L-II. Nuclear extracts were obtained and incubated with ^{32}P -labeled NF-κB probe and appropriate competitors and were resolved on a 6% polyacrylamide gel. TNF-α, nuclear extracts from cells treated with 20 ng/ml of TNF-α, for 3h. “Probe (-)”-NF-κB DNA primers labeled with ^{32}P ; “S (specific competitor)”-unlabeled NF-κB probe (20-fold excess); “NS (Non-specific competitor)”- unlabeled mutant NF-κB probe (20-fold excess).

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B.



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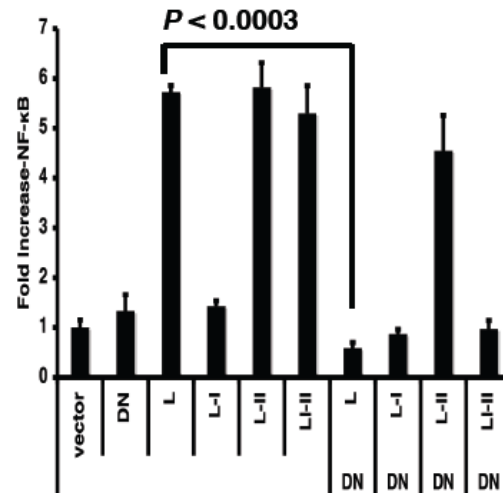
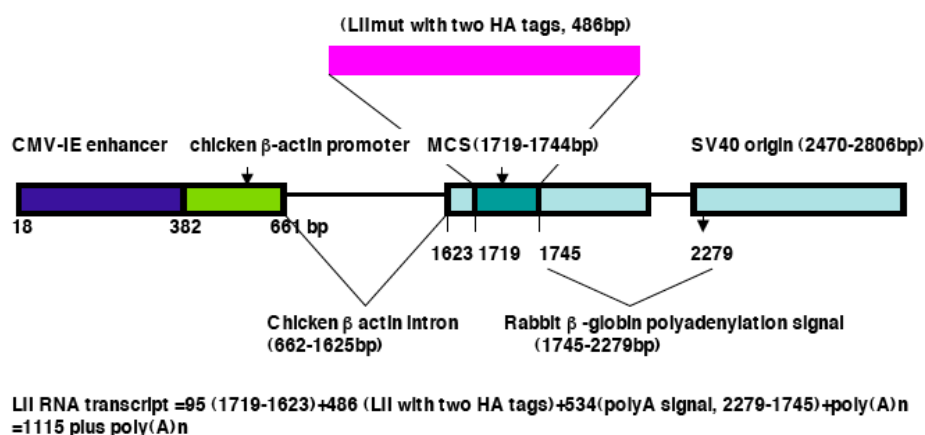
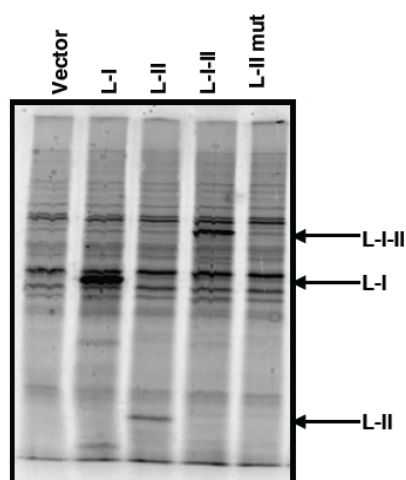


Figure 4-2. Activation of NF-κB by region II of the L gene in an AKT-independent manner (A) Domain I of L is important for interaction with AKT1. ³⁵S-labeled L-I and L-II were synthesized by *in vitro* transcription and translation. AKT1 was obtained from cells transfected with AKT1 expression plasmid. ³⁵S-labeled L-I or L-II was mixed with cell lysate containing AKT1 and immunoprecipitated with anti-AKT1 antibody. **(B and C) Activation of NF-κB by the L-II region.** A dual luciferase assay was performed as described for Fig. 4.1A in the presence of AKT IV inhibitor (Inh) (0.5 μM) (Calbiochem) or vehicle [dimethyl sulfoxide (DMSO)] **(B)** or in the presence of AKT1 dominant negative (DN) **(C)**.

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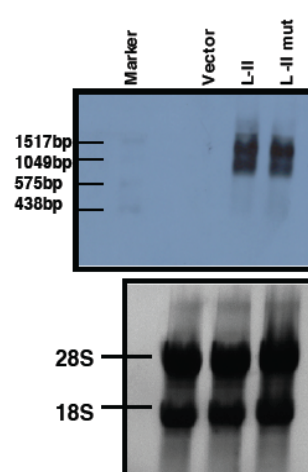
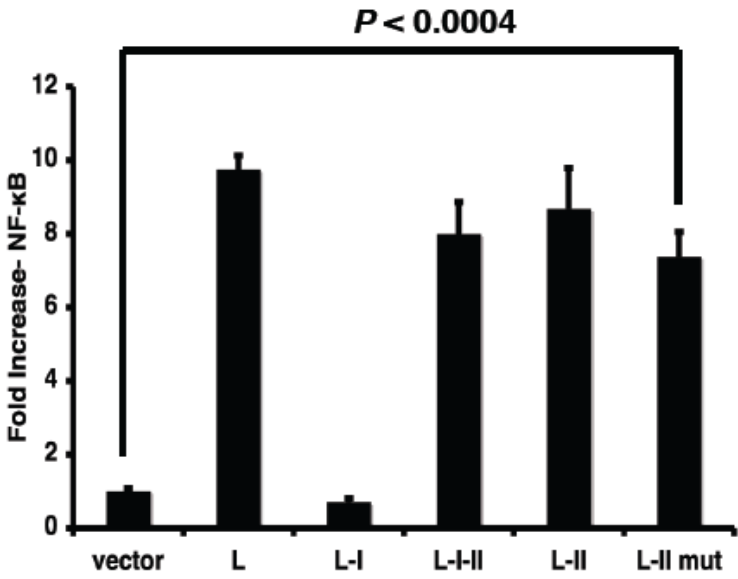
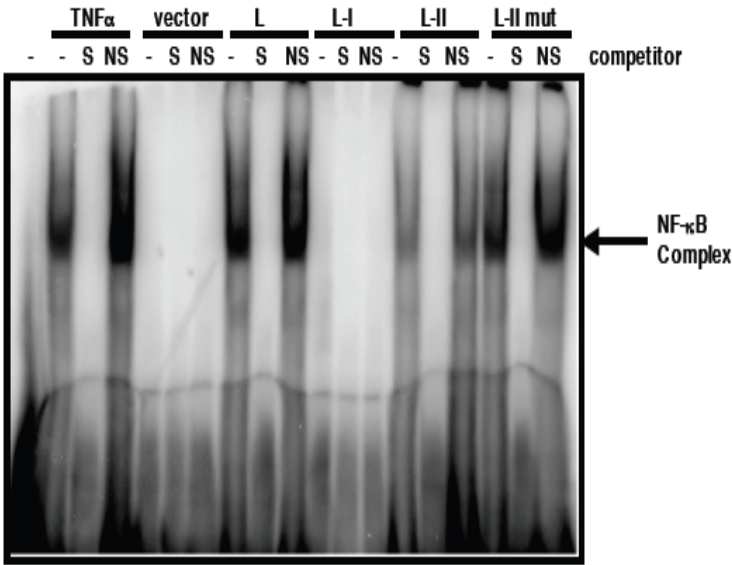


Figure 4-3 The cloning of L-II mutant plasmid and its expression. (A) Schematic of the plasmid expressing L-II mutant RNA. The L-II region was amplified using PCR primers that add two copies of HA tags at the C-terminal of L-II region and subcloned into EcoRI and NheI site of the vector pCAGGS (239, 272). The size of the L-II RNA transcript is about 1,000 nt without poly(A). **(B) Expression of L mutants.** The cells were transfected with vector, L-I, L-II, L-I-II, or L-II mut, and immunoprecipitation was performed to analyze the expression levels of the protein. **(C) Expression of the L RNA.** The amount of L-II and L-II mut RNA were compared using northern blot with anti-L-II antisense DIG-labeled RNA probe. Methylene blue staining was used to indicate the total RNA levels of the samples. “Marker” indicates the DIG-labeled RNA molecular weight marker.

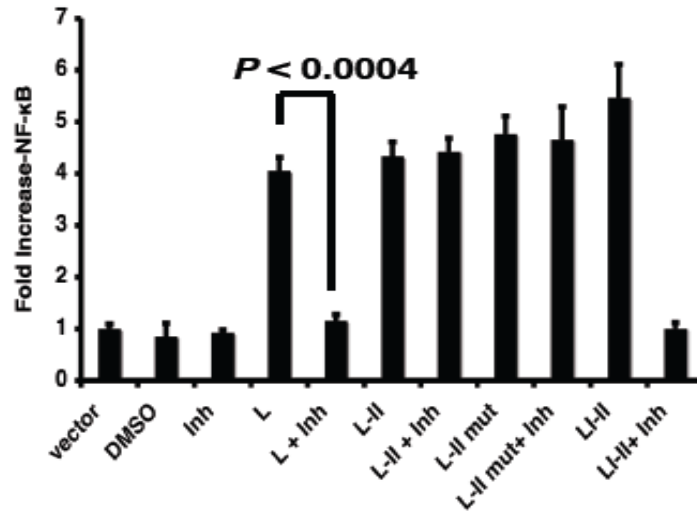
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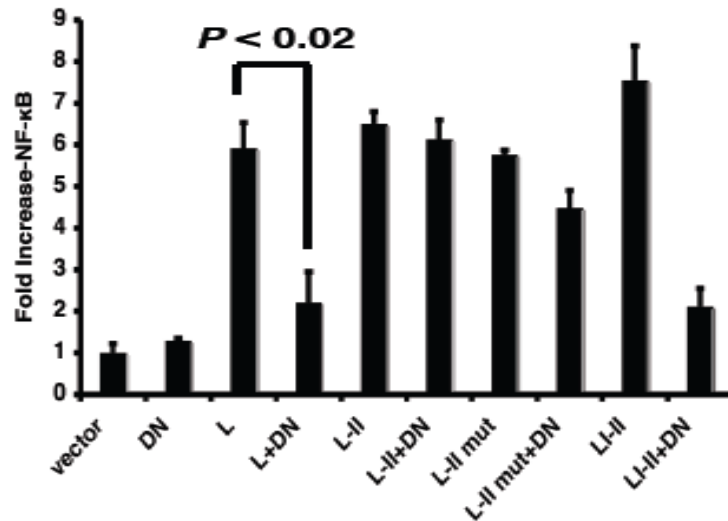
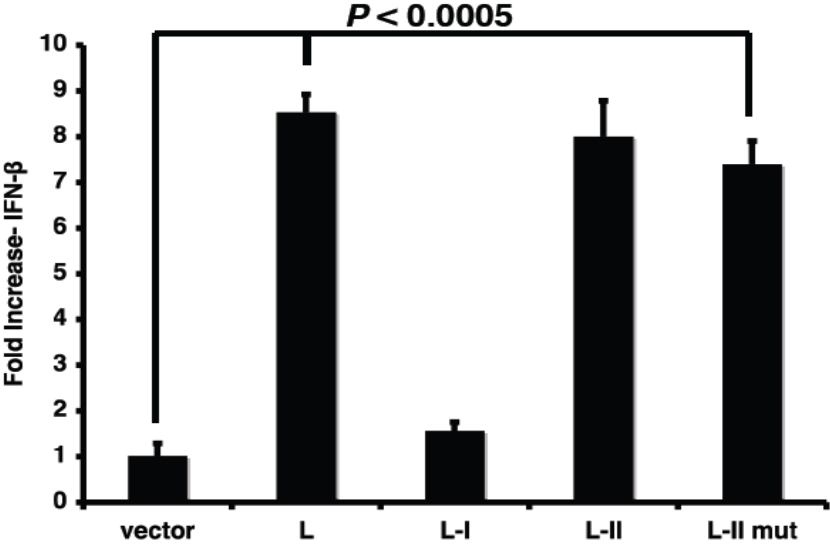
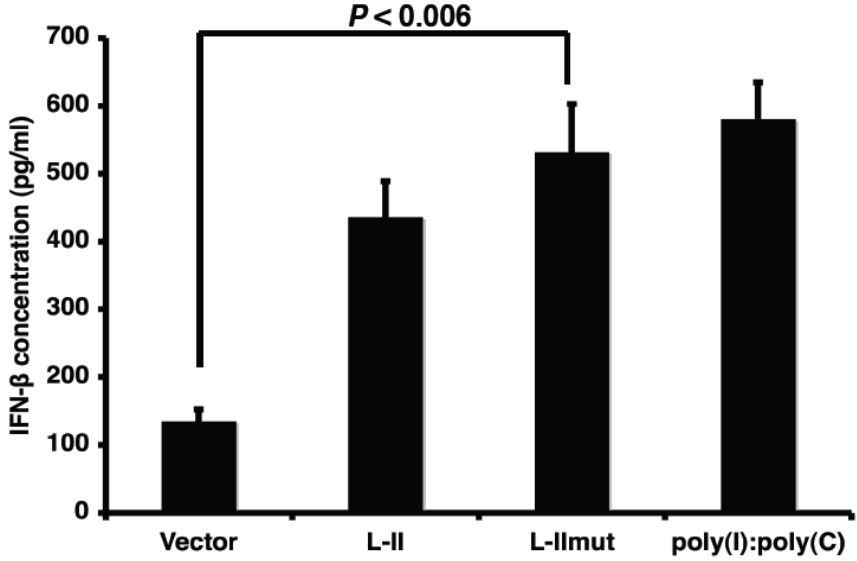


Figure 4-4. The L-II RNA activates NF-κB in an AKT1 independent manner. (A) Activation of NF-κB by the L-II mut. The L-II mutant contains a stop codon in place of the start codon of L-II. The reporter gene assay was performed as described in Fig.1A. **(B) EMSA.** The gel shift experiment was performed as described in Fig.4.1B using appropriate competitors. **(C and D) Activation of NF-κB by L-II RNA is independent of AKT1.** The dual luciferase experiment was performed using AKT1 inhibitor (Inh) **(C)** or AKT1 DN, AKT-AH **(D)** along with L, L-I-II, L-II, or L-II mutant plasmids as described in Fig. 4.2 B and C. All transfections were carried out in replicates of four and error bars indicate SD.

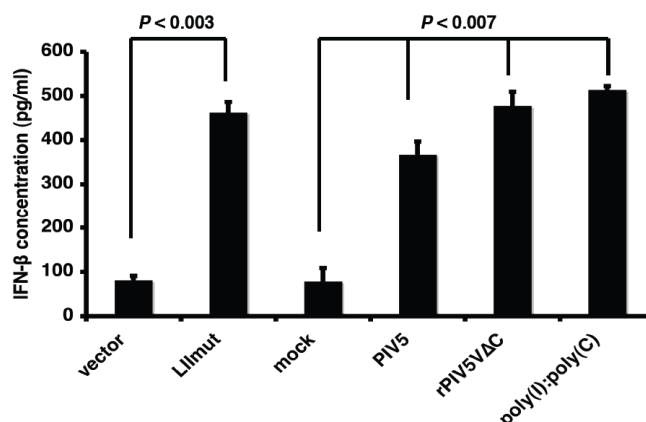
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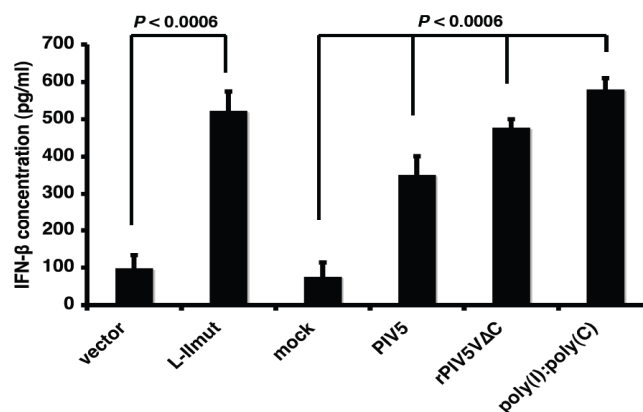
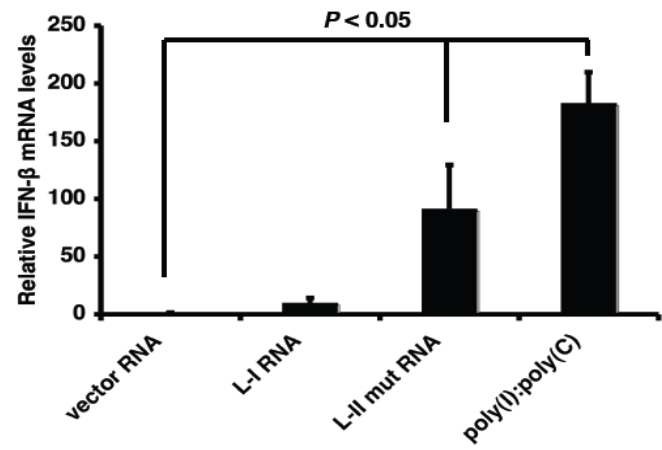
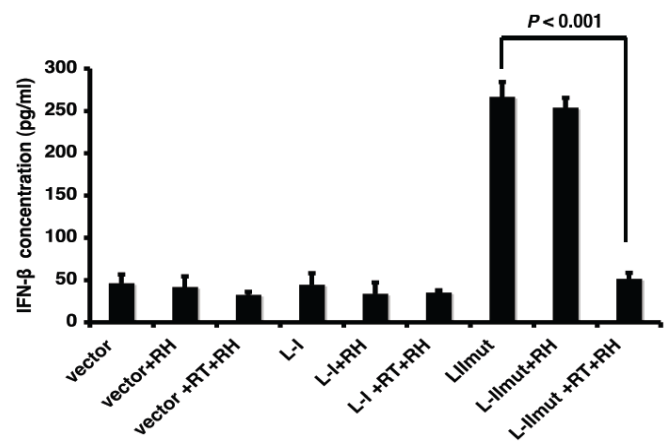


Figure 4-5. The L-II RNA activated IFN- β expression. (A) Activation of IFN- β promoter by the L-II mut. A dual luciferase assay was performed as described in Fig. 4.1A. A plasmid containing F-Luc under control of an IFN- β promoter was used in place of a NF- κ B- containing promoter as described in Fig. 4.1A. **(B) Induction of IFN- β production by L-II RNA.** Plasmids encoding L-II or L-II mut were transfected into HEK293T cells and the amount of IFN- β in the media were measured using ELISA at 1 day post-transfection. **(C) Activation of IFN- β by purified RNA.** Vero cells were transfected with plasmids containing L, L-I, L-II, or L-II mut, or infected with wild type PIV5, rPIV5VΔC, or mock-infected. Total RNAs were purified from transfected or infected cells. The purified RNAs were then transfected into 293T cells and concentrations of IFN- β in the media were measured using ELISA after 1 day. **(D) Activation of IFN- β by purified mRNA.** The Vero cells were transfected with empty vector or the plasmid containing L-II mut, or infected with wild-type PIV5, rPIV5VΔC, or mock infected. mRNAs were purified and transfected into 293T cells and IFN- β concentrations after one day were measured using ELISA. The graphs are average of three independent experiments and error bars represent SD.

