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Accessory proteins 8b and 8ab of severe acute respiratory syndrome coronavirus suppress the interferon signaling pathway by mediating ubiquitin-dependent rapid degradation of interferon regulatory factor 3

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ABSTRACT

Severe acute respiratory syndrome coronavirus (SARS-CoV) is an inefficient inducer of interferon (IFN) response. It expresses various proteins that effectively circumvent IFN production at different levels via distinct mechanisms. Through the construction of recombinant IBV expressing proteins 8a, 8b and 8ab encoded by SARS-CoV ORF8, we demonstrate that expression of 8b and 8ab enables the corresponding recombinant viruses to partially overcome the inhibitory actions of IFN activation to achieve higher replication efficiencies in cells. We also found that proteins 8b and 8ab could physically interact with IRF3. Overexpression of 8b and 8ab resulted in the reduction of poly (I:C)-induced IRF3 dimerization and inhibition of the IFN-β signaling pathway. This counteracting effect was partially mediated by protein 8b/8ab-induced degradation of IRF3 in a ubiquitin-proteasome-dependent manner. Taken together, we propose that SARS-CoV may exploit the unique functions of proteins 8b and 8ab as novel mechanisms to overcome the effect of IFN response during virus infection.

1. Introduction

When challenged by a viral infection, the host mounts an immediate innate immune response, leading to the production of type I interferons (IFN-α and IFN-β) and the expression of hundreds of downstream IFN-stimulated genes (ISGs) (Stetson and Medzhitov, 2006; Suhara et al., 2002). Central to the induction of type I IFN is interferon regulatory factor 3 (IRF3) (Hiscott, 2007; Taniguchi et al., 2001). The initial step of the signaling cascade leading to IRF3 activation is recognition of specific viral pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs) in two major pathways. These include cell surface toll-like receptors (TLRs), such as TLR3, TLR7, TLR8 and TLR9, which sense viral components (Kumagai et al., 2008; Kumar et al., 2009a, 2009b), and cytosolic RNA helicases such as retinoic acid-inducible gene I (RIG-I) and/or melanoma differentiation-associated gene 5 (MDA5), which detect viral RNA (Loo et al., 2008; Onomoto et al., 2007). Upon binding to their ligands, PRRs recruit adaptor proteins to set off a series of signaling cascades to phosphorylate and dimerize IRF3 (Fitzgerald et al., 2003). The activated IRF3 homodimer then translocates to the nucleus, switching on IFN synthesis (Lin et al., 1998; Suhara et al., 2002; Thanos and Maniatis, 1995). Many viruses have also evolved strategies to counteract the IFN action, including mechanisms that allow virus to evade recognition by the immune surveillance system, so as to inhibit IFN induction by hijacking molecules involved in IFN activation pathways or by inhibiting downstream signal transduction (Goodbourn et al., 2000; Versteeg et al., 2007; Weber et al., 2003).

Severe acute respiratory syndrome coronavirus (SARS-CoV) was the etiological agent of the SARS epidemic in 2003 (Guan et al., 2003; Marra et al., 2003). Apart from four typical structural proteins, nucleocapsid (N), envelope (E), membrane (M) and spike (S) proteins, and approximately 16 non-structural proteins (Nsp1-16) involved in viral replication, SARS-CoV encodes an exceptionally high number of accessory proteins that bear little resemblance to accessory genes of other coronaviruses (Liu et al., 2014; Narayanan et al., 2008b). Similar to other coronaviruses, SARS-CoV is an inefficient inducer of IFN-β

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response in cell culture system (Spiegel et al., 2005) and is sensitive to the antiviral state induced by IFNs (Spiegel et al., 2004; Zheng et al., 2004). Its genome may therefore encode proteins that allow this virus to effectively circumvent IFN production in order to overcome limitations imposed by the IFN action.

