Hepaciviral NS3/4A proteases interfere with MAVS signalling of their cognate animal hosts and also with human MAVS: implications for zoonotic transmission

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Running title: Hepaciviral proteases cleave cognate and human MAVS

Key words: MAVS, HCV, innate immune signalling, NS3/4A protease, viral evasion

Word count, abstract: 207

Word count, text: 4750
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Financial support: Supported by a grant from European Research Council ERC-2011-StG_281473-(VIRAFRONT) and by a grant from the Helmholtz Association SO-024 to T.P.
ABSTRACT

Multiple novel members of the genus Hepacivirus have recently been discovered in diverse mammalian species. However, to date, their replication mechanisms and zoonotic potential have not been explored in detail. The NS3/4A serine protease of HCV is critical for cleavage of the viral polyprotein. It also cleaves the cellular innate immune adaptor MAVS, thus decreasing IFN production and contributing to HCV persistence in the human host.

To investigate conservation of fundamental aspects of the hepaciviral life-cycle, we explored if MAVS cleavage and suppression of innate immune signaling represents a common mechanism employed across different clades of the genus Hepacivirus to enhance viral replication. To estimate the zoonotic potential of these non-human hepaciviruses, we assessed if their NS3/4A proteases were capable of cleaving human MAVS.

NS3/4A proteases of viruses infecting Colobus monkeys, rodents, horses, and cows cleaved the MAVS protein of their cognate hosts and interfered with its ability to induce the IFN-β promoter. All NS3/4A proteases from non-human viruses readily cleaved human MAVS. Thus, NS3/4A-dependent cleavage of MAVS is a conserved replication strategy across multiple clades within the genus Hepacivirus. Human MAVS is susceptible to cleavage by these non-human viral proteases indicating that it does not pose a barrier for zoonotic transmission of these viruses to humans.
Virus infection is recognized by cellular sensor proteins triggering innate immune signaling and antiviral defenses. While viruses have evolved strategies to thwart these antiviral programs in their cognate host species, these evasion mechanisms are often ineffective in a novel host, thus limiting viral transmission across species. HCV, the best characterized member of the genus Hepacivirus within the family Flaviviridae, uses its NS3/4A protease to disrupt innate immune signaling by cleaving the cellular adaptor protein MAVS. Recently, a large number of HCV-related viruses were discovered in various animal species including wild, live-stock and companion animals. We show that the NS3/4A proteases of these hepaciviruses from different animals and representing various clades of the genus cleave their cognate host MAVS proteins, in addition to human MAVS. Therefore, cleavage of MAVS is a common strategy of hepaciviruses and human MAVS is likely unable to limit replication of these non-human viruses upon zoonotic exposure.
INTRODUCTION

Viral zoonotic infections are responsible for numerous emerging infectious diseases (1, 2). Although biological infection barriers (e.g., viral host factor usage, host restriction factors) can limit viral cross-species transmission, recent epidemics of Ebola virus, Zika virus and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in West Africa, Latin America and the Middle East respectively, highlight that RNA viruses frequently overcome these barriers, spread to humans and cause severe disease. Human contacts with wild, livestock and companion animals combined with increasing density of human populations, rising mobility, and climatic changes, facilitate increased human exposure to novel viral pathogens and subsequent spread within human populations. Thus, understanding biological barriers of viral cross-species transmission has high priority to estimate risks of viral transmission to humans and to enable development of preventive and/or therapeutic measures. Moreover, detailed information about species-specific viral replication barriers facilitates development of animal models for human pathogens: for example hepatitis C virus (HCV).

HCV is a plus-strand RNA virus and a major human pathogen causing chronic liver disease in more than 146 million individuals worldwide (3). It possesses a 9.6 kb genome encoding a polyprotein consisting of 10 viral proteins including the structural proteins core, E1 and E2, the p7 ion channel protein, and the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B. It is parenterally transmitted among humans primarily through needle sharing, unsafe medical procedures and use of medical equipment. Although HCV is generally considered to have a narrow species tropism, naturally only infecting humans (4), chimpanzees are susceptible to experimental infection. HCV was discovered more than 25 years ago (5), and initially recognized as the sole member of the genus Hepacivirus within the family Flaviviridae. Subsequently, numerous additional viruses were discovered and assigned to the genus...
Hepacivirus including the GB virus (GBV, previously known as GBV-B; (6)) with unknown natural host species and a large number of HCV-related viruses with different mammalian host species including wild rodents and bats (7, 8), Colobus monkeys and domesticated animals living in close contact with humans (cattle, dogs, and horses) (9-13). In parallel, several new viruses were discovered and assigned to the related genus Pegivirus within the Flaviviridae. These viruses include Simian Pegivirus SPgV (formerly GBV-A) naturally infecting New World monkeys, the non-pathogenic human Pegivirus virus HPgV (formerly GBV-C) and the bat Pegivirus BPgV (formerly GBV-D) (6). More recently, a second human Pegivirus, tentatively named HPgV-2, was discovered associated with HCV co-infection, and a human Hepegivirus that shares features between Hepaci- and Pegiviruses was found in blood transfusion recipients (7, 14). The pathogenic potential of these two latter viruses is currently unknown. Finally, in bats more than 80 different Hepaci- and Pegiviruses were identified highlighting the extraordinary diversity of these viruses (8). Nevertheless, the evolutionary origin of HCV in humans remains unknown. Moreover, the potential for cross-species transmission of these novel viruses between animals and to humans is also unknown. In addition, whether key replication and immune evasion mechanisms are conserved across the genus Hepacivirus remains unexplored.