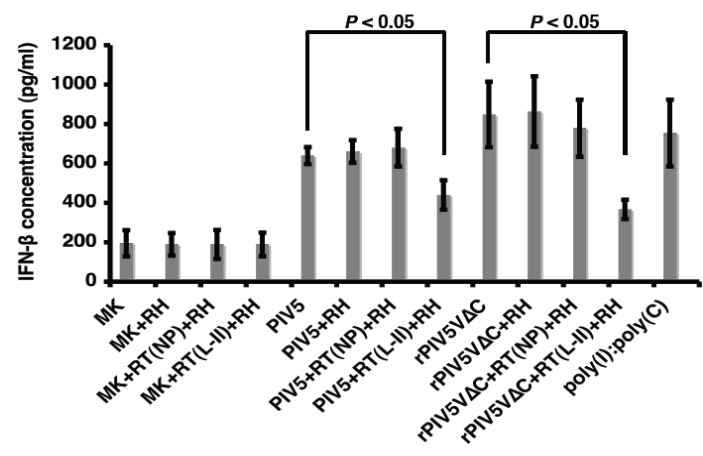
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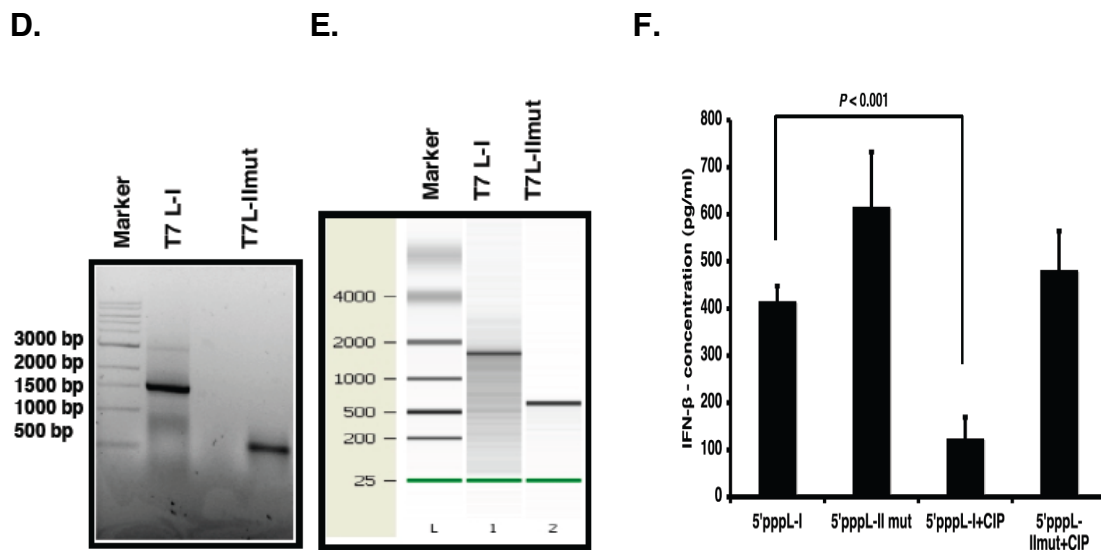


Figure 4-6. L-II mRNA stimulates the activation of IFN- β . (A) **Activation of IFN- β by the L-II in the presence of CHX.** The 293T cells in 6-well plates were transfected with 1 μ g of RNA or 250 ng of poly(I):poly(C) and incubated with CHX (20 μ g/ml) for 16 hours. The total RNAs were purified and subjected to reverse transcription and then real time PCR analysis. The relative IFN- β mRNA level represents the normalized values to vector. The ratio IFN- β mRNA and β -actin mRNA of vector transfected cells was set to 1. The samples were run in three replicates and error bars are SD. (B) **Lack of activation of IFN- β in the absence of the L-II mRNA.** The purified L-II RNA was reverse transcribed using a L-II sequence-specific primer with a reverse transcriptase (RT). The product and/or purified L-II RNA were treated or untreated with RNase H (RH). The purified products were then transfected into 293T cells and IFN- β concentrations after one day were determined using ELISA. (C) **Lack of activation of IFN- β in the absence of the L mRNA.** The same experiment as in Fig. 4.4C was carried out using RNAs purified from infected cells. RT(NP) indicates reverse transcription using NP-specific primer; RT(L-II) indicates reverse transcription using L-specific primer (region II). (D) **Analyzing the size of T7 RNA transcripts.** The size of the purified *in vitro* T7 RNA transcripts used in Fig. 4.6F were analyzed on (D) **Agarose gel (1%).** (E) **RNA chip using an Agilent Bioanalyzer.** Size markers are indicated. (F) **Induction of IFN- β production by *in vitro* transcribed L-II RNA.** The L-I and L-II RNA were *in vitro* synthesized using Riboprobe *in vitro* transcription systems (Promega). The RNA transcripts were treated or untreated with CIP to remove 5'-triphosphate and transfected into 293T cells. At one day post-transfection, IFN- β concentrations in the medium were measured using ELISA. The graphs are average of three independent experiments and error bars represent SD.

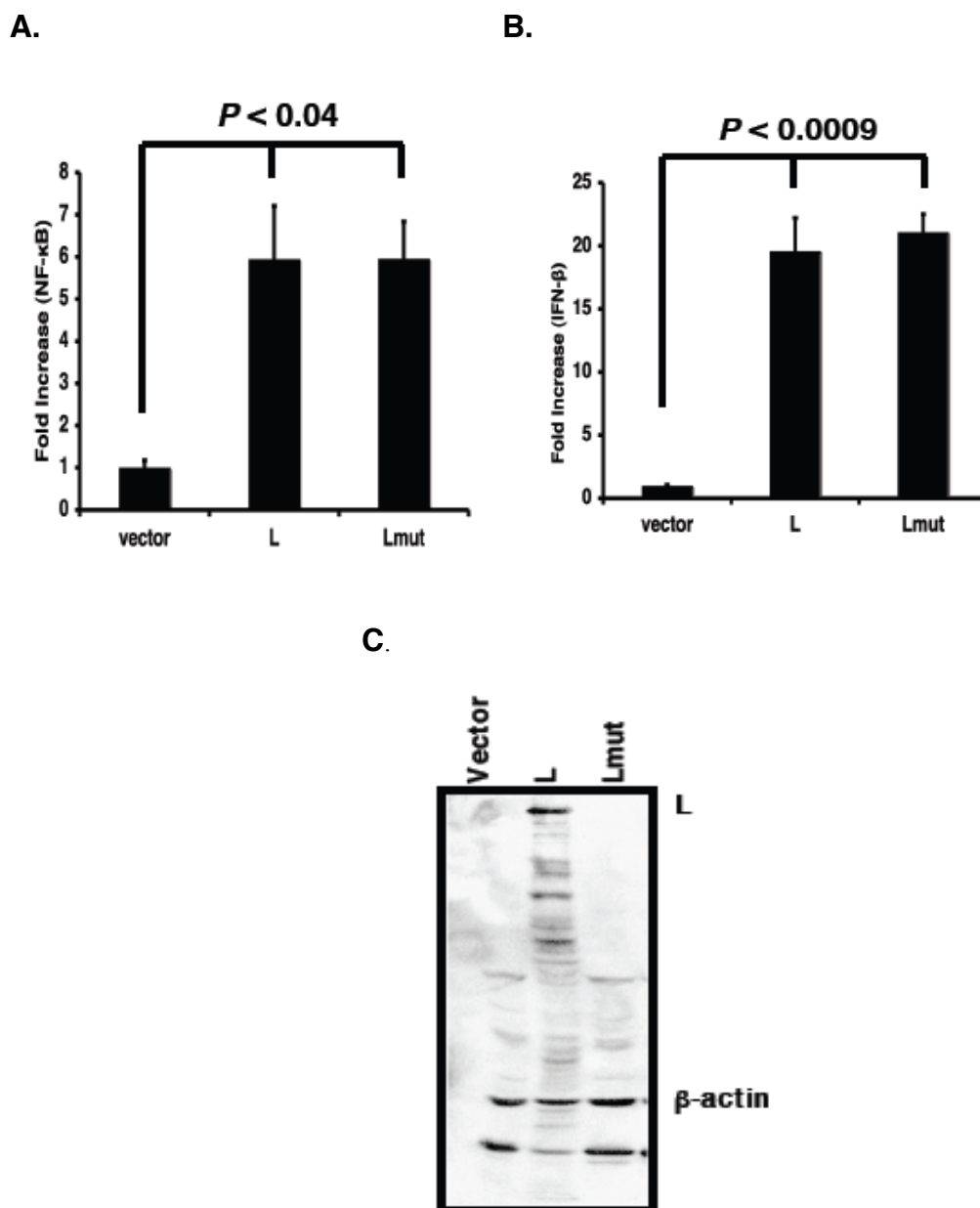
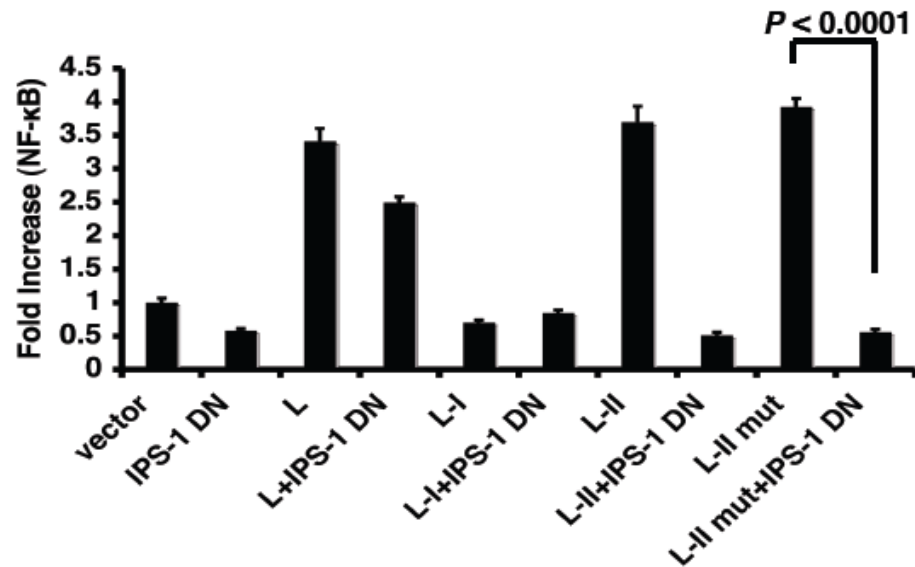


Figure 4-7. Activation of NF- κ B and IFN- β by the L mRNA. (A) Activation of NF- κ B by the L mut. A mutant L gene with two in-frame stop codons at 6 nts downstream of L start codon was generated (L mut). The reporter gene assay was performed as described in Fig. 4.1A. **(B) Activation of IFN- β promoter by the L mut.** A dual luciferase assay was performed as described in Fig. 4.5A **(C) Expression of L mutants.** The cells were transfected with vector, L or L mut, and immunoblotting was performed to analyze the expression levels of the proteins.

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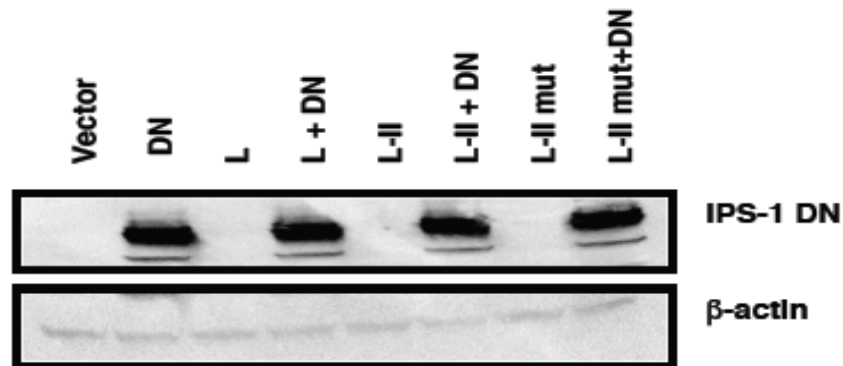
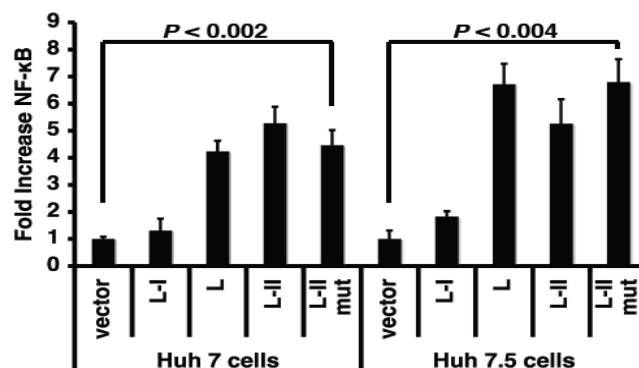
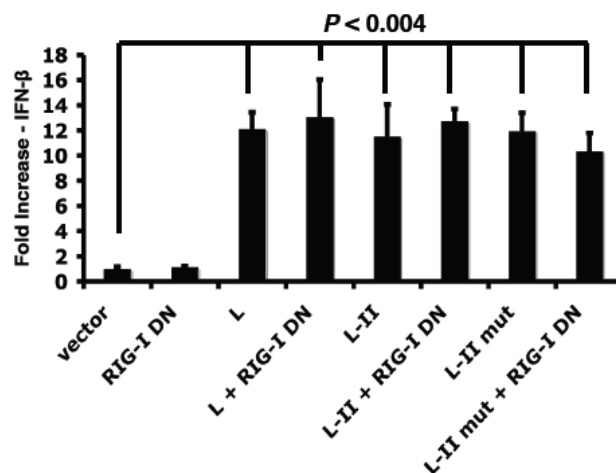


Figure 4-8. The role of IPS-1 in activation of NF-κB and IFN-β by L-II RNA. (A) Effect of IPS-1 DN on activation of NF-κB by L RNA. A dual luciferase experiment was performed, as previously described in Fig. 4.1A using IPS-1 DN with a Flag tag (500 ng/μl). **(B) Immunoblotting.** An immunoblotting experiment was performed to examine the expression of IPS-1-DN using anti-FLAG and anti-β-actin antibody. All transfections were carried out in replicates of four and error bars represent SD.

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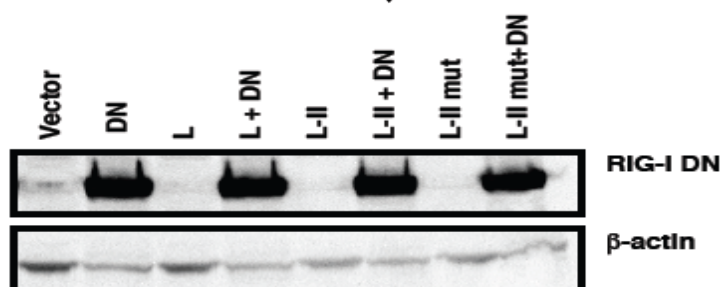
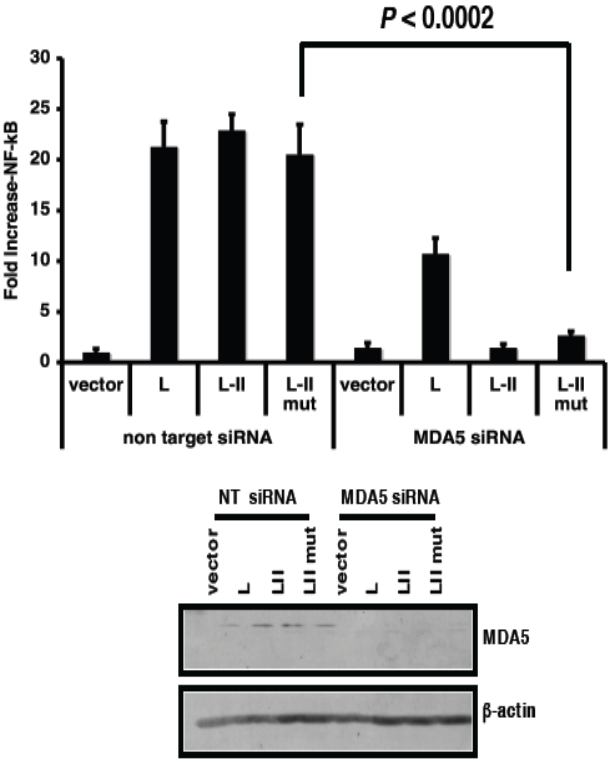
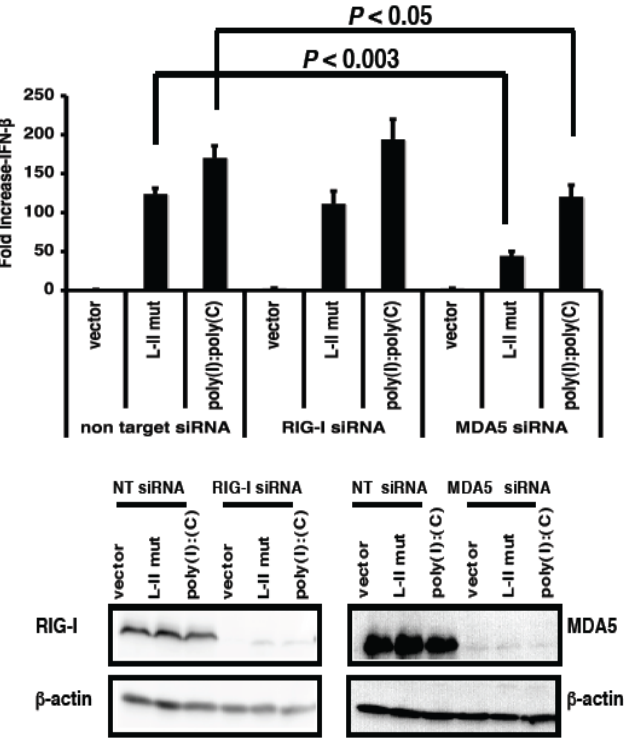


Figure 4-9. The role of RIG-I in activation of NF-κB and IFN-β by L-II RNA. (A) Activation of NF-κB by the L-II RNA in a RIG-I independent manner. At 18-20h after transfection, a dual luciferase assay was performed using lysates from Huh7 or Huh7.5 cells (RIG-I defective due to a T to I mutation at amino acid residue 55) transfected with vector, L, L-I, L-II, or L-II mutant. **(B) Effect of RIG-I DN on activation of NF-κB by the L-II RNA.** A reporter gene assay was performed using a plasmid expressing RIG-I DN with a Flag tag (500 ng/μl) along with the plasmids indicated. **(C) Immunoblotting.** An immunoblotting experiment was performed to examine the expression of RIG-I-DN using anti-FLAG and anti-β-actin antibody. All transfections were carried out in replicates of four and error bars represent SD.

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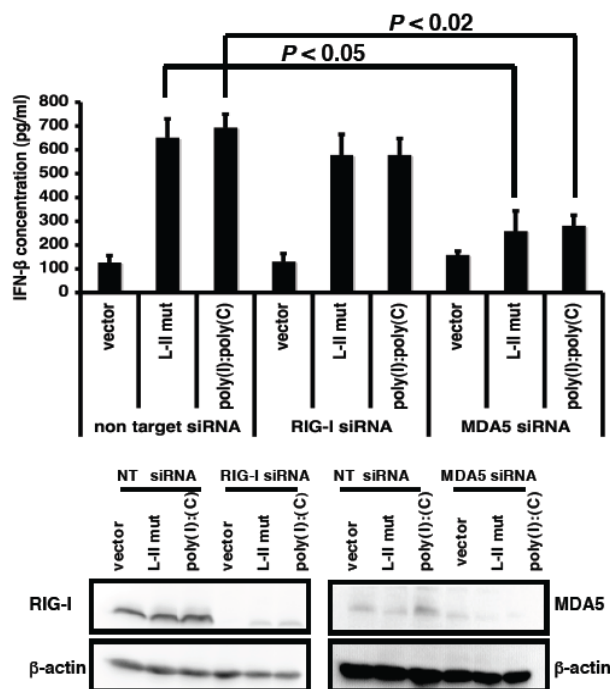
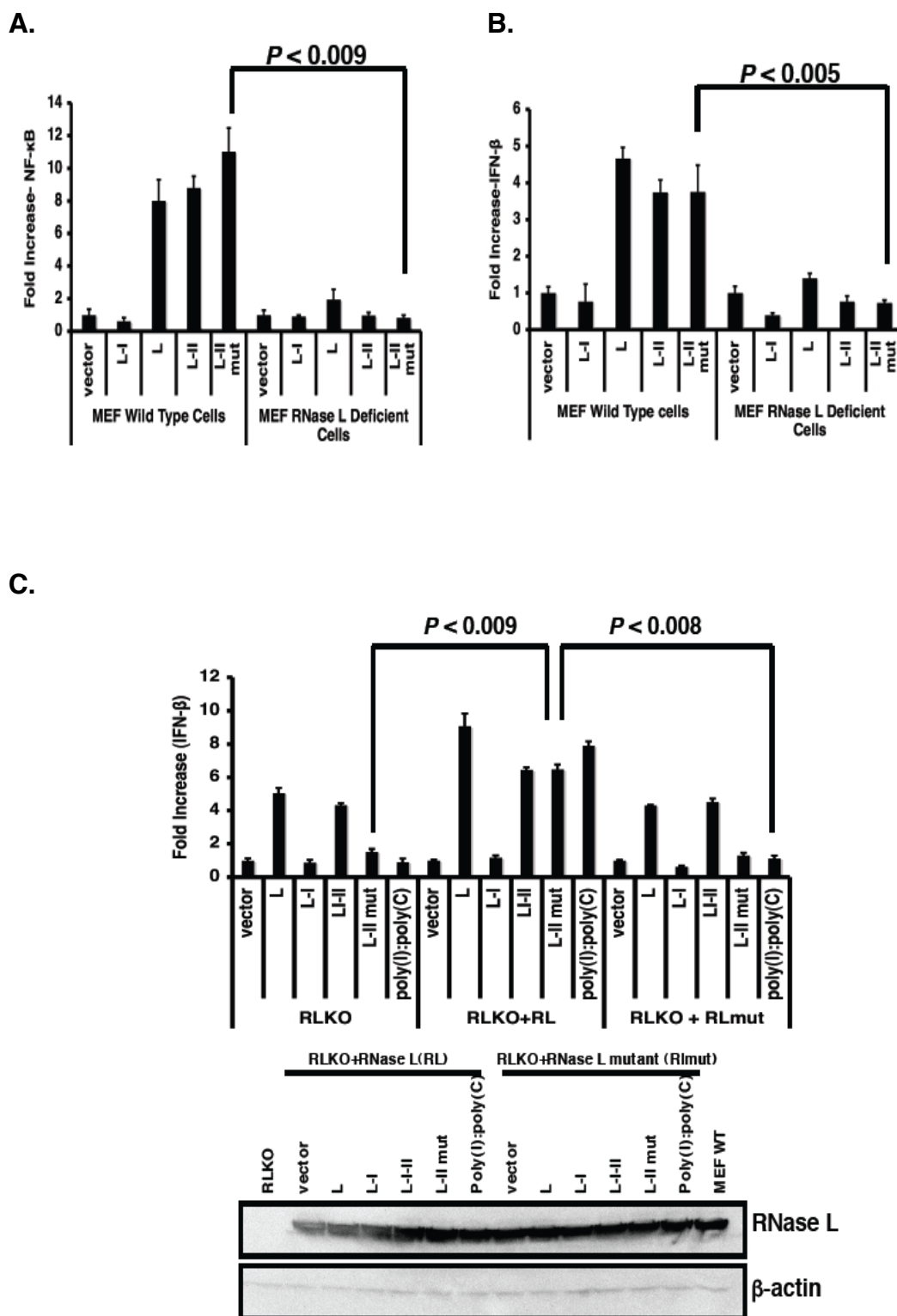


Figure 4-10. MDA5 plays a critical role in activation of NF- κ B and IFN- β by viral mRNA. (A) siRNA. The cells were transfected with siRNA targeting MDA5 or with control siRNA (NT siRNA). At 48h after siRNA transfection, the cells were transfected with plasmids encoding L, L-II, or L-II mut, along with reporter luciferase genes for NF- κ B and renilla luciferase. Luciferase activities were measured at 24h after transfection. The amounts of MDA5 and β -actin in the lysates from the dual-luciferase assay were measured by immunoblot analysis. **(B) The role of RIG-I and MDA5 in activation of the IFN- β promoter by viral mRNA.** 293T cells were transfected with siRNA targeting RIG-I, MDA5 or with non-target (NT) siRNA. 48h post-transfection of siRNA, the cells were transfected with vector, L-II mut, or with poly(I):poly(C), along with the luciferase reporter plasmids (for IFN- β and renilla). The luciferase activity was measure at 18-20h post-transfection. The amounts of MDA5 and actin in the lysates from the dual luciferase assay were examined by immunoblotting with anti-MDA5 and anti- β -actin antibodies. **(C) The role of RIG-I and MDA5 in activation of IFN- β by viral mRNA.** siRNA transfection was performed as described in Fig 4.10B in 293T cells and at 48h post-transfection of siRNA, the cells were transfected with vector, L-II mut, or with poly(I):poly(C). After 18-20h, amounts of IFN- β in the medium of different samples were measured using ELISA. Expression levels of RIG-I and MDA5 were examined by immunoblotting.