Together with a few structural proteins and Nsps, many coronavirus accessory proteins could suppress IFN production by targeting different aspects of the IFN signaling cascade (Lim et al., 2016; Liu et al., 2014; Zhong et al., 2012). For instance, SARS-CoV Papain-like protease (PLpro) attenuates IFN synthesis by abrogating IRF3 phosphorylation and nuclear translocation by physically interacting with IRF3 (Devaraj et al., 2007). IFN induction mediated by a constitutively active IRF3 is also inhibited by the de-ubiquitination activity of SARS-CoV PLpro (Matthews et al., 2014). On the other hand, Nsp1 of PEDV does not interfere IRF3 phosphorylation and nuclear translocation, but interrupts the enhanceosome assembly of IRF3 and CREB-binding protein (CBP) by promoting proteasomal degradation of CBP (Zhang et al., 2016). Targeting further upstream, SARS-CoV M protein prevents IRF3 phosphorylation by inhibiting the assembly of TBK1/IKK complex (Siu et al., 2009), whereas MERS-CoV M protein interacts with TRAF3 and disrupts TRAF3-TBK1 association, leading to reduced IRF3 phosphorylation (Lui et al., 2016). Likewise, the accessory protein ORF4b of MERS-CoV has been shown to specifically bind to TBK1 and IKKε, thereby inhibiting IRF3 phosphorylation and IFN-β production (Yang et al., 2015).

Signaling molecules downstream of IFN synthesis are also targets of SARS-CoV proteins. For instance, SARS-CoV Nsp1 inhibits STAT1-mediated transcription of ISGs by inhibiting its phosphorylation (Wathelet et al., 2007), apart from inducing degradation of a wide range of host mRNAs (Kamitani et al., 2006; Narayanan et al., 2008a). SARS-CoV ORF6 blocks STAT1 nuclear translocation by trapping the nuclear import factors in the endoplasmic reticulum and Golgi apparatus (Frieman et al., 2007). Among other IFN antagonists identified are nucleocapsid (N) protein of SARS-CoV and PEDV (Kopecky-Bromberg et al., 2007; Ding et al., 2014), accessory protein 4a of MERS-CoV (Siu et al., 2014), and PLpro domain 2 (PLP2) of MHV-A59 (Wang et al., 2011). IFN antagonism mediated by SARS-CoV N protein seemed to target a very early step of RNA recognition (Lu et al., 2011). Overexpression of SARS-CoV accessory protein 3a was found to down-regulate type I IFN receptor by promoting its ubiquitination and subsequent degradation via the lysosomal pathway (Minakshi et al., 2009).

Although both 8b and 8ab are encoded by SARS-CoV ORF8, they are expressed under distinct conditions. 8ab is expressed as a single protein encoded by the single continuous ORF (ORF8ab) found in SARS-CoV isolated from animals and early stage human isolates. In contrast, as a consequence of a 29-nt deletion that results in two separate overlapping ORFs (ORF8a / ORF8b), most human isolates obtained at the middle to later phase of the epidemic encode instead 8a and 8b as two distinct proteins (Guan et al., 2003; Oostra et al., 2007). ORF8 is presumably acquired from SARS-related coronavirus from greater horseshoe bats through recombination (Lau et al., 2015), and the 29-nt deletion may be an evolutionary adaptation for enhancing viral pathogenesis in the human host. Proteins 8a, 8b and 8ab may possess different biochemical properties and possibly cellular functions (Law et al., 2006; Le et al., 2007). Protein 8ab was shown to be a glycosylated ER resident protein which can activate ATF6 to modulate the unfolded protein response (Sung et al., 2009). Protein 8a was found to enhance viral replication and induce cell death (C.Y. Chen et al., 2007), while 8b induces DNA synthesis and down-regulates SARS-CoV E protein via a proteasome-independent pathway (Keng et al., 2006). SARS-CoV 8b and 8ab were also shown to bind to both mono- and poly-ubiquitin when expressed in cell culture (Keng et al., 2006). Whether these ubiquitin-binding properties allow them to interact with host cell proteins remains unknown. Interestingly, when an in vivo attenuated recombinant SARS-CoV lacking the full-length E gene is passaged in mice, the ORF8 sequence mutates to encode a PDZ-binding motif in protein 8a, and the virus regained virulence (Jimenez-Guardeño et al., 2015).

