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mda-5, but not RIG-I, is a common target for paramyxovirus V proteins

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Abstract

The induction of IFN-β by the paramyxovirus PIV5 (formerly known as SV5) is limited by the action of the viral V protein that targets the cellular RNA helicase mda-5. Here we show that 12 other paramyxoviruses also target mda-5 by a direct interaction between the conserved cysteine-rich C-terminus of their V proteins and the helicase domain of mda-5. The inhibition of IFN-β induction is not species-restricted, being observed in a range of mammalian cells as well as in avian cells, and we show that the inhibition of mda-5 function is also not restricted to mammalian cells. In contrast, the V proteins do not bind to the related RNA helicase RIG-I and do not inhibit its activity. The relative contributions of mda-5 and RIG-I to IFN-β induction are discussed.

Keywords: Interferon; Paramyxoviruses; PIV5; mda-5; RIG-I; V proteins

Introduction

The *Paramyxoviridae* are a family of enveloped viruses with a single-stranded, negative sense RNA genome of between 15 and 19 kb (reviewed by Lamb and Kolakofsky, 2001) and are members of the virus order *Mononegavirales*, which also contains the *Filoviridae* and *Rhabdoviridae*. Several members of the *Paramyxoviridae* family cause significant disease, including measles virus (MeV), mumps virus (MuV) and human respiratory syncytial virus (HRSV) of man and rinderpest virus, Newcastle disease virus (NDV) and bovine respiratory syncytial virus (BRSV) of animals. Recently, paramyxoviruses have been shown to have zoonotic potential with the emergence of Hendra virus (HeV) and Nipah virus (NiV) in Australia and Asia, respectively (reviewed by Eaton et al., 2006).

All viruses must have some form of immune evasion mechanism in order to replicate in the face of the host immune response, and like many other viruses (reviewed by Goodbourn et al., 2000; Hengel et al., 2005) members of the *Paramyxoviridae* have mechanisms for the evasion of the Interferon (IFN) system, a major component of the innate, anti-viral immune response (reviewed by Stock et al., 2004; Conzelmann, 2005). Many members of the *Paramyxovirinae* subfamily use one or more products of their P/V/C gene to antagonize IFN signaling by various mechanisms, including the targeted degradation of STAT proteins by the V proteins of Rubulaviruses such as simian virus 5 (SV5, recently renamed as parainfluenza virus 5, PIV5; Chatziandreou et al., 2004), human parainfluenza virus type 2 (hPIV2) and MuV, disruption of STAT phosphorylation by Sendai virus (SeV) C proteins, and sequestration of STATs in high molecular weight complexes observed for the V, W and P proteins of HeV and NiV.

Many paramyxoviruses also target the production of type I IFN. The V proteins of PIV5, hPIV2 and SeV have been shown to limit IFN-β induction by the synthetic dsRNA, poly(rI)–poly(rC) (Poole et al., 2002), and this property is shared by the SeV C protein (Strahle et al., 2003; Komatsu et al., 2004), and the

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NS1/NS2 pairing in RSV (Bossert et al., 2003; Spann et al., 2004; Spann et al., 2005). IFN-β induction has been studied in great detail and requires the activation of NF-κB and IRF-3 from cytoplasmic pools that relocate to the nucleus and associate on the IFN-β promoter with other architectural factors to form the enhanceosome complex (reviewed by Merika and Thanos, 2001). Two signaling pathways have been identified that mediate dsRNA responses upstream of the IRF-3 and NF-κB activation node (reviewed by Kawai and Akira, 2006). Extracellular dsRNA, or dsRNA present in endosomal compartments, recognizes and activates a transmembrane protein called Toll-like receptor 3 (TLR3) that recruits an adaptor molecule called TRIF. TRIF in turn recruits NAP1 (Sasai et al., 2005), a protein that can act as an adaptor for the recruitment of kinases named TBK-1 and IKKi that can interact with and phosphorylate IRF-3 (Fitzgerald et al., 2003; Sharma et al., 2003). TRIF also interacts with TRAF6 and RIP to activate NF-κB. In addition, cells possess a TLR3-independent pathway (Li et al., 2005a; Melchjorsen et al., 2005; Schroder and Bowie, 2005) that responds to dsRNA apparently generated in the cytoplasm by viral replication. This pathway utilizes two RNA helicase molecules (retinoic acid-inducible gene-I, RIG-I and melanoma differentiation-associated gene-5, mda-5; Andrejeva et al., 2004; Yoneyama et al., 2004; Yoneyama et al., 2005; Kato et al., 2005) that can directly recognize dsRNA. When activated by dsRNA they recruit and activate an adaptor called Cardif/VISA/MAVS/IPS-1 (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005; reviewed by Hiscott et al., 2006; Johnson and Gale, 2006). Like TRIF, this adaptor is upstream of the IRF-3 and NF-κB pathways, but the details of activation are currently unclear.

We previously demonstrated that PIV5 (strain W3A) infections are poor at inducing IFN-β, and that the V protein inhibits the activation of both IRF-3 and NF-κB in response to dsRNA (He et al., 2002; Poole et al., 2002). We also demonstrated that the V proteins of hPIV2 and SeV which have highly conserved cysteine-rich carboxy-termini, have similar properties. Subsequently, we identified mda-5 as an interaction partner for the PIV5 V protein as well as for the V proteins of hPIV2, SeV, MuV and HeV (Andrejeva et al., 2004). Interaction with the cysteine-rich C-terminus of PIV5 V blocks the ability of mda-5 to stimulate IRF-3 and NF-κB activation both constitutively and in response to dsRNA. mda-5 (also known as HELICARD, RH116 and IFN-inducible helicase 1; IFIH-1) is a member of the DExD/H-box RNA helicase family that can bind to dsRNA and has two CARD homologies at its N-terminus. Its closest relative, RIG-I, has a similar arrangement of N-terminal CARD homologies and a C-terminal dsRNA binding RNA helicase domain and has also been demonstrated to promote the activation of IRF-3 and NF-κB in response to dsRNA (Yoneyama et al., 2004). To date, no viral inhibitors have been reported for RIG-I. Studies on HCV have identified the downstream adaptor protein of RIG-I and mda-5, termed Cardif/VISA/MAVS/IPS-1, as a target for viral interference, and in this case the protein is directly proteolytically cleaved by the NS3/4a protease (Li et al., 2005b; Meylan et al., 2005), causing release from mitochondrial membranes and loss of association with RIG-I (Lin et al., 2006; Loo et al., 2006). Since Cardif/VISA/MAVS/IPS-1 is downstream of both mda-5 and RIG-I, HCV prevents activation by both pathways. In the case of paramyxoviruses, blocking mda-5 could leave the possibility of IFN-β induction in response to RIG-I activation. Although we did not detect RIG-I interaction with the PIV5 V protein in our previous study (Andrejeva et al., 2004), we did not specifically investigate this possibility. In this paper, we demonstrate that interaction of paramyxovirus V proteins with mda-5 is seen in every case examined, and moreover that this interaction inhibits mda-5 function. In contrast, none of the V proteins examined interacted with or inhibited RIG-I.