D.

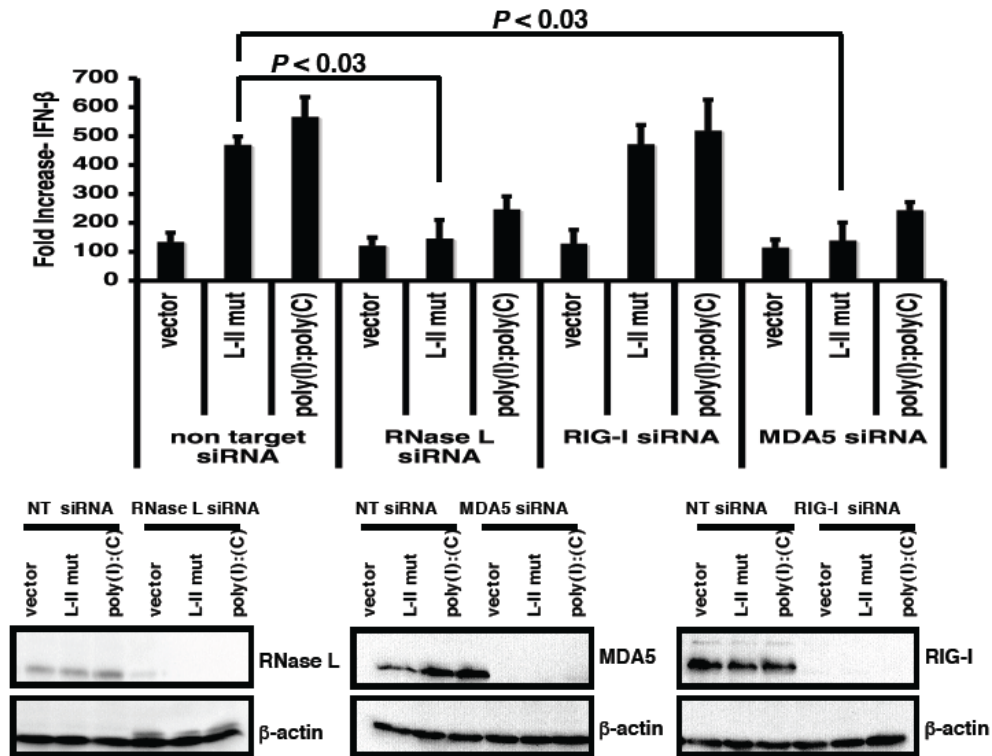


Figure 4-11. RNase L is critical for the activation of NF-κB and IFN-β by viral mRNA. (A) The role of RNase L in activating NF-κB. A dual luciferase assay for NF-κB activation was performed as described in Fig. 4.1A using wild-type (WT) or RNase L-deficient MEFs (RLKO). (B) The role of RNase L in activating the IFN-β promoter. A dual luciferase assay for IFN-β promoter activation was performed as described in Fig. 4.5A using wild-type (WT) or RNase L-deficient MEFs. (C) Restoration of IFN-β activation in RNase L^{-/-} MEF. NF-κB activation after knocking in RNase L cDNA in RNase L-deficient MEFs was examined by dual luciferase experiment in RNase L-deficient (RLKO). RLKO were transiently transfected with RNase L cDNA or inactive RNase L mutant (R667A) cDNA. At 18h after transfection, the cells were transfected with 1 μg/μl of vector, L mutants, or L RNA along with reporter plasmids. At one day post-transfection, the luciferase assay was performed. Amounts of RNase L and β-actin were examined by immunoblotting with anti-RNase L (Abnova) and anti-β-actin antibodies (Sigma). All transfections were carried out in replicates of four and error bars represent SD. (D) The role of RNase L in activating IFN-β expression. 293T cells were transfected with siRNA targeting RIG-I, MDA5, RNase L or control siRNA. IFN-β production was measured using ELISA. The expression of RIG-I, MDA5 and RNase L was examined by immunoblotting using the respective antibodies and β-actin as the loading control. The graph is average of three independent experiments and error bars represent SD.

4.4 DISCUSSION

Recently, it has been reported that the viral genomic RNA of negative-stranded viruses, such as influenza virus and Sendai virus, which has 5'-triphosphate that can activate IFN expression via a RIG-I-dependent pathway (157, 201, 277). Unlike these negative-stranded viruses, PIV5, a negative-stranded RNA virus, activated IFN- β expression through a viral mRNA-induced, RNase L/MDA5-dependent pathway. PIV5 replicates entirely in the cytoplasm (175) and the viral mRNA would be readily accessed by RNase L/MDA5 proteins. Previous reports indicate that PIV5 and some paramyxoviruses activate IFN expression via MDA5 (108, 382). Because dsRNA is a known trigger of MDA5, it is speculated that dsRNA generated during viral genome replication might activate MDA5.

Viral genomic RNA of paramyxovirus is tightly encapsidated by nucleocapsid protein and is resistant to RNase digestion, and inaccessible to other host proteins such as MDA5 (175). Therefore it is not surprising that no double-stranded RNA (dsRNA) was detected in paramyxovirus infection using dsRNA-recognizing antibody (365). In addition, since the nascent genome RNA is encapsidated, the double-stranded region of the viral RNA genome within the replication/transcription complex is small (greater than 2 kb is thought to be a MDA5 trigger). Pichlmair *et. al.* reported that long dsRNA was not sufficient to activate IFN expression through a MDA5-dependent pathway (261) and higher-order RNA structures containing both dsRNA and ssRNA are activators of

MDA5-dependent IFN expression. The nature of the higher-order RNA structure remains unidentified. Our identification of a viral mRNA that activates IFN- β expression through MDA5 is consistent with these results.

We propose that RNase L plays a role in sensing viral RNA by MDA5, leading to activation of NF- κ B as well as IFN- β . RNase L is an antiviral protein activated by 2'-5' oligoadenylate (2-5A). 2-5A is produced by 2'-5' oligoadenylate synthetase (2-5 OAS), the expression of which is induced by IFN- β (315). Activated RNase L cleaves viral mRNA and prevents viral replication (315). RNase L has recently been reported to amplify MDA5-dependent IFN expression through cleavage of cellular RNA (205). We speculate that RNase L recognizes the viral mRNA and processes it into an activator of MDA5, leading to expression of IFN, since siRNAs targeting RNase L and MDA5 reduced activation of IFN- β expression by the viral mRNA. Alternatively, it is possible that viral mRNA activates MDA5-dependent IFN expression and RNase L plays a role in amplifying IFN production. It is known that wild-type PIV5 induces low levels of IFN expression and rPIV5V Δ C infection produces high levels of IFN (125, 268). Interestingly, RNAs purified from both virus-infected cells induced expression of high levels of IFN- β . This result is consistent with previous reports that the V protein of PIV5 can block induction of IFN induced by PIV5 infection (125, 268).

We have mapped the RNA sequence to a 432 nts long region. Preliminary analysis using RNA structure prediction programs indicates potential secondary structures within the sequence. Further detailed structure and function analysis of the

sequence will define the sequence element and structure within the viral mRNA that activates IFN- β expression through RNase L and MDA5. This sequence and structure may serve as a prototype for searching other natural triggers of MDA5. This work has not only identified a novel trigger for MDA5, but also may lead to the discovery of small RNA molecules capable of activating IFN- β expression which might be useful in antiviral therapy.

Chapter 5

CONCLUSIONS AND SUMMARY

Innate immunity is the first line of defense to pathogen intrusion. This is important not only to limit initial virus replication but also to help in establishment of an effective adaptive immunity in the host. Interferons confer an antiviral state in the host cells. One of the early steps of the signaling response of IFN expression is activation of transcription factors, NF- κ B and IRF-3. Viruses have evolved sophisticated mechanisms to subvert the expression of IFNs. In this battlefield of survival between host and pathogen, the outcome is determined by the ability of virus to regulate the innate immune signaling pathways. One of the critical pathways manipulated by the viruses for their own advantage is the NF- κ B-dependent signaling pathway. The understanding of this host-virus relationship in terms of modulation of immune responses can lead to discovery of novel targets for antiviral therapies.

The establishment of an efficient antiviral innate immune response requires detection of the infectious pathogens through host receptors leading to stimulation of an innate immune signaling cascade for interferon production. Understanding the molecular mechanisms by which viruses modulate these innate immune responses is crucial to develop new strategies to control virus infections. Our work investigated the important aspects of innate immune signaling pathways critical for viral pathogenesis:

understanding the role of viral genes in the activation of innate immune responses, characterizing the signature viral molecules recognized by DExD/H box RNA helicases, RIG-I and MDA5 and defining the underlying mechanism of interferon expression by the viral molecules.

5.1 Viruses and NF- κ B Activation

NF- κ B has an extensive role in the immune responses and acts as a key regulator of this system. It is one of the critical transcriptional regulators which can promote expression of several genes involved in the host innate immune responses, including cytokines, chemokines, adhesion receptors, growth factors and proto-oncogenes (106). Its role has also been identified in cancer where it has been shown to be involved in oncogenesis (274) and programmed cell death (23).

To modulate the host immune responses, the NF- κ B pathway represents a good target for viruses. NF- κ B activation is one of the early steps that is triggered within minutes of virus infection that does not require any protein synthesis and results in a robust stimulation of several cellular genes (215). In the case of measles virus infection in glial cells, the phosphorylation of I κ B can be seen within five minutes of infection, suggesting that upstream components upon virus entry are sufficient to induce the activation of immune responses (80). Because of the profound role of NF- κ B in controlling the cell cycle through regulation of a wide array of target genes, NF- κ B

serves as an attractive target for the viruses to modulate. The viruses utilize this pathway for their own advantages either by manipulating the antiapoptotic properties of NF- κ B to evade host immune response or by causing apoptosis to enhance virus spread (131). Interestingly, in some cancers, viruses maintain the activation of NF- κ B to promote oncogenesis (154).

NF- κ B is activated in response to many stimuli including viruses, stress and cytokines. NF- κ B exists as a homo or heterodimer bound by I κ B in an unstimulated cell. This prevents translocation of NF- κ B to the nucleus and maintains it in an inactive form (248). Its activation requires phosphorylation of I κ B at serine 32 and 36 by the I κ B kinase (IKK) complex. This phosphorylation results in ubiquitination of this inhibitory subunit and its subsequently targeted for 26S proteosomal degradation. This allows release of the NF- κ B protein which then translocates to the nucleus and stimulates transcription of NF- κ B responsive genes containing a consensus decameric sequence, 5'-GGGRNWYYCC-3', (where 'R', A or G; N, any nucleotide; W, A or T; Y, C or T) (120). The NF- κ B family of proteins consist of five subunits (c-Rel, RelA, RelB, p50/p105 and p52/p100) that share a conserved 300-residue long Rel homology domain at their N-terminus which plays important roles in dimerization, DNA binding and nuclear transport. The C-terminal domain of c-Rel, RelA and RelB acts as the transactivation domain (TAD). In the case of p100 and p105, the C-terminus domain lacks the TAD and inhibits transcription unless associated with TAD-containing NF- κ B family members or proteins consisting of co-activator (122).

The viruses can utilize their multifunctional protein or dsRNA to interfere with NF- κ B pathway and stimulate NF- κ B activation for promoting their own replication, modulating cellular apoptosis and promoting cell proliferation. However, persistent activation of NF- κ B due to virus infections has also been related to inflammation and malignancies (154). Many viruses including HIV-1, HTLV-1, Epstein-Barr virus, hepatitis B virus (HBV) and cytomegalovirus have gene promoters with NF- κ B binding sites which require NF- κ B activation for their own life cycle (131). These viruses not only utilize different viral components to stimulate this response but also involve different mechanisms for this activation.

In HIV-1, the promoter proximal (enhancer) region of the long terminal repeat (LTR) contains two inducible NF- κ B binding sites that are important for regulating HIV-1 gene expression (6, 146). Constitutive activation of NF- κ B has been observed in primary monocytes and myeloid cell lines that are chronically infected with HIV-1, permitting high HIV-1 gene expression and replication (14, 77, 78). Different mechanisms have been proposed for this activation, in most cases involving the activation of IKK complex. The binding of HIV-1 to the CD-4 receptor through its envelope glycoprotein gp120 can lead to NF- κ B activation through the Lck/Raf and the PI3K/AKT pathway, which can stimulate IKK (98, 240, 247, 279). HIV regulatory elements or accessory proteins have also been reported to stimulate NF- κ B activation. The HIV TAT protein has been found to activate NF- κ B through the T-cell specific

tyrosine kinase p56^{lck} dependent pathway (207). The virion derived accessory protein Vpr promotes activation of IL-8 in T cells through a mechanism dependent on NF- κ B and NF-IL-6 (284). HIV Nef protein stimulates IL-2 expression and mediates both NFAT and NF- κ B activation. This role of the Nef protein has been found to be important for its function in HIV-1 transcription, replication and virus spread (359). In some studies, HIV-1 transcription itself has been related to activation of cytokines such as TNF- α , IL-1, IL-6, and IFN- β that can activate NF- κ B through IKK (282). The role of dsRNA generated during HIV-1 replication is also reported to stimulate IFN activation through its recognition by PKR which can also stimulate NF- κ B activation (384).

HTLV-1-Tax protein has been found to induce NF- κ B activation (263). The HBx protein of HBV, which promotes hepatocellular carcinoma in chronic HBV infection, can also stimulate NF- κ B activation by activation of MEKK1 (MAPK pathway) and PKC, which causes degradation of I κ B and p105 (366). The highly pathogenic avian influenza virus has also been reported to activate NF- κ B through its NP, M and HA proteins which involve generation of oxidative radicals and endoplasmic reticulum (ER) overload (249). The induction of IKK activation has also been studied in the case of paramyxoviruses. The Sendai virus infection can promote degradation of I κ B, hence it can activate NF- κ B (8). The infection of airway epithelial cells with RSV also results in persistent activation of NF- κ B (95).

However, some viruses can exploit NF- κ B activation to promote apoptosis which

can be useful for virus spread. In the case of encephalomyocarditis virus (EMCV) infection, the p50 knockout mice show increased apoptosis but survival of EMCV infection, whereas the normal mice die (283). While some viruses encode viral proteins that can inhibit NF- κ B activation. The core protein of hepatitis C virus is reported to regulate NF- κ B activation to modulate both apoptotic and antiapoptotic pathways during virus infection (332, 380). The A238L and A224L proteins of African swine fever virus can inhibit NF- κ B activation (269). In the case of paramyxoviruses, measles and mumps virus, they fail to phosphorylate and degrade I κ B in neuronal cells, which may help the virus to escape immune response and establish a persistent infection (8).

Viruses face selective pressure from the immune system and therefore have evolved to evade these responses or utilize them to favor their own replication. The above-mentioned studies and several other reports have identified roles of NF- κ B as a crucial target for virus interference. NF- κ B signaling is not only important with respect to virus infections but is also linked with cancers, inflammatory diseases and neurodegenerative disorders, which make NF- κ B a very promising therapeutic target. Even though tremendous progress has been made in understanding the NF- κ B pathway but still the activation and regulation of this pathway by viruses have not been well characterized.

Paramyxoviruses are the leading cause of severe animal and human diseases. The human respiratory syncytial virus and human parainfluenza virus (hPIV) types 1-3 are the

most common cause of acute respiratory tract infections in young children and the elderly (69, 113, 367). There are currently no vaccines for these viruses. Although, there are vaccines available for measles and mumps viruses, these viruses are still a big health concern in the developing countries. Even with vaccination programs, outbreaks have occurred in Canada, United Kingdom and United States (74, 75, 138, 139). Hendra and Nipah virus infections can lead to vasculitis and encephalitis accounting for a mortality rate of about 40% (37, 90). These viruses have evolved mechanisms to control their RNA synthesis and also to escape the immune response system. Apart from encoding genes important for virus replication, they also encode genes that can interfere with the host immune system. These virally encoded antagonist proteins play a vital role in understanding the virus regulation of immune system. Discovering how paramyxoviruses regulate immune responses has been of prime importance in this research field.

Our work has been focused on understanding regulation of the NF- κ B pathway using parainfluenza virus 5 (PIV5), a prototypical paramyxovirus as a model system. PIV5 is a poor inducer of the antiviral responses, and a viral protein, V is known to block the signaling pathways leading to cytokine expression. A recombinant PIV5 lacking the conserved region of the V protein (rPIV5V Δ C) activates expression of cytokines IFN- β and IL-6 by mediating the activation of transcription factor NF- κ B, indicating that the cellular factors can sense the presence of the virus and induce cytokine expression in PIV5 infected cells (10, 197). Infection with wild type PIV5 induces very little if any, expression of cytokines such as IL-6 and IFN- β . Activation of IL-6 by rPIV5V Δ C was found to be mediated through NF- κ B (197). Interestingly, the V protein only blocked

rPIV5VAC-activated IL-6 expression and had no effect on LPS-activated IL-6 production or TNF α -activated NF- κ B stimulation. This suggests that the V protein is involved in blocking a virus specific pathway that leads to NF- κ B activation and IL-6 expression (197). The virus can mediate activation of NF- κ B through different mechanisms including expression of a viral protein, activation of membrane receptors such as TLRs or by its dsRNA which are generated as a replication intermediate.

In Chapter 3, we explored the role of PIV5 proteins in activation of NF- κ B and investigated the mechanism for this activation. By performing reporter gene assays and gel shift assays for NF- κ B activation using PIV5 proteins, we observed that expression of the large (L) protein of PIV5 was sufficient for this activation. The L protein is the polymerase protein of paramyxoviruses, and plays important roles for viral RNA replication and transcription. It is about 250 kDa and consists of six domains that are conserved in this family of viruses. We mapped the region that is critical for the NF- κ B activation by making deletion mutants of L. We identified that L-mut plasmid expressing domains I and II of the L gene is sufficient to activate NF- κ B. We also found that the L protein expression can stimulate activation of cytokines such as IL-6 and IFN- β . The inhibitory role of the V protein was also identified in L-mediated activation of NF- κ B which is in agreement with studies performed previously with rPIV5VAC (197). This is the first report that identifies a novel role of the polymerase protein of paramyxovirus, demonstrating the L protein of PIV5 regulates immune responses during virus infection.

To further investigate the mechanism of activation of NF- κ B by L, we examined the role of AKT1, a serine/threonine kinase. AKT has been reported to play an important role in viral RNA synthesis of NNSV (329), a process in which L is also important and its known role in activation of NF- κ B (279), made it a good target for our study. AKT participates in many cellular pathways and is activated in response to many stimuli including growth factors and cellular stress in a PI3K dependent manner (38). Its kinase activity is important for regulating cellular processes including cell survival, cell proliferation, cell cycle progression and angiogenesis, cell metabolism and cell migration (52, 151). In some cells, AKT can prevent apoptosis through mediating inhibition of proapoptotic molecules such as Bad and caspase-9 (73). While in some circumstances, AKT can also regulate the expression of genes with antiapoptotic activity by mediating the activation of transcription factors such as CREB, and the I κ B kinase (IKK), a positive regulator of NF- κ B (152, 279). Its role in cell cycle progression is associated with regulation of cyclin D functions (391). The AKT pathway has also been linked to many cancers as well, including pancreatic, colon, ovarian, lung, breast, prostate and leukemia (102).

We have found that the inhibitor of AKT1 as well as dominant negative mutant of AKT1 blocked the activation of NF- κ B by L, indicating that AKT1 plays a critical role in activation of NF- κ B by L. AKT normally exists in an inactive form in the untransformed cells. Phosphorylation is required for its activation (31). We further analyzed the role of L in AKT activation and found that expression of the L protein enhanced phosphorylation of AKT1 and also it interacts with AKT1 where domain I of the L protein was found to

be important for its interaction with AKT1 and the domain II being important for the activation of AKT1. We conclude that the PIV5 L protein can activate NF- κ B through an AKT1-mediated pathway.