To successfully infect a new host, zoonotic viruses must cross not only the entry barrier but also subvert the innate immune response raised upon sensing of viral danger signals (15, 16). In the case of HCV, the viral NS3/4A serine protease can disrupt innate immune sensing by cleaving human mitochondrial antiviral signaling protein (MAVS) (17) and human TIR-domain-containing adaptor-inducing interferon-β (TRIF) (18), two critical adaptor proteins that link recognition of viral double-stranded RNA with interferon induction. Intriguingly, MAVS has been under strong positive selection pressure during primate evolution and acquired resistance to HCV protease cleavage in three independent primate species (19). Moreover, hepaciviral GBV,
and pegiviral SPgV, HPgV, and BPgV proteases also cleave MAVS, indicating that primates have likely been under constant exposure to ancient hepac- and/or pegiviruses (19). Of note, expression of poorly cleavable, non-human MAVS variant limits HCV replication indicating that resistance of MAVS to hepaciviral protease cleavage is a determinant of cross-species transmission (19, 20). In this regard it is worth mentioning that the NS3/4A protease of the HCV-related Non-primate Hepacivirus (NPHV; formerly canine hepacivirus CHV) cleaves human MAVS (19, 21, 22), suggesting that in principle this virus, which circulates among horses, may be able to overcome the species barrier imposed by MAVS upon transmission to humans. At the outset of this investigation it was unclear if hepacviruses from non-human hosts are typically capable of cleaving human MAVS. Moreover, it was uncertain if present-day hepacviruses cleave their cognate host MAVS proteins.
MATERIALS AND METHODS

Sequence acquisition and alignment. Representative hepaciviral genome sequences and their cognate host MAVS sequences were downloaded from GenBank (See tables 1 and 2). Where cognate host MAVS sequences were not available (Otomops martiensseni, Hipposideros vittatus, Myodes glareolus, Peromyscus maniculatus, and Rhabdomys pumilio), MAVS sequences from the most closely related species deposited on GenBank were utilized as surrogates (Mus musculus, Eptesicus fuscus, and Myotis brandti). Nucleotide sequences were trimmed and translated and amino acid sequences were aligned using the Clustal W tool in MEGA5. Amino acid conservation plots for NS3/4A and MAVS were calculated using a 10-amino acid sliding window in CLC Genomics Workbench v8.1.

Molecular phylogenetic analysis. The evolutionary history of hepaciviral NS3/4A and mammalian MAVS nucleotide sequences were inferred using the Maximum Likelihood method implemented in MEGA5 based on the Data specific model (23). To assess the significance of clades, the bootstrap approach was employed whereby 1000 pseudo-replicate trees were generated. Grouping of sequences was considered significant if the percentage of trees in which the associated taxa clustered together was >70%. For each phylogeny generated, the tree with the highest log likelihood is presented, with significant bootstrap values assigned to the corresponding tree nodes. Trees were generated under a GTR+Γ+I model of substitution: discrete Gamma distribution (Γ) was used to model evolutionary rate differences among sites (4 rate categories) and proportion of invariant sites (I) was also incorporated into the tree building process. The trees are drawn to scale, with branch lengths proportional to the number of substitutions per site. All positions containing gaps and missing data were eliminated from the analysis. For hepaciviral NS3/4A, the analysis incorporated nucleotide sequences from nine viral
isolates and a total of 1950 nucleotide positions were included in the final analysis. For MAVS, the analysis involved nucleotide sequences from nine mammalian species and a total of 1416 nucleotide positions were included in the final analysis.

Cell culture. HEK 293T wild-type (WT), HEK 293T-MAVS−/− (24), and Huh7-Lunet hCD81 (25) cells used in this study were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 2 mM L-glutamine, non-essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal calf serum (FCS) at 37°C and with 5% CO2.

Plasmid construction. Lentiviral pWPI plasmids, expressing either the WT or the modified NS3/4A or MAVS variants tagged with either a tandem N-terminal HA-tag “2xHA-tag” (pWPI-HA) or a triple N-terminal FLAG-tag “3xFLAG-tag” (pWPI-FLAG) respectively, were created using either PCR-based cloning strategies or synthesized gene fragments (gBlocks, IDT). To generate the protease-inactive mutants, PCR-based site-directed mutagenesis was performed on our panel of hepaciviral NS3/4As to abrogate the catalytic activity of the protease (S139A). The pWPI vectors expressing either human (Hu) or mouse (Mus musculus; Mu) MAVS were created previously (24). The rhesus macaque (Macaca mulatta, Rhe) and other non-human MAVS were generated by PCR using total cDNA from primary macaque hepatocytes or gBlocks as the PCR template. Each amplicon was then cloned into pWPI-FLAG. PCR-based site-directed mutagenesis was also performed to generate a protease-resistant human MAVS (C508R). To create the RFP-based reporter constructs, the lentiviral-based pTRIP-RFP-NLS-Hu MAVS (kindly provided by Charles Rice) was modified by using the Gibson assembly cloning method (NEB) to replace the C-terminal Hu MAVS gene fragment with gBlocks encoding the C-terminal MAVS fragments from each indicated non-human mammals. More detailed cloning strategies are...
available upon request. The IFN-β promoter reporter plasmid (pGL3b-IFN-β promoter-Firefly luciferase) was kindly provided by Stefan Lienenklaus (Institute for Laboratory Animal Science, Hannover Medical School, Hannover, Germany).

