BIOCHEMICAL PHARMACOLOGY 72 (2006) 1477–1484

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Recruitment of an interferon molecular signaling complex to the mitochondrial membrane: Disruption by hepatitis C virus NS3-4A protease

John Hiscott *, *Judith Lacoste*, *Rongtuan Lin*

Lady Davis Institute for Medical Research-Jewish General Hospital, Departments of Microbiology & Immunology and Medicine, McGill University, Montreal, Canada H3T 1E2

ARTICLE INFO

Article history:
Received 2 May 2006
Accepted 21 June 2006

Keywords:
Hepatitis C
Interferon
RIG-I
Viral evasion
IRF

ABSTRACT

Recent advances in the understanding of the signaling pathways leading to the host antiviral response to hepatitis C virus (HCV), the mechanisms used by HCV to evade the immune response, and the development of small molecule inhibitors of HCV have generated optimism that novel therapeutic approaches to control HCV disease may soon be available. HCV infection is detected by the cytoplasmic, RNA helicase RIG-I that plays an essential role in signaling to the host antiviral response. Recently the adapter molecule that links RIG-I sensing of incoming viral RNA to downstream signaling and gene activation events was characterized by four different groups: MAVS/IPS-1-1/VISA/Cardif contains an amino-terminal CARD domain and carboxyl-terminal mitochondrial transmembrane sequence that localizes to the mitochondrial membrane. Furthermore, the hepatitis C virus NS3-4A protease complex specifically targets MAVS/IPS-1/VISA/Cardif for cleavage as part of its immune evasion strategy. Using a combination of biochemical analysis, subcellular fractionation and confocal microscopy, we demonstrate that: (1) NS3-4A cleavage of MAVS/IPS-1/VISA/Cardif causes relocation from the mitochondrial membrane to the cytosolic fraction, resulting in disruption of signaling to the antiviral immune response; (2) disruption requires a function NS3-4A protease; (3) a point mutant of MAVS/IPS-1/VISA/Cardif (Cys508Ala) is not cleaved from the mitochondria by active protease; and (4) the virus-induced IKKε kinase, but not TBK1, co-localizes strongly with MAVS at the mitochondrial membrane and the localization of both molecules is disrupted by NS3-4A expression. These observations provide an outline of the mechanism by which HCV evades the IFN antiviral response.

1. Introduction

The hepatitis C virus (HCV) is an important cause of human chronic liver diseases [13,28] and is a major public health problem. More than 170 million people worldwide are infected with HCV [64]. HCV is an enveloped virus classified in the *Flaviviridae* family [50]. The positive-stranded viral RNA genome encodes a single polyprotein precursor that is processed into structural proteins (core, envelope protein 1 (E1) and 2 (E2), p7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) by host and viral proteases (reviewed in Refs. [49,65]). Although cellular and humoral immune responses are present during acute and chronic HCV infection [6], the immune response is rarely effective in eradicating the

* *Corresponding author.*
E-mail address: john.hiscott@mcgill.ca (J. Hiscott).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.06.030

virus. The majority of HCV-infected subjects develop chronic liver infection, demonstrating that HCV may have evolved strategies to overcome or evade efficient innate immune responses of the host (reviewed in Ref. [12]).

Little is known of the mechanisms or host functions involved in virus entry, uncoating, trafficking, virus assembly and egress. The recent development of cell culture systems for HCV infection and replication [36,63,69] ushers in a new era of advancement in HCV research. Understanding the viral life cycle will assist in the development of antiviral drugs and will help in the analysis of potential candidate vaccines.

1.1. *The host innate antiviral response*

Pathogen-associated molecular patterns (PAMPs) presented by the infecting viruses or other pathogens activate multiple signaling cascades through Toll-like receptor-dependent and independent pathways, culminating in the production of cytokines and chemokines. Rapid induction of type I interferon (IFN) expression is a central event in establishing the host innate antiviral response (reviewed in Refs. [30,54,60]), culminating in the activation of NF-κB and IRF-3 transcription factors which trigger the release of IFNβ and IFNα1. Once produced, secreted IFN acts in a paracrine fashion to induce gene expression in neighboring cells through engagement of cell surface IFN receptors. Among the hundreds of IFN-stimulated genes, transcriptional upregulation of the IRF-7 gene is also observed, and in response to virus infection, IRF-7 and IRF-3 contribute to further amplification of the IFN response through the induction of multiple species of IFN-α, leading to a second wave of IFN expression in response to pathogen.

Interferon regulatory factor (IRF)-3 and IRF-7 play essential roles in the virus-induced type I IFN gene activation following virus infection [5,32,33,35,38,51,52,68]. IRF-3 is activated by C-terminal phosphorylation which promotes dimerization, cytoplasmic to nuclear translocation, DNA binding, association with CBP/p300 histone acetyltransferases and transactivation of downstream early genes such as IFNβ, IFNα1 and RANTES. In contrast, IRF-7 protein is synthesized *de novo* upon IFN stimulation and contributes to the expression of delayed-type genes including other IFNα subtypes. As with IRF-3, virus infection induces C-terminal phosphorylation of IRF-7 [38,53]. The IKK-related kinases – IKKε [46] and TBK-1 [9,48,62] – were shown to be essential signaling components required for IRF-3 and IRF-7 phosphorylation [15,39,56].