Viruses can stimulate activation of AKT but the mechanism is not known. The human T cell lymphotropic virus type 1 (HTLV-1) is the etiologic agent of adult T cell leukemia and chronic inflammatory diseases and encodes a 40 kDa protein, Tax which plays important roles in viral replication and gene regulation (2, 246). The Tax protein has been reported to activate AKT, through targeting multiple molecules which involve phosphorylation of Bad, interaction with the p85 molecule of PI3K, mediating NF- κ B activation and p53 inhibition (147-149). This process is speculated to help viruses in suppressing apoptosis and preventing elimination of virus infected cells and hence promoting virus replication and cell survival (149).

Activation of AKT has been found to be critical for regulation of viral RNA synthesis by nonsegmented negative stranded RNA viruses through phosphorylation of the P protein which is an essential cofactor for the viral RNA polymerase complex (329). The L protein plays an important role in transcription and replication of the viruses but the functions of each domain are not well defined. Our studies suggest a possible role for the domain II in AKT activation. Whether the activation is direct or indirect is not clear. However, the inhibitor against PI3K did not affect activation of NF- κ B by L suggesting that AKT activation is independent of PI3 kinase activity. In our study we have identified that the L protein is involved in phosphorylation of AKT, and we speculate that L may

have intrinsic kinase activity or may be associated with a kinase to initiate AKT activation. Further studies are necessary to determine the role of L in AKT phosphorylation. There are reports suggesting that the L may have kinase activity or may be associated with a host kinase (20, 21, 92, 114). We speculate that the L protein regulation of cytokine expression is a byproduct of AKT activation. The role of L in NF- κ B activation is blocked following the expression of the V protein likely through interaction of V with AKT. This work suggests a novel role of L in regulation of innate immune responses and a possible mechanism of virus spread.

5.2 Viruses and Type I interferon Induction

NF- κ B signaling can lead to the establishment of antiviral responses through production of interferon (IFN) and pro-inflammatory cytokines. The IFN expression is triggered by recognition of specific viral components, pathogen associated molecular pattern (PAMPs) by cellular receptors, pathogen recognition receptors (PRRs). The effective sensing of PAMPs results in the robust activation of signaling pathways and culminates in induction of IFN production, thus facilitating the clearance of the intruder. The TLRs and RLRs along with interferon stimulated host antiviral proteins such as PKR and RNase L, form a network of an effective defense system of the host. They work through recognition of different viral PAMPs and initiate a signaling cascade that leads to IFN expression (290). The viral PAMPs, mostly viral nucleic acids or proteins can either associate with TLRs present on endosomes or with RLRs, RIG-I and MDA5 in the cytoplasm (348). RIG-I primarily responds to uncapped ssRNAs containing terminal 5'

triphosphorylates but no 5'-OH or 5'-methyl guanosine cap and at least 23 nucleotides long with sequence rich in uridine and adenosine (70, 132, 260, 334), and short dsRNA molecules (156). The cellular RNA generally have different structures, lengths and most importantly their 5'-ends are modified due to capping and methylation processes, so they are not detected by RIG-I, thus governing specificity of RIG-I to only viral RNA (334).

MDA5 has been reported to detect the long dsRNA molecules (more than 2kb) (156). However, how MDA5 distinguishes between cellular and viral RNA is not clear. Once RIG-I and MDA5 are activated through RNA, they mediate interaction with a common adaptor IPS-1, through a N-terminal CARD domain. IPS-1 recruitment initiates a signaling cascade that involves the activation of the transcription factors, IRF-3 and NF- κ B which results in inducing the production of interferon and pro-inflammatory cytokines (159). Intriguingly, activity of an endonuclease, RNase L can enhance the magnitude of IFN induction which degrades both cellular and viral RNAs (205). These cleaved RNA products generated by RNase L have a 5'-OH group and a 3'-monophosphate and are speculated to consist of ssRNA and dsRNA molecules that can engage both the MDA5 and RIG-I pathway for IFN induction. The mechanisms of recognition of viral PAMPs by these sensors and nature of the ligands detected by them are not well understood. The main focus of our study was to understand the regulation of the immune responses by viruses, especially understanding the roles of viral RNA ligands and cellular sensors in the IFN stimulation.

In Chapter 4, our studies investigated the role of domain II of the L gene (L-II) in

stimulating immune responses. Surprisingly, we found that L-II polypeptide was sufficient to activate NF- κ B in an AKT1 independent pathway and also stimulated interferon production. Due to the well-established role of viral RNA as the trigger for NF- κ B and IFN- β activation, we hypothesized that RNA encoded by the domain II of the L gene is sufficient to activate interferon production. We generated a L-II mutant plasmid, whose translation was abolished by introduction of a stop codon in the place of a start codon in the L-II sequence. Interestingly, we found that the L-II RNA is capable of activating NF- κ B in an AKT independent manner and also activates IFN- β expression. We further characterized the role of L-II RNA in stimulating IFN induction by purifying the RNA and mRNA from L-II transfected cells and measured the induction of IFN- β with these RNAs using ELISA assays. We have found that the L-II RNA induced robust IFN- β expression. Our results suggest that the putative mRNA of domain II of L gene is critical for IFN- β activation. Our data indicates that the L-II RNA acts as a potent inducer of IFN- β , however specific sequence and structure element within this L-II region (432 nucleotides) that could stimulate this response, still remain to be identified.

We were also interested in understanding the mechanism of IFN- β activation by the L-II RNA. The expression of interferon requires the recognition of viral RNA by host sensors. We focused our studies on understanding the roles of RNA helicases, RIG-I and MDA5 in the activation of IFN by the L-II RNA. Both these helicases have different substrate specificity but signal through a common adaptor protein, IPS-1. A dominant negative mutant of IPS-1 inhibited the activation of NF- κ B by L-II mutant transfected

cells, indicating that RIG-I/MDA5 may be critical for this activation. However, activation of NF- κ B by L-II was not affected in a cell line defective in RIG-I or by expressing a dominant negative mutant of RIG-I, suggesting that RIG-I is not important for this activation. We further investigated the role of MDA5. Knocking down the expression of MDA5 using siRNA reduced the activation of NF- κ B and IFN- β , indicating that MDA5 is critical for activation of the interferon response by the L-II RNA. We have examined how this L-II RNA is recognized by MDA5. Previously, it has been reported that RNase L plays an important role in IFN expression through degradation of mRNA (205). Using RNase L knockout cells and siRNA against RNase L, we have found that RNase L is critical for activation of NF- κ B and IFN- β by L-II RNA.

Knowledge about the natural ligands for these helicases is incomplete. These RNA sensors are reported to be involved in the recognition of different viruses. Studies using MDA5 and RIG-I knockout mice have revealed that MDA5 is more sensitive to detection of picornavirus infections such as EMCV and poliovirus which produce long dsRNA species and stimulates IFN- β activation (108, 157). On the other hand, RIG-I is effective in activating IFN production in response to paramyxoviruses, influenza viruses where 5'ppp was identified as an important feature for viral RNA recognition (132, 157, 260). Interestingly, there are examples where both RIG-I and MDA5 being found to play important roles in virus detection including viruses from the paramyxovirus family such as Sendai virus and Newcastle disease virus and flaviviruses such as West Nile virus (10, 101, 109, 222, 379). This suggests that viruses are capable of generating multiple PAMPs

that can engage different sensors for their recognition. But what makes one sensor to dominate over the other in the cellular system is not clear.

The intriguing question is why viruses consist of these PAMPs that can result in their own demise. One of the possibilities could be that PAMPs are generated due to errors during virus replication as viruses have a tendency to mutate over time or they could be the result of an internal defect during virus life cycle. One of the interesting features of the paramyxoviruses is the generation of defective interfering (DI) virus particles. These DI viruses are replication deficient and are classified into three different forms: the DI genomes with internal deletions which lack the segments of viral genome, 5'-terminal panhandle structures which have completely complementary 5'-3' ends and hairpin or snapback DI genomes which consist of dsRNA hairpin structures (179, 308). The virus stocks of paramyxoviruses including SeV, measles, PIV5 and VSV are found to be rich in these DI particles and induce high amount of IFN induction (162, 166, 311, 325). In the studies with SeV, it has been shown that IFN production is highly correlated to presence of these copyback genomes which can induce both RIG-I and MDA5 mediated antiviral responses (326, 382) whereas using the plaque purified viruses lacking these DI particles are found to be a poor inducer of IFN (150). The presence of these DI particles adds more complexity to our understanding about the IFN inducers. It remains unclear what are the potent inducers of IFNs during virus infections, the PAMPs generated as a result of normal virus replication or due to aberrant virus replication.

The negative stranded RNA viruses try to protect their genome and antigenome from being exposed to the host sensors by tightly encapsidating it with the nucleocapsid protein (110). The process of transcription and replication are also highly controlled by these viruses, thereby limiting the generation of any PAMPs, that may otherwise activate IFN (196, 209). In this scenario, the concept of DI particles is quite enticing to serve as a possible PAMP. But in lieu with these studies, our work has identified the role viral mRNA as a probable IFN inducer.

Other than regulating the replication and transcription processes, viruses have strategically designed their defense system, which include encoding genes that can interfere with the host immune responses and also that can block host transcription and/or protein synthesis. In paramyxoviruses, the proteins products of the P/V/C gene are recognized as virally encoded IFN antagonist proteins (176). In Sendai virus, the C protein functions to block IFN signaling likely through its interaction with the signal transducer and activator of transcription 1 (STAT1) in the infected cells by interfering with STAT1 phosphorylation or stability (81, 111). The V proteins of mumps, HPIV2 virus and PIV5 act as an IFN antagonist (252, 381). The V protein of PIV5 targets STAT1 for degradation and blocks IFN- α/β and IFN- γ signaling in the infected cells (81). Interestingly, the role of the V protein has also been identified in blocking dsRNA induced IFN- β promoter activation through its interaction with MDA5 (10). This interaction also exists in other members of paramyxoviruses such as SeV, measles, mumps, Nipah, Hendra and HPIV2 (10). The V proteins of paramyxoviruses mediate this interaction through their conserved cysteine rich C-terminal and bind to the helicase

domain of MDA5 (10, 56). This interaction results in inhibition of MDA5 activation by blocking the binding of dsRNA to helicase domain and its subsequent oligomerization (57). The role of the V protein has also been identified as a competitive inhibitor of TBK-1 and thus can block IRF-3 phosphorylation (202). The PIV5-infected cells limit the production of IFN expression which we speculate could be likely through the action of the V protein thus making the activation of IFN by the L gene in the PIV5-infected cells immaterial. The V protein also plays a role in regulating viral RNA synthesis of PIV5 (196), so it is possible that making deletions in the V protein can result in imbalance of this V mediated control, thus making the virus more prone to producing PAMPs or exposing the regions that can serve as active PAMPs, hence resulting in IFN activation. We speculate this could likely be the reason that rPIV5-V Δ C virus, the recombinant virus having the deletion of the cysteine-rich domain at the C-terminus of the V protein, induces high IFN activation. However, there are reports suggesting that PAMPs are not generated or exposed during normal PIV5 replication and it is likely the presence of DI particles that induces IFN activation (55, 166). This was supported by the study utilizing a cell line capable of inducing IFN- β promoter at single cell level (55) and found that rPIV5-V Δ C virus does not induces IFN- β promoter activation in the majority of the infected cells (166). However, we cannot rule out the possibility of the existence of DI particles and their role in the IFN induction during the virus infection but as in our study we have utilized purified mRNA from both L-II transfected and the virus-infected cells, the likelihood of interference by the DI particles in our system is very small. Overall, our work does add a new paradigm molecule “a viral mRNA” that acts as a potent inducer of the IFN activation through MDA5 and RNase L.

We hypothesize that the activation of interferon responses by the L-II RNA is the coordinated work of RNase L and MDA5 since siRNA knock down of RNase L or MDA5 expression results in reduction of IFN production by the L-II RNA. We speculate that this process of RNA recognition could be initiated by RNase L which degrades the L-II RNA. Subsequently, the cleaved RNA product is recognized by MDA5 and stimulates the downstream signaling for IFN production. However, it is also possible that direct recognition of L-II RNA by MDA5 is responsible for activating interferon signaling where RNase L is involved in amplifying the interferon response. We hypothesize that both RNase L and MDA5 significantly contribute to the activation of IFN by the L-II RNA. The working model illustrating the roles of the L gene in stimulating innate immune responses is represented in **Figure 5**.

In summary, we have identified novel roles of the L gene in regulating the host innate immune responses. We have reported that the L protein is capable of inducing activation of NF- κ B through a protein kinase, AKT1. We have also identified viral RNA (putative PAMP) encoded by the L gene that can be recognized by RNase L-MDA5. We have mapped the region of L-II that is critical for this response to a 432 nt long sequence. Further studies are needed to characterize the nature of this RNA region. This work helps in our understanding of the molecular pathways involved in induction of innate immune responses by virus infections and also suggests targets for discovery of small molecules activators of RNA sensors which can lead to development of novel antivirals.

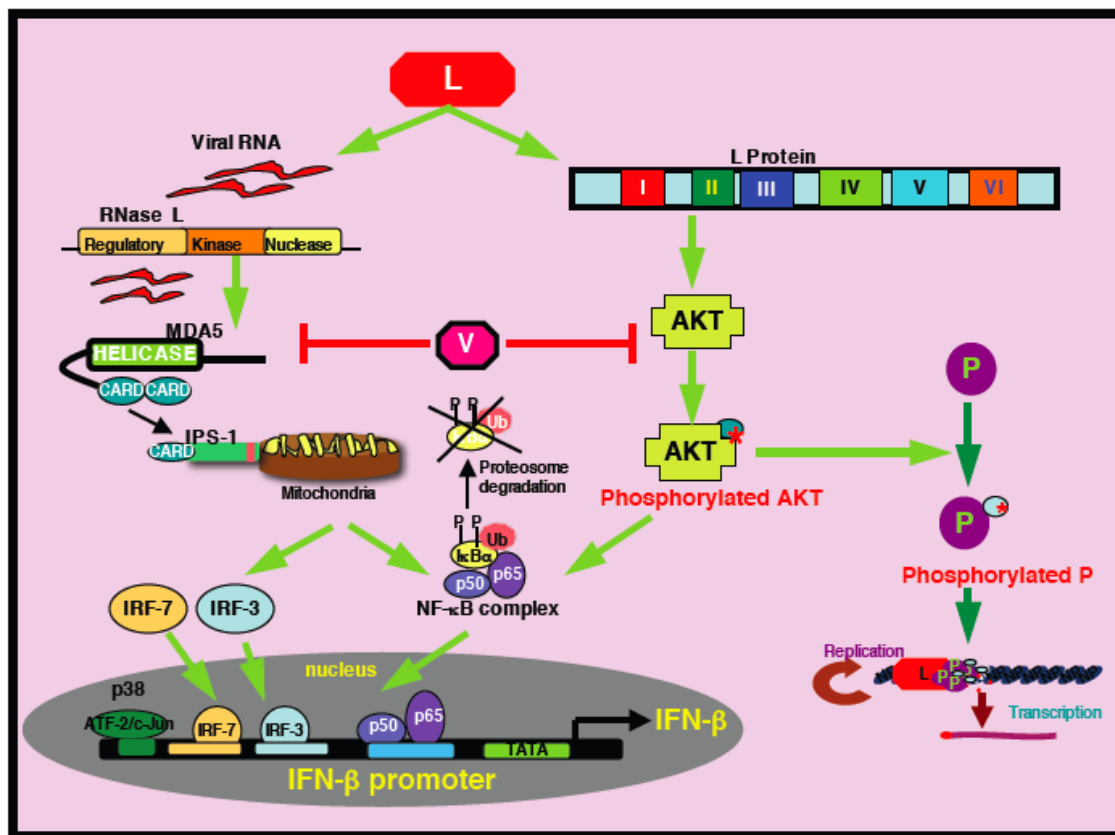


Figure 5. Proposed model for the roles of the L gene in activation of host signaling pathways. The novel roles of the L gene of PIV5 have been identified in the innate immune signaling besides its known role in virus replication and transcription. The L gene can lead to activation of NF-κB and IFN-β through two independent mechanisms, where both RNA and protein play important roles. We hypothesize that the L mRNA can be recognized and cleaved by 2-5A activated RNase L and is further detected by MDA5. MDA5 through its CARD domain mediates interaction with CARD containing adaptor protein, IPS-1 present on the outer mitochondrial membrane. IPS-1 gets activated and stimulates the downstream signaling leading to activation of transcription factors, NF-κB and IRF-3. The activated transcription factors translocate to the nucleus and stimulate the expression of IFN-β gene. Additionally, the expression of the L protein can also activate NF-κB but through different mechanism which is dependent on a host protein kinase, AKT. The L protein can mediate this activation through its interaction and promoting the phosphorylation of AKT. The domain I and II of the L protein together are sufficient for this process, where domain I mediates interaction with AKT and domain II is important for promoting activation of AKT. Activated AKT plays critical role in the viral RNA synthesis likely through phosphorylation of the P protein and also stimulates NF-κB and interferon activation. However, the V protein acts as an antagonist for the L-mediated interferon pathways, which we speculate is likely through its interaction with MDA5 and AKT.

Bibliography

1. Abraham, G., and A. K. Banerjee. 1976. Sequential transcription of the genes of vesicular stomatitis virus. *Proc Natl Acad Sci U S A* 73:1504-8.
2. Akagi, T., H. Ono, and K. Shimotohno. 1995. Characterization of T cells immortalized by Tax1 of human T-cell leukemia virus type 1. *Blood* 86:4243-9.
3. Akira, S., and S. Sato. 2003. Toll-like receptors and their signaling mechanisms. *Scand J Infect Dis* 35:555-62.
4. Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nat Rev Immunol* 4:499-511.
5. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124:783-801.
6. Alcamí, J., T. Lain de Lera, L. Folgueira, M. A. Pedraza, J. M. Jacque, F. Bachelierie, A. R. Noriega, R. T. Hay, D. Harrich, R. B. Gaynor, and et al. 1995. Absolute dependence on kappa B responsive elements for initiation and Tat-mediated amplification of HIV transcription in blood CD4 T lymphocytes. *EMBO J* 14:1552-60.
7. Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413:732-8.
8. Algarte, M., H. Nguyen, C. Heylbroeck, R. Lin, and J. Hiscott. 1999. IkappaB-mediated inhibition of virus-induced beta interferon transcription. *J Virol* 73:2694-702.
9. Andrejeva, J., K. S. Childs, D. F. Young, T. S. Carlos, N. Stock, S. Goodbourn, and R. E. Randall. 2004. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN- β promoter. *Proc Natl Acad Sci U S A* 101:17264-17269.
10. Andrejeva, J., K. S. Childs, D. F. Young, T. S. Carlos, N. Stock, S. Goodbourn, and R. E. Randall. 2004. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN- β promoter. *Proc Natl Acad Sci U S A* 101:17264-9.
11. Ank, N., H. West, C. Bartholdy, K. Eriksson, A. R. Thomsen, and S. R. Paludan. 2006. Lambda interferon (IFN- λ), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. *J Virol* 80:4501-9.
12. Ank, N., H. West, and S. R. Paludan. 2006. IFN- λ : novel antiviral cytokines. *J Interferon Cytokine Res* 26:373-9.
13. Armstrong, R. T., A. S. Kushnir, and J. M. White. 2000. The transmembrane domain of influenza hemagglutinin exhibits a stringent length requirement to support the hemifusion to fusion transition. *J Cell Biol* 151:425-37.
14. Asin, S., J. A. Taylor, S. Trushin, G. Bren, and C. V. Paya. 1999. IkappaB mediates NF-kappaB activation in human immunodeficiency virus-infected cells. *J Virol* 73:3893-903.
15. Bach, E. A., M. Aguet, and R. D. Schreiber. 1997. The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol* 15:563-91.