Results

The V proteins of thirteen paramyxoviruses inhibit IFN-β induction

We have previously shown that the V protein of PIV5 could limit the induction of IFN-β both in the context of virus infection and in response to the synthetic dsRNA polyinosinic. polycytidylic acid (poly(rI)–poly(rC); He et al., 2002; Poole et al., 2002). We reasoned that this inhibition was operating at the level of an upstream signal transduction component since the activation of both IRF-3 and NF-κB was affected. We subsequently demonstrated that the V protein of PIV5 interacted with the RNA helicase mda-5 and inhibited its function as an activator of endogenous IFN-β gene transcription by preventing the activation of both NF-κB and IRF-3 (Andrejeva et al., 2004).

In addition to PIV5, we demonstrated that the V proteins of the closely related Rubulavirus hPIV2 and the Respirovirus SeV could also limit poly(rI)–poly(rC) signaling (Poole et al., 2002) and that the V proteins of a number of paramyxoviruses (specifically PIV5, hPIV2, SeV, MuV and HeV) could interact with mda-5. To test whether this binding in all cases correlated with inhibition of IFN-β induction, we first investigated whether expression of the V proteins was sufficient to interfere with efficient activation of the IFN-β promoter. Fig. 1A shows that the V proteins of PIV5, hPIV2, SeV, MuV and HeV all restricted induction of a co-transfected IFN-β promoter reporter plasmid by poly(rI)–poly(rC) in Vero cells. Additionally, we made similar observations for the V proteins of eight other paramyxoviruses (Measles, Nipah, NDV, Porcine Rubulavirus-PoRV, Menangle, Mapuera, Tioman and Salem; Fig. 1A). As previously seen for the V protein of PIV5, the inhibition appeared to be maximal at 80–85% and we were unable to increase this by further overexpression of any V protein (data not shown). We had also previously shown that the PIV5-mediated block was a property of the cysteine-rich C-terminus of the V protein. We investigated whether similar conclusions could be drawn for other V proteins and Fig. 1B shows that the same effect is seen for MuV, MapV and NiV. Thus inhibition is a property of the C-terminal region and can be mapped down to a domain of no more than 60 amino acids, a cysteine-rich domain that is the most highly conserved region amongst paramyxoviral V proteins (Fig. 1C).

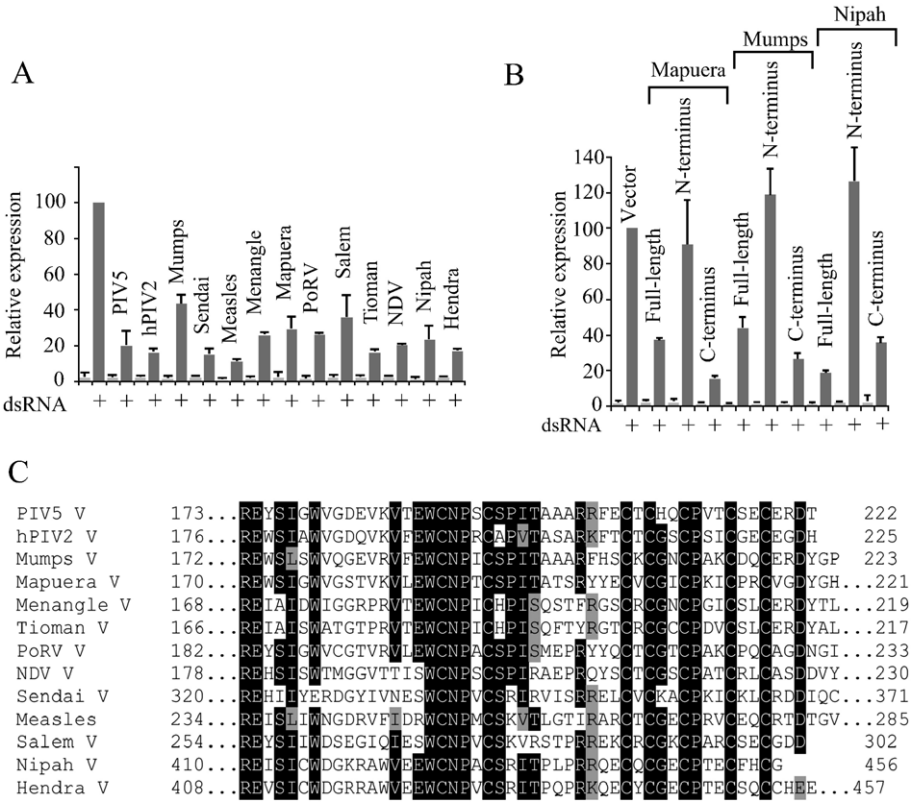

Fig. 1. Paramyxovirus V proteins inhibit IFN-β induction. (A) Vero cells were transfected with a reporter for IFN-β promoter activity, (pIFΔ(−116)lucter), the β-galactosidase expression vector pJATlac and either an expression plasmid driving the overexpression of the indicated paramyxovirus V protein, or the control "empty vector" pEF.plink2. Transfected cells were either mock-treated or transfected with the synthetic dsRNA poly(rI)–poly(rC) and cell extracts prepared. (B) Experiments were performed identically to (A) except that N- or C-terminal truncations of Mapuera, Mumps or Nipah V proteins were used as effectors. For each of panels (A) and (B) luciferase and β-galactosidase activities were determined from cellular extracts and relative expression values calculated accordingly. A reference value of 100 has been assigned to the level of expression seen with poly(rI)–poly(rC)-treated control cells. Values shown represent data from at least three independent experiments with the standard error of the mean indicated. (C) Comparison of conserved cysteine-rich C-terminal domains of paramyxovirus V proteins. Amino acids highlighted in black are conserved in at least seven V proteins, while amino acids highlighted in grey are conservative changes.

The V proteins of all paramyxoviruses tested interact with mda-5

We next extended our observations to look at the ability of the V proteins to interact with mda-5 by using both the yeast two-hybrid assay and co-immunoprecipitation of tagged proteins from transfected cells. The yeast two-hybrid data are shown in Fig. 2A and demonstrate interaction between mda-5 and all 13 paramyxovirus V proteins tested. Fig. 2B shows an example of co-immunoprecipitation between a protein with the electrophoretic mobility of mda-5 and the V proteins of Menangle and Tioman viruses. We have previously shown interactions between mda-5 and the V proteins of PIV5, hPIV2, SeV, MuV and HeV (Andrejeva et al., 2004) by immunoprecipitation, and we have also demonstrated interactions between mda-5 and the V proteins of MeV, NDV, Nipah, Mapuera, Salem and PoRV using this technique (data not shown). Thus using both assays all 13 V proteins have been shown to interact with mda-5, suggesting that this interaction may be universal.