1.2. *Triggering the host response to HCV infection*

For RNA viruses, protein and nucleic acid products of infection or replication, including ssRNA and dsRNA, have been identified as viral PAMPs that are engaged by specific TLRs or nucleic acid-binding proteins that serve as PAMP receptors [24]. HCV nonstructural proteins and viral positive-stranded RNA form membrane-associated replication complexes in the cytoplasm of the cell that may be sensed by TLR-dependent or independent mechanisms. These replication complexes then transcribe negative-stranded RNA intermediates from which positive-stranded RNA molecular are generated [43]. Several reports indicate that HCV infection triggers the host cell to activate multiple signaling cascades, culminating in the production of cytokines and chemokines that could potentially disrupt virus replication and initiate innate and adaptive immune responses. How sensing occurs *in vivo* remains an interesting and poorly understood aspect of the early host response to infection.

1.3. *Evasion of the host response by HCV*

The innate immune response is the earliest phase of immune defense and also regulates the adaptive immune response [7]. Since triggering the IFN antiviral response in HCV-infected cells would limit virus replication, HCV strategies to block the innate immune response are crucial for the establishment of a microenvironment conducive to HCV infection and replication. Several HCV structural and nonstructural proteins, including E2, Core and NS5A proteins, have been shown to inhibit the innate immune response [1,40,42,57,61]. Among these HCV immunsuppressive proteins, NS5A has the ability to modulate a number of cell-cycle regulatory genes [20,21], and has been implicated in the interference of IFN-mediated antiviral functions [59]. E2 and NS5A have been shown to bind to the kinase domain of PKR and inhibit of IRF-1 activation [17,19,47,61]. Furthermore, the interferon signaling pathway is inhibited by HCV induced upregulation of protein phosphatase PP2A.

1.4. *RIG-I pathway*

HCV and many viral infections are detected by the host cell through the presence of viral nucleic acids; extracellular viral dsRNA is recognized by the Toll-like receptor 3 (TLR3) [2,3], whereas intracellular viral dsRNA is detected by two recently characterized DExD/H box RNA helicases, RIG-I [67] and/or Mda5 [4,25]. The importance of the RIG-I pathway was confirmed with the generation of RIG-I-deficient mice [26], which revealed that RIG-I and not the TLR system played an essential role in the IFN antiviral response in most cell types—fibroblastic, epithelial and conventional dendritic cells. In contrast, plasmacytoid dendritic cells (pDCs) utilize TLR mediated signaling in preference to RIG-I.

Upon dsRNA recognition and binding by its RNA helicase activity, RIG-I dimerizes and undergoes conformational alterations that enable the N-terminal CARD domain to interact with other downstream adapter protein(s). RIG-I signaling ultimately engages the IKK kinase complex, the stress activated kinases, as well as the IKK-related kinases TBK1 and IKKε, leading to phosphorylation and activation of NF-κB, ATF-2/c-jun and IRF-3 transcription factors, respectively [37]. Coordinated activation of these factors results in the formation of a transcriptionally competent enhanceosome that triggers IFN-β production [44].

Recent studies demonstrated that HCV gene product NS3-4A protease complex, a multifunctional serine protease, efficiently blocked RIG-I signaling pathway and contributed to virus persistence by enabling HCV to escape the IFN antiviral response. Nevertheless, RIG-I was not a direct target of NS3-4A and likewise, the kinases TBK1 and IKKε were not subject to proteolytic cleavage by NS3-4A [10,16,58]. Interestingly, the NS3-4A protease appears to target the TRIF/TICAM adapter of the TLR3 pathway and causes specific proteolytic

cleavage of TRIF, although this pathway appears to have a minimal role in triggering the IFN antiviral response. Additional evidence for the importance of RIG-I comes from studies demonstrating that permissiveness for HCV RNA replication in Huh7.5 [8] cells is due to mutational inactivation of RIG-I protein [58]. Thus, RIG-I signaling appears to be an essential pathway regulating cellular permissiveness to HCV replication.

1.5. MAVS/IPS/VISA/Cardif

The adaptor molecule that links RIG-I sensing of incoming viral RNA and downstream activation events was recently elucidated by four independent groups [27,41,55,66]. MAVS/IPS-1/VISA/Cardif contains an amino-terminal CARD domain and a carboxyl-terminal mitochondrial transmembrane sequence that localizes this protein to the mitochondrial membrane, thus suggesting a novel role for mitochondrial signaling in the cellular innate response [55]. Under the name of Cardif, this protein was described by Meylan et al. to interact with RIG-I and recruits IKKα, IKKβ and IKKε kinases through its C-terminal region. Importantly, Cardif was cleaved at its C-terminal end – adjacent to the mitochondrial targeting domain – by the NS3-4A protease of hepatitis C virus [41]. Li et al. subsequently demonstrated that NS3-4A cleavage of MAVS/IPS-1/VISA/Cardif resulted in its dissociation from the mitochondrial membrane and disruption of signaling to the antiviral immune response [31].