16. Bach, E. A., J. W. Tanner, S. Marsters, A. Ashkenazi, M. Aguet, A. S. Shaw, and R. D. Schreiber. 1996. Ligand-induced assembly and activation of the gamma interferon receptor in intact cells. *Mol Cell Biol* 16:3214-21.
17. Ball, L. A., and C. N. White. 1976. Order of transcription of genes of vesicular stomatitis virus. *Proc Natl Acad Sci U S A* 73:442-6.
18. Bamming, D., and C. M. Horvath. 2009. Regulation of signal transduction by enzymatically inactive antiviral RNA helicase proteins MDA5, RIG-I, and LGP2. *J Biol Chem* 284:9700-12.
19. Bankamp, B., S. M. Horikami, P. D. Thompson, M. Huber, M. Billeter, and S. A. Moyer. 1996. Domains of the measles virus N protein required for binding to P protein and self-assembly. *Virology* 216:272-7.
20. Barik, S., and A. K. Banerjee. 1992. Phosphorylation by cellular casein kinase II is essential for transcriptional activity of vesicular stomatitis virus phosphoprotein P. *Proc Natl Acad Sci U S A* 89:6570-4.
21. Barik, S., and A. K. Banerjee. 1992. Sequential phosphorylation of the phosphoprotein of vesicular stomatitis virus by cellular and viral protein kinases is essential for transcription activation. *J Virol* 66:1109-18.
22. Barik, S., T. McLean, and L. C. Dupuy. 1995. Phosphorylation of Ser232 directly regulates the transcriptional activity of the P protein of human respiratory syncytial virus: phosphorylation of Ser237 may play an accessory role. *Virology* 213:405-12.
23. Barkett, M., and T. D. Gilmore. 1999. Control of apoptosis by Rel/NF-kappaB transcription factors. *Oncogene* 18:6910-24.
24. Barral, P. M., D. Sarkar, P. B. Fisher, and V. R. Racaniello. 2009. RIG-I is cleaved during picornavirus infection. *Virology* 391:171-6.
25. Barral, P. M., D. Sarkar, Z. Z. Su, G. N. Barber, R. DeSalle, V. R. Racaniello, and P. B. Fisher. 2009. Functions of the cytoplasmic RNA sensors RIG-I and MDA-5: key regulators of innate immunity. *Pharmacol Ther* 124:219-34.
26. Basler, C. F., and A. Garcia-Sastre. 2007. Sensing RNA virus infections. *Nat Chem Biol* 3:20-1.
27. Bauer, S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci U S A* 98:9237-42.
28. Baum, A., and A. Garcia-Sastre. Induction of type I interferon by RNA viruses: cellular receptors and their substrates. *Amino Acids* 38:1283-99.
29. Bayard, B., P. Bette-Bobillo, and S. Aliau. 1994. Affinity purification and characterization of (2'-5')oligo(adenylate)-dependent RNase from mouse spleen. *Eur J Biochem* 223:403-10.
30. Beg, A. A., S. M. Ruben, R. I. Scheinman, S. Haskill, C. A. Rosen, and A. S. Baldwin, Jr. 1992. I kappa B interacts with the nuclear localization sequences of the subunits of NF-kappa B: a mechanism for cytoplasmic retention. *Genes Dev* 6:1899-913.
31. Bellacosa, A., C. C. Kumar, A. Di Cristofano, and J. R. Testa. 2005. Activation of AKT kinases in cancer: implications for therapeutic targeting. *Adv Cancer Res* 94:29-86.
32. Bisbal, C., B. Bayard, M. Silhol, L. Leserman, and B. Lebleu. 1985. 2'5' Oligoadenylate analogues:synthesis, biological activities and intracellular delivery. *Prog Clin Biol Res* 202:89-95.

33. Bisbal, C., C. Martinand, M. Silhol, B. Lebleu, and T. Salehzada. 1995. Cloning and characterization of a RNase L inhibitor. A new component of the interferon-regulated 2-5A pathway. *J Biol Chem* 270:13308-17.
34. Bisbal, C., T. Salehzada, B. Lebleu, and B. Bayard. 1989. Characterization of two murine (2'-5')(A)n-dependent endonucleases of different molecular mass. *Eur J Biochem* 179:595-602.
35. Bisbal, C., M. Silhol, H. Laubenthal, T. Kaluza, G. Carnac, L. Milligan, F. Le Roy, and T. Salehzada. 2000. The 2'-5' oligoadenylate/RNase L/RNase L inhibitor pathway regulates both MyoD mRNA stability and muscle cell differentiation. *Mol Cell Biol* 20:4959-69.
36. Bisbal, C., and R. H. Silverman. 2007. Diverse functions of RNase L and implications in pathology. *Biochimie* 89:789-98.
37. Bishop, K. A., A. C. Hickey, D. Khetawat, J. R. Patch, K. N. Bossart, Z. Zhu, L. F. Wang, D. S. Dimitrov, and C. C. Broder. 2008. Residues in the stalk domain of the hendra virus g glycoprotein modulate conformational changes associated with receptor binding. *J Virol* 82:11398-409.
38. Brazil, D. P., and B. A. Hemmings. 2001. Ten years of protein kinase B signalling: a hard Akt to follow. *Trends Biochem Sci* 26:657-64.
39. Buchholz, C. J., C. Retzler, H. E. Homann, and W. J. Neubert. 1994. The carboxy-terminal domain of Sendai virus nucleocapsid protein is involved in complex formation between phosphoprotein and nucleocapsid-like particles. *Virology* 204:770-6.
40. Buchholz, C. J., D. Spehner, R. Drillien, W. J. Neubert, and H. E. Homann. 1993. The conserved N-terminal region of Sendai virus nucleocapsid protein NP is required for nucleocapsid assembly. *J Virol* 67:5803-12.
41. Buchholz, U. J., S. Finke, and K. K. Conzelmann. 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *J Virol* 73:251-9.
42. Buetti, E., and P. W. Choppin. 1977. The transcriptase complex of the paramyxovirus SV5. *Virology* 82:493-508.
43. Calain, P., and L. Roux. 1993. The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *J Virol* 67:4822-30.
44. Carpten, J., N. Nupponen, S. Isaacs, R. Sood, C. Robbins, J. Xu, M. Faruque, T. Moses, C. Ewing, E. Gillanders, P. Hu, P. Bujnovszky, I. Makalowska, A. Baffoe-Bonnie, D. Faith, J. Smith, D. Stephan, K. Wiley, M. Brownstein, D. Gildea, B. Kelly, R. Jenkins, G. Hostetter, M. Matikainen, J. Schleutker, K. Klinger, T. Connors, Y. Xiang, Z. Wang, A. De Marzo, N. Papadopoulos, O. P. Kallioniemi, R. Burk, D. Meyers, H. Gronberg, P. Meltzer, R. Silverman, J. Bailey-Wilson, P. Walsh, W. Isaacs, and J. Trent. 2002. Germline mutations in the ribonuclease L gene in families showing linkage with HPC1. *Nat Genet* 30:181-4.
45. Castelli, J. C., B. A. Hassel, A. Maran, J. Paranjape, J. A. Hewitt, X. L. Li, Y. T. Hsu, R. H. Silverman, and R. J. Youle. 1998. The role of 2'-5' oligoadenylate-activated ribonuclease L in apoptosis. *Cell Death Differ* 5:313-20.
46. Castelli, J. C., B. A. Hassel, K. A. Wood, X. L. Li, K. Amemiya, M. C. Dalakas, P. F. Torrence, and R. J. Youle. 1997. A study of the interferon antiviral mechanism: apoptosis activation by the 2-5A system. *J Exp Med* 186:967-72.
47. Cayley, P. J., J. A. Davies, K. G. McCullagh, and I. M. Kerr. 1984. Activation of the ppp(A2'p)nA system in interferon-treated, herpes simplex virus-infected cells and

- evidence for novel inhibitors of the ppp(A2'p)nA-dependent RNase. *Eur J Biochem* 143:165-74.
48. Cevik, B., S. Smallwood, and S. A. Moyer. 2003. The L-L oligomerization domain resides at the very N-terminus of the sendai virus L RNA polymerase protein. *Virology* 313:525-36.
 49. Chan, T. O., S. E. Rittenhouse, and P. N. Tsichlis. 1999. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem* 68:965-1014.
 50. Chan, Y. P., C. L. Koh, S. K. Lam, and L. F. Wang. 2004. Mapping of domains responsible for nucleocapsid protein-phosphoprotein interaction of Henipaviruses. *J Gen Virol* 85:1675-84.
 51. Chandrasekaran, K., Z. Mehrabian, X. L. Li, and B. Hassel. 2004. RNase-L regulates the stability of mitochondrial DNA-encoded mRNAs in mouse embryo fibroblasts. *Biochem Biophys Res Commun* 325:18-23.
 52. Chang, F., J. T. Lee, P. M. Navolanic, L. S. Steelman, J. G. Shelton, W. L. Blalock, R. A. Franklin, and J. A. McCubrey. 2003. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. *Leukemia* 17:590-603.
 53. Chatziandreou, N., N. Stock, D. Young, J. Andrejeva, K. Hagmaier, D. J. McGeoch, and R. E. Randall. 2004. Relationships and host range of human, canine, simian and porcine isolates of simian virus 5 (parainfluenza virus 5). *J Gen Virol* 85:3007-16.
 54. Chen, L., J. J. Gorman, J. McKimm-Breschkin, L. J. Lawrence, P. A. Tulloch, B. J. Smith, P. M. Colman, and M. C. Lawrence. 2001. The structure of the fusion glycoprotein of Newcastle disease virus suggests a novel paradigm for the molecular mechanism of membrane fusion. *Structure* 9:255-66.
 55. Chen, S., J. A. Short, D. F. Young, M. J. Killip, M. Schneider, S. Goodbourn, and R. E. Randall. Heterocellular induction of interferon by negative-sense RNA viruses. *Virology* 407:247-55.
 56. Childs, K., N. Stock, C. Ross, J. Andrejeva, L. Hilton, M. Skinner, R. Randall, and S. Goodbourn. 2007. mda-5, but not RIG-I, is a common target for paramyxovirus V proteins. *Virology* 359:190-200.
 57. Childs, K. S., J. Andrejeva, R. E. Randall, and S. Goodbourn. 2009. Mechanism of mda-5 Inhibition by paramyxovirus V proteins. *J Virol* 83:1465-73.
 58. Choppin P.W., C. R. W. 1975. Reproduction of paramyxoviruses, p. 95-178. *In* W. R. Fraenkel-Conrat H (ed.), *Comprehensive Virology*, vol. 4. Plenum Press, New York.
 59. Clemens, M. J., and C. M. Vaquero. 1978. Inhibition of protein synthesis by double-stranded RNA in reticulocyte lysates: evidence for activation of an endoribonuclease. *Biochem Biophys Res Commun* 83:59-68.
 60. Clemens, M. J., and B. R. Williams. 1978. Inhibition of cell-free protein synthesis by pppA2'p5'A2'p5'A: a novel oligonucleotide synthesized by interferon-treated L cell extracts. *Cell* 13:565-72.
 61. Cohn, M. L., E. D. Robinson, D. Thomas, M. Faerber, S. Carey, R. Sawyer, K. K. Goswami, A. H. Johnson, and J. R. Richert. 1996. T cell responses to the paramyxovirus simian virus 5: studies in multiple sclerosis and normal populations. *Pathobiology* 64:131-135.
 62. Cohn, M. L., E. D. Robinson, D. Thomas, M. Faerber, S. Carey, R. Sawyer, K. K. Goswami, A. H. Johnson, and J. R. Richert. 1996. T cell responses to the

- paramyxovirus simian virus 5: studies in multiple sclerosis and normal populations. *Pathobiology* 64:131-5.
63. Colby, C., Jr. 1971. The induction of interferon by natural and synthetic polynucleotides. *Prog Nucleic Acid Res Mol Biol* 11:1-32.
 64. Colby, C., C. Jurale, and J. R. Kates. 1971. Mechanism of synthesis of vaccinia virus double-stranded ribonucleic acid in vivo and in vitro. *J Virol* 7:71-6.
 65. Colby, C., and M. J. Morgan. 1971. Interferon induction and action. *Annu Rev Microbiol* 25:333-60.
 66. Colby, C., B. D. Stollar, and M. I. Simon. 1971. Interferon induction: DNA-RNA hybrid or double stranded RNA? *Nat New Biol* 229:172-4.
 67. Colman, P. M., and M. C. Lawrence. 2003. The structural biology of type I viral membrane fusion. *Nat Rev Mol Cell Biol* 4:309-19.
 68. Coronel, E. C., T. Takimoto, K. G. Murti, N. Varich, and A. Portner. 2001. Nucleocapsid incorporation into parainfluenza virus is regulated by specific interaction with matrix protein. *J Virol* 75:1117-23.
 69. Counihan, M. E., D. K. Shay, R. C. Holman, S. A. Lowther, and L. J. Anderson. 2001. Human parainfluenza virus-associated hospitalizations among children less than five years of age in the United States. *Pediatr Infect Dis J* 20:646-53.
 70. Cui, S., K. Eisenacher, A. Kirchhofer, K. Brzozka, A. Lammens, K. Lammens, T. Fujita, K. K. Conzelmann, A. Krug, and K. P. Hopfner. 2008. The C-terminal regulatory domain is the RNA 5'-triphosphate sensor of RIG-I. *Mol Cell* 29:169-79.
 71. Curran, J., H. Homann, C. Buchholz, S. Rochat, W. Neubert, and D. Kolakofsky. 1993. The hypervariable C-terminal tail of the Sendai paramyxovirus nucleocapsid protein is required for template function but not for RNA encapsidation. *J Virol* 67:4358-64.
 72. Das, T., A. Schuster, S. Schneider-Schaulies, and A. K. Banerjee. 1995. Involvement of cellular casein kinase II in the phosphorylation of measles virus P protein: identification of phosphorylation sites. *Virology* 211:218-26.
 73. Datta, S. R., A. Brunet, and M. E. Greenberg. 1999. Cellular survival: a play in three Acts. *Genes Dev* 13:2905-27.
 74. Dayan, G. H., M. P. Quinlisk, A. A. Parker, A. E. Barskey, M. L. Harris, J. M. Schwartz, K. Hunt, C. G. Finley, D. P. Leschinsky, A. L. O'Keefe, J. Clayton, L. K. Kightlinger, E. G. Dietle, J. Berg, C. L. Kenyon, S. T. Goldstein, S. K. Stokley, S. B. Redd, P. A. Rota, J. Rota, D. Bi, S. W. Roush, C. B. Bridges, T. A. Santibanez, U. Parashar, W. J. Bellini, and J. F. Seward. 2008. Recent resurgence of mumps in the United States. *N Engl J Med* 358:1580-9.
 75. Dayan, G. H., and S. Rubin. 2008. Mumps outbreaks in vaccinated populations: are available mumps vaccines effective enough to prevent outbreaks? *Clin Infect Dis* 47:1458-67.
 76. De, B. P., S. Gupta, and A. K. Banerjee. 1995. Cellular protein kinase C isoform zeta regulates human parainfluenza virus type 3 replication. *Proc Natl Acad Sci U S A* 92:5204-8.
 77. DeLuca, C., H. Kwon, R. Lin, M. Wainberg, and J. Hiscott. 1999. NF-kappaB activation and HIV-1 induced apoptosis. *Cytokine Growth Factor Rev* 10:235-53.
 78. DeLuca, C., L. Petropoulos, D. Zmeureanu, and J. Hiscott. 1999. Nuclear I kappa B beta maintains persistent NF-kappaB activation in HIV-1-infected myeloid cells. *J Biol Chem* 274:13010-6.

79. Desai, S. Y., R. C. Patel, G. C. Sen, P. Malhotra, G. D. Ghadge, and B. Thimmapaya. 1995. Activation of interferon-inducible 2'-5' oligoadenylate synthetase by adenoviral VAI RNA. *J Biol Chem* 270:3454-61.
80. Dhib-Jalbut, S., J. Xia, H. Rangaviggula, Y. Y. Fang, and T. Lee. 1999. Failure of measles virus to activate nuclear factor-kappa B in neuronal cells: implications on the immune response to viral infections in the central nervous system. *J Immunol* 162:4024-9.
81. Didcock, L., D. F. Young, S. Goodbourn, and R. E. Randall. 1999. The V protein of simian virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. *J Virol* 73:9928-33.
82. Diebold, S. S. 2008. Recognition of viral single-stranded RNA by Toll-like receptors. *Adv Drug Deliv Rev* 60:813-23.
83. Dong, B., M. Niwa, P. Walter, and R. H. Silverman. 2001. Basis for regulated RNA cleavage by functional analysis of RNase L and Ire1p. *RNA* 7:361-73.
84. Dong, B., and R. H. Silverman. 1995. 2-5A-dependent RNase molecules dimerize during activation by 2-5A. *J Biol Chem* 270:4133-7.
85. Dong, B., and R. H. Silverman. 1997. A bipartite model of 2-5A-dependent RNase L. *J Biol Chem* 272:22236-42.
86. Dong, B., L. Xu, A. Zhou, B. A. Hassel, X. Lee, P. F. Torrence, and R. H. Silverman. 1994. Intrinsic molecular activities of the interferon-induced 2-5A-dependent RNase. *J Biol Chem* 269:14153-8.
87. Du, K., and P. N. Tsichlis. 2005. Regulation of the Akt kinase by interacting proteins. *Oncogene* 24:7401-9.
88. Durbin, A. P., J. W. Siew, B. R. Murphy, and P. L. Collins. 1997. Minimum protein requirements for transcription and RNA replication of a minigenome of human parainfluenza virus type 3 and evaluation of the rule of six. *Virology* 234:74-83.
89. Earp, L. J., S. E. Delos, H. E. Park, and J. M. White. 2005. The many mechanisms of viral membrane fusion proteins. *Curr Top Microbiol Immunol* 285:25-66.
90. Eaton, B. T., C. C. Broder, D. Middleton, and L. F. Wang. 2006. Hendra and Nipah viruses: different and dangerous. *Nat Rev Microbiol* 4:23-35.
91. Einberger, H., R. Mertz, P. H. Hofschneider, and W. J. Neubert. 1990. Purification, renaturation and reconstituted protein kinase activity of the Sendai virus large (L) protein: L protein phosphorylates the NP and P proteins in vitro. *J. Virol.* 64:4274-4280.
92. Einberger, H., R. Mertz, P. H. Hofschneider, and W. J. Neubert. 1990. Purification, renaturation, and reconstituted protein kinase activity of the Sendai virus large (L) protein: L protein phosphorylates the NP and P proteins in vitro. *J Virol* 64:4274-80.
93. Emerson, S. U. 1982. Reconstitution studies detect a single polymerase entry site on the vesicular stomatitis virus genome. *Cell* 31:635-42.
94. Emerson, S. U., and Y. Yu. 1975. Both NS and L proteins are required for in vitro RNA synthesis by vesicular stomatitis virus. *J Virol* 15:1348-56.
95. Fiedler, M. A., and K. Wernke-Dollries. 1999. Incomplete regulation of NF-kappaB by IkappaBalpha during respiratory syncytial virus infection in A549 cells. *J Virol* 73:4502-7.
96. Field, A. K., A. A. Tytell, G. P. Lampson, and M. R. Hilleman. 1967. Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. *Proc Natl Acad Sci U S A* 58:1004-10.

97. Flodstrom-Tullberg, M., M. Hultcrantz, A. Stotland, A. Maday, D. Tsai, C. Fine, B. Williams, R. Silverman, and N. Sarvetnick. 2005. RNase L and double-stranded RNA-dependent protein kinase exert complementary roles in islet cell defense during coxsackievirus infection. *J Immunol* 174:1171-7.
98. Flory, E., C. K. Weber, P. Chen, A. Hoffmeyer, C. Jassoy, and U. R. Rapp. 1998. Plasma membrane-targeted Raf kinase activates NF-kappaB and human immunodeficiency virus type 1 replication in T lymphocytes. *J Virol* 72:2788-94.
99. Floyd-Smith, G., E. Slattery, and P. Lengyel. 1981. Interferon action: RNA cleavage pattern of a (2'-5')oligoadenylate--dependent endonuclease. *Science* 212:1030-2.
100. Floyd-Smith, G., O. Yoshie, and P. Lengyel. 1982. Interferon action. Covalent linkage of (2'-5')pppApApA(32P)pCp to (2'-5')(A)n-dependent ribonucleases in cell extracts by ultraviolet irradiation. *J Biol Chem* 257:8584-7.
101. Fredericksen, B. L., B. C. Keller, J. Fornek, M. G. Katze, and M. Gale, Jr. 2008. Establishment and maintenance of the innate antiviral response to West Nile Virus involves both RIG-I and MDA5 signaling through IPS-1. *J Virol* 82:609-16.
102. Fresno Vara, J. A., E. Casado, J. de Castro, P. Cejas, C. Belda-Iniesta, and M. Gonzalez-Baron. 2004. PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev* 30:193-204.
103. Fuentes, S., K. C. Tran, P. Luthra, M. N. Teng, and B. He. 2007. Function of the respiratory syncytial virus small hydrophobic protein. *J Virol* 81:8361-6.
104. Gao, Y., and J. Lenard. 1995. Multimerization and transcriptional activation of the phosphoprotein (P) of vesicular stomatitis virus by casein kinase-II. *EMBO J* 14:1240-7.
105. Gao, Y., and J. Lenard. 1995. Multimerization and transcriptional activation of the phosphoprotein (P) of vesicular stomatitis virus by casein kinase-II. *EMBO J* 14:101-108.
106. Ghosh, S., M. J. May, and E. B. Kopp. 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 16:225-60.
107. Gilmore, T. D. 2006. Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene* 25:6680-4.
108. Gitlin, L., W. Barchet, S. Gilfillan, M. Cella, B. Beutler, R. A. Flavell, M. S. Diamond, and M. Colonna. 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc Natl Acad Sci U S A* 103:8459-64.
109. Gitlin, L., L. Benoit, C. Song, M. Cella, S. Gilfillan, M. J. Holtzman, and M. Colonna. Melanoma differentiation-associated gene 5 (MDA5) is involved in the innate immune response to Paramyxoviridae infection in vivo. *PLoS Pathog* 6:e1000734.
110. Goodbourn, S., and R. E. Randall. 2009. The regulation of type I interferon production by paramyxoviruses. *J Interferon Cytokine Res* 29:539-47.
111. Gotoh, B., K. Takeuchi, T. Komatsu, and J. Yokoo. 2003. The STAT2 activation process is a crucial target of Sendai virus C protein for the blockade of alpha interferon signaling. *J Virol* 77:3360-70.
112. Grdzlishvili, V. Z., S. Smallwood, D. Tower, R. L. Hall, D. M. Hunt, and S. A. Moyer. 2005. A single amino acid change in the L-polymerase protein of vesicular stomatitis virus completely abolishes viral mRNA cap methylation. *J Virol* 79:7327-37.