**Generation of lentiviral pseudoparticles (PP).** Lentiviral pseudotype particles with envelope glycoproteins from vesicular stomatitis virus (VSV-G) were created as previously described (24). In short, HEK 293T WT cells were transfected with envelope glycoprotein expression constructs pcz-VSV-G, lentiviral gag-pol expression construct pCMVΔR.74, and lentiviral genomic backbone (pWPI or pTRIP) encoding desired gene expression using Polyethylenimine (PEI) transfection method (Sigma-Aldrich). The medium was changed 24 h later and the pseudoparticles were harvested, filtered, and directly used for gene transduction on the next day.

**Transfection and IFN-β promoter reporter assay.** To analyze protein expression, HEK 293T WT or HEK 293T-MAVS⁻/⁻ cells were transfected with a combination of 100 ng (or as further indicated in the figure legends) plasmid DNA expressing the indicated NS3/4A protease plus 100 ng plasmid DNA expressing the full-length MAVS or RFP-based reporter using Lipofectamine 2000 (Invitrogen) and then seeded in poly-L-lysine (Sigma-Aldrich) coated 24-well plate. The cells were then lysed 48 h later prior to Western blotting analysis. For IFN-β promoter firefly luciferase reporter assays, 100 ng IFN-β promoter reporter plasmid was transected into HEK 293T-MAVS⁻/⁻ cells in 24-well format together with plasmid DNA expressing the indicated protease and MAVS. For protease inhibitor assay, the drugs (telaprevir or boceprevir) were given at 24 hours post-transfection. At 48 hours post-transfection, cells were lysed in 350 µl Passive Lysis Buffer (Promega) and the firefly luciferase was activity measured. Results were shown as normalized values against each indicated MAVS challenged by mock plasmid DNA. The
telaprevir and boceprevir were a kind gift from Dr Marc Windisch (Institute Pasteur Korea, Seongnam, South Korea).

**Western blotting.** Cell monolayers were lysed in RIPA buffer (0.3 M NaCl, 20 mM TrisHCl (pH 8), 1% sodium deoxycholate, 0.1% SDS and 1% Triton X-100). Each sample was mixed with 5× denaturing protein sample buffer (200 mM Tris-Cl [pH 8.8], 5 mM EDTA, 0.1% bromophenol blue, 10% sucrose, 3.3% sodium dodecyl sulfate [SDS], 2% 2-mercaptoethanol [2-ME]), heated for 5 minutes at 95°C, loaded onto an SDS-PAGE gel and resolved by electrophoresis. Subsequently, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare) which was then treated with SuperSignal Western Blot Enhancer (Thermo) followed by blocking with 5% milk in PBS containing 0.5% Tween (PBS-T) for 1 h at RT. The membrane was then incubated with either α-HA tag mAb (16B12, 1:300, BioLegends), α-FLAG tag mAb (M2, 1:500, Sigma-Aldrich), α-Hu MAVS mAb (E-3, 1:300, Santa Cruz), α-Mu MAVS mAb (E-6, 1:300, Santa Cruz), α-turboRFP mAb (1:1000, Evrogen) or α-β-actin-HRP mAb (1:1000, Sigma-Aldrich) followed by incubation with secondary antibody coupled to horseradish peroxidase (Sigma-Aldrich). Bound antibodies were detected with the ECL Plus detection system (GE Healthcare) after mixing with SuperSignal Femto Substrate (Thermo) in a 10:1 ratio.

**Immunofluorescence and confocal analysis.** Huh7-LunethCD81 cells (8 x 10^4 cells/well) were seeded on coverslips in 24-well plate followed by overnight transduction with lentiviral pseudoparticles expressing the indicated genes in presence of 4μg/mL polybrene (Sigma-Aldrich). 48 h later, cells were fixed and permeabilized. Immunostaining was then performed using α-HA tag mAb (1:500) alone or in combination with α-Hu MAVS mAb (1:500) or α-Hu Calnexin mAb (1:500; Abcam) as indicated followed by incubation with secondary antibody.
coupled to either Alexa 488 (Green; Sigma-Aldrich) or 647 (Red; Sigma-Aldrich). Nuclear DNA was stained using DAPI at a dilution of 1:3000. Coverslips were then mounted with Fluoromount-G (Southern Biotech) and processed for confocal analysis using a laser-scanning confocal microscope (Olympus). A 100x magnification lens was used for all pictures. ImageJ software was used to create overlaid pictures.