We have previously shown that alanine substitution of cysteines 193 or 207 in the C-terminus of the PIV5 V protein severely impaired the inhibition of IFN-β induction (Poole et al., 2002). To address the significance of the interaction between V and mda-5, we investigated whether these point mutations affect mda-5 binding. Fig. 3 shows that alanine substitution of cysteines 193 or 207 abolishes mda-5 binding, and that this correlates with the inability of these mutants to limit IFN-β induction by mda-5.

V proteins limit the induction of IFN-β in response to poly(rI)–poly(rC) in cells from a variety of species

Given that all the V proteins we examined, including NDV (an avian virus), interacted with human mda-5, we reasoned that it is unlikely that the species tropism seen with these viruses could be due to species differences in their ability to inhibit mda-5 activation of IFN-β production (in contrast to the demonstrated properties of the V proteins to block IFN signaling). To test this directly, we examined the ability of the V protein of PIV5 to block induction of a co-transfected IFN-β promoter in cells derived from a variety of species. Fig. 4 shows that it was able to limit induction in cells from human (Fig. 4A), murine (Fig. 4B), bovine (Fig. 4C) and avian sources (Fig. 4D); we have also shown that the PIV5 V protein

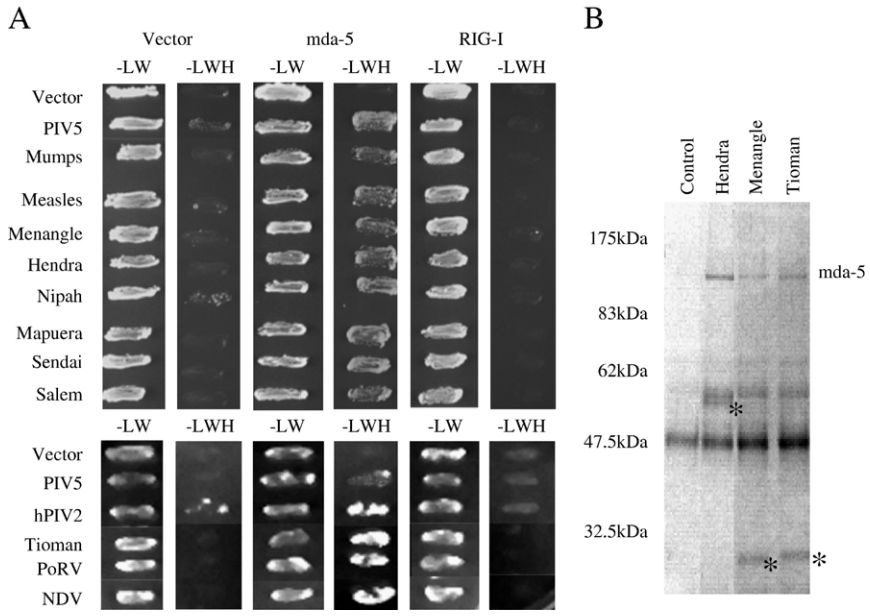

Fig. 2. Paramyxovirus V proteins interact with mda-5 but not RIG-I. (A) Interactions between viral V proteins and either mda-5 or RIG-I were analyzed in the yeast two-hybrid system. Yeast cells were transformed with a plasmid expressing mda-5 or RIG-I fused to the Gal4 DNA binding domain (DBD) or the empty vector pGBKT7 expressing the Gal4 DBD alone, and a plasmid expressing the indicated paramyxovirus V protein fused to the Gal4 activation domain (AD) or the empty vector pGADT7 expressing the Gal4AD alone. Growth on synthetic drop-out medium lacking leucine and tryptophan (−LW) is indicative of successful transformation of yeast by both GAL4 DBD plasmids (bait) and GAL4 AD plasmids (prey). Growth on synthetic drop-out medium lacking leucine, tryptophan and histidine and containing 10 mM 3-aminotriazole (−LWH) is indicative of a productive interaction between bait and prey. The top panel shows interactions in the yeast strain PJ69-4α, and the indicated viral V proteins gave little or no background transactivation in this strain. Since the V proteins of hPIV2, Tioman, Porcine Rubulavirus (PoRV) and NDV gave limited background transactivation (data not shown), we were unable to rigorously test interactions in PJ69-4α; the lower panel shows the interactions in yeast strain CG1945, where background transactivation was not observed. (B) 293 cells were co-transfected with a plasmid that expresses mda-5 (pEF.mda-5) and a plasmid that expresses the myc-tagged V proteins of Hendra, Menangle or Tioman virus, or the empty vector (pEF.plink2-control). Cell extracts were subjected to immunoprecipitation with the 9E10 monoclonal antibody to the myc tag. After separation on a 4–12% gradient SDS-PA gel, the total polypeptides present in the immune precipitates were visualized by Coomassie stain. The mobility of the mda-5 protein is indicated to the right of the panel and the V proteins are highlighted with an asterisk.

can limit induction of a co-transfected IFN-β promoter by transfected poly(rI)–poly(rC) in all other human cell lines tested (HeLa, 293, 293TLR3, HEp2, HEC-1b, unpublished data). We additionally tested the properties of NDV V in avian cells (Fig. 4D) and showed that it is also capable of limiting the poly(rI)–poly(rC) response. Thus inhibition of IFN-β induction appears a common feature of paramyxoviral V proteins. However, it is also of note that in all cases tested IFN-β induction could not be reduced to zero (even with saturating amounts of V; data not shown), and that the magnitude of the inhibition varied between cell lines, although it was reproducible within a cell type. These data suggest that the target for the V proteins is not the only route to IFN-β induction in response to poly(rI)–poly(rC).

Paramyxovirus V proteins inhibit the activity of mda-5 in both mammals and birds

The above data demonstrated that the V proteins are capable of limiting poly(rI)–poly(rC) responses in a manner that is not restricted to the host species. We next investigated whether the V proteins were able to inhibit direct IFN-β promoter transactivation by mda-5. Fig. 5A shows that activation by human mda-5 in Vero cells could be blocked by the V proteins of all paramyxoviruses tested, including from the avian virus NDV. Interestingly, when we attempted to test whether the V proteins could block mda-5 activity in avian cells, we discovered that human mda-5 was unable to activate the chicken IFN-2 promoter in these cells (Fig. 5B). Thus, we could not initially determine whether the limitation of poly (rI)–poly(rC) signaling seen in avian cells (Fig. 4D) was a result of blocking mda-5. We hypothesized that the failure of human mda-5 to function in avian cells reflected sequence differences between human and avian mda-5s such that the former could not signal effectively. Consistent with this, when we cloned and sequenced chicken mda-5, we noted considerable sequence divergence between the human and avian N-terminal effector CARD domains (43% amino acid identity; see Fig. 5D) that interact with Cardif/VISA/MAVS/IPS-1; in contrast, the C-terminal helicase domains showed a relatively high degree of conservation (67% amino acid identity with extended regions of over 80%). When avian mda-5 was overexpressed in avian cells, it could indeed transactivate the IFN-2 promoter and, significantly, this activity could be inhibited by the V proteins of both PIV5 and NDV (Fig. 5B). Interestingly, similar to the inability of human mda-5 to function in avian cells, the avian mda-5 was unable to function in Vero cells (Fig. 5C). Thus, although human and avian mda-5s activate the IFN promoter in a species-specific manner, they are blocked equally well by the V proteins of human and avian