In this study, we show proteins 8b and 8ab as novel IFN antagonists. Evidence presented supports the direct interaction between these two proteins and IRF3. The two proteins were also found to partially suppress IFN induction by limiting IRF3 activation and/or promoting the proteasome-mediated degradation of IRF3.

2. Materials and methods

2.1. Cell culture

African green monkey kidney Cos-7 and Vero cells, human non-small cell lung carcinoma H1299 cells and human hepatocellular carcinoma Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Hyclone) and 1% penicillin/streptomycin DMEM (Invitrogen) and maintained at 37 °C with 5% CO_2. To inhibit the proteasome activity, MG132 (Sigma) at a final concentration of 10 μM was added to cells 2 h prior to harvest.

2.2. Virus growth curve and plaque assay

To compare the growth kinetics of various recombinant virus strains, Vero cells were infected with the respective recombinant IBV strains at a multiplicity of infection (MOI) of 0.5, and harvested at a 4 h interval within 20 h post-infection for virus titration through plaque assay.

A monolayer of Vero cells seeded on 6-well plates a day prior to infection was infected with 200 μl of 10-fold serially diluted virus stock. After 1 h of incubation at 37 °C with regular shaking to ensure even distribution of the virus, cells were washed with PBS and cultured in 3 ml of DMEM containing 1% carboxymethyl cellulose (CMC) for 3 days. The cells were then fixed with 4% paraformaldehyde and stained with 0.1% toluidine. The number of plaques was counted in duplicates and the virus titer was calculated as plaque-forming unit (Pfu) per ml.

2.3. Plasmid Construction

The pKTO-Flag-8b and pKTO-Flag-8ab plasmids were previously described (Le et al., 2007). The coding sequences of 8b or 8ab were also subcloned to the pXJ40-Flag vector, which contains the CMV promoter for expression in mammalian cell lines. For the construction of the pXJ40-Myc-IRF3 plasmid, human IRF3 gene was amplified from cDNA of H1299 cells using the forward primer 5′-AACGCCTCGACGGAACCC CAAAGCCACGGAT-3′and the reverse primer 5′-GCCGGTACCTTATTG GTTGAGGTGGTGGGG-3′ prior to ligation into pXJ40-My plasmid at *Xho*I and *Kpn*I restriction sites. Truncated mutants were then constructed based on the pXJ40-Myc-IRF3 construct using the following primers for PCR amplification: for IRF3 (1 − 133), Forward 5′-CCGCT CGAGCGGATGATGGGAACCCCAAAGCCACG−3′and Reverse 5′–GGGG TACCCCTCAAGAAGTACTGCCTCCACCAT–3′; for IRF3 (399-379), Forward 5′-CCGCTCGAGCGGATGGATACCCAGGAAGACATTCT-3′ and Reverse 5′-GGGGTACCCCTCATCCAGGCAGCGTCCTGTCTC-3′; for IRF3 (241 − 427), Forward 5′-CCGCTCGAGCGGATGTGGCCAGTCACACTGC CAGA-3′ and Reverse 5′-GGGGTACCCCTCAGCTCTCCCCAGGGCCCT-3′; for IRF3 (134 − 427): Forward 5′-CCGCTCGAGCGGATGGATACCCAGG AAGACATTCT-3′ and Reverse 5′-GGGGTACCCCTCAGCTCTCCCCAGG GCCCT-3′. Constitutively active mutant pXJ40-Myc-IRF3-5D was generated by performing sequential site-directed mutagenesis PCR (Quik-Change II site-directed mutagenesis kit; Stratagene) to replace amino acids at positions 396, 398, 402, 404, and 405 with the phosphomimetic aspartic acid.

2.4. Western blot analysis

Cells were lysed in RIPA buffer in the presence of protease inhibitors (Roche Diagnostics) and phosphatase inhibitors (Pierce). Protein lysates were separated by electrophoresis in 8% SDS polyacrylamide gels and