**Statistical methods.** GraphPad Prism 6 software was used for data analysis using a one-way ANOVA adjusted with Sidak’s multiple comparison tests. P values < 0.05 (*) were considered statistically significant whereas p values < 0.01 (**), <0.001 (***) and <0.0001 (****) were considered highly significant.
RESULTS

Amino acid diversity, evolutionary relationships and conserved functional motifs in the predicted hepaciviral NS3/4A and mammalian MAVS proteins

We investigated the properties of hepaciviral NS3/4A proteases and selected eight isolates from each distinct clade within the genus *Hepacivirus* representing viruses isolated from horse/dog (26), rodent (7, 27), bat (8), non-human primate (13), and cattle (9, 11) (Table. 1). The amino acid similarity plot (Fig. 1A; top panel) revealed that the helicase domain of NS3 was more conserved compared to the protease domain and the NS4A transmembrane co-factor. Nevertheless, the catalytic triad of the NS3 protease was completely conserved among all hepaciviruses, suggesting that the encoded proteins possess serine protease activity (see Fig. 1a, bottom panels). Interestingly, although the predicted protease cleavage site at the NS3 C-terminus was not fully conserved, the conserved -EEC sequence clearly marked the NS4A terminus of all hepaciviral isolates analyzed (Fig. 1A, bottom right panel). Phylogenetic analysis of NS3/4A nucleotide sequences revealed a clade containing HCV, NPHV/CHV, BHV-C, BHV-D, and RHV-1 which was well supported, with significant internal branch lengths: the isolates within this cluster are more closely related to HCV NS3/4A (Fig. 1A, bottom left panel). The remainder of the tree was characterized by long internal branch-lengths and non-significant bootstrap values suggesting these isolates are genetically divergent from HCV and from each other. These results support previous reports that the NPHV/CHV NS3/4A is the most closely related hepaciviral protease to HCV. Indeed, amino acid similarity to HCV NS3/4A is greatest for NPHV/CHV (56%) and as low as 37% identity in case of Cattle-HV (Fig. 1A).

To explore if MAVS antagonism through NS3/4A protease-dependent cleavage is a conserved strategy among all hepaciviruses, we cloned MAVS variants originating from species harboring the above mentioned viruses. As NPHV/CHV was initially found in a dog (*Canis lupus*; Can),
and subsequently shown to be circulating among horses (*Equus caballus*; Equ), the MAVS proteins from both species were included. However, since some viruses included in this study were isolated from poorly characterized species within the very large orders Rodentia and Chiroptera (Table 1), for some of these host species there was no MAVS sequence information deposited on sequence databases. Consequently, we substituted with the closest available species: mouse (*Mus musculus*; Mu) MAVS was used as the surrogate for rodents and big brown bat (*Eptesicus fuscus*; Fus) and Brandt’s bat (*Myotis brandtii*; Brn) MAVS variants for bat species (Table. 2). Rhesus macaque (*Macaca mulatta*; Rhe) MAVS was included to provide an example of a MAVS variant that is resistant to hepaciviral protease cleavage (HCV NS3/4A; (19)).

Comparison of the viral NS3/4A and host-species MAVS phylogenies (Fig. 1A and B) revealed a striking lack of congruence between tree topologies: for instance, the Colobus monkey (*Colobus guereza*; Gue), which is closely related to humans, hosts a virus (GHV) only distantly related to HCV, whereas NPHV/CHV, the virus most closely related to HCV infects horses which are more distantly related to humans than primates. These data indicate that hepacviruses did not co-speciate with their cognate host from a single ancestral virus and indicate multiple cross-species transmissions characterize the evolution of the Hepaciviral genus. Overall, sequence similarity between MAVS proteins was most pronounced within the CARD domain and ranged from 85% amino acid conservation (rhesus macaque) to 48% (mouse) when comparing full length proteins to human MAVS. Interestingly, the C-terminus portion of all MAVS proteins investigated comprise a conserved and highly-hydrophobic region (Fig. 1B and Fig. 2C) which likely serves as a transmembrane domain (TMD) for anchoring in the mitochondrial membrane (28). Using the TMpred program (28) the presence of a TMD was confirmed for all MAVS proteins (Fig. 1C). Cleavage of human MAVS by the HCV NS3/4A protease is dependant on residue Cysteine 508 within a protein region partially matching the HCV consensus cleavage site.
Interestingly, the standard HCV NS3/4A protease consensus cleavage site which includes E/D at P6, T/C at P1 and A/S at position P1’ is partially conserved among MAVS variants and only fully matched by the mouse MAVS protein (Fig. 1B) (30-32). Even when using less stringent and more accurate cleavage prediction rules dog, horse and cattle (Bos taurus; Bos) MAVS proteins are not predicted to be cleaved (32). Thus, MAVS polymorphisms could affect hepacivirus cleavage in a species-specific fashion and potentially contribute to species tropism.

Expression of representative hepaciviral NS3/4A and mammalian MAVS trans-membrane domains (TMDs)

Lentiviral vectors expressing N-terminally HA-tagged NS3/4A proteins (Fig. 2A) from the above mentioned hepaciviruses were transduced and subsequent expression and subcellular localization monitored in Huh7-Lunet hCD81 cells (Fig. 2B). All hepaciviral proteases expressed displayed a cytoplasmic and reticular staining that largely overlapped with the ER marker protein calnexin and was comparable to the staining pattern of the HCV NS3/4A protease (Fig. 2B).