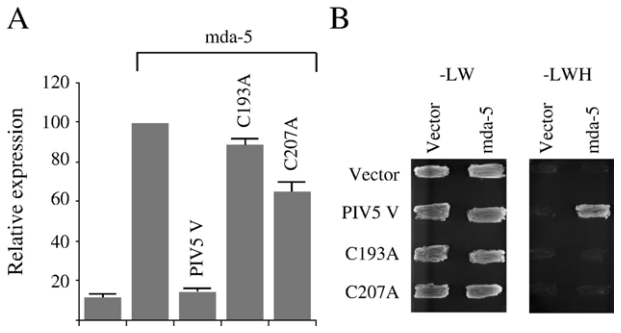

Fig. 3. The conserved cysteines of PIV5 V are required for interaction with and inhibition of mda-5. (A) Vero cells were transfected with a reporter for IFN-β promoter activity, (pIFΔ(−116)lucter), the β-galactosidase expression vector pJATlac, an expression plasmid for mda-5 (pEF.mda-5) and either a plasmid driving the expression of the wild-type PIV5 V or PIV5 V containing cysteine/alanine substitutions, or the control "empty vector" pEF.plink2. Cell extracts were prepared after 48 h and luciferase and β-galactosidase activities were determined and relative expression values calculated accordingly. A reference value of 100 has been assigned to the level of expression seen in mda-5 transfected cells. Values shown represent data from at least three independent experiments with the standard error of the mean indicated. (B) Interactions between the wild-type PIV5 V protein or PIV5 V containing cysteine/alanine substitutions and mda-5 were analyzed in the yeast two-hybrid system. Yeast cells (strain PJ69-4α) were transformed with a plasmid expressing mda-5 fused to the Gal4 DBD, or the empty vector pGBKT7, and a plasmid expressing the indicated V protein fused to the Gal4 AD or the empty vector pGADT7. Growth on synthetic drop-out medium lacking leucine and tryptophan (−LW) is indicative of successful transformation of yeast by GAL4 DBD plasmids (bait) and GAL4 AD plasmids (prey). Growth on synthetic drop-out medium lacking leucine, tryptophan and histidine and containing 10 mM 3-aminotriazole (−LWH) is indicative of productive interaction between bait and prey.

paramyxoviruses. These results underline the importance of mda-5 as a target for viral interference.

Paramyxovirus V proteins do not interact with or inhibit the activity of RIG-I

Since our original observations on the ability of the PIV5 V protein to block mda-5 function, it has become clear that another RNA helicase, RIG-I (also called DDX58), can also play a role in IFN induction. We were therefore interested in determining whether the paramyxovirus V proteins also targeted RIG-I. We thus cloned the cDNA for human RIG-I and co-transfected it into Vero cells with an IFN-β promoter reporter plasmid. Fig. 6A shows that RIG-I strongly transactivated the IFN-β reporter in Vero cells and that this effect was enhanced in the presence of poly(rI)–poly(rC). Furthermore, there were no significant differences between the properties of RIG-I and mda-5 in these cells. To investigate the effects of V proteins on RIG-I function, we co-expressed the V proteins in transient transfections and showed that mostly they were unable to exert a significant block on IFN-β induction by RIG-I; the possible exception to this is the V protein of SeV, which lowered RIG-I function to about 60% (Fig. 6B). Consistent with the inability to block RIG-I function, we were unable to detect an interaction between RIG-I and any of the V proteins tested (see Fig. 2). In contrast to the V proteins, the NS3/4a gene product of Hepatitis C Virus lowered the activity of RIG-I, as expected for a protein that targets Cardif//VISA/MAVS/IPS-1 the downstream adaptor for both mda-5 and RIG-I.

The V protein of PIV5 targets the helicase domain of mda-5

Previous observations on the properties of mda-5 suggest a model in which dsRNA binding to the helicase domain triggers activation of the effector CARD domain (Andrejeva et al., 2004; Yoneyama et al., 2005). Since the V proteins prevent activation of mda-5, it seemed likely that the V proteins interact with the helicase domain of mda-5. To test this directly, we performed either immunoprecipitation experiments on co-transfected cells (Fig. 7A) or yeast two-hybrid assays (Fig. 7B). Both approaches demonstrated that the C-terminal helicase domain of human mda-5 is sufficient for strong interaction with the V protein of PIV5; we were unable to detect interaction with the CARD domain in either assay (data not shown). These data offer an explanation of our observations that while avian mda-5 and human mda-5 demonstrate species-specificity in their ability to activate the IFN-β promoter, they are equally sensitive to the inhibitory effects of V proteins since the conserved C-terminal regions of the mda-5s would facilitate interactions with V.

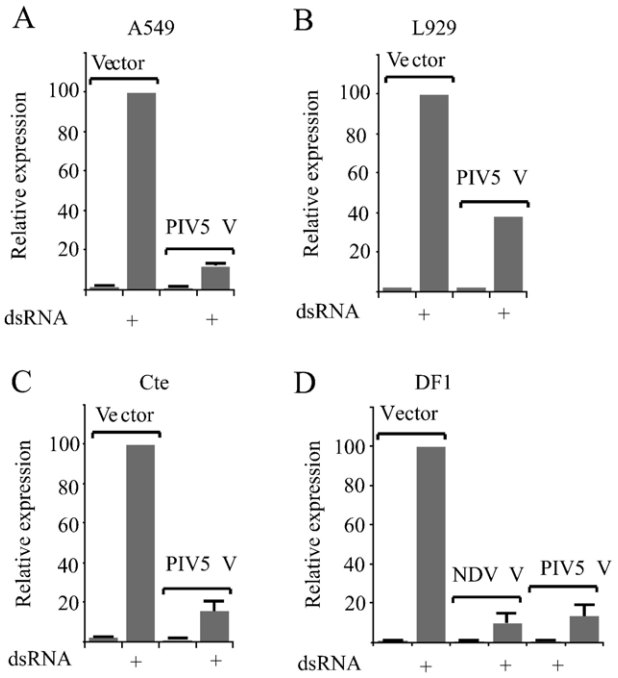

Fig. 4. V proteins limit the induction of IFN-β in response to poly(rI)–poly(rC) in cells from a variety of species. (A) A549 cells (human), (B) mouse L929 cells, (C) calf testes (Cte) cells and (D) chicken DF1 cells were transfected with a reporter for IFN-β promoter activity, (pIFΔ(−116)lucter) (or the chicken IFN-2 promoter reporter pCh.IFN-2IFNΔlucter in the case of DF1 cells), the β-galactosidase expression vector pJATlac, and either an expression plasmid driving the overexpression of the indicated paramyxovirus V protein, or the control "empty vector" pEF.plink2. Transfected cells were either mock-treated or transfected with poly(rI)–poly(rC) and cell extracts prepared. Luciferase and β-galactosidase activities were determined from cellular extracts and relative expression values calculated accordingly. A reference value of 100 has been assigned to the level of expression seen with poly(rI)–poly(rC)-treated control cells. Values shown represent data from at least three independent experiments with the standard error of the mean indicated.

