Virus Research 172 (2013) 75–80

Contents lists available at SciVerse ScienceDirect

Virus Research

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

Short communication

Influenza A polymerase subunit PB2 possesses overlapping binding sites for polymerase subunit PB1 and human MAVS proteins

Deendayal Patel[a,b], L. Wayne Schultz[a,b], Timothy C. Umland[a,b,*]

[a] Hauptman-Woodward Medical Research Institute, 700 Ellicott Street, Buffalo, NY 14203, USA
[b] Department of Structural Biology, University at Buffalo-State University of New York, 700 Ellicott St., Buffalo, NY 14203, USA

ARTICLE INFO

Article history:
Received 15 August 2012
Received in revised form 9 November 2012
Accepted 3 December 2012
Available online 12 December 2012

Keywords:
Influenza A virus
Polymerase subunit PB2
Polymerase subunit PB1
Mitochondrial antiviral signaling protein (MAVS)
Protein–protein interaction (PPI)
Host tropism

ABSTRACT

Influenza A virus is an important human pathogen accounting for widespread morbidity and mortality, with new strains emerging from animal reservoirs possessing the potential to cause pandemics. The influenza A RNA-dependent RNA polymerase complex consists of three subunits (PA, PB1, and PB2) and catalyzes viral RNA replication and transcription activities in the nuclei of infected host cells. The PB2 subunit has been implicated in pathogenicity and host adaptation. This includes the inhibition of type I interferon induction through interaction with the host's mitochondrial antiviral signaling protein (MAVS), an adaptor molecule of RIG-I-like helicases. This study reports the identification of the cognate PB2 and MAVS interaction domains necessary for complex formation. Specifically, MAVS residues 1–150, containing both the CARD domain and the N-terminal portion of the proline rich-region, and PB2 residues 1–37 are essential for PB2–MAVS virus–host protein–protein complex formation. The three α-helices constituting PB2 (1–37) were tested to determine their relative influence in complex formation, and Helix3 was observed to promote the primary interaction with MAVS. The PB2 MAVS-binding domain unexpectedly coincided with its PB1-binding domain, indicating an important dual functionality for this region of PB2. Analysis of these interaction domains suggests both virus and host properties that may contribute to host tropism. Additionally, the results of this study suggest a new strategy to develop influenza A therapeutics by simultaneously blocking PB2–MAVS and PB2–PB1 protein–protein interactions and their resulting activities.

The RNA-dependent RNA polymerase (RdRP) of influenza A virus is a heterotrimeric protein complex comprised of three virally-encoded protein subunits, designated PA, PB1, and PB2 (Area et al., 2004; Lamb and Krug, 2003). The RdRP is a central component for both replication of the negative strand viral RNA (vRNA) via a complementary RNA (cRNA) intermediate, and for vRNA transcription yielding viral mRNAs for use in protein synthesis. These essential roles make the RdRP complex an attractive target for the development of novel antivirals. PA has been implicated in a diverse range of functions including endonuclease and protease activities, additionally participating in vRNA/cRNA promoter binding (Dias et al., 2009; Maier et al., 2008; Sanz-Ezquerro et al., 1996; Yuan et al., 2009). PB1 contains the polymerase active site (Biswas and Nayak, 1994; Gonzalez and Ortin, 1999) forming the core of the RdRP complex and interacting with both PA (PB1 N-terminus) (Perez and Donis, 1995, 2001) and PB2 (PB1 C-terminus) (Deng et al., 2005). PB2 has a cap-binding motif that binds to the 5′ cap of host-cell mRNAs to facilitate a cap-snatching mechanism (Guilligay et al., 2008; Ulmanen et al., 1981). Additionally, PB2 displays a bipartite nuclear localization signal and is transported to the nucleus independently from PA and PB1 (MacDonald et al., 2012; Mukaigawa and Nayak, 1991).

Molecular signatures have been identified within the RdRP complex correlating with pathogenicity and adaptation of avian influenza viruses to mammalian hosts, the majority being present in the PB2 subunit (Boivin et al., 2010). Sequence changes in PB2 were shown to be important for overcoming the species barrier (Mehle and Doudna, 2009; Salomon et al., 2006), but in many cases the mechanism is unknown. PB2 was recently observed to interact with the mitochondrial antiviral signaling protein (MAVS; also referred to as IPS1, VISA, and Cardif), thereby inhibiting type I interferon (IFN) induction (Graef et al., 2010; Iwai et al., 2010). MAVS is

Abbreviations: CARD, caspase activation and recruitment domain; co-IP, co-immunoprecipitation; cRNA, complementary RNA; GST, glutathione-*S*-transferase; HA, hemagglutinin epitope tag; IFN, interferon; MBP, maltose binding protein; MAVS, mitochondrial antiviral signaling protein; MTS, mitochondrial targeting signal sequence; PRR, proline rich region; PPI, protein–protein interaction; RLH, RIG-I-like helicases; RdRP, RNA-dependent RNA polymerase; TM, transmembrane domain; vRNA, viral RNA.