Moreover, we created expression constructs for fusion proteins between a red fluorescent protein (RFP) carrying a nuclear localization signal (RFP-NLS) and the C-terminal portion of above mentioned MAVS variants as has been described for human MAVS by Jones et al. (33). Here, the RFP-NLS reporter was fused with ± 75 C-terminal amino acid residues from each indicated MAVS, including the putative hepaciviral protease recognition site and the predicted TMDs (Fig. 2C). In this context, cleavage of these fusion proteins at the predicted hepaciviral cleavage site will detach the RFP-NLS protein from the MAVS TMD and thus result in nuclear localization of the fluorescent protein. After transduction of Huh7-Lunet hCD81 with the expression constructs of the RFP-NLS-MAVS reporter proteins, a strong colocalization between endogenous human
MAVS and the respective fusion proteins was observed (Fig. 2D) indicating proper expression and subcellular localization at the mitochondrial surface.

Hepaciviral NS3/4A serine proteases cleave MAVS proteins of their cognate (or surrogate) host species

To monitor cleavage of these MAVS fusion proteins by hepaciviral proteases Huh7-Lunet hCD81 were simultaneously transduced with indicated NS3/4A proteases and MAVS fusion proteins (Fig. 2E). As expected co-transduction of these cells with the RFP-NLS human MAVS TMD construct and the HCV NS3/4A protease resulted in cleavage of the MAVS reporter protein which was clearly evident by the nuclear localization of the RFP-NLS protein (Fig. 2E). In contrast, the RFP-NLS Rhe MAVS TMD construct, which carries a single amino acid difference to the human cleavage site (V506G; (19)), was not cleaved by the HCV protease, as is evidenced by reticular cytoplasmic staining of the RFP-NLS protein in cells co-expressing the HCV protease (Fig. 2E; 2nd column). Interestingly, the colobus monkey, horse, dog, and cattle MAVS C-termini were found to be susceptible to cleavage by their cognate hepaciviral NS3/4A proteases. Similarly, the surrogate mouse, big brown bat, and Brandt's bat MAVS C-termini proteins were cleaved by the representative rodent and bat hepaciviral NS3/4A proteases (Fig. 2E).

To confirm these observations, we also analyzed cleavage of full-length MAVS proteins by selected hepaciviral proteases (Fig. 3). To ensure specificity of the cleavage NS3/4A protease expression constructs carrying an inactivating mutation of the catalytic serine residue (S139A) were used in parallel (Fig. 3A). As both big brown bat and Brandt's bat MAVS come from bats within the same family, the Brandt's bat MAVS was selected for further analysis and since the predicted colobus monkey MAVS cleavage site matches the human counterpart this construct
was not further analyzed. An N-terminal triple FLAG tag (3xFLAG) was added to MAVS variants with no specific antibody available (horse, dog, cattle, and Brandt's bat MAVS) (Fig. 3A). Subsequently, the indicated wild-type or mutant NS3/4A proteases and MAVS expression plasmids were co-transfected into HEK 293T-MAVS-/- cells, lacking endogenous expression of human MAVS (24). To directly monitor the impact of MAVS cleavage on innate immune signaling, a reporter plasmid encoding a firefly luciferase under the control of the IFN-β promoter was co-transfected and luciferase activity was monitored in the lysates of transfected cells. Interestingly, transient expression of either human or non-human MAVS in these HEK 293T-MAVS-/- cells triggered comparable downstream IFN-β promoter activity (evidenced by similar luciferase activity; Fig. 3). This indicates that each non-human MAVS protein is capable of innate immune signaling in human cells which likely reflects the high conservation of the CARD-like domain among all MAVS variants. The co-expression of HCV and all rodent hepaciviral NS3/4A proteins resulted in efficient cleavage of and signaling interference with mouse MAVS as is evidenced by the detection of a truncated MAVS-species and significantly reduced IFN-β promoter activity (Fig. 3B). Both cleavage and interference with signaling was specific to hepaciviral NS3/4A protease activity since co-expression of protease variants with inactive catalytic triad did not reduce MAVS signaling and ablated detection of a truncated MAVS variant. Notably, the HCV NS3/4A cleaved and interfered with the signaling of horse MAVS (Fig. 3C), although the predicted cleavage site of this MAVS variant does not fully match the HCV cleavage site rules reported by Rögnvaldsson et al. (32). In contrast, no cleavage and signaling interference was observed in case of cattle MAVS (Fig. 3E), and only modest cleavage in the absence of overt signaling interference was visible for dog MAVS (Fig. 3D). Both of these MAVS variants are not predicted to be cleaved by the HCV NS3/4A protease according to the Rögnvaldsson rules. Notably, these MAVS variants were susceptible to cleavage and signaling
interference by their cognate hepaciviral NS3/4A proteases (NPHV; Fig. 3D and Cattle-HV; Fig. 3E) which is in full agreement with the RFP-NLS reporter assays depicted in Figure 2. The Brandt's bat MAVS protein was resistant to the HCV NS3/4A protease (Fig. 3F), and only modest cleavage and weak or no signaling interference was observed for the BHV-C and BHV-D NS3/4A proteases, respectively. Notably, the BHV-C original host belongs to the *Molossidae* family, which is very distantly related to the BHV-D host belonging to the *Hipposideridae* family. Moreover, the animal host of BHV-C is more closely related to the surrogate Brandt's bat family, *Vespertilionidae*, compared to the host of BHV-D which may explain the relatively inefficient interference with Brandt's bat MAVS and the slightly better interference by the BHV-C NS3/4A protease (34).