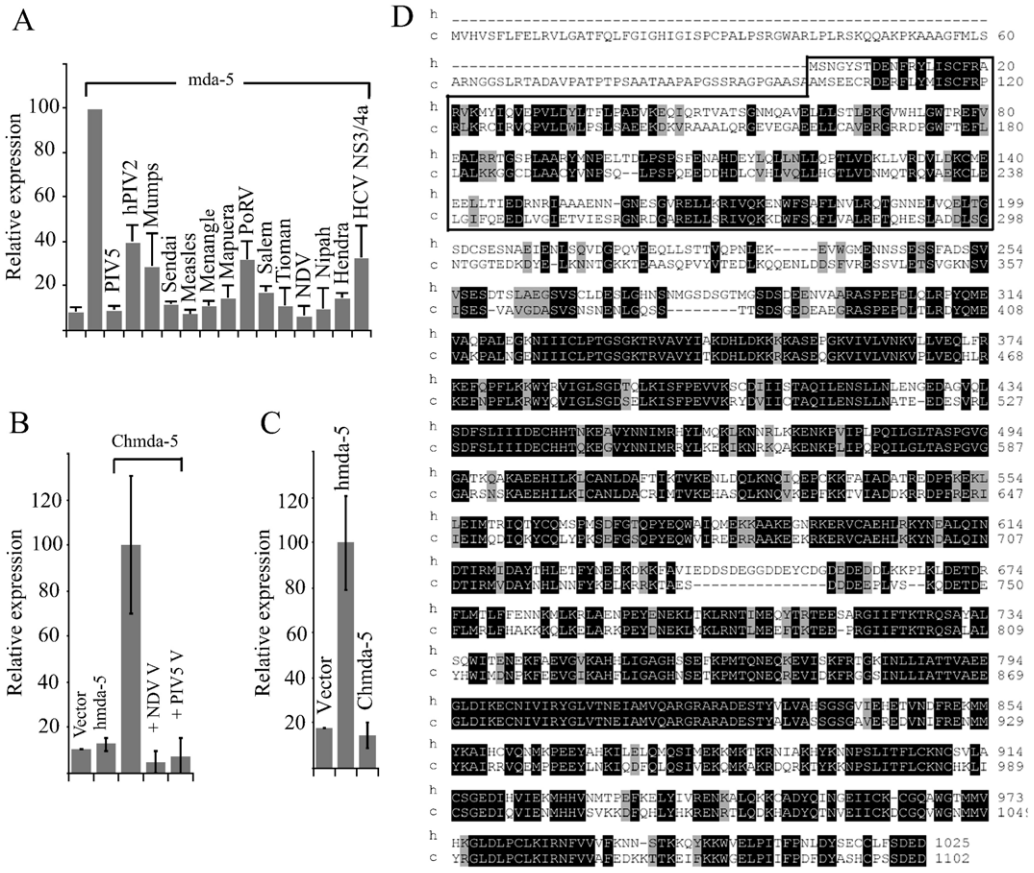

Fig. 5. Paramyxovirus V proteins inhibit the activity of human and avian mda-5. (A) Vero cells were transfected with a reporter for IFN-β promoter activity, (pIFΔ(−116)lucter), the β-galactosidase expression vector pJATlac, an expression plasmid for mda-5 (pEF.mda-5) and either a plasmid driving the expression of the indicated V protein, HCV NS3/4a or the control "empty vector" pEF.plink2. (B) Chicken DF1 fibroblasts were transfected with a reporter for avian IFN-2 promoter activity, (pCh.IFN-2IFNΔlucter), the β-galactosidase expression vector pJATlac, an expression plasmid for human mda-5 (pEF.mda-5) or chicken mda-5 (pEF. Chmda-5) and either an expression plasmid for NDV V or PIV5 V or the control "empty vector" pEF.plink2. (C) Vero cells were transfected with a reporter for IFN-β promoter activity, (pIFΔ(−116)lucter), the β-galactosidase expression vector pJATlac, an expression plasmid for human mda-5 (pEF.mda-5) or chicken mda-5 (pEF. Chmda-5) or the control "empty vector" pEF.plink2. For each of panel A, B and C, cell extracts were prepared after 48 h and luciferase and β-galactosidase activities were determined and relative expression values calculated accordingly. A reference value of 100 has been assigned to the level of expression seen in mda-5 transfected cells. Values shown represent data from at least three independent experiments with the standard error of the mean indicated. (D) A comparison of human (h) and chicken (c) mda-5 ORFs; amino acids highlighted in black are conserved, while amino acids highlighted in grey are conservative changes. The CARD domain is boxed.

Since the V proteins are able to block the activation of IFN-β by mda-5, but not by RIG-I, and yet both of the helicase proteins signal to a common adaptor (Cardif/VISA/MAVS/IPS-1) through their N-terminal CARD domains, it was of interest to determine the properties of mda-5/RIG-I chimaeras. We therefore constructed chimaeras in which the CARD domains were swapped (Fig. 7C). Fig. 7D shows that chimaeras comprising the effector CARD domain of mda-5 with the rest of the protein derived from RIG-I (m/R in Fig. 7C) or comprising the effector CARD domain of RIG-I with the rest of the protein derived from mda-5 (R/m in Fig. 7C) were as effective as intact mda-5 or RIG-I at stimulating basal or poly(rI)–poly(rC)-responsive IFN-β promoter activity. However, when we tested the sensitivity of the chimaeras to inhibition by the V protein of PIV5, we discovered that only the R/m version was affected (Fig. 7E). These data indicate that the CARD domains of mda-5 and RIG-I are fully interchangeable with respect to their ability to stimulate IFN-β expression, but only the C-terminus of mda-5 is sensitive to inhibition by the PIV5 V protein, consistent with the location of the site of protein–protein interaction.

Discussion

The data presented here show that the V proteins of all thirteen paramyxoviruses tested interact with the cellular RNA helicase mda-5 via their highly conserved cysteine-rich C-terminal domains. The consequence of this interaction is to inhibit the known function of this helicase, namely the activation of transcription of the IFN-β gene. In contrast, the V proteins do not interact with the related helicase RIG-I and nor do they inhibit its activity.