113. Hall, C. B., G. A. Weinberg, M. K. Iwane, A. K. Blumkin, K. M. Edwards, M. A. Staat, P. Auinger, M. R. Griffin, K. A. Poehling, D. Erdman, C. G. Grijalva, Y. Zhu, and P. Szilagyi. 2009. The burden of respiratory syncytial virus infection in young children. *N Engl J Med* 360:588-98.
114. Hammond, D. C., B. E. Haley, and J. A. Lesnaw. 1992. Identification and characterization of serine/threonine protein kinase activity intrinsic to the L protein of vesicular stomatitis virus New Jersey. *J Gen Virol* 73 (Pt 1):67-75.
115. Han, J. Q., and D. J. Barton. 2002. Activation and evasion of the antiviral 2'-5' oligoadenylate synthetase/ribonuclease L pathway by hepatitis C virus mRNA. *RNA* 8:512-25.
116. Han, J. Q., G. Wroblewski, Z. Xu, R. H. Silverman, and D. J. Barton. 2004. Sensitivity of hepatitis C virus RNA to the antiviral enzyme ribonuclease L is determined by a subset of efficient cleavage sites. *J Interferon Cytokine Res* 24:664-76.
117. Hanada, M., J. Feng, and B. A. Hemmings. 2004. Structure, regulation and function of PKB/AKT--a major therapeutic target. *Biochim Biophys Acta* 1697:3-16.
118. Harrison, M. S., T. Sakaguchi, and A. P. Schmitt. Paramyxovirus assembly and budding: building particles that transmit infections. *Int J Biochem Cell Biol* 42:1416-29.
119. Hassel, B. A., A. Zhou, C. Sotomayor, A. Maran, and R. H. Silverman. 1993. A dominant negative mutant of 2-5A-dependent RNase suppresses antiproliferative and antiviral effects of interferon. *EMBO J* 12:3297-304.
120. Hayden, M. S., and S. Ghosh. 2004. Signaling to NF-kappaB. *Genes Dev* 18:2195-224.
121. Hayden, M. S., A. P. West, and S. Ghosh. 2006. NF-kappaB and the immune response. *Oncogene* 25:6758-80.
122. Hayden, M. S., A. P. West, and S. Ghosh. 2006. SnapShot: NF-kappaB signaling pathways. *Cell* 127:1286-7.
123. He, B., G. P. Leser, R. G. Paterson, and R. A. Lamb. 1998. The paramyxovirus SV5 small hydrophobic (SH) protein is not essential for virus growth in tissue culture cells. *Virology* 250:30-40.
124. He, B., G. Y. Lin, J. E. Durbin, R. K. Durbin, and R. A. Lamb. 2001. The SH integral membrane protein of the paramyxovirus simian virus 5 is required to block apoptosis in MDBK cells. *J Virol* 75:4068-79.
125. He, B., R. G. Paterson, N. Stock, J. E. Durbin, R. K. Durbin, S. Goodbourn, R. E. Randall, and R. A. Lamb. 2002. Recovery of paramyxovirus simian virus 5 with a V protein lacking the conserved cysteine-rich domain: the multifunctional V protein blocks both interferon-beta induction and interferon signaling. *Virology* 303:15-32.
126. Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 303:1526-9.
127. Hiebert, S. W., C. D. Richardson, and R. A. Lamb. 1988. Cell surface expression and orientation in membranes of the 44-amino-acid SH protein of simian virus 5. *J Virol* 62:2347-57.
128. Higuchi, Y., Y. Miyahara, M. Kawano, M. Tsurudome, H. Matsumura, S. Kusagawa, H. Komada, M. Nishio, and Y. Ito. 1992. Sequence analysis of the large (L) protein of simian virus 5. *J Gen Virol* 73 (Pt 4):1005-10.
129. Hiscott, J. 2001. Introduction--cytokine receptors, signaling pathways and viruses. *Cytokine Growth Factor Rev* 12:129-31.

130. Hiscott, J., H. Kwon, and P. Genin. 2001. Hostile takeovers: viral appropriation of the NF-kappaB pathway. *J Clin Invest* 107:143-51.
131. Hiscott, J., T. L. Nguyen, M. Arguello, P. Nakhaei, and S. Paz. 2006. Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. *Oncogene* 25:6844-67.
132. Hornung, V., J. Ellegast, S. Kim, K. Brzozka, A. Jung, H. Kato, H. Poeck, S. Akira, K. K. Conzelmann, M. Schlee, S. Endres, and G. Hartmann. 2006. 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 314:994-7.
133. Horvath, C. M., and R. A. Lamb. 1992. Studies on the fusion peptide of a paramyxovirus fusion glycoprotein: roles of conserved residues in cell fusion. *J Virol* 66:2443-55.
134. Hovanessian, A. G., and J. N. Wood. 1980. Anticellular and antiviral effects of pppA(2'p5'A)n. *Virology* 101:81-90.
135. Hsiung, G. D., P. W. Chang, R. R. Cuadrado, and P. Isacson. 1965. Studies of parainfluenza viruses. III. Antibody responses of different animal species after immunization. *J. Immunol.* 94:67-73.
136. Hsuing, G. D. 1972. Parainfluenza-5 virus: infection of man and animal. *Prog. Med. Virol.* 14:241-247.
137. Huntley, C. C., B. P. De, N. R. Murray, A. P. Fields, and A. K. Banerjee. 1995. Human parainfluenza virus type 3 phosphoprotein: identification of serine 333 as the major site for PKC zeta phosphorylation. *Virology* 211:561-7.
138. Hviid, A., and M. Melbye. 2008. Measles-mumps-rubella vaccination and asthma-like disease in early childhood. *Am J Epidemiol* 168:1277-83.
139. Hviid, A., S. Rubin, and K. Muhlemann. 2008. Mumps. *Lancet* 371:932-44.
140. Isaacs, A., and J. Lindenmann. 1957. Virus interference. I. The interferon. *Proc R Soc Lond B Biol Sci* 147:258-67.
141. Isaacs, A., J. Lindenmann, and R. C. Valentine. 1957. Virus interference. II. Some properties of interferon. *Proc R Soc Lond B Biol Sci* 147:268-73.
142. Iverson, L. E., and J. K. Rose. 1982. Sequential synthesis of 5'-proximal vesicular stomatitis virus mRNA sequences. *J Virol* 44:356-65.
143. Iwasaki, M., M. Takeda, Y. Shirogane, Y. Nakatsu, T. Nakamura, and Y. Yanagi. 2009. The matrix protein of measles virus regulates viral RNA synthesis and assembly by interacting with the nucleocapsid protein. *J Virol* 83:10374-83.
144. Jack, P. J., D. B. Boyle, B. T. Eaton, and L. F. Wang. 2005. The complete genome sequence of J virus reveals a unique genome structure in the family Paramyxoviridae. *J Virol* 79:10690-700.
145. Jacques, J. P., and D. Kolakofsky. 1991. Pseudo-templated transcription in prokaryotic and eukaryotic organisms. *Genes Dev* 5:707-13.
146. Jeeninga, R. E., M. Hoogenkamp, M. Armand-Ugon, M. de Baar, K. Verhoef, and B. Berkhout. 2000. Functional differences between the long terminal repeat transcriptional promoters of human immunodeficiency virus type 1 subtypes A through G. *J Virol* 74:3740-51.
147. Jeong, S. J., A. Dasgupta, K. J. Jung, J. H. Um, A. Burke, H. U. Park, and J. N. Brady. 2008. PI3K/AKT inhibition induces caspase-dependent apoptosis in HTLV-1-transformed cells. *Virology* 370:264-72.
148. Jeong, S. J., C. A. Pise-Masison, M. F. Radonovich, H. U. Park, and J. N. Brady. 2005. A novel NF-kappaB pathway involving IKKbeta and p65/RelA Ser-536 phosphorylation results in p53 inhibition in the absence of NF-kappaB transcriptional activity. *J Biol Chem* 280:10326-32.

149. Jeong, S. J., C. A. Pise-Masison, M. F. Radonovich, H. U. Park, and J. N. Brady. 2005. Activated AKT regulates NF-kappaB activation, p53 inhibition and cell survival in HTLV-1-transformed cells. *Oncogene* 24:6719-28.
150. Johnston, M. D. 1981. The characteristics required for a Sendai virus preparation to induce high levels of interferon in human lymphoblastoid cells. *J Gen Virol* 56:175-84.
151. Kandel, E. S., and N. Hay. 1999. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp Cell Res* 253:210-29.
152. Kane, L. P., V. S. Shapiro, D. Stokoe, and A. Weiss. 1999. Induction of NF-kappaB by the Akt/PKB kinase. *Curr Biol* 9:601-4.
153. Kang, D. C., R. V. Gopalkrishnan, Q. Wu, E. Jankowsky, A. M. Pyle, and P. B. Fisher. 2002. mda-5: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. *Proc Natl Acad Sci U S A* 99:637-42.
154. Karin, M., Y. Cao, F. R. Greten, and Z. W. Li. 2002. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2:301-10.
155. Kato, A., K. Kiyotani, Y. Sakai, T. Yoshida, and Y. Nagai. 1997. The paramyxovirus, Sendai virus, V protein encodes a luxury function required for viral pathogenesis. *EMBO J* 16:578-87.
156. Kato, H., O. Takeuchi, E. Mikamo-Satoh, R. Hirai, T. Kawai, K. Matsushita, A. Hiiragi, T. S. Dermody, T. Fujita, and S. Akira. 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *J Exp Med* 205:1601-10.
157. Kato, H., O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K. J. Ishii, O. Yamaguchi, K. Otsu, T. Tsujimura, C. S. Koh, C. Reis e Sousa, Y. Matsuura, T. Fujita, and S. Akira. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441:101-5.
158. Kawai, T., and S. Akira. 2006. Innate immune recognition of viral infection. *Nat Immunol* 7:131-7.
159. Kawai, T., and S. Akira. 2006. [Role of IPS-1 in type I IFN induction]. *Nippon Rinsho* 64:1231-5.
160. Kawai, T., and S. Akira. 2007. SnapShot: Pattern-recognition receptors. *Cell* 129:1024.
161. Kawai, T., and S. Akira. 2007. TLR signaling. *Semin Immunol* 19:24-32.
162. Keene, J. D., I. M. Chien, and R. A. Lazzarini. 1981. Vesicular stomatitis virus defective interfering particle containing a muted internal leader RNA gene. *Proc Natl Acad Sci U S A* 78:2090-4.
163. Keller, M. A., S. K. Murphy, and G. D. Parks. 2001. RNA replication from the simian virus 5 antigenomic promoter requires three sequence-dependent elements separated by sequence-independent spacer regions. *J Virol* 75:3993-8.
164. Khabar, K. S., Y. M. Siddiqui, F. al-Zoghaibi, L. al-Haj, M. Dhalla, A. Zhou, B. Dong, M. Whitmore, J. Paranjape, M. N. Al-Ahdal, F. Al-Mohanna, B. R. Williams, and R. H. Silverman. 2003. RNase L mediates transient control of the interferon response through modulation of the double-stranded RNA-dependent protein kinase PKR. *J Biol Chem* 278:20124-32.
165. Khwaja, A., P. Rodriguez-Viciana, S. Wennstrom, P. H. Warne, and J. Downward. 1997. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J* 16:2783-93.

166. Killip, M. J., D. F. Young, C. S. Ross, S. Chen, S. Goodbourn, and R. E. Randall. Failure to activate the IFN-beta promoter by a paramyxovirus lacking an interferon antagonist. *Virology* 415:39-46.
167. Kingston, R. L., W. A. Baase, and L. S. Gay. 2004. Characterization of nucleocapsid binding by the measles virus and mumps virus phosphoproteins. *J Virol* 78:8630-40.
168. Kolakofsky, D., T. Pelet, D. Garcin, S. Hausmann, J. Curran, and L. Roux. 1998. Paramyxovirus RNA synthesis and the requirement for hexamer genome length: the rule of six revisited. *J Virol* 72:891-9.
169. Komuro, A., and C. M. Horvath. 2006. RNA- and virus-independent inhibition of antiviral signaling by RNA helicase LGP2. *J Virol* 80:12332-42.
170. Kubota, K., K. Nakahara, T. Ohtsuka, S. Yoshida, J. Kawaguchi, Y. Fujita, Y. Ozeki, A. Hara, C. Yoshimura, H. Furukawa, H. Haruyama, K. Ichikawa, M. Yamashita, T. Matsuoka, and Y. Iijima. 2004. Identification of 2'-phosphodiesterase, which plays a role in the 2-5A system regulated by interferon. *J Biol Chem* 279:37832-41.
171. Kumar, H., T. Kawai, and S. Akira. Pathogen recognition by the innate immune system. *Int Rev Immunol* 30:16-34.
172. Kurt-Jones, E. A., L. Popova, L. Kwinn, L. M. Haynes, L. P. Jones, R. A. Tripp, E. E. Walsh, M. W. Freeman, D. T. Golenbock, L. J. Anderson, and R. W. Finberg. 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* 1:398-401.
173. Lamb, R. A., and D. Kolakofsky. . 2001. Paramyxoviridae: The viruses and their replication. *In* I. D. M. K. a. P. M. Howley (ed.), *Fields Virology* (Fourth Edition). Lippincott, Williams and Wilkins, Philadelphia.
174. Lamb, R. A., and T. S. Jardetzky. 2007. Structural basis of viral invasion: lessons from paramyxovirus F. *Curr Opin Struct Biol* 17:427-36.
175. Lamb, R. A., and D. Kolakofsky. 2001. *Paramyxoviridae: The viruses and their replication. In* D. M. Knipe and P. M. Howley (ed.), *Fields Virology* (Fourth Edition). Lippincott, Williams and Wilkins, Philadelphia.
176. Lamb, R. A., Parks, G.D. 2007. Paramyxoviridae: The viruses and their replication, p. 1449 -1496. *In* D. M. Knipe, Howley, P.M. (ed.), *Fields Virology*, 5th ed, vol. 1. Wolter Kluwer , Lippincott Williams and Wilkins.
177. Lamb, R. A., R. G. Paterson, and T. S. Jardetzky. 2006. Paramyxovirus membrane fusion: lessons from the F and HN atomic structures. *Virology* 344:30-7.
178. Lampson, G. P., A. A. Tytell, A. K. Field, M. M. Nemes, and M. R. Hilleman. 1967. Inducers of interferon and host resistance. I. Double-stranded RNA from extracts of *Penicillium funiculosum*. *Proc Natl Acad Sci U S A* 58:782-9.
179. Lazzarini, R. A., J. D. Keene, and M. Schubert. 1981. The origins of defective interfering particles of the negative-strand RNA viruses. *Cell* 26:145-54.
180. Le Roy, F., C. Bisbal, M. Silhol, C. Martinand, B. Lebleu, and T. Salehzada. 2001. The 2-5A/RNase L/RNase L inhibitor (RLI) [correction of (RNI)] pathway regulates mitochondrial mRNAs stability in interferon alpha-treated H9 cells. *J Biol Chem* 276:48473-82.
181. Le Roy, F., T. Salehzada, C. Bisbal, J. P. Dougherty, and S. W. Peltz. 2005. A newly discovered function for RNase L in regulating translation termination. *Nat Struct Mol Biol* 12:505-12.
182. Li, J., E. C. Fontaine-Rodriguez, and S. P. Whelan. 2005. Amino acid residues within conserved domain VI of the vesicular stomatitis virus large polymerase protein essential for mRNA cap methyltransferase activity. *J Virol* 79:13373-84.

183. Li, J., A. Rahmeh, M. Morelli, and S. P. Whelan. 2008. A conserved motif in region v of the large polymerase proteins of nonsegmented negative-sense RNA viruses that is essential for mRNA capping. *J Virol* 82:775-84.
184. Li, M., P. T. Schmitt, Z. Li, T. S. McCrory, B. He, and A. P. Schmitt. 2009. Mumps virus matrix, fusion, and nucleocapsid proteins cooperate for efficient production of virus-like particles. *J Virol* 83:7261-72.
185. Li, X., C. T. Ranjith-Kumar, M. T. Brooks, S. Dharmaiah, A. B. Herr, C. Kao, and P. Li. 2009. The RIG-I-like receptor LGP2 recognizes the termini of double-stranded RNA. *J Biol Chem* 284:13881-91.
186. Li, X. L., J. A. Blackford, and B. A. Hassel. 1998. RNase L mediates the antiviral effect of interferon through a selective reduction in viral RNA during encephalomyocarditis virus infection. *J Virol* 72:2752-9.
187. Li, X. L., J. A. Blackford, C. S. Judge, M. Liu, W. Xiao, D. V. Kalvakolanu, and B. A. Hassel. 2000. RNase-L-dependent destabilization of interferon-induced mRNAs. A role for the 2-5A system in attenuation of the interferon response. *J Biol Chem* 275:8880-8.
188. Li, Z., J. Xu, J. Patel, S. Fuentes, Y. Lin, D. Anderson, K. Sakamoto, L. F. Wang, and B. He. Function of the small hydrophobic protein of J paramyxovirus. *J Virol* 85:32-42.
189. Li, Z., M. Yu, H. Zhang, D. E. Magoffin, P. J. Jack, A. Hyatt, H. Y. Wang, and L. F. Wang. 2006. Beilong virus, a novel paramyxovirus with the largest genome of non-segmented negative-stranded RNA viruses. *Virology* 346:219-28.
190. Lin, A., B. Devaux, A. Green, C. Sagerstrom, J. Elliott, and M. Davis. 1990. Expression of T cell antigen receptor heterodimers in a lipid-linked form. *Science* 249:677-679.
191. Lin, A. Y., B. Devaux, A. Green, C. Sagerstrom, J. F. Elliott, and M. M. Davis. 1990. Expression of T cell antigen receptor heterodimers in a lipid-linked form. *Science* 249:677-9.
192. Lin, G. Y., and R. A. Lamb. 2000. The paramyxovirus simian virus 5 V protein slows progression of the cell cycle. *J Virol* 74:9152-66.
193. Lin, G. Y., R. G. Paterson, and R. A. Lamb. 1997. The RNA binding region of the paramyxovirus SV5 V and P proteins. *Virology* 238:460-469.
194. Lin, G. Y., R. G. Paterson, and R. A. Lamb. 1997. The RNA binding region of the paramyxovirus SV5 V and P proteins. *Virology* 238:460-9.
195. Lin, Y., A. C. Bright, T. A. Rothermel, and B. He. 2003. Induction of apoptosis by paramyxovirus simian virus 5 lacking a small hydrophobic gene. *J Virol* 77:3371-83.
196. Lin, Y., F. Horvath, J. A. Aligo, R. Wilson, and B. He. 2005. The role of simian virus 5 V protein on viral RNA synthesis. *Virology* 338:270-80.
197. Lin, Y., M. Sun, S. M. Fuentes, C. D. Keim, T. Rothermel, and B. He. 2007. Inhibition of interleukin-6 expression by the V protein of parainfluenza virus 5. *Virology* 368:262-72.
198. Ling, Z., K. C. Tran, and M. N. Teng. 2009. Human respiratory syncytial virus nonstructural protein NS2 antagonizes the activation of beta interferon transcription by interacting with RIG-I. *J Virol* 83:3734-42.
199. Liston, P., and D. J. Briedis. 1994. Measles virus V protein binds zinc. *Virology* 198:399-404.
200. Liu, W., S. L. Liang, H. Liu, R. Silverman, and A. Zhou. 2007. Tumour suppressor function of RNase L in a mouse model. *Eur J Cancer* 43:202-9.