**Human MAVS-dependent innate immune signaling is susceptible to interference by non-human hepaciviral NS3/4A proteases**

To explore the potential for zoonotic transmission of non-human hepaciviruses to humans, we next explored the ability of the non-human hepaciviral NS3/4A proteases to cleave human MAVS. To this end Huh7-Lunet hCD81 were transduced with indicated full length non-human hepaciviral NS3/4A protease expression constructs and subsequently stained for endogenous MAVS expression and ectopic NS3/4A expression (Fig. 4A). Interestingly, in all cells expressing hepaciviral protease, more diffuse or lower endogenous MAVS signals were observed, suggesting that all non-human NS3/4A proteases investigated at least partially cleaved human MAVS (Fig. 4A). To confirm this observation and to obtain more quantitative results on human MAVS cleavage and signaling interference by these non-human viral proteases, we next co-expressed human MAVS with the indicated proteases in the context of HEK 293T-MAVS<sup>-/-</sup> cells. Human MAVS was susceptible to cleavage and signaling interference by all hepaciviral NS3/4A
proteases and only the RHV isolate yielded a modest interference (Fig. 4B). MAVS signaling interference could be ablated by addition of boceprevir or telaprevir in the case of HCV NS3/4A protease expression. However, MAVS interference by none of the other hepaciviral proteases was ablated by these protease inhibitors indicating that these drugs do not interfere with these proteases (Fig. 4E and F). When residue Cysteine 508 of human MAVS, which is critical for cleavage by HCV (17, 29), was mutated to Arginine (Fig. 4C) human MAVS was resistant to cleavage by all tested non-human hepaciviral NS3/4A proteases (Fig. 4D), indicating that these viral proteases cleave human MAVS at the same site as HCV. Taken together these results indicate that the NS3/4A serine proteases of non-human hepaciviruses examined here not only cleave MAVS of their cognate/surrogate animal hosts, but they also cleave and interfere with signaling of human MAVS (Fig. 5).
DISCUSSION

Recently, numerous new members of the genus *Hepacivirus* were discovered in diverse mammalian species. However, presently there is very limited information regarding their host range, their pathogenic potential and their ability to transmit between different animal species and to humans. To address some of these open questions, in this study we explored if these novel viruses cleave the innate immunity signaling adaptor protein MAVS of their cognate animal hosts, and if so, whether they are capable of interfering also with human MAVS. Previous reports had shown that the HCV protease NS3/4A efficiently cleaves human MAVS, thus dampening innate immune signaling via RIG-I dependent double-stranded RNA sensing (17, 35). In contrast, HCV is unable to interfere with rhesus macaque-derived MAVS (19), and since HCV replication in primary macaque hepatocytes is partially controlled by IFN-dependent mechanisms, this indicates that the inability of HCV to overcome rhesus macaque MAVS, at least in part, limits HCV replication in these cells (20). On the contrary, we and others have observed that HCV readily cleaves mouse MAVS (24, 36). Collectively, these results indicated that inefficient MAVS cleavage by HCV represents one of many biological barrier to HCV transmission between hosts and that the HCV protease cleaves and interferes with MAVS proteins in a highly species-specific manner.

To explore if cleavage of MAVS is a common strategy of hepacviruses we co-expressed NS3/4A proteases of representative hepacviruses from each clade together with their cognate - or where necessary due to the lack of deposited sequences - surrogate host-derived MAVS proteins. By monitoring MAVS cleavage via an RFP-NLS-relocalization assay (33) or directly through measuring proteolytic cleavage and in parallel innate immune signaling to the IFN-β promotor, we provide strong evidence that all examined hepacviruses encode serine proteases that cleave their cognate/surrogate host-derived MAVS proteins. In case of BHV-C and BHC-D, two bat-
derived viruses, we noted relatively inefficient or even absent interference with innate immune
signaling, respectively. However, in both cases a low amount of cleaved bat-derived MAVS was
detectable (Fig. 3), suggesting that cleavage - albeit inefficiently - does occur. It is possible that
bat-tropic hepaciviruses poorly cleave their natural hosts’ MAVS proteins. However, we rather
believe that poor bat MAVS cleavage demonstrated here is due to the genetic distance between
the cognate host bat species from which the respective viruses were isolated, and the surrogate
bat species used for our assays. To ultimately clarify this question and also to address subtle
differences between cleavage efficiency, future work involving characterization of MAVS and
permissive cells from these species are required. Nevertheless, collectively these results highlight
that cleavage of MAVS is a conserved strategy across all currently known clades of the genus
Hepacivirus. This conclusion is in agreement with and extends the report by Patel and colleagues
who had previously shown that NPHV/CHV and a few members of the genus Pegivirus are
capable of cleaving human MAVS (19).