2. Results

2.1. Identification of K1271/MAVS

The observations that RIG-I, TBK1 and IKKε are not proteolytic substrates of NS3-4A indicated that an unidentified adaptor(s) between RIG-I and the kinases may be a target for NS3-4A cleavage [10,16]. A search program was written in python language www.biopython.org; a database search was performed that identified an uncharacterized protein – KIAA1271 (K1271) – containing a single CARD-like domain at the N-terminus and a Leu-Val rich C-terminus (Fig. 1). This molecule was identical to the MAVS/IPS/VISA/Cardif adapter [27,41,55,66]. Co-expression of K1271/MAVS strongly activated the IFN-β promoter and appeared to be the adapter linking RIG-I sensing to downstream signaling events. Both the N- and C-termini were essential for transactivation function, since deletion of either end of the protein eliminated transactivation (Fig. 1). Interestingly, an expression construct that contained the N- and C-terminal domains but lacked the region between aa150 and aa467 also stimulated the IFN-β promoter (Fig. 1). As shown by Meylan et al. [41], K1271/MAVS is a direct target of the HCV protease NS3-4A, with cleavage occurring adjacent to the transmembrane domain at Cys508 (Fig. 1).

2.2. Inhibition of downstream IFN activation

NS3-4A expression had profound effects on downstream IFN gene activation events. Co-expression of increasing amounts of NS3/4A strongly inhibited K1271/MAVS-mediated activation of IFN-β promoter, whereas a truncated form of K1271/MAVS (K1271 aa1-508) was unable to stimulate the IFN-β promoter and was also not inhibited by NS3/4A co-expression. Co-expression of the viral NS3/4A protease completely inhibited K1271/MAVS-induced IRF-3 and IRF-7 phosphorylation, and as a measure of the antiviral response, it was also demonstrated that endogenous ISG56 expression was blocked in NS3-4A expressing cells [34].

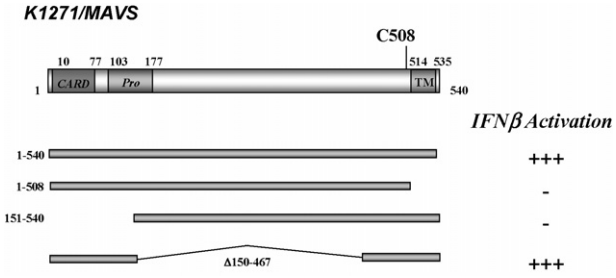

Fig. 1 – Schematic representation of the RIG-I adapter K1271/MAVS. The 540aa MAVS/IPS-1/VISA/Cardif molecule is shown schematically. The location of the CARD domain, proline rich region that interacts with TRAF6 and the C-terminal mitochondrial membrane region (TM) is shown. Also illustrated is the region adjacent to the TM containing cysteine 508, the target residues for the HCV NS3/4A protease. Different N- and C-terminal deletions used in co-transfection experiments to induce the IFN-β-pGL3 reporter plasmid are shown below the schematic. +++ indicates a 150–250-fold induction of the IFNβ promoter; − indicates a less than 10-fold induction relative to control [34].

2.3. Distinct subcellular localizations of TBK1 and IKKε: recruitment of IKKε to K1271/MAVS localized at the mitochondrial membrane

To determine if localization and/or recruitment of the virus-activated kinases TBK1 and IKKε could be observed in association with mitochondrial localized K1271/MAVS, endogenous K1271/MAVS and IKKε were visualized by confocal microscopy in lung epithelial A549 cells. In cells infected with VSV for 1 h, perinuclear IKKε was detected (Fig. 2, top green panel) that co-localized with K1271/MAVS (Fig. 2, top red panel). The merge of the respective signals illustrated a strong co-localization and close association between these two proteins (Fig. 2, top right panel). Interestingly, VSV-induced IKKε localized almost exclusively with mitochondria (detected by MTO) in the reticulotubular mitochondrial network. A similar co-localization was not observed between TBK1 and mitochondria; only a portion of the detected TBK-1 appeared to be associated with the mitochondrial fraction; ongoing studies are investigating the localization and recruitment of TBK-1 to the K1271/MAVS and the mitochondria (Fig. 2, lower panels).