201. Loo, Y. M., J. Fornek, N. Crochet, G. Bajwa, O. Perwitasari, L. Martinez-Sobrido, S. Akira, M. A. Gill, A. Garcia-Sastre, M. G. Katze, and M. Gale, Jr. 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *J Virol* 82:335-45.
202. Lu, L. L., M. Puri, C. M. Horvath, and G. C. Sen. 2008. Select paramyxoviral V proteins inhibit IRF3 activation by acting as alternative substrates for inhibitor of kappaB kinase epsilon (IKKe)/TBK1. *J Biol Chem* 283:14269-76.
203. Luthra, P., D. Sun, M. Wolfgang, and B. He. 2008. AKT1-dependent activation of NF-kappaB by the L protein of parainfluenza virus 5. *J Virol* 82:10887-95.
204. Maitra, R. K., N. A. McMillan, S. Desai, J. McSwiggen, A. G. Hovanessian, G. Sen, B. R. Williams, and R. H. Silverman. 1994. HIV-1 TAR RNA has an intrinsic ability to activate interferon-inducible enzymes. *Virology* 204:823-7.
205. Malathi, K., B. Dong, M. Gale, Jr., and R. H. Silverman. 2007. Small self-RNA generated by RNase L amplifies antiviral innate immunity. *Nature* 448:816-9.
206. Malur, A. G., N. K. Gupta, P. De Bishnu, and A. K. Banerjee. 2002. Analysis of the mutations in the active site of the RNA-dependent RNA polymerase of human parainfluenza virus type 3 (HPIV3). *Gene Expr* 10:93-100.
207. Manna, S. K., and B. B. Aggarwal. 2000. Differential requirement for p56lck in HIV-tat versus TNF-induced cellular responses: effects on NF-kappa B, activator protein-1, c-Jun N-terminal kinase, and apoptosis. *J Immunol* 164:5156-66.
208. Manning, B. D., and L. C. Cantley. 2007. AKT/PKB signaling: navigating downstream. *Cell* 129:1261-74.
209. Manuse, M. J., and G. D. Parks. 2009. Role for the paramyxovirus genomic promoter in limiting host cell antiviral responses and cell killing. *J Virol* 83:9057-67.
210. Marie, I., J. E. Durbin, and D. E. Levy. 1998. Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. *EMBO J* 17:6660-9.
211. Martinand, C., C. Montavon, T. Salehzada, M. Silhol, B. Lebleu, and C. Bisbal. 1999. RNase L inhibitor is induced during human immunodeficiency virus type 1 infection and down regulates the 2-5A/RNase L pathway in human T cells. *J Virol* 73:290-6.
212. Martinand, C., T. Salehzada, M. Silhol, B. Lebleu, and C. Bisbal. 1998. RNase L inhibitor (RLI) antisense constructions block partially the down regulation of the 2-5A/RNase L pathway in encephalomyocarditis-virus-(EMCV)-infected cells. *Eur J Biochem* 254:248-55.
213. Massey, D. M., N. Deans, and J. Lenard. 1990. Phosphorylation of NS protein by vesicular stomatitis virus nucleocapsids: lack of effect during RNA synthesis and separation of kinase from L protein. *J Virol* 64:3259-64.
214. Mathur, M., T. Das, and A. K. Banerjee. 1996. Expression of L protein of vesicular stomatitis virus Indiana serotype from recombinant baculovirus in insect cells: requirement of a host factor(s) for its biological activity in vitro. *J Virol* 70:2252-9.
215. May, M. J., and S. Ghosh. 1998. Signal transduction through NF-kappa B. *Immunol Today* 19:80-8.
216. Mayo, M. A. 2002. A summary of taxonomic changes recently approved by ICTV. *Arch Virol* 147:1655-63.
217. Mazumder, B., G. Adhikary, and S. Barik. 1994. Bacterial expression of human respiratory syncytial viral phosphoprotein P and identification of Ser237 as the site of phosphorylation by cellular casein kinase II. *Virology* 205:93-103.
218. Mazumder, B., and S. Barik. 1994. Requirement of casein kinase II-mediated phosphorylation for the transcriptional activity of human respiratory syncytial viral

- phosphoprotein P: transdominant negative phenotype of phosphorylation-defective P mutants. *Virology* 205:104-11.
219. McCandlish, I. A., H. Thompson, H. J. Cornwell, and N. G. Wright. 1978. A study of dogs with kennel cough. *Vet Rec* 102:293-301.
 220. McCartney, S. A., L. B. Thackray, L. Gitlin, S. Gilfillan, H. W. Virgin, and M. Colonna. 2008. MDA-5 recognition of a murine norovirus. *PLoS Pathog* 4:e1000108.
 221. Meager, A., K. Visvalingam, P. Dilger, D. Bryan, and M. Wadhwa. 2005. Biological activity of interleukins-28 and -29: comparison with type I interferons. *Cytokine* 31:109-18.
 222. Melchjorsen, J., S. B. Jensen, L. Malmgaard, S. B. Rasmussen, F. Weber, A. G. Bowie, S. Matikainen, and S. R. Paludan. 2005. Activation of innate defense against a paramyxovirus is mediated by RIG-I and TLR7 and TLR8 in a cell-type-specific manner. *J Virol* 79:12944-51.
 223. Merika, M., and D. Thanos. 2001. Enhanceosomes. *Curr Opin Genet Dev* 11:205-8.
 224. Meurs, E., K. Chong, J. Galabru, N. S. Thomas, I. M. Kerr, B. R. Williams, and A. G. Hovanessian. 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. *Cell* 62:379-90.
 225. Min, J. Y., and R. M. Krug. 2006. The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. *Proc Natl Acad Sci U S A* 103:7100-5.
 226. Mosavi, L. K., T. J. Cammett, D. C. Desrosiers, and Z. Y. Peng. 2004. The ankyrin repeat as molecular architecture for protein recognition. *Protein Sci* 13:1435-48.
 227. Muller, R., O. Poch, M. Delarue, D. H. Bishop, and M. Bouloy. 1994. Rift Valley fever virus L segment: correction of the sequence and possible functional role of newly identified regions conserved in RNA-dependent polymerases. *J Gen Virol* 75 (Pt 6):1345-52.
 228. Murali, A., X. Li, C. T. Ranjith-Kumar, K. Bhardwaj, A. Holzenburg, P. Li, and C. C. Kao. 2008. Structure and function of LGP2, a DEX(D/H) helicase that regulates the innate immunity response. *J Biol Chem* 283:15825-33.
 229. Murphy, S. K., Y. Ito, and G. D. Parks. 1998. A functional antigenomic promoter for the paramyxovirus simian virus 5 requires proper spacing between an essential internal segment and the 3' terminus. *J Virol* 72:10-9.
 230. Murphy, S. K., and G. D. Parks. 1997. Genome nucleotide lengths that are divisible by six are not essential but enhance replication of defective interfering RNAs of the paramyxovirus simian virus 5. *Virology* 232:145-57.
 231. Murphy, S. K., and G. D. Parks. 1999. RNA replication for the paramyxovirus simian virus 5 requires an internal repeated (CGNNNN) sequence motif. *J Virol* 73:805-9.
 232. Nakanishi, M., Y. Goto, and Y. Kitade. 2005. 2-5A induces a conformational change in the ankyrin-repeat domain of RNase L. *Proteins* 60:131-8.
 233. Nallagatla, S. R., R. Toroney, and P. C. Bevilacqua. 2008. A brilliant disguise for self RNA: 5'-end and internal modifications of primary transcripts suppress elements of innate immunity. *RNA Biol* 5:140-4.
 234. Ng, D. T., R. E. Randall, and R. A. Lamb. 1989. Intracellular maturation and transport of the SV5 type II glycoprotein hemagglutinin-neuraminidase: specific and transient association with GRP78-BiP in the endoplasmic reticulum and extensive internalization from the cell surface. *J Cell Biol* 109:3273-89.
 235. Nilsen, T. W., and C. Baglioni. 1979. Mechanism for discrimination between viral and host mRNA in interferon-treated cells. *Proc Natl Acad Sci U S A* 76:2600-4.

236. Nilsen, T. W., S. G. Weissman, and C. Baglioni. 1980. Role of 2',5'-oligo(adenylic acid) polymerase in the degradation of ribonucleic acid linked to double-stranded ribonucleic acid by extracts of interferon-treated cells. *Biochemistry* 19:5574-9.
237. Nilsen, T. W., D. L. Wood, and C. Baglioni. 1982. Presence of 2',5'-oligo(A) and of enzymes that synthesize, bind, and degrade 2',5'-oligo(A) in HeLa cell nuclei. *J Biol Chem* 257:1602-5.
238. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants by a novel eukaryotic vector. *Gene* 108:193-200.
239. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108:193-9.
240. Norris, J. L., and A. S. Baldwin, Jr. 1999. Oncogenic Ras enhances NF-kappaB transcriptional activity through Raf-dependent and Raf-independent mitogen-activated protein kinase signaling pathways. *J Biol Chem* 274:13841-6.
241. Novick, D., B. Cohen, and M. Rubinstein. 1994. The human interferon alpha/beta receptor: characterization and molecular cloning. *Cell* 77:391-400.
242. Ogino, T., and A. K. Banerjee. The HR motif in the RNA-dependent RNA polymerase L protein of Chandipura virus is required for unconventional mRNA-capping activity. *J Gen Virol* 91:1311-4.
243. Ogino, T., M. Kobayashi, M. Iwama, and K. Mizumoto. 2005. Sendai virus RNA-dependent RNA polymerase L protein catalyzes cap methylation of virus-specific mRNA. *J Biol Chem* 280:4429-35.
244. Ogino, T., S. P. Yadav, and A. K. Banerjee. Histidine-mediated RNA transfer to GDP for unique mRNA capping by vesicular stomatitis virus RNA polymerase. *Proc Natl Acad Sci U S A* 107:3463-8.
245. Onoguchi, K., M. Yoneyama, A. Takemura, S. Akira, T. Taniguchi, H. Namiki, and T. Fujita. 2007. Viral infections activate types I and III interferon genes through a common mechanism. *J Biol Chem* 282:7576-81.
246. Osame, M., K. Usuku, S. Izumo, N. Ijichi, H. Amitani, A. Igata, M. Matsumoto, and M. Tara. 1986. HTLV-I associated myelopathy, a new clinical entity. *Lancet* 1:1031-2.
247. Ozes, O. N., L. D. Mayo, J. A. Gustin, S. R. Pfeffer, L. M. Pfeffer, and D. B. Donner. 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 401:82-5.
248. Pahl, H. L. 1999. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 18:6853-66.
249. Pahl, H. L., and P. A. Baeuerle. 1997. The ER-overload response: activation of NF-kappa B. *Trends Biochem Sci* 22:63-7.
250. Pantua, H. D., L. W. McGinnes, M. E. Peeples, and T. G. Morrison. 2006. Requirements for the assembly and release of Newcastle disease virus-like particles. *J Virol* 80:11062-73.
251. Parisien, J. P., D. Bammig, A. Komuro, A. Ramachandran, J. J. Rodriguez, G. Barber, R. D. Wojahn, and C. M. Horvath. 2009. A shared interface mediates paramyxovirus interference with antiviral RNA helicases MDA5 and LGP2. *J Virol* 83:7252-60.
252. Parisien, J. P., J. F. Lau, J. J. Rodriguez, B. M. Sullivan, A. Moscona, G. D. Parks, R. A. Lamb, and C. M. Horvath. 2001. The V protein of human parainfluenza virus 2 antagonizes type I interferon responses by destabilizing signal transducer and activator of transcription 2. *Virology* 283:230-9.

253. Parisien, J. P., J. F. Lau, J. J. Rodriguez, C. M. Ulane, and C. M. Horvath. 2002. Selective STAT protein degradation induced by paramyxoviruses requires both STAT1 and STAT2 but is independent of alpha/beta interferon signal transduction. *J Virol* 76:4190-8.
254. Parks, G. D. 1994. Mapping of a region of the paramyxovirus L protein required for the formation of a stable complex with the viral phosphoprotein P. *J Virol* 68:4862-72.
255. Patch, J. R., G. Cramer, L. F. Wang, B. T. Eaton, and C. C. Broder. 2007. Quantitative analysis of Nipah virus proteins released as virus-like particles reveals central role for the matrix protein. *Virol J* 4:1.
256. Paterson, R. G., G. P. Leser, M. A. Shaughnessy, and R. A. Lamb. 1995. The paramyxovirus SV5 V protein binds two atoms of zinc and is a structural component of virions. *Virology* 208:121-31.
257. Paterson, R. G., C. J. Russell, and R. A. Lamb. 2000. Fusion protein of the paramyxovirus SV5: destabilizing and stabilizing mutants of fusion activation. *Virology* 270:17-30.
258. Pei, Z., Y. Bai, and A. P. Schmitt. PIV5 M protein interaction with host protein angiotensin-like 1. *Virology* 397:155-66.
259. Pei, Z., M. S. Harrison, and A. P. Schmitt. Parainfluenza virus 5 m protein interaction with host protein 14-3-3 negatively affects virus particle formation. *J Virol* 85:2050-9.
260. Pichlmair, A., O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 314:997-1001.
261. Pichlmair, A., O. Schulz, C. P. Tan, J. Rehwinkel, H. Kato, O. Takeuchi, S. Akira, M. Way, G. Schiavo, and C. Reis e Sousa. 2009. Activation of MDA5 requires higher-order RNA structures generated during virus infection. *J Virol* 83:10761-9.
262. Pippig, D. A., J. C. Hellmuth, S. Cui, A. Kirchhofer, K. Lammens, A. Lammens, A. Schmidt, S. Rothenfusser, and K. P. Hopfner. 2009. The regulatory domain of the RIG-I family ATPase LGP2 senses double-stranded RNA. *Nucleic Acids Res* 37:2014-25.
263. Pise-Masison, C. A., R. Mahieux, H. Jiang, M. Ashcroft, M. Radonovich, J. Duvall, C. Guillermin, and J. N. Brady. 2000. Inactivation of p53 by human T-cell lymphotropic virus type 1 Tax requires activation of the NF-kappaB pathway and is dependent on p53 phosphorylation. *Mol Cell Biol* 20:3377-86.
264. Platanius, L. C. 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* 5:375-86.
265. Poch, O., B. M. Blumberg, L. Bougueleret, and N. Tordo. 1990. Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. *J. Gen. Virol.* 71:1153-1162.
266. Poch, O., B. M. Blumberg, L. Bougueleret, and N. Tordo. 1990. Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. *J Gen Virol* 71 (Pt 5):1153-62.
267. Pohl, C., W. P. Duprex, G. Krohne, B. K. Rima, and S. Schneider-Schaulies. 2007. Measles virus M and F proteins associate with detergent-resistant membrane fractions and promote formation of virus-like particles. *J Gen Virol* 88:1243-50.

268. Poole, E., B. He, R. A. Lamb, R. E. Randall, and S. Goodbourn. 2002. The V proteins of simian virus 5 and other paramyxoviruses inhibit induction of interferon-beta. *Virology* 303:33-46.
269. Powell, P. P., L. K. Dixon, and R. M. Parkhouse. 1996. An IkappaB homolog encoded by African swine fever virus provides a novel mechanism for downregulation of proinflammatory cytokine responses in host macrophages. *J Virol* 70:8527-33.
270. Randall, R. E., and A. Bermingham. 1996. NP:P and NP:V interactions of the paramyxovirus simian virus 5 examined using a novel protein:protein capture assay. *Virology* 224:121-9.
271. Randall, R. E., and A. Bermingham. 1996. NP:P and NP:V interactions of the paramyxovirus simian virus 5 examined using a novel protein:protein capture assay. *Virology* 224:121-129.
272. Randall, R. E., and S. Goodbourn. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol* 89:1-47.
273. Ravid, D., G. P. Leser, and R. A. Lamb. A role for caveolin 1 in assembly and budding of the paramyxovirus parainfluenza virus 5. *J Virol* 84:9749-59.
274. Rayet, B., and C. Gelinas. 1999. Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* 18:6938-47.
275. Rebouillat, D., and A. G. Hovanessian. 1999. The human 2',5'-oligoadenylate synthetase family: interferon-induced proteins with unique enzymatic properties. *J Interferon Cytokine Res* 19:295-308.
276. Redaelli, C., F. Granucci, L. De Gioia, and L. Cipolla. 2006. Synthesis and biological activity of Akt/PI3K inhibitors. *Mini Rev Med Chem* 6:1127-36.
277. Rehwinkel, J., C. P. Tan, D. Goubau, O. Schulz, A. Pichlmair, K. Bier, N. Robb, F. Vreede, W. Barclay, E. Fodor, and C. Reis e Sousa. RIG-I detects viral genomic RNA during negative-strand RNA virus infection. *Cell* 140:397-408.
278. Rice, A. P., and I. M. Kerr. 1984. Interferon-mediated, double-stranded RNA-dependent protein kinase is inhibited in extracts from vaccinia virus-infected cells. *J Virol* 50:229-36.
279. Romashkova, J. A., and S. S. Makarov. 1999. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 401:86-90.
280. Roth-Cross, J. K., S. J. Bender, and S. R. Weiss. 2008. Murine coronavirus mouse hepatitis virus is recognized by MDA5 and induces type I interferon in brain macrophages/microglia. *J Virol* 82:9829-38.
281. Rothenfusser, S., N. Goutagny, G. DiPerna, M. Gong, B. G. Monks, A. Schoenemeyer, M. Yamamoto, S. Akira, and K. A. Fitzgerald. 2005. The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *J Immunol* 175:5260-8.
282. Roulston, A., R. Lin, P. Beauparlant, M. A. Wainberg, and J. Hiscott. 1995. Regulation of human immunodeficiency virus type 1 and cytokine gene expression in myeloid cells by NF-kappa B/Rel transcription factors. *Microbiol Rev* 59:481-505.
283. Roulston, A., R. C. Marcellus, and P. E. Branton. 1999. Viruses and apoptosis. *Annu Rev Microbiol* 53:577-628.
284. Roux, P., C. Alfieri, M. Hrimech, E. A. Cohen, and J. E. Tanner. 2000. Activation of transcription factors NF-kappaB and NF-IL-6 by human immunodeficiency virus type 1 protein R (Vpr) induces interleukin-8 expression. *J Virol* 74:4658-65.

285. Rusch, L., A. Zhou, and R. H. Silverman. 2000. Caspase-dependent apoptosis by 2',5'-oligoadenylate activation of RNase L is enhanced by IFN-beta. *J Interferon Cytokine Res* 20:1091-100.
286. Russell, C. J., T. S. Jardetzky, and R. A. Lamb. 2004. Conserved glycine residues in the fusion peptide of the paramyxovirus fusion protein regulate activation of the native state. *J Virol* 78:13727-42.
287. Russell, C. J., T. S. Jardetzky, and R. A. Lamb. 2001. Membrane fusion machines of paramyxoviruses: capture of intermediates of fusion. *EMBO J* 20:4024-34.
288. Russell, C. J., K. L. Kantor, T. S. Jardetzky, and R. A. Lamb. 2003. A dual-functional paramyxovirus F protein regulatory switch segment: activation and membrane fusion. *J Cell Biol* 163:363-74.
289. Russell, C. J., and L. E. Luque. 2006. The structural basis of paramyxovirus invasion. *Trends Microbiol* 14:243-6.
290. Saito, T., and M. Gale, Jr. 2008. Differential recognition of double-stranded RNA by RIG-I-like receptors in antiviral immunity. *J Exp Med* 205:1523-7.
291. Saito, T., R. Hirai, Y. M. Loo, D. Owen, C. L. Johnson, S. C. Sinha, S. Akira, T. Fujita, and M. Gale, Jr. 2007. Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. *Proc Natl Acad Sci U S A* 104:582-7.
292. Saito, T., D. M. Owen, F. Jiang, J. Marcotrigiano, and M. Gale, Jr. 2008. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. *Nature* 454:523-7.
293. Samal, S. K., and P. L. Collins. 1996. RNA replication by a respiratory syncytial virus RNA analog does not obey the rule of six and retains a nonviral trinucleotide extension at the leader end. *J Virol* 70:5075-82.
294. Samuel, M. A., K. Whitby, B. C. Keller, A. Marri, W. Barchet, B. R. Williams, R. H. Silverman, M. Gale, Jr., and M. S. Diamond. 2006. PKR and RNase L contribute to protection against lethal West Nile Virus infection by controlling early viral spread in the periphery and replication in neurons. *J Virol* 80:7009-19.
295. Sanchez, A., B. P. De, and A. K. Banerjee. 1985. In vitro phosphorylation of NS protein by the L protein of vesicular stomatitis virus. *J Gen Virol* 66 (Pt 5):1025-36.
296. Sarbassov, D. D., D. A. Guertin, S. M. Ali, and D. M. Sabatini. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307:1098-101.
297. Sato, M., N. Hata, M. Asagiri, T. Nakaya, T. Taniguchi, and N. Tanaka. 1998. Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. *FEBS Lett* 441:106-10.
298. Satoh, T., H. Kato, Y. Kumagai, M. Yoneyama, S. Sato, K. Matsushita, T. Tsujimura, T. Fujita, S. Akira, and O. Takeuchi. LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proc Natl Acad Sci U S A* 107:1512-7.
299. Scherbik, S. V., J. M. Paranjape, B. M. Stockman, R. H. Silverman, and M. A. Brinton. 2006. RNase L plays a role in the antiviral response to West Nile virus. *J Virol* 80:2987-99.
300. Schlee, M., E. Hartmann, C. Coch, V. Wimmenauer, M. Janke, W. Barchet, and G. Hartmann. 2009. Approaching the RNA ligand for RIG-I? *Immunol Rev* 227:66-74.
301. Schlee, M., A. Roth, V. Hornung, C. A. Hagmann, V. Wimmenauer, W. Barchet, C. Coch, M. Janke, A. Mihailovic, G. Wardle, S. Juranek, H. Kato, T. Kawai, H. Poeck, K. A. Fitzgerald, O. Takeuchi, S. Akira, T. Tuschl, E. Latz, J. Ludwig, and G. Hartmann. 2009. Recognition of 5' triphosphate by RIG-I helicase requires short

- blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* 31:25-34.
302. Schmidt, A., T. Schwerd, W. Hamm, J. C. Hellmuth, S. Cui, M. Wenzel, F. S. Hoffmann, M. C. Michallet, R. Besch, K. P. Hopfner, S. Endres, and S. Rothenfusser. 2009. 5'-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proc Natl Acad Sci U S A* 106:12067-72.
 303. Schmitt, A. P., B. He, and R. A. Lamb. 1999. Involvement of the cytoplasmic domain of the hemagglutinin-neuraminidase protein in assembly of the paramyxovirus simian virus 5. *J Virol* 73:8703-12.
 304. Schmitt, A. P., and R. A. Lamb. 2004. Escaping from the cell: assembly and budding of negative-strand RNA viruses. *Curr Top Microbiol Immunol* 283:145-96.
 305. Schmitt, A. P., G. P. Leser, E. Morita, W. I. Sundquist, and R. A. Lamb. 2005. Evidence for a new viral late-domain core sequence, FPIV, necessary for budding of a paramyxovirus. *J Virol* 79:2988-97.
 306. Schmitt, A. P., G. P. Leser, D. L. Waning, and R. A. Lamb. 2002. Requirements for budding of paramyxovirus simian virus 5 virus-like particles. *J Virol* 76:3952-64.
 307. Schmitt, P. T., G. Ray, and A. P. Schmitt. The C-terminal end of parainfluenza virus 5 NP protein is important for virus-like particle production and M-NP protein interaction. *J Virol* 84:12810-23.
 308. Schubert, M., and R. A. Lazzarini. 1981. Structure and origin of a snapback defective interfering particle RNA of vesicular stomatitis virus. *J Virol* 37:661-72.
 309. Seth, R. B., L. Sun, C. K. Ea, and Z. J. Chen. 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 122:669-82.
 310. Sharp, T. V., D. A. Raine, D. R. Gewert, B. Joshi, R. Jagus, and M. J. Clemens. 1999. Activation of the interferon-inducible (2'-5') oligoadenylate synthetase by the Epstein-Barr virus RNA, EBER-1. *Virology* 257:303-13.
 311. Shingai, M., T. Ebihara, N. A. Begum, A. Kato, T. Honma, K. Matsumoto, H. Saito, H. Ogura, M. Matsumoto, and T. Seya. 2007. Differential type I IFN-inducing abilities of wild-type versus vaccine strains of measles virus. *J Immunol* 179:6123-33.
 312. Sidhu, M. S., J. P. Menonna, S. D. Cook, P. C. Dowling, and S. A. Udem. 1993. Canine distemper virus L gene: sequence and comparison with related viruses. *Virology* 193:50-65.
 313. Silverman, R. H. 1985. Functional analysis of 2-5A-dependent RNase and 2-5a using 2',5'-oligoadenylate-cellulose. *Anal Biochem* 144:450-60.
 314. Silverman, R. H. 2003. Implications for RNase L in prostate cancer biology. *Biochemistry* 42:1805-12.
 315. Silverman, R. H. 2007. Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response. *J Virol* 81:12720-9.
 316. Silverman, R. H., D. D. Jung, N. L. Nolan-Sorden, C. W. Dieffenbach, V. P. Kedar, and D. N. SenGupta. 1988. Purification and analysis of murine 2-5A-dependent RNase. *J Biol Chem* 263:7336-41.
 317. Silverman, R. H., J. J. Skehel, T. C. James, D. H. Wreschner, and I. M. Kerr. 1983. rRNA cleavage as an index of ppp(A2'p)nA activity in interferon-treated encephalomyocarditis virus-infected cells. *J Virol* 46:1051-5.
 318. Skehel, J. J., and D. C. Wiley. 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem* 69:531-69.