The relationship between mda-5, RIG-I and the Cardif/VISA/MAVS/IPS-1 adaptor protein is the subject of intense investigation, the key question being whether the helicases are redundant parallel sensors or whether they sense different viral-derived signals. Consistent with the results described above, in

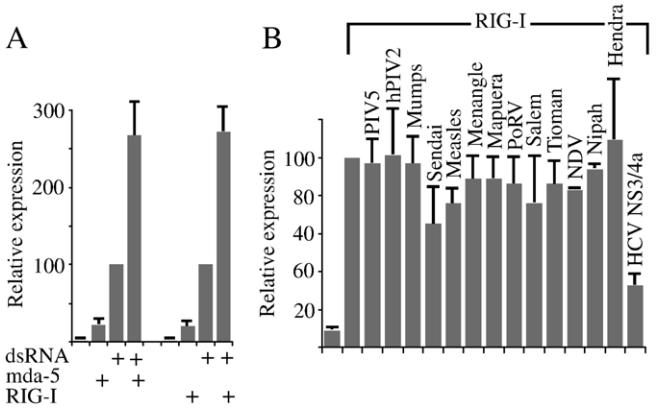

Fig. 6. Paramyxovirus V proteins do not inhibit RIG-I function. (A) Vero cells were transfected with a reporter for IFN-β promoter activity, (pIFΔ(−116) lucter), the β-galactosidase expression vector pJATlac and either a plasmid driving the expression of mda-5 (pEF.mda-5) or RIG-I (pEF.RIG-I), or the control "empty vector" pEF.plink2. Transfected cells were either mock-treated or transfected with poly(rI)–poly(rC) and cell extracts prepared. (B) Vero cells were transfected with a reporter for IFN-β promoter activity, (pIFΔ(−116) lucter), the β-galactosidase expression vector pJATlac, an expression plasmid for RIG-I (pEF.RIG-I) and either a plasmid driving the expression of the indicated V protein, HCV NS3/4a, or the control "empty vector" pEF.plink2. Cell extracts were prepared after 48 h and luciferase and β-galactosidase activities were determined and relative expression values calculated accordingly. A reference value of 100 has been assigned to the level of expression seen in poly(rI)–poly(rC)-treated cells (A) or RIG-I-transfected cells (B). Values shown represent data from at least three independent experiments with the standard error of the mean indicated.

cell culture systems, both mda-5 and RIG-I have been reported to signal responses to transfected poly(rI)–poly(rC) (Andrejeva et al., 2004; Yoneyama et al., 2004; Yamashita et al., 2005; Yoneyama et al., 2005; Cardenas et al., 2006; Taima et al., 2006). Indeed, in the results reported here, we cannot distinguish between the properties of mda-5 and RIG-I with respect to their ability to signal in response to transfected poly (rI)–poly(rC), and in agreement with this chimaeras between mda-5 and RIG-I with swapped CARD effector domains are similarly indistinguishable. Thus it seems likely that signaling through RIG-I is responsible for the poly(rI)–poly(rC)-induced activity that is resistant to blocking by the V protein of PIV5 in tissue culture cells. However, studies on knockout mice indicate that mda-5 plays a much more important role than RIG-I in the regulation of total serum type I IFN levels in response to injected poly(rI)–poly(rC) (Kato et al., 2006), in IFN-β production in poly(rI)–poly(rC)-treated dendritic cells or macrophages (Gitlin et al., 2006; Kato et al., 2006), and in poly(rI)–poly(rC)-transfected embryo-derived fibroblasts (Kato et al., 2006), but curiously RIG-I appears to play a limited role in poly(rI)–poly(rC) responses in these systems (Kato et al., 2006). This discrepancy with the cell culture results remains unresolved and is something we are actively addressing.

In contrast to poly(rI)–poly(rC), the relative roles of mda-5 and RIG-I in signaling responses to viral infections are less clear. Ectopic overexpression experiments or gene knockouts achieved in cell culture using siRNA or dominant interfering forms of factors have shown a role for both RIG-I and mda-5 in IFN induction by NDV and Sendai virus (Andrejeva et al., 2004; Yoneyama et al., 2004; Yoneyama et al., 2005; Melchjorsen et al., 2005), while mda-5, but not RIG-I, has been shown to be required for IFN-β induction by measles virus (Berghall et al., 2006) and picornaviruses (Kato et al., 2006). Furthermore, it has recently been shown that West Nile Virus replication is still controlled in the absence of RIG-I (Fredericksen and Gale, 2006), although this has yet to be linked to signaling through mda-5. Interestingly, the available data on a direct comparison suggest that inhibiting RIG-I has a greater effect than inhibiting mda-5 on limiting the production of IFN. Thus RIG-I inhibition causes a greater block to NDV-induced IFN yield than a block to mda-5 function (Yoneyama et al., 2005). Our own observations on an extensive panel of RNA viruses would suggest that impairing either mda-5 or RIG-I leads to a reduction in IFN yield, although with many viruses disruption of RIG-I function has a greater impact, although the reasons for this remain unclear (unpublished data). Experiments on knock-out mice show that mda-5 is essential for IFN production in response to picornaviruses (Gitlin et al., 2006; Kato et al., 2006), but may play a less important role in responses to other virus types, where RIG-I is critical (Kato et al., 2006). However, such results need to be interpreted cautiously since the viruses under study will often encode inhibitors of IFN induction. Indeed, in the case of many paramyxovirus infections (including NDV, Sendai and measles) the expression of the V protein will give the misleading impression that mda-5 makes only a limited contribution to IFN induction.

It is also important to stress that different viruses, or different virus preparations, might produce distinct types of IFN inducing signal, and if so, that this might influence the apparent contribution of RIG-I and mda-5 to IFN induction. It has been commonly held since the mid-1970s that the proximal inducer for IFN is dsRNA (reviewed in Marcus, 1983), produced as a result of viral transcription or replication. However, it is clear from studies on paramyxoviruses that different preparations of viruses vary significantly in their potential for IFN induction with optimal induction being a property of defective interfering (DI) particles (Johnston, 1981; Poole et al., 2002; Strahle et al., 2006). Significantly, a recent study has shown that maximal induction of IFN by SeV is achieved by preparations rich in copyback genomes that self-anneal and form dsRNA (Strahle et al., 2006). By contrast, preparations that are low in DI particles are poor inducers (Johnston, 1981; Strahle et al., 2006). Thus the contribution of distinct sensors to IFN inducers will differ according to the nature of the virus preparation examined, especially if RIG-I and mda-5 are activated by RNA molecules with distinct structures, as recently suggested (Kato et al., 2006).