2.4. Cleavage of the K1271/MAVS–IKKε complex from the mitochondria by NS3-4A

To address the fate of the K1271/MAVS–IKKε in the presence of NS3-4A protease, transient expression studies were

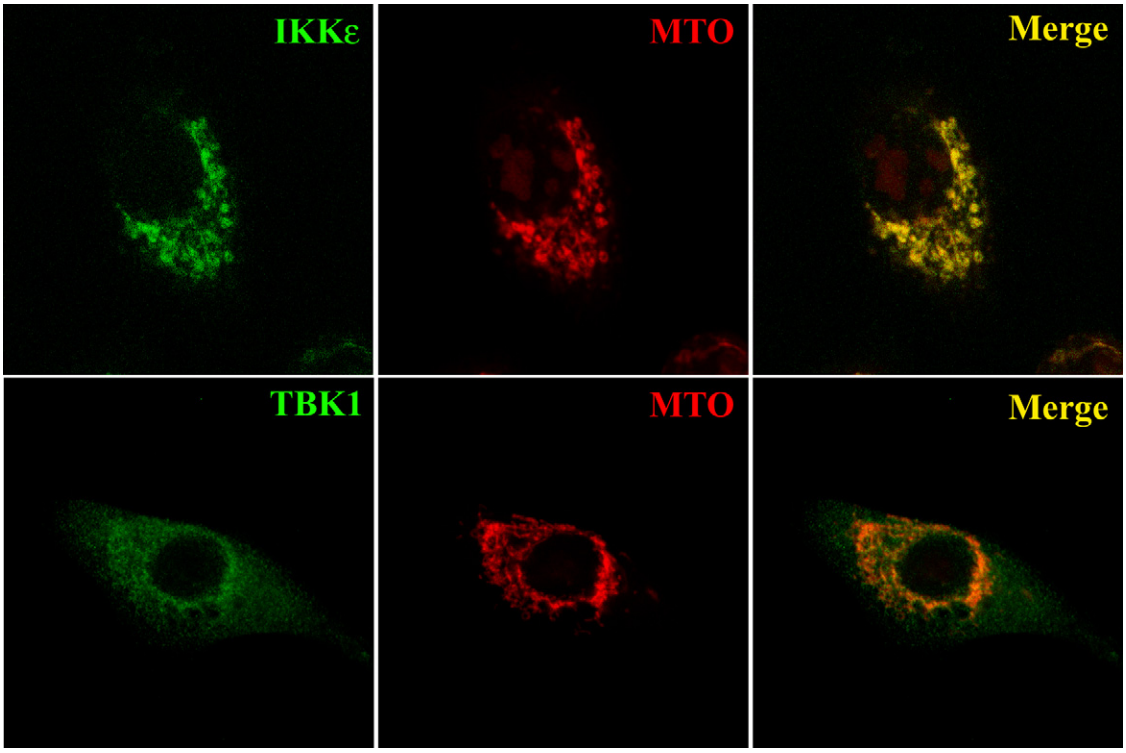

Fig. 2 – Distinct subcellular localization of IKKε and TBK1. VSV-infected A549 cells were treated with MitoTracker Orange (MTO, in red), fixed and stained for either IKKε (upper panels) or TBK1 (lower panels). The confocal fluorescent images were merged. IKKε was detected using mouse anti-IKKε and anti-mouse AF488 antibodies, while TBK1 was detected using rabbit anti-TBK1 and anti-rabbit AF488 antibodies.

undertaken in primate Cos-7 cells. As observed for the endogenous molecules, co-staining of transfected K1271/MAVS and IKKε revealed a strong co-localization with mitochondria (Fig. 3). Importantly, NS3-4A protease co-expression was sufficient to disrupt the mitochondrial localization of K1271/MAVS–IKKε, resulting in a diffuse cytoplasmic staining pattern (Fig. 4, upper panels). A protease inactive form of NS3/4A was unable to cleave the K1271/MAVS–IKKε molecular complex from the mitochondrial surface (Fig. 4, lower panels). When K1271 (C508A) was used in similar experiments, K1271/MAVS–IKKε retained a mitochondrial-like staining pattern (data not shown). Furthermore, these observations support earlier studies characterizing the expression of K1271/MAVS in human hepatoma Huh7 and the HCV replicon expressing Huh8 cells [34]. Full length K1271/MAVS was detected in extracts of Huh7 cells in association with the mitochondrial membrane, whereas the predominant form of K1271/MAVS in Huh8 cells was truncated and was diffusely localized within the cytoplasm [34].

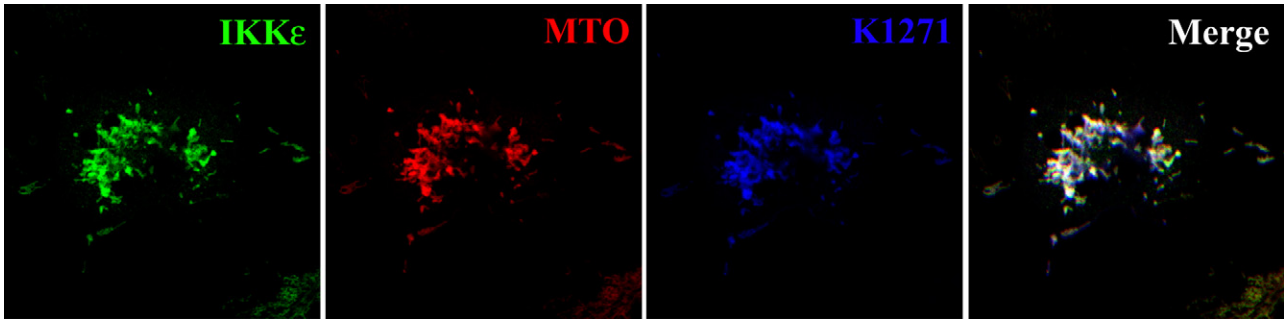

Fig. 3 – Co-localization of mitochondria and transfected IKKε and K1271. COS-7 cells were transfected with IKKε and K1271 expression plasmids, treated with MTO (in red), fixed and stained for either IKKε (in green) or K1271 (in blue). The confocal fluorescent images were merged. IKKε was detected as described above. K1271 was detected using guinea pig anti-K1271 and anti-guinea pig AF647 antibodies.