319. Slattery, E., N. Ghosh, H. Samanta, and P. Lengyel. 1979. Interferon, double-stranded RNA, and RNA degradation: activation of an endonuclease by (2'-5')An. *Proc Natl Acad Sci U S A* 76:4778-82.
320. Smallwood, S., C. D. Eason, J. A. Feller, S. M. Horikami, and S. A. Moyer. 1999. Mutations in conserved domain II of the large (L) subunit of the Sendai virus RNA polymerase abolish RNA synthesis. *Virology* 262:375-383.
321. Smallwood, S., T. Hovel, W. J. Neubert, and S. A. Moyer. 2002. Different substitutions at conserved amino acids in domains II and III in the Sendai L RNA polymerase protein inactivate viral RNA synthesis. *Virology* 304:135-45.
322. Srinivas, H., D. Xia, N. L. Moore, I. P. Uray, H. Kim, L. Ma, N. L. Weigel, P. H. Brown, and J. M. Kurie. 2006. Akt phosphorylates and suppresses the transactivation of retinoic acid receptor alpha. *Biochem J* 395:653-62.
323. Stambolic, V., and J. R. Woodgett. 2006. Functional distinctions of protein kinase B/Akt isoforms defined by their influence on cell migration. *Trends Cell Biol* 16:461-6.
324. Stokes, H. L., A. J. Easton, and A. C. Marriott. 2003. Chimeric pneumovirus nucleocapsid (N) proteins allow identification of amino acids essential for the function of the respiratory syncytial virus N protein. *J Gen Virol* 84:2679-83.
325. Strahle, L., D. Garcin, and D. Kolakofsky. 2006. Sendai virus defective-interfering genomes and the activation of interferon-beta. *Virology* 351:101-11.
326. Strahle, L., J. B. Marq, A. Brini, S. Hausmann, D. Kolakofsky, and D. Garcin. 2007. Activation of the beta interferon promoter by unnatural Sendai virus infection requires RIG-I and is inhibited by viral C proteins. *J Virol* 81:12227-37.
327. Sumpter, R., Jr., Y. M. Loo, E. Foy, K. Li, M. Yoneyama, T. Fujita, S. M. Lemon, and M. Gale, Jr. 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol* 79:2689-99.
328. Sun, D., P. Luthra, Z. Li, and B. He. 2009. PLK1 down-regulates parainfluenza virus 5 gene expression. *PLoS Pathog* 5:e1000525.
329. Sun, M., S. M. Fuentes, K. Timani, D. Sun, C. Murphy, Y. Lin, A. August, M. N. Teng, and B. He. 2008. Akt plays a critical role in replication of nonsegmented negative-stranded RNA viruses. *J Virol* 82:105-14.
330. Sun, M., T. A. Rothermel, L. Shuman, J. A. Aligo, S. Xu, Y. Lin, R. A. Lamb, and B. He. 2004. Conserved cysteine-rich domain of paramyxovirus simian virus 5 V protein plays an important role in blocking apoptosis. *J Virol* 78:5068-78.
331. Sun, S. C., J. Elwood, C. Beraud, and W. C. Greene. 1994. Human T-cell leukemia virus type I Tax activation of NF-kappa B/Rel involves phosphorylation and degradation of I kappa B alpha and RelA (p65)-mediated induction of the c-rel gene. *Mol Cell Biol* 14:7377-84.
332. Tai, D. I., S. L. Tsai, Y. M. Chen, Y. L. Chuang, C. Y. Peng, I. S. Sheen, C. T. Yeh, K. S. Chang, S. N. Huang, G. C. Kuo, and Y. F. Liaw. 2000. Activation of nuclear factor kappaB in hepatitis C virus infection: implications for pathogenesis and hepatocarcinogenesis. *Hepatology* 31:656-64.
333. Takahasi, K., H. Kumeta, N. Tsuduki, R. Narita, T. Shigemoto, R. Hirai, M. Yoneyama, M. Horiuchi, K. Ogura, T. Fujita, and F. Inagaki. 2009. Solution structures of cytosolic RNA sensor MDA5 and LGP2 C-terminal domains: identification of the RNA recognition loop in RIG-I-like receptors. *J Biol Chem* 284:17465-74.

334. Takahasi, K., M. Yoneyama, T. Nishihori, R. Hirai, H. Kumeta, R. Narita, M. Gale, Jr., F. Inagaki, and T. Fujita. 2008. Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses. *Mol Cell* 29:428-40.
335. Takeda, K., and S. Akira. 2007. Toll-like receptors. *Curr Protoc Immunol Chapter* 14:Unit 14 12.
336. Takeuchi, K., K. Tanabayashi, M. Hishiyama, and A. Yamada. 1996. The mumps virus SH protein is a membrane protein and not essential for virus growth. *Virology* 225:156-62.
337. Takeuchi, K., K. Tanabayashi, M. Hishiyama, A. Yamada, and A. Sugiura. 1991. Variations of nucleotide sequences and transcription of the SH gene among mumps virus strains. *Virology* 181:364-6.
338. Takimoto, T., K. G. Murti, T. Bousse, R. A. Scroggs, and A. Portner. 2001. Role of matrix and fusion proteins in budding of Sendai virus. *J Virol* 75:11384-91.
339. Takimoto, T., G. L. Taylor, H. C. Connaris, S. J. Crennell, and A. Portner. 2002. Role of the hemagglutinin-neuraminidase protein in the mechanism of paramyxovirus-cell membrane fusion. *J Virol* 76:13028-33.
340. Tanaka, N., M. Nakanishi, Y. Kusakabe, Y. Goto, Y. Kitade, and K. T. Nakamura. 2004. Structural basis for recognition of 2',5'-linked oligoadenylates by human ribonuclease L. *EMBO J* 23:3929-38.
341. tenOever, B. R., M. J. Servant, N. Grandvaux, R. Lin, and J. Hiscott. 2002. Recognition of the measles virus nucleocapsid as a mechanism of IRF-3 activation. *J Virol* 76:3659-69.
342. tenOever, B. R., S. Sharma, W. Zou, Q. Sun, N. Grandvaux, I. Julkunen, H. Hemmi, M. Yamamoto, S. Akira, W. C. Yeh, R. Lin, and J. Hiscott. 2004. Activation of TBK1 and IKKvarepsilon kinases by vesicular stomatitis virus infection and the role of viral ribonucleoprotein in the development of interferon antiviral immunity. *J Virol* 78:10636-49.
343. Thomas, K. W., M. M. Monick, J. M. Staber, T. Yarovinsky, A. B. Carter, and G. W. Hunninghake. 2002. Respiratory syncytial virus inhibits apoptosis and induces NF-kappa B activity through a phosphatidylinositol 3-kinase-dependent pathway. *J Biol Chem* 277:492-501.
344. Thomas, S. M., R. A. Lamb, and R. G. Paterson. 1988. Two mRNAs that differ by two nontemplated nucleotides encode the amino coterminal proteins P and V of the paramyxovirus SV5. *Cell* 54:891-902.
345. Thompson, S. D., W. G. Laver, K. G. Murti, and A. Portner. 1988. Isolation of a biologically active soluble form of the hemagglutinin-neuraminidase protein of Sendai virus. *J Virol* 62:4653-60.
346. Timani, K. A., D. Sun, M. Sun, C. Keim, Y. Lin, P. T. Schmitt, A. P. Schmitt, and B. He. 2008. A single amino acid residue change in the P protein of parainfluenza virus 5 elevates viral gene expression. *J Virol* 82:9123-33.
347. Tytell, A. A., G. P. Lampson, A. K. Field, and M. R. Hilleman. 1967. Inducers of interferon and host resistance. 3. Double-stranded RNA from reovirus type 3 virions (reo 3-RNA). *Proc Natl Acad Sci U S A* 58:1719-22.
348. Uematsu, S., and S. Akira. 2006. [Innate immune recognition of viral infection]. *Uirusu* 56:1-8.
349. Uematsu, S., and S. Akira. 2006. [Innate immunity and toll-like receptor]. *Nippon Naika Gakkai Zasshi* 95:1115-21.
350. Uematsu, S., and S. Akira. 2007. Toll-like receptors and Type I interferons. *J Biol Chem* 282:15319-23.

351. Ulane, C. M., and C. M. Horvath. 2002. Paramyxoviruses SV5 and HPIV2 assemble STAT protein ubiquitin ligase complexes from cellular components. *Virology* 304:160-6.
352. Unterholzner, L., and A. G. Bowie. 2008. The interplay between viruses and innate immune signaling: Recent insights and therapeutic opportunities. *Biochem Pharmacol* 75:589-602.
353. Uze, G., and D. Monneron. 2007. IL-28 and IL-29: newcomers to the interferon family. *Biochimie* 89:729-34.
354. Uze, G., G. Schreiber, J. Piehler, and S. Pellegrini. 2007. The receptor of the type I interferon family. *Curr Top Microbiol Immunol* 316:71-95.
355. Vanden Berghe, W., K. De Bosscher, E. Boone, S. Plaisance, and G. Haegeman. 1999. The nuclear factor-kappaB engages CBP/p300 and histone acetyltransferase activity for transcriptional activation of the interleukin-6 gene promoter. *J Biol Chem* 274:32091-8.
356. Vanden Berghe, W., S. Plaisance, E. Boone, K. De Bosscher, M. L. Schmitz, W. Fiers, and G. Haegeman. 1998. p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-kappaB p65 transactivation mediated by tumor necrosis factor. *J Biol Chem* 273:3285-90.
357. Venkataraman, T., M. Valdes, R. Elsby, S. Kakuta, G. Caceres, S. Saijo, Y. Iwakura, and G. N. Barber. 2007. Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. *J Immunol* 178:6444-55.
358. Villanueva, N., J. Navarro, E. Mendez, and I. Garcia-Albert. 1994. Identification of a protein kinase involved in the phosphorylation of the C-terminal region of human respiratory syncytial virus P protein. *J Gen Virol* 75 (Pt 3):555-65.
359. Wang, J. K., E. Kiyokawa, E. Verdin, and D. Trono. 2000. The Nef protein of HIV-1 associates with rafts and primes T cells for activation. *Proc Natl Acad Sci U S A* 97:394-9.
360. Wang, L.-F., Chua, K.B., Yu, M. and Eaton, B.T. 2003. Genome Diversity of Emerging Paramyxoviruses. *Current Genomics* 4:263-273.
361. Waning, D. L., A. P. Schmitt, G. P. Leser, and R. A. Lamb. 2002. Roles for the cytoplasmic tails of the fusion and hemagglutinin-neuraminidase proteins in budding of the paramyxovirus simian virus 5. *J Virol* 76:9284-97.
362. Wansley, E. K., and G. D. Parks. 2002. Naturally occurring substitutions in the P/V gene convert the noncytopathic paramyxovirus simian virus 5 into a virus that induces alpha/beta interferon synthesis and cell death. *J Virol* 76:10109-21.
363. Waris, G., K. D. Tardif, and A. Siddiqui. 2002. Endoplasmic reticulum (ER) stress: hepatitis C virus induces an ER-nucleus signal transduction pathway and activates NF-kappaB and STAT-3. *Biochem Pharmacol* 64:1425-30.
364. Watling, D., H. T. Serafinowska, C. B. Reese, and I. M. Kerr. 1985. Analogue inhibitor of 2-5A action: effect on the interferon-mediated inhibition of encephalomyocarditis virus replication. *EMBO J* 4:431-6.
365. Weber, F., V. Wagner, S. B. Rasmussen, R. Hartmann, and S. R. Paludan. 2006. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. *J Virol* 80:5059-64.
366. Weil, R., H. Sirma, C. Giannini, D. Kremsdorf, C. Bessia, C. Dargemont, C. Brechot, and A. Israel. 1999. Direct association and nuclear import of the hepatitis B virus X protein with the NF-kappaB inhibitor IkappaBalpha. *Mol Cell Biol* 19:6345-54.

367. Welliver, R. C. 2003. Respiratory syncytial virus and other respiratory viruses. *Pediatr Infect Dis J* 22:S6-10; discussion S10-2.
368. Whelan, S. P., J. N. Barr, and G. W. Wertz. 2004. Transcription and replication of nonsegmented negative-strand RNA viruses. *Curr Top Microbiol Immunol* 283:61-119.
369. Williams, B. R., R. R. Golgher, R. E. Brown, C. S. Gilbert, and I. M. Kerr. 1979. Natural occurrence of 2-5A in interferon-treated EMC virus-infected L cells. *Nature* 282:582-6.
370. Wilson, R. L., S. M. Fuentes, P. Wang, E. C. Taddeo, A. Klatt, A. J. Henderson, and B. He. 2006. Function of small hydrophobic proteins of paramyxovirus. *J Virol* 80:1700-9.
371. Wreschner, D. H., T. C. James, R. H. Silverman, and I. M. Kerr. 1981. Ribosomal RNA cleavage, nuclease activation and 2-5A(ppp(A2'p)nA) in interferon-treated cells. *Nucleic Acids Res* 9:1571-81.
372. Xiang, Y., Z. Wang, J. Murakami, S. Plummer, E. A. Klein, J. D. Carpten, J. M. Trent, W. B. Isaacs, G. Casey, and R. H. Silverman. 2003. Effects of RNase L mutations associated with prostate cancer on apoptosis induced by 2',5'-oligoadenylates. *Cancer Res* 63:6795-801.
373. Xu, P., Z. Li, D. Sun, Y. Lin, J. Wu, P. A. Rota, and B. He. Rescue of wild-type mumps virus from a strain associated with recent outbreaks helps to define the role of the SH ORF in the pathogenesis of mumps virus. *Virology*.
374. Yang, Z. Z., O. Tschopp, M. Hemmings-Mieszczak, J. Feng, D. Brodbeck, E. Perentes, and B. A. Hemmings. 2003. Protein kinase B alpha/Akt1 regulates placental development and fetal growth. *J Biol Chem* 278:32124-31.
375. Yin, H. S., R. G. Paterson, X. Wen, R. A. Lamb, and T. S. Jardetzky. 2005. Structure of the uncleaved ectodomain of the paramyxovirus (hPIV3) fusion protein. *Proc Natl Acad Sci U S A* 102:9288-93.
376. Yin, H. S., X. Wen, R. G. Paterson, R. A. Lamb, and T. S. Jardetzky. 2006. Structure of the parainfluenza virus 5 F protein in its metastable, prefusion conformation. *Nature* 439:38-44.
377. Yoeli-Lerner, M., and A. Toker. 2006. Akt/PKB signaling in cancer: a function in cell motility and invasion. *Cell Cycle* 5:603-5.
378. Yoneyama, M., M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K. Taira, E. Foy, Y. M. Loo, M. Gale, Jr., S. Akira, S. Yonehara, A. Kato, and T. Fujita. 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 175:2851-8.
379. Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 5:730-7.
380. You, L. R., C. M. Chen, and Y. H. Lee. 1999. Hepatitis C virus core protein enhances NF-kappaB signal pathway triggering by lymphotoxin-beta receptor ligand and tumor necrosis factor alpha. *J Virol* 73:1672-81.
381. Young, D. F., L. Didcock, S. Goodbourn, and R. E. Randall. 2000. Paramyxoviridae use distinct virus-specific mechanisms to circumvent the interferon response. *Virology* 269:383-90.
382. Yount, J. S., L. Gitlin, T. M. Moran, and C. B. Lopez. 2008. MDA5 participates in the detection of paramyxovirus infection and is essential for the early activation of

- dendritic cells in response to Sendai Virus defective interfering particles. *J Immunol* 180:4910-8.
383. Yuan, P., T. B. Thompson, B. A. Wurzburg, R. G. Paterson, R. A. Lamb, and T. S. Jardetzky. 2005. Structural studies of the parainfluenza virus 5 hemagglutinin-neuraminidase tetramer in complex with its receptor, sialyllactose. *Structure* 13:803-15.
 384. Zamanian-Daryoush, M., T. H. Mogensen, J. A. DiDonato, and B. R. Williams. 2000. NF-kappaB activation by double-stranded-RNA-activated protein kinase (PKR) is mediated through NF-kappaB-inducing kinase and IkappaB kinase. *Mol Cell Biol* 20:1278-90.
 385. Zhang, X., J. M. Bourhis, S. Longhi, T. Carsillo, M. Buccellato, B. Morin, B. Canard, and M. Oglesbee. 2005. Hsp72 recognizes a P binding motif in the measles virus N protein C-terminus. *Virology* 337:162-74.
 386. Zhang, X., C. Glendening, H. Linke, C. L. Parks, C. Brooks, S. A. Udem, and M. Oglesbee. 2002. Identification and characterization of a regulatory domain on the carboxyl terminus of the measles virus nucleocapsid protein. *J Virol* 76:8737-46.
 387. Zheng, X., R. H. Silverman, A. Zhou, T. Goto, B. S. Kwon, H. E. Kaufman, and J. M. Hill. 2001. Increased severity of HSV-1 keratitis and mortality in mice lacking the 2-5A-dependent RNase L gene. *Invest Ophthalmol Vis Sci* 42:120-6.
 388. Zhong, H., R. E. Voll, and S. Ghosh. 1998. Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol Cell* 1:661-71.
 389. Zhou, A., B. A. Hassel, and R. H. Silverman. 1993. Expression cloning of 2-5A-dependent RNAase: a uniquely regulated mediator of interferon action. *Cell* 72:753-65.
 390. Zhou, A., J. Paranjape, T. L. Brown, H. Nie, S. Naik, B. Dong, A. Chang, B. Trapp, R. Fairchild, C. Colmenares, and R. H. Silverman. 1997. Interferon action and apoptosis are defective in mice devoid of 2',5'-oligoadenylate-dependent RNase L. *EMBO J* 16:6355-63.
 391. Zhou, B. P., Y. Liao, W. Xia, B. Spohn, M. H. Lee, and M. C. Hung. 2001. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat Cell Biol* 3:245-52.
 392. Zhou, Z., O. J. Hamming, N. Ank, S. R. Paludan, A. L. Nielsen, and R. Hartmann. 2007. Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. *J Virol* 81:7749-58.

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PUBLICATIONS

- Sun, D., **Luthra, P.**, Xu, P., Yoon, H. and He, B. **Identification of a phosphorylation site of the P protein important for PIV5 mRNA transcription and growth.** 2011 (Journal of Virology, published online ahead of print).
- **Luthra, P.**, Sun, D., Silverman, R.H. and He, B. **Activation of Interferon β expression by a viral mRNA through RNase L and MDA5.** PNAS 108(5): 2118-2123, February 2011.
- Sun, D., **Luthra, P.**, Li, Z. and He, B. **PLK1 down-regulates Parainfluenza Virus 5 gene expression.** PLoS Pathogens 5(7): e1000525, July 2009.
- **Luthra, P.**, Sun, D., Wolfgang, M. and He, B. **AKT-1 dependent activation of NF- κ B by the L protein of parainfluenza virus 5.** Journal of Virology 82(21): 10887-10895, November 2008.
- Fuentes, S., Tran, K. C., **Luthra, P.**, Teng, M. N. and He, B. **Function of the Respiratory Syncytial Virus Small Hydrophobic protein.** Journal of Virology 81(15): 8361- 8366, May 2007.

ACCOMPLISHMENTS

- “The center for vaccine and Immunology Award” at 4th Atlanta Paramyxer, Emory University. 2010
- “Veterinary Medicine Symposium Award”, University of Georgia. 2010
- Travel fellowship from American Society for Virology, 2008-2010
- “Huck Institute of Life Sciences Fellowship”, Pennsylvania State University 2005-2006
- Honors in Biotechnology, GGS Indraprastha University, Delhi, India, 2005

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Protein and Molecular cloning (BMB 432), The Pennsylvania State University
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