It is worth mentioning that the HCV NS3/4A also cleaves TRIF (18). Although the relevance of
TRIF cleavage for HCV replication and persistence is discussed (37), it will be interesting to
demonstrate whether TRIF cleavage is unique to HCV or also common to other members of this
virus genus. Moreover, the HCV NS3/4A protease also cleaves several human host factors that
are unrelated to innate immune sensing including T-cell protein tyrosine phosphatase (TC-PTP)
(38), UV-damage DNA-binding protein 1 (DDB1) (39) and the membrane associated peroxidase
GPx8 (40). At least in part, these cleavage events are important for full replication of HCV in
human cells (38, 40). It will be intriguing to find out if the respective non-human orthologs of
these host factors are also cleaved by the respective non-human hepaciviruses and whether this is
critical to support replication of these viruses. Unfortunately, infection systems for these novel
hepaciviruses are currently not available, making it difficult to explore this question. However, in
the case of NPHV, the recently established infectious molecular clone will be instrumental to develop such systems and to subsequently explore this question (22).

If cleavage of these additional host factors is conserved and functionally relevant among other hepaciviruses, it will be important to analyze if these non-human viral NS3/4A proteases also cleave the human orthologs in order to fully assess the zoonotic potential of these viruses. In this study we show that human MAVS is readily susceptible to cleavage by all hepaciviruses examined. These results suggest that unlike rhesus macaque MAVS for HCV, human MAVS would not be able to relay antiviral signaling upon infection of human cells by these HCV-related viruses. Clearly, numerous other host factors co-determine viral species tropism, most notably entry factors (41). Therefore, future work should address if cell entry factor usage is conserved between hepaciviruses. Besides important information regarding the potential zoonotic relevance of these new viruses, these studies certainly will provide important new insights into the infection and replication strategies of this diverse group of viruses.

ACKNOWLEDGEMENT

We are very grateful to Charles Rice for the pTRIP-RFP-NLS-Hu MAVS plasmid. We also thank all members of the Institute for Experimental Virology at TWINCORE for helpful comments and discussions of this work.


FIGURE LEGENDS

Table 1: List of the representative hepaciviral isolates.

Table 2: List of mammalian MAVS used in the study.

Figure 1. Amino acid diversity, evolutionary relationships and conserved functional motifs in hepaciviral NS3/4A and mammalian MAVS proteins. (A) Amino acid similarity plot of nine full-length hepaciviral NS3/4A proteins is shown (top panel), with relative similarity shown on the y-axis and amino acid position in the encoded proteins presented on the x-axis. For the purpose of positional referencing, a cartoon of the NS3/4A protein is located directly below, with the protease and helicase domains of NS3 colored in light and dark blue respectively (middle panel). The NS4A protein and N-terminus of NS4B are colored pink and grey respectively. Black scissors positioned below the cartoon represent the NS2 cleavage site while white scissors represent putative substrates for cleavage by the NS3 protease. Magnification zooms positioned below the cartoon depict amino acid conservation of the NS3 protease catalytic triad and adjacent residues (three alignment blocks on the left: turquoise around amino acid H57, red (D81) and green (S139)) and putative cleavage substrates for the NS3/4A (two alignment blocks on the right: NS3/4A junction and NS4A/4B junction) in the representative hepaciviral proteins (bottom middle panels). Red-colored and underlined amino acid residues show common consensus cleavage sequence for HCV NS3/4A. A phylogenetic tree depicting the evolutionary relationships of the included hepaciviral NS3/4A sequences is positioned to the left of these alignments, with significant groupings depicted by an asterisk. Percentage amino acid similarities of non-human hepaciviral NS3/4A proteins with HCV NS3/4A are indicated to the right. (B) Amino acid similarity plot of nine mammalian MAVS proteins is shown (top panel), with relative
similarity shown on the y-axis and amino acid position in the encoded protein presented on the x-axis. For the purpose of positional referencing, a cartoon of the MAVS protein is located directly below, with the card-like and proline-rich domains colored green and the transmembrane domain (TMD) colored yellow (middle panel). White scissors positioned below the cartoon represent putative substrate recognition sites for cleavage by hepatic viral NS3/4A proteases. Magnification zoom positioned below the cartoon depicts amino acid conservation in the putative cleavage site and adjacent residues in the representative mammalian MAVS proteins (bottom middle panel). Red-colored and underlined amino acid residues highlight residues that conform to the classical HCV NS3/4A consensus cleavage site. A phylogenetic tree depicting the evolutionary relationships of the included mammalian MAVS sequences is positioned to the left of this magnified alignment, with significant groupings depicted by an asterisk. Percentage amino acid similarities of mammalian MAVS proteins with human MAVS are located to the right. (C) Transmembrane domain prediction of the nine mammalian MAVS protein. The hydrophobicity-hydrophilicity analysis was performed using the TMPred server (http://www.ch.embnet.org/software/TMPRED_form.html). The listing gives both inside to outside (i → o) and outside to inside (o → i) transmembrane helix orientations. A greater than 500 score (dotted-line) predicts a highly-hydrophobic transmembrane region.