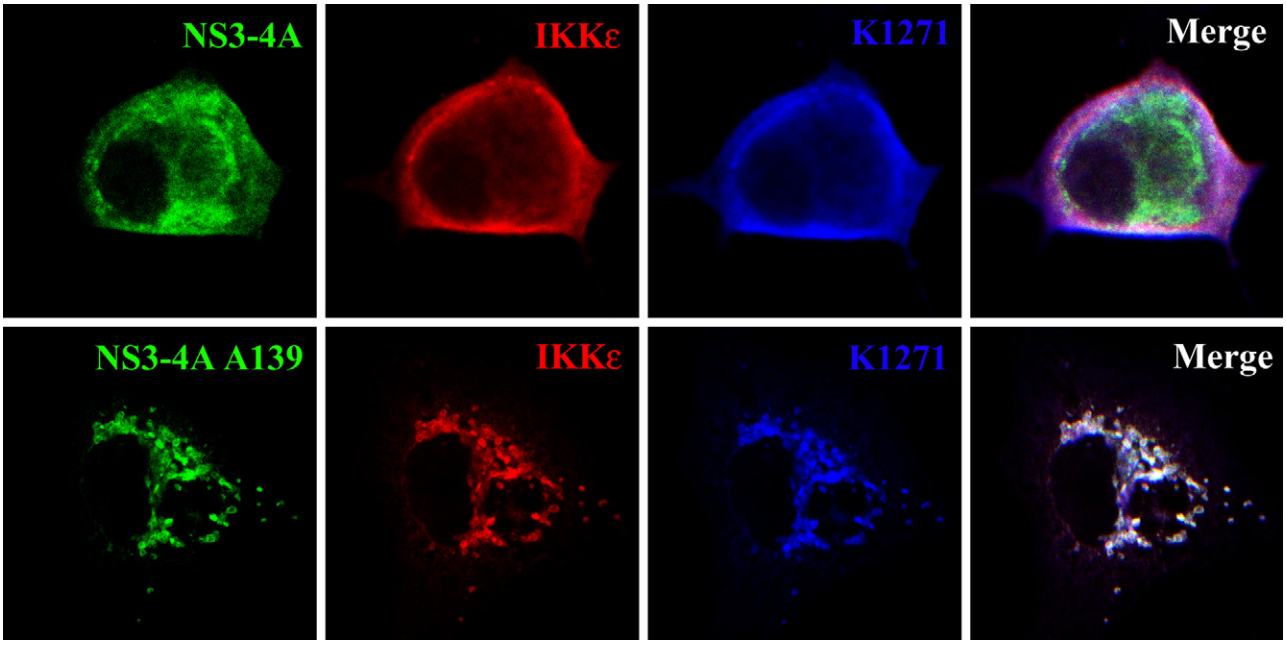

Fig. 4 – The protease activity of NS3-4A is required to disrupt the IKKε-K1271 complex localization. COS-7 cells transfected with IKKε-myc and K1271 expression plasmids, and with a construct encoding wild type (upper panels) or protease inactive (lower panels) NS3-4A-flag, were stained for flag (in green), IKKε (in red), K1271 (in blue), and confocal fluorescent images were merged. Flag-tagged NS3-4A was detected using rabbit anti-flag and anti-rabbit AF488 antibodies. Myc-tagged IKKε was detected using mouse anti-myc (clone 9E10) and anti-mouse Cy3 antibodies. K1271 was detected as described in Fig. 3.

3. Discussion

The DExD/H box RNA helicase RIG-I plays an essential role in the sensing of incoming of virus infection and directly relays regulatory signals to the host antiviral response. The adaptor molecule providing a link between RIG-I sensing of incoming virus particles and downstream activation events was recently elucidated; MAVS/IPS-1-1/VISA/Cardif [27,41,55,66] localizes to the mitochondrial membrane suggesting a link between recognition of viral infection, the development of innate immunity and mitochondrial function [55]. The IKK-related kinases – TBK1 and IKKε – are critical downstream components of the activation of the interferon antiviral response, through their ability to phosphorylate the C-terminal domains of IRF-3 and IRF-7. Interestingly, the hepatitis C virus NS3-4A protease activity specifically cleaved the MAVS/IPS-1-1/VISA/Cardif–IKKε molecular complex from the mitochondria as part of its immune evasion strategy. Furthermore, a protease-inactive NS3-4A mutant was unable to affect the MAVS/IPS-1/VISA/Cardif protein subcellular localization and its association with IKKε. Disruption of the mitochondrial localization of MAVS/IPS-1-1/VISA/Cardif–IKKε also ablated downstream signaling to the IFN antiviral response. These observations provide the outline of the mechanism by which HCV evades the IFN antiviral response. Furthermore, recent biochemical evidence using ultracentrifuge purified mitochondria and immunoblot analysis also reveals that IKKε is inducibly recruited to the mitochondria in virus-infected cells, while TBK1 remains partitioned in the cytosol and only a small amount appears to be recruited to the mitochondria. We are currently evaluating the significance of the recruitment of IKKε to the MAVS/IPS-1-1/VISA/Cardif complex.

