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Middle East Respiratory Syndrome Coronavirus 4a Protein Is a Double-Stranded RNA-Binding Protein That Suppresses PACT-Induced Activation of RIG-I and MDA5 in the Innate Antiviral Response

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ABSTRACT

Middle East respiratory syndrome coronavirus (MERS-CoV) is an emerging pathogen that causes severe disease in human. MERS-CoV is closely related to bat coronaviruses HKU4 and HKU5. Evasion of the innate antiviral response might contribute significantly to MERS-CoV pathogenesis, but the mechanism is poorly understood. In this study, we characterized MERS-CoV 4a protein as a novel immunosuppressive factor that antagonizes type I interferon production. MERS-CoV 4a protein contains a double