In the context of paramyxovirus infection, our results suggest that it is more important for them to inhibit mda-5 function than RIG-I. Indeed, a critical role for mda-5 is supported by the observation that viruses that are defective in their expression of the V protein make a substantially increased yield of IFN (He et al., 2002; Poole et al., 2002; Komatsu et al., 2004). Since available evidence suggests that RIG-I can play a role in IFN induction by paramyxoviruses (Andrejeva et al., 2004; Yoneyama et al., 2004; Melchjorsen et al., 2005; Yoneyama et al., 2005, and our unpublished data), it is intriguing that many

paramyxoviruses do not appear to inhibit RIG-I, at least through their V proteins. There are a number of potential explanations for these observations. Firstly, paramyxoviruses may have other as yet uncharacterized mechanisms to limit the production of IFN. Secondly, paramyxoviruses may be able to survive and propagate in an environment in which limited amounts of IFN are made in a presumably RIG-I-dependent manner; in support of this is the fact that most paramyxoviruses encode mechanisms to disable IFN signaling, and in the case of PIV5 can even dismantle a pre-established IFN-dependent anti-viral state (Didcock et al., 1999; Carlos et al., 2005). Thirdly, both mda-5 and RIG-I are IFN-inducible genes (Kang et al., 2002; Berghall et al., 2006; Matikainen et al., 2006; Siren et al., 2006; Veckman et al., 2006) and thus by blocking IFN signaling paramyxoviruses will prevent the IFN-dependent upregulation of both RIG-I and mda-5 (as well as other members of the IFN induction cascade) and will severely limit the eventual production of IFN by an infected cell.

Materials and methods

Cells, transfections and inductions

Vero, 293FT, A549, L929, DF1 and primary calf testes cells were maintained in Dulbecco's modification of Eagle's medium + 10% fetal bovine serum and penicillin/streptomycin.

Transfections were carried out in 6 well tissue culture dishes using either Lipofectamine (InVitrogen) or Polyfect (Qiagen) with a total of 0.5 μg or 1.5 μg of DNA, respectively, under the manufacturer's recommended conditions. For induction of cells by synthetic dsRNA, poly(rI)–poly(rC) (Amersham Biosciences) was diluted to a final concentration of 2 μg/ml in serum-free medium containing 0.8% Lipofectamine, and added to cells for 2 h. After this period, the medium was replaced with medium containing 2% fetal bovine serum and induction continued for a further 13 h before harvesting.

Plasmids

The reporter plasmids for the IFN-β promoter, pIFΔ(−116) lucter (King and Goodbourn, 1994) and the constitutive β-galactosidase reporter plasmid, pJATlacZ (Masson et al., 1992) have been previously described. The chicken IFN-2 promoter reporter (pChIFN-2lucter) was constructed from Chick Embryo Fibroblast genomic DNA by PCR using Accuprime Pfx DNA polymerase (InVitrogen) using primers that permitted amplification of a fragment from −158 to +14 of the chicken IFN-2 promoter (Sick et al., 1998). Primers incorporated *Bgl*II and

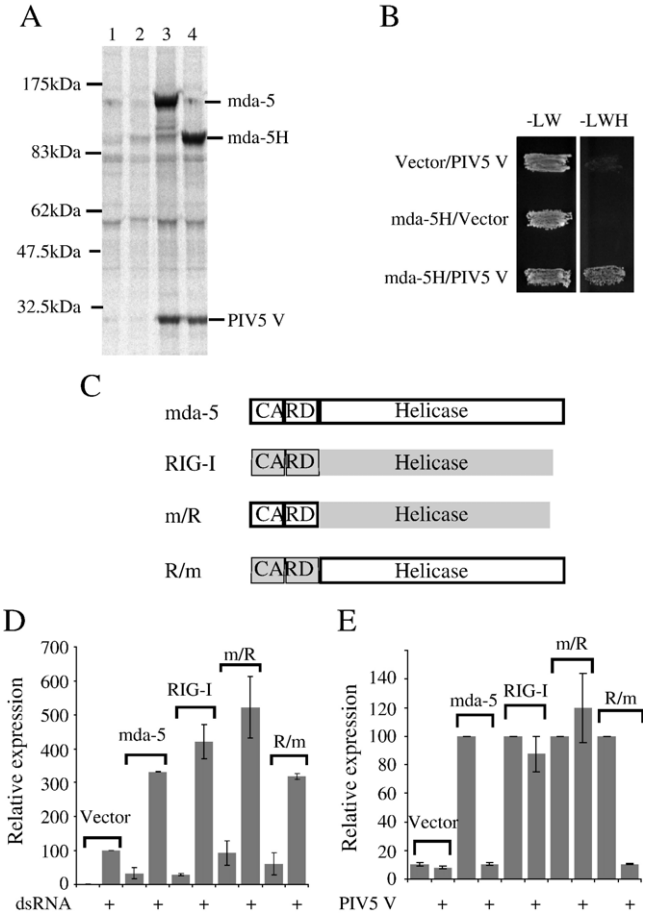

Fig. 7. The V protein of PIV5 targets the helicase domain of mda-5. (A) 293 cells were co-transfected with either a plasmid that expresses full-length mda-5 (pEF.mda-5, lanes 1 and 3), or the helicase domain of mda-5 (mda-5H; pEF.mda-5ΔN—Andrejeva et al., 2002, lanes 2 and 4) and a plasmid that allows expression of the V protein of PIV5 (pEF.SV5/V). Transfected cells were metabolically labeled with L-(^{35}S)methionine and subjected to immunoprecipitation with either a control antibody (lanes 1 and 2) or an antibody (anti-Pk) to the V protein (lanes 3 and 4). After separation on a 4–12% gradient SDS-PA gel, the labeled polypeptides were visualized by autoradiography. The mobilities of the full-length mda-5 protein, the mda-5 helicase domain (mda-5H) and the PIV5 V protein are indicated to the right of the panel. (B) Interactions between the PIV5 V protein and either mda-5 or the mda-5 helicase domain were analyzed in the yeast two-hybrid system. PJ69-4α cells were transformed with a plasmid expressing the mda-5 helicase domain (mda-5H) fused to the Gal4 DNA binding domain (DBD) or the empty vector pGBKT7 expressing the Gal4 DBD alone, and a plasmid expressing the PIV5 V protein fused to the Gal4 activation domain (AD) or the empty vector pGADT7 expressing the Gal4AD alone. Growth on synthetic drop-out medium lacking leucine and tryptophan (−LW) is indicative of successful transformation of yeast by both GAL4 DBD plasmids (bait) and GAL4 AD plasmids (prey). Growth on synthetic drop-out medium lacking leucine, tryptophan and histidine and containing 10 mM 3-aminotriazole (−LWH) is indicative of a productive interaction between bait and prey. (C) Arrangement of the mda-5/RIG-I chimaeras used in panels D and E. (D) Vero cells were transfected with a reporter for IFN-β promoter activity, (pIFΔ(−116) lucter), the β-galactosidase expression vector pJATlac, a plasmid driving the expression of either mda-5 (pEF.mda-5), RIG-I (pEF.RIG-I), or the indicated mda-5/RIG-I chimaeras, or the control "empty vector" pEF.plink2. Transfected cells were either mock-treated or transfected with poly(rI)–poly(rC) and cell extracts prepared. (E) Vero cells were transfected with a reporter for IFN-β promoter activity, (pIFΔ(−116)lucter), the β-galactosidase expression vector pJATlac, a plasmid driving the expression of either mda-5 (pEF.mda-5), RIG-I (pEF.RIG-I), or the indicated mda-5/RIG-I chimaeras, and either the control "empty vector" pEF.plink2 or the PIV5 V-expressing plasmid pEF.SV5/V. Cell extracts were prepared after 48 h. For panels D and E, luciferase and β-galactosidase activities were determined and relative expression values calculated accordingly. A reference value of 100 has been assigned to the level of expression seen in poly(rI)–poly(rC)-treated cells transfected with the control vector (D) or seen in mda-5-transfected cells (E). Values shown represent data from at least three independent experiments with the standard error of the mean indicated.