The strong and apparently selective recruitment of IKKε to the mitochondria in association with MAVS/IPS-1-1/VISA/Cardif is enigmatic, given that TBK1 is principally involved in downstream signaling to IRF-3 and IRF-7 phosphorylation and development of the antiviral response [14,39,56]. Studies in TBK1 and IKKε knockout mice demonstrate a clear role for TBK1 in the generation of the antiviral response, with only an accessory role associated to date with IKKε [23,45]. Given that IKKε knockout mice do not appear to have major defects in IFN induction, targeting an IKKε dependent pathway does not explain the inhibition of IFN induction by the hepatitis C protease. Selective recruitment of IKKε may reflect a distinct functional role for this kinase activity in the host response to virus infection, perhaps at the level of coordinating mitochondrial dependent cell death in virus-infected cells. Moreover, while the majority of TBK1 was detected throughout the cytoplasm, a fraction did appear to co-localize with mitochondria; biochemical studies demonstrated that a small proportion of total TBK1 is present in highly purified mitochondrial fractions (unpublished data). Whether this fraction is enzymatically active and/or physically associated with MAVS/IPS-1-1/VISA/Cardif remains to be determined. Although, co-localization of TBK1 with the mitochondria and MAVS/IPS-1-1/VISA/Cardif could be transient and not easily detected by confocal analysis, the observation that IKKε is so intimately associated with MAVS/IPS-1-1/VISA/Cardif is not easily resolved at this point. However, in support of a functional role of IKKε in HCV pathogenesis, previous experiments demonstrated that IKKε overexpression, but not the expression of TBK1 or other signaling adapters, partially reversed HCV protease mediated inhibition of IFN induction [10].

The localization of this CARD domain-containing adaptor to the mitochondrial membrane is highly strategic and may help the host cell sense incoming viral challenge and coordinate an immune or apoptotic response, depending on the pathogen. Many viruses replicate in intracellular organelles such as the endoplasmic reticulum; a good example is HCV which replicates in the membranous web that connects the ER to the mitochondria. dsRNA structures, possibly within replicating ribonucleoprotein complexes, may be recognized by RIG-I and/or Mda-5, resulting in downstream signaling through MAVS. Mitochondria may be at the center of a delicate balancing act between the host immune response and virus-induced apoptosis. In the case of HCV infection, cleavage of MAVS by the NS3-4A protease appears to tip the balance, resulting in disruption of innate immune responses and establishment of chronic HCV persistence [18].

The identification of MAVS/IPS-1/VISA/Cardif, its role in innate signaling, and its characterization as the physiologically relevant target of the NS3-4A protease is an important step in the complete understanding of the mechanisms by which HCV evades the early host response. The essential localization of this CARD domain-containing adapter to the mitochondria furthermore suggests an important function in the coordination of the innate immune and apoptotic responses.

Lamarre et al. originally identified a new class of potent macrocyclic NS3 protease inhibitors, the prototype of which is BILN 2061 [29], a small molecule inhibitor that is biologically available through oral ingestion. Administration of BILN 2061 to patients infected with HCV genotype 1 for 2 days resulted in a two–three log reduction of HCV RNA plasma levels, and established proof-of-concept in humans for an HCV NS3 protease inhibitor. VX-950 is another HCV NS3-4A protease inhibitor advanced to clinical studies [11]. While the challenges of designing potent inhibitors of the viral protease have been solved, as highlighted by BILN 2061 and VX-950, it remains unclear whether these efforts will yield promising drug candidates, given that problems associated with the emergence of viral resistance or unanticipated side effects in clinical trials may be significant problems [22]. The implications for the study of HCV pathogenesis are particularly profound, given that experimental compounds such as BILN2061 and VX-950 that block NS3-4A protease activity may accomplish two goals: inhibition of virus multiplication and processing, as well as restoration of the early innate immune response that is critical to the development of a robust adaptive response in patients [11,29].

Acknowledgements

The authors wish to thank Hong-Bing Shu, Zhijian Chen, Ganes Sen and Michael Gale for reagents used in this study and members of the Molecular Oncology Group, Lady Davis Institute, for helpful discussions. This research was supported by grants from Canadian Institutes of Health Research (J.H. and R.L.) and by the National Cancer Institute of Canada, with the support of the Canadian Cancer Society (J.H.). R.L. was supported by a FRSQ Chercheur-boursier and J.H. by a CIHR Senior Investigator Award.