Figure 2: Expression of the predicted hepatic viral NS3/4A proteases and of mammalian MAVS transmembrane domains (TMDs). (A) Diagram illustrating the N-terminally HA-tagged hepatic viral NS3/4A constructs. (B) Detection of the HA-NS3 expression in Huh7-Lunet hCD81 cells transduced by lentiviral pseudoparticles (PP) harboring each distinct non-human hepatic viral NS3/4A construct. The expression of HA-tagged HCV NS3/4A (1st column) serves as comparison control and human Calnexin (2nd row) serves as an ER marker. The shown images
are representatives of two individual experiments. (C) Diagram illustrating the RFP – nuclear localization signal (NLS) reporter constructs. The dotted lines indicate the MAVS C-terminal domain from each indicated MAVS species fused to the reporter C-terminus. The red arrow and yellow box indicate the predicted cleavage site and hydrophobic TMD of MAVS, respectively, from each indicated species. (D) Expression profile of reporter construct harboring the C-terminal domain and TMD from each indicated species (2nd row) in comparison with the endogenous Hu MAVS expression stained with its N-terminal specific antibody (1st row) in Huh7-Lunet hCD81 cells. (E) Expression profile of the indicated reporter construct (2nd row) in the presence of HCV or non-human hepaciviral NS3/4A protease (1st row). The cleavage of MAVS TMD by the viral NS3/4A proteases causes relocalization of the RFP-NLS reporter to the nuclei. The dotted white-line outlines the HA-positive (NS3/4A-expressing) cells. The shown images are representatives of two individual experiments.

Figure 3: Effect of hepaciviral NS3/4A expression on non-human full-length MAVS expression and downstream IFN-β promoter induction. (A) Diagram illustrating wild-type (W) or S139A mutant (m) NS3/4A proteases from hepaciviral isolates tested against FLAG-tagged or non-tagged full-length MAVS from each indicated species. (B) Effect of NS3/4A from rodent hepacivirus against surrogate mouse (Mu) MAVS. (C & D) Effect of NS3/4A from horse / dog hepacivirus against horse (Equ) or dog (Can) MAVS. (E) Effect of NS3/4A from cattle hepacivirus against the cattle (Bos) MAVS. (F) Effect of bat hepacivirus NS3/4A against surrogate Brand’s bat (Brn) MAVS. The expression of HCV NS3/4A serves as comparison control. WB analysis (upper panel; red arrow-head indicates cleaved MAVS species) and MAVS dependent IFN-β promoter reporter assay (lower panel) were performed as described in the
materials and methods. WB images are representatives of at least two individual experiments and all graphical data presented are mean values ± SD of at least three independent experiments.

Figure 4: Non-human hepaciviral NS3/4A proteases cleave and inhibit signalling of human MAVS. (A) Localization and expression of endogenous Hu MAVS in Huh7-Lunet hCD81 cells is influenced by the presence of non-human hepaciviral NS3/4A protease. The cells were transduced by lentiviral pseudoparticles (PP) harboring the indicated HA-tagged NS3/4A construct. The expression of HCV NS3/4A (1st column) serves as comparison control. The dotted white-line outlines the HA-positive (NS3/4A-expressing) cells. The confocal immunofluorescence analysis was conducted using HA-tag and Hu MAVS specific antibodies.

(B) Effect of wild-type (WT) or S139A mutant non-human hepaciviral NS3/4A proteases on Hu MAVS expression (upper panel) and downstream MAVS-dependent signaling to the IFN-β promoter (lower panel). HEK 293T MAVS−/− cells were transfected with indicated expression constructs or mock transfected. Subsequently, protein expression was monitored by Western blotting using HA-, actin and Hu MAVS specific antibodies. IFN-β promoter activity was quantified using luciferase assays. (C) Diagram illustrating wild-type or C508R mutant human MAVS proteins. (D) Effect of wild-type (WT) non-human hepaciviral NS3/4A proteases on wild-type or C508R mutant Hu MAVS expression (upper panel) and downstream MAVS dependent IFN-β promoter activity (lower panel). HCV NS3/4A expression serves as comparison control.

Red arrow-heads in WB panels indicate MAVS cleavage products. (E & F) The effect of HCV protease inhibitors (boceprevir [B] or telaprevir [T]) against the indicated hepaciviral proteases (P). Either Hu MAVS (E) or Mu MAVS (F) was used as the protease substrate. The expression of the MAVS (M) in absence of protease (P) serves as untreated control. Each drug was given at a final dose of 2.5 µM. WB analysis and MAVS dependent IFN-β promoter reporter assay were
done as explained in materials and methods. WB images are representatives of at least two individual experiments and all graphical data are shown as mean values ± SD of at least three independent experiments.

**Figure 5:** Schematic summary of hepaciviral NS3/4A protease interference with mammalian MAVS function. The phylogenetic trees highlight the relatedness among nucleotide sequence of the analysed proteins. Green box = interference. Red box = no interference. * = cognate species. S = surrogate species. White box = untested. Interference was scored when both cleavage of MAVS was detectable and at the same time IFN promoter activity was significantly reduced under the chosen experimental conditions.
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Figure 2

A) NS3

B) MCV HA-HIS/5A

C) MAVS

D) MCV HA-HIS/5A

E) MCV HA-HIS/5A