*Mlu*I sites and the *Bgl*II/*Mlu*I fragment was inserted between the *Bam*HI and *Mlu*I sites of ptkΔ(−39)lucter (King and Goodbourn, 1994).

Expression vectors were based on the EF1α promoter vector, pEF.plink2 (Marais et al., 1995). A modified version of this vector (pEF.myc.plink2) was constructed by the addition of the myc epitope tag sequence directly upstream of the multiple cloning site such that ORFs inserted at the *Nco*I site in the MCS are in-frame with the myc epitope tag sequence. pEF.myc.IRES.neo was similarly constructed from pEF.IRES.neo (Andrejeva et al., 2002). The PIV5 V expression construct, pEF.SV5-V (Didcock et al., 1999), the hPIV2 V and Sendai virus V expression constructs, pEF.hPIV2-V and pEF.SeV-V, and the PIV5 V constructs directing expression of cysteine substitutions in PIV5 V, pEF.SV5-V[C193A] and pEF.SV5-V[C207A] (Poole et al., 2002), and mda-5 expression constructs, pEF.mda-5 and pEF.mda-5.c-*myc* (Andrejeva et al., 2004) have been previously described. For expression vectors driving the synthesis of RIG-I (pEF.RIG-I) and chicken mda-5 (pEF.Chmda-5), full-length cDNAs were assembled from fragments generated from IFN-treated 293 cells or chick embryo fibroblasts respectively using sequential reverse transcription (SuperscriptIII-InVitrogen) and PCR (Accuprime-InVitrogen). All fragments were sequenced before gene assembly. The plasmid expressing the mda-5 CARD domain fused to the RIG-I helicase domain was created by replacing nucleotides 862–3078 of the mda-5 coding sequence in pEF.mda-5 with nucleotides 673–2778 of the RIG-I coding sequence. This was achieved by fusing a filled-in *Nco*I site within the mda-5 cDNA to a filled-in *Eco*RI site within the RIG-I cDNA; the resultant chimaera has the first 287 amino acids of mda-5 and amino acids 225–925 of RIG-I. The plasmid expressing the RIG-I CARD domain fused to the mda-5 helicase domain was created by cloning an *Nco*I-*Bsr*GI filled in fragment of RIG-I and an *Xmn*I–*Bam*HI fragment of mda-5 into the *Nco*I–*Bam*HI sites of pEFplink2, such that the resulting plasmid contained nucleotides 1–714 of the RIG-I coding sequence, and nucleotides 868–3078 of the mda-5 coding sequence; the resultant chimaera has the first 238 amino acids of RIG-I and amino acids 289–1025 of mda-5. For expression vectors for viral V proteins (pEF.x-V), full-length V ORFs derived by PCR (see below) were cloned between the *Nco*I and *Xba*I restriction endonuclease recognition sites of pEF. myc.plink2 (HeV, MenV, MuV, MeV, NiV, NDV and TiV) or pEF. myc IRES.neo (MapV, PoRV and SalV) by incorporating these sites into the PCR primers. Plasmids containing ORFs for HeV P, NiV P, MapV V, MenV V, PoRV V and TiV V were provided by Dr. Lin-Fa Wang, CSIRO, Geelong, Australia, the Sal V plasmid was provided by Dr. Randall Renshaw, Cornell University, and the MuV plasmid was provided by Professor Bert Rima, Queen's University, Belfast, Northern Ireland. The ORFs from these plasmids were amplified using Vent polymerase (New England Biolabs). As the plasmid templates for HeV and NiV both encoded the P ORF, it was necessary to introduce an additional, non-templated G residue at the editing site of both P ORFs by PCR mutagenesis. The ORFs for MeV V and NDV V proteins were obtained from viral RNA by reverse transcriptase and PCR as described above. Specifically, MeV V was obtained from an RNA sample from 2fTGH cells infected with the persistent hamster neurotropic strain, HNT-PI (Burnstein et al., 1964; Rammohan et al., 1983); GenBank Accession number L36043), and NDV V was obtained from an RNA sample from eggs infected with the LaSota strain of NDV (a kind gift of Dr. Elizabeth Aldous, VLA Weybridge). All fragments were sequenced before gene assembly and primer details and sequence information are available on request. The HCV NS3/4a gene (genotype 4a) was cloned, following PCR amplification, into a bicistronic expression vector derived from the self-inactivating lentivirus vector pHR-SIN-CSGW as previously described (Hilton et al., in press). V truncation constructs were generated by PCR using full-length V constructs as a template and primers complementary to either the start and end of the V/P shared region (to create Vn constructs) or the start and end of the V-unique sequence (to create Vc constructs). These PCR products incorporated *Nco*I and *Xba*I restriction endonuclease sites as described for the full-length clones and were subsequently cloned into pEF.myc.plink2.

The plasmids directing the expression in yeast of the GAL4 DNA binding domain fusion to full-length mda-5, pGBKT7.mda-5, and of the GAL4 activation domain fusion to full-length PIV5 V, pGADT7.SV5-V have been previously described (Andrejeva et al., 2004). A plasmid directing the expression in yeast of the GAL4 DNA binding domain fusion to full-length RIG-I, pGBKT7.RIG-I, was constructed by transferring the full-length RIG-I ORF from pEF.RIG-I into pGBKT7 (Clontech). Plasmids directing the synthesis of the GAL4 activation domain fusion to full-length ORFs from the panel of paramyxoviral V proteins were constructed by transferring the ORF fragments from the relevant pEF.x-V vectors described above into pGADT7 (Clontech) to generate pGADT7.x-V.

Immunoprecipitations

Extracts from transfected cells were subjected to immunoprecipitation with the 9E10 monoclonal antibody to the myc tag, or with an antibody to the PIV5 V protein (anti-Pk; Randall et al., 1987) as previously described (Andrejeva et al., 2002).

Yeast two-hybrid

Combinations of GAL4 DNA binding domain and activation domain fusion plasmids were introduced into *Saccharomyces cerevisiae* strains PJ69-4α or CG1945 (Clontech) as indicated in Fig. 2A, and positive transformants were selected by growth at 30 °C on synthetic dropout medium lacking leucine and tryptophan (SD-LW). Double transformants were then streaked sequentially onto SD plates lacking leucine and tryptophan, and lacking leucine, tryptophan, and histidine and containing 10 mM 3-aminotriazole. Growth was monitored at 30 °C.

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