REFERENCES

[1] Aizaki H, Saito S, Ogino T, Miyajima N, Harada T, Matsuura Y, et al. Suppression of interferon-induced antiviral activity in cells expressing hepatitis C virus proteins. J Interferon Cytokine Res 2000;20:1111–20.
[2] Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004;4:499–511.
[3] Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732–8.
[4] Andrejeva J, Childs KS, Young DF, Carlos TS, Stock N, Goodbourn S, et al. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. Proc Natl Acad Sci USA 2004;101:17264–9.
[5] Au WC, Moore PA, LaFleur DW, Tombal B, Pitha PM. Characterization of the interferon regulatory factor-7 and its potential role in the transcription activation of interferon A genes. J Biol Chem 1998;273:29210–7.
[6] Battaglia AM, Hagmeyer KO. Combination therapy with interferon and ribavirin in the treatment of chronic hepatitis C infection. Ann Pharmacother 2000;34:487–94.
[7] Biron CA. Role of early cytokines, including alpha and beta interferons (IFN-alpha/beta), in innate and adaptive immune responses to viral infections. Semin Immunol 1998;10:383–90.
[8] Blight KJ, McKeating JA, Marcotrigiano J, Rice CM. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J Virol 2003;77:3181–90.
[9] Bonnard M, Mirtsos C, Suzuki S, Graham K, Huang J, Ng M, et al. Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-kappaB-dependent gene transcription. EMBO J 2000;19:4976–85.
[10] Breiman A, Grandvaux N, Lin R, Ottone C, Akira S, Yoneyama M, et al. Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKepsilon. J Virol 2005;79:3969–78.
[11] Chen SH, Tan SL. Discovery of small-molecule inhibitors of HCV NS3-4A protease as potential therapeutic agents against HCV infection. Curr Med Chem 2005;12:2317–42.
[12] Chisari FV. Unscrambling hepatitis C virus–host interactions. Nature 2005;436:930–2.
[13] Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 1989;244:359–62.
[14] Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, et al. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003;4:491–6.
[15] Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, et al. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med 2003;198:1043–55.
[16] Foy E, Li K, Sumpter Jr R, Loo YM, Johnson CL, Wang C, et al. Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signalling. Proc Natl Acad Sci USA 2005;79:2689–99.
[17] Gale Jr M, Blakely CM, Kwieciszewski B, Tan SL, Dossett M, Tang NM, et al. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. Mol Cell Biol 1998;18:5208–18.
[18] Gale Jr M, Foy EM. Evasion of intracellular host defence by hepatitis C virus. Nature 2005;436:939–45.