* Corresponding author at: Hauptman-Woodward Medical Research Institute, 700 Ellicott Street, Buffalo, NY 14203, USA. Tel.: +1 716 898 8632; fax: +1 716 898 8660.
E-mail address: umland@hwi.buffalo.edu (T.C. Umland).

0168-1702/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2012.12.003

an adaptor molecule of RIG-I-like helicases (RLH), and is localized primarily to the outer mitochondrial membrane via a C-terminal transmembrane domain (Dixit et al., 2010; Seth et al., 2005). MAVS participates in an intracellular signaling cascade responsive to cytoplasmic viral RNA, prompting the production of type I IFN. This innate immunity pathway provides early antiviral responses, and not surprisingly viruses have developed multiple systems to overcome or modulate this host response. Both PB2 and MAVS are large, multi-domain proteins and outside of a course screening of PB2 fragments that identified the N-terminal residues 1–256 as important (Iwai et al., 2010), the localized sites comprising the PB2–MAVS interface have not been previously reported. Furthermore, details of the manner by which PB2 binding to MAVS suppresses IFN induction are unknown.

In the present study, we identified the domains within both PB2 and human MAVS, respectively, responsible for formation of this virus–host protein–protein interaction (PPI). Human MAVS is a 540 amino acid protein that contains an N-terminal caspase activation and recruitment domain (CARD; residues 10–93), a proline rich region (PRR, residues 103–173), and a C-terminal transmembrane helical domain (TM; residues 514–535). The extended region between the PRR and the TM is predicted to be intrinsically disordered. To determine the specific region of the MAVS protein that participates in the PB2–MAVS PPI, a series of deletions to the MAVS gene were constructed. To prepare the truncated MAVS constructs, fragments encoding amino acids 1–110, 1–130, 1–150, 1–160, 1–175, and 1–512 were PCR amplified from a pDONR221 plasmid containing the complete human MAVS gene (DNASU HsCD00296475) and cloned into a pCAGEN-based vector (Addgene plasmid 11160; Matsuda and Cepko, 2004) modified with a C-terminal hemagglutinin (HA) fusion tag. MAVS (1–512) was used as a surrogate for full-length MAVS in order to avoid potential non-specific hydrophobic interactions promoted by the MAVS TM helix that may yield misleading PPI results. The full-length A/WSN/1933 (H1N1) PB2 gene was PCR amplified from the pCAGGS-WSN-PB2 plasmid (kindly provided by Dr. Yoshihiro Kawaoka, University of Wisconsin–Madison) and an N-terminal triple-FLAG (3x-FLAG) tag was generated by overlapping PCR to construct the pCAGEN-3x-FLAG-PB2 plasmid. Each truncated MAVS construct (pCAGEN-MAVS (1–93)/(1–110)/(1–130)/(1–150)/(1–160)/(1–175)/(1–512)-HA) was co-transfected with pCAGEN-3x-FLAG-PB2 into HEK293 cells using Lipofectamine LTX (Invitrogen). Cell extracts were prepared 24 h post-transfection and co-immunoprecipitation (co-IP) assays were performed to determine the presence of interactions between MAVS fragments and full-length PB2. Co-IP was performed using the Pierce Direct IP Kit (Thermo Fisher Scientific), coupling the resin with an anti-PB2 polyclonal antibody, generated in rabbit against the PB2 peptide ^{330}FKRTSGSSVKREEE^{343} (GenScript), or with anti-FLAG M2 antibody (Stratagene). Similar co-IP results were obtained for both antibodies, further suggesting that the observed interactions represented a specific PB2–MAVS complex. MAVS that co-immunoprecipitated with PB2 was then detected by Western blotting using a monoclonal anti-HA antibody (Sigma). The co-IP results demonstrated that MAVS fragments 1–150, 1–160, 1–175, and 1–512 but not MAVS fragments 1–110 or 1–130 formed a complex with PB2 (Fig. 1A and Fig. S1). All of the MAVS and the PB2 constructs expressed well as determined by Western blotting using cell lysate and antibodies against HA and FLAG epitope tags, respectively. Each of the co-IP experiments in this report, unless otherwise noted, used lysate from co-transfected cells expressing both proteins being tested for interactions, and so these Western blots also confirmed antibody specificity. No antibody cross reactivity was observed for any of the antibody–fusion protein combinations. To further map the region of interaction within MAVS, a series of three sequentially truncated mutants was generated (MAVS(1–135), MAVS(1–140), and MAVS(1–145)). HEK293 cells were co-transfected with each new MAVS truncation mutant and the full-length 3x-FLAG-PB2 vectors. No significant interactions were detected by co-IP for PB2 with MAVS(1–135) or MAVS(1–140). However, a weak interaction, as compared to the PB2–MAVS (1–150) interaction, was observed between MAVS(1–145) and PB2 (Fig. S2). Relative complex formation with PB2 by MAVS(1–135), MAVS(1–140), and MAVS(1–145) was <0.1%, 0.0%, and 44%, respectively, compared to that of MAVS(1–150), as estimated by densitometry of the Western blot (AlphaImager AlphaEaseFC software). We selected MAVS(1–150) and MAVS(1–175) constructs for use in further studies. These results support that the MAVS N-terminal fragment 1–150 forms a stable complex with PB2, and that MAVS residues 93–150 (the N-terminal two-thirds of the PRR domain) play a necessary role in the virus–host PPI.