[19] Gale Jr MJ, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, et al. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology 1997;230:217–27.
[20] Ghosh AK, Majumder M, Steele R, Yaciuk P, Chrivia J, Ray R, et al. Hepatitis C virus NS5A protein modulates transcription through a novel cellular transcription factor SRCAP. J Biol Chem 2000;275:7184–8.
[21] Ghosh AK, Steele R, Meyer K, Ray R, Ray RB. Hepatitis C virus NS5A protein modulates cell cycle regulatory genes and promotes cell growth. J Gen Virol 1999;80(Pt 5):1179–83.
[22] Goudreau N, Llinas-Brunet M. The therapeutic potential of NS3 protease inhibitors in HCV infection. Expert Opin Investig Drugs 2005;14:1129–44.
[23] Hemmi H, Takeuchi O, Sato S, Yamamoto M, Kaisho T, Sanjo H, et al. The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. J Exp Med 2004;199:1641–50.
[24] Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004;5:987–95.
[25] Kang DC, Gopalkrishnan RV, Wu Q, Jankowsky E, Pyle AM, Fisher PB. mda-5: an interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. Proc Natl Acad Sci USA 2002;99:637–42.
[26] Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, et al. Cell type-specific involvement of RIG-I in antiviral response. Immunity 2005;23:19–28.
[27] Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, et al. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol 2005;6:981–8.
[28] Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 1990;12:671–5.
[29] Lamarre D, Anderson PC, Bailey M, Beaulieu P, Bolger G, Bonneau P, et al. An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. Nature 2003;426:186–9.
[30] Levy DE. Whence interferon? Variety in the production of interferon in response to viral infection. J Exp Med 2002;195:F15–8.
[31] Li XD, Sun L, Seth RB, Pineda G, Chen ZJ. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc Natl Acad Sci USA 2005;102:17717–22.
[32] Lin R, Heylbroeck C, Genin P, Pitha PM, Hiscott J. Essential role of interferon regulatory factor 3 in direct activation of RANTES chemokine transcription. Mol Cell Biol 1999;19:959–66.
[33] Lin R, Heylbroeck C, Pitha PM, Hiscott J. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. Mol Cell Biol 1998;18:2986–96.
[34] Lin R, Lacoste J, Nakhaei P, Sun Q, Yang L, Paz S, et al. Dissociation of a MAVS/IPS-1/VISA/Cardif-IKKε molecular complex from the mitochondrial outer membrane by Hepatitis C virus NS3-4A proteolytic cleavage. J Virol 2006;80:6071–83.
[35] Lin R, Mamane Y, Hiscott J. Structural and functional analysis of interferon regulatory factor 3: localization of the transactivation and autoinhibitory domains. Mol Cell Biol 1999;19:2465–74.
[36] Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623–6.
[37] Maniatis T, Falvo JV, Kim TH, Kim TK, Lin CH, Parekh BS, et al. Structure and function of the interferon-beta enhanceosome. Cold Spring Harb Symp Quant Biol 1998;63:609–20.
[38] Marie I, Durbin JE, Levy DE. Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. EMBO J 1998;17:6660–9.
[39] McWhirter SM, Fitzgerald KA, Rosains J, Rowe DC, Golenbock DT, Maniatis T. IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. Proc Natl Acad Sci USA 2004;101:233–8.
[40] Melen K, Fagerlund R, Nyqvist M, Keskinen P, Julkunen I. Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs. J Med Virol 2004;73:536–47.
[41] Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, et al. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 2005;437:1167–72.
[42] Miller K, McArdle S, Gale Jr MJ, Geller DA, Tenoever B, Hiscott J, et al. Effects of the hepatitis C virus core protein on innate cellular defense pathways. J Interferon Cytokine Res 2004;24:391–402.
[43] Moradpour D, Brass V, Bieck E, Friebe P, Gosert R, Blum HE, et al. Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication. J Virol 2004;78:13278–84.
[44] Munshi N, Yie J, Merika M, Senger K, Lomvardas S, Agalioti T, et al. The IFN-beta enhancer: a paradigm for understanding activation and repression of inducible gene expression. Cold Spring Harb Symp Quant Biol 1999;64.
[45] Perry AK, Chow EK, Goodnough JB, Yeh WC, Cheng G. Differential requirement for TANK-binding kinase-1 in type I interferon responses to toll-like receptor activation and viral infection. J Exp Med 2004;199:1651–8.
[46] Peters RT, Liao SM, Maniatis T. IKKepsilon is part of a novel PMA-inducible IkappaB kinase complex. Mol Cell 2000;5:513–22.
[47] Pflugheber J, Fredericksen B, Sumpter Jr R, Wang C, Ware F, Sodora DL, et al. Regulation of PKR and IRF-1 during hepatitis C virus RNA replication. Proc Natl Acad Sci USA 2002;99:4650–5.
[48] Pomerantz JL, Baltimore D. NF-kappaB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase. EMBO J 1999;18:6694–704.
[49] Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol 2005;5:215–29.
[50] Rosenberg S. Recent advances in the molecular biology of hepatitis C virus. J Mol Biol 2001;313:451–64.
[51] Sato M, Hata N, Asagiri M, Nakaya T, Taniguchi T, Tanaka N. Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. FEBS Lett 1998;441:106–10.
[52] Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, et al. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity 2000;13:539–48.
[53] Sato M, Tanaka N, Hata N, Oda E, Taniguchi T. Involvement of the IRF family transcription factor IRF-3 in virus-induced activation of the IFN-beta gene. FEBS Lett 1998;425:112–6.
[54] Servant MJ, Grandvaux N, Hiscott J. Multiple signaling pathways leading to the activation of interferon regulatory factor 3. Biochem Pharmacol 2002;64:985–92.
[55] Seth RB, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral

signaling protein that activates NF-kappaB and IRF 3. Cell 2005;122:669–82.
[56] Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. Science 2003;300:1148–51.
[57] Song J, Fujii M, Wang F, Itoh M, Hotta H. The NS5A protein of hepatitis C virus partially inhibits the antiviral activity of interferon. J Gen Virol 1999;80(Pt 4):879–86.
[58] Sumpter Jr R, Loo YM, Foy E, Li K, Yoneyama M, Fujita T, et al. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J Virol 2005;79:2689–99.
[59] Tan SL, Katze MG. How hepatitis C virus counteracts the interferon response: the jury is still out on NS5A. Virology 2001;284:1–12.
[60] Taniguchi T, Ogasawara K, Takaoka A, Tanaka N. Irf family of transcription factors as regulators of host defense. Annu Rev Immunol 2001;19:623–55.
[61] Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM. Inhibition of the interferon-inducible protien kinase PKR by HCV E2 protein. Science 1999;285:107–10.
[62] Tojima Y, Fujimoto A, Delhase M, Chen Y, Hatakeyama S, Nakayama K, et al. NAK is an IkappaB kinase-activating kinase. Nature 2000;404:778–82.
[63] Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791–6.
[64] Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin Liver Dis 2000;20:1–16.
[65] Wieland SF, Chisari FV. Stealth and cunning: hepatitis B and hepatitis C viruses. J Virol 2005;79:9369–80.
[66] Xu LG, Wang YY, Han KJ, Li LY, Zhai Z, Shu HB. VISA is an adapter protein required for virus-triggered IFN-beta signaling. Mol Cell 2005;19:727–40.
[67] Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 2004;5:730–7.
[68] Yoneyama M, Suhara W, Fukuhara Y, Fukada M, Nishida E, Fujita T. Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. EMBO J 1998;17:1087–95.
[69] Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci USA 2005;102:9294–9.