To further examine if the MAVS CARD or PRR domains alone were sufficient for the PB2 interaction we cloned these fragments (residues 1–93 and 93–175, respectively) into pCAGEN-based vectors. To enhance expression levels and stability, pCAGEN was modified to include a N-terminal six-histidine-glutathione-*S*-transferase (GST) fusion tag, based on a previously reported expression strategy (Potter et al., 2008). HEK293 cells were co-transfected with plasmids pCAGEN-3x-FLAG-PB2 and pCAGEN-GST-MAVS(1–93) or pCAGEN-GST-MAVS(93–175). Co-IP analysis of cell extracts with anti-FLAG antibody (binding to PB2) followed by Western blotting using anti-GST polyclonal antibody (Millipore; to identify MAVS constructs) established that neither isolated MAVS CARD or PRR domains were capable of forming a stable complex with PB2 (Fig. 1B). These results demonstrated that MAVS residues 1–150, including both the CARD domain and the N-terminal two-thirds of the PRR domain, were required for a stable interaction with PB2. The requirement of the MAVS CARD-PRR fragment for PB2-binding suggests that MAVS may undergo a conformational change upon PB2-binding or that MAVS' PB2-binding interface is composed of residues from both the CARD and PRR domains.

Truncated PB2 constructs were then prepared to identify the minimal fragment required for interaction with MAVS. Initial screening used three PB2 fragments based upon known functional domains, specifically a fragment including the PB1-binding domain (residues 1–177) (Sugiyama et al., 2009), the cap binding domain (residues 318–483) (Das et al., 2010), and the RNA binding and nuclear import domain (residues 535–757) (Kuzuhara et al., 2009). Each PB2 construct was cloned into the pCAGEN-3x-FLAG vector. HEK293 cells were co-transfected with pCAGEN-3x-FLAG-PB2 (1–177), (318–483), or (535–757) vectors and pCAGEN-MAVS (1–175)-HA. Cell extracts were subjected to co-IP analysis using anti-HA antibody to immunoprecipitate MAVS and Western blotting with anti-FLAG antibody to identify co-precipitated PB2 constructs. This revealed a strong interaction between MAVS and PB2(1–177) and a weak interaction between MAVS and PB2(535–757) (Fig. 2).

Based on the observed strong interaction with MAVS, we selected PB2(1–177) for finer screening to identify the minimal fragment required for the interaction with MAVS. Expression vectors encoding the fragments PB2(1–37), PB2(1–86), and PB2(37–177) were prepared in a pCAGEN vector modified to include an N-terminal 6xHis-maltose binding protein (MBP) fusion tag to enhance expression and stability. HEK293 cells were co-transfected with pCAGEN-MBP-PB2(1–37), (1–86), or (37–177) and pCAGEN-MAVS (1–150)-HA vectors. A control experiment demonstrated no interaction between MBP alone and MAVS (1–150)-HA (data not shown). Co-IP was conducted with anti-HA monoclonal antibody (Sigma; binding to the MAVS construct) followed by Western blotting using anti-MBP rabbit polyclonal antibody (New England Biolabs) to identify PB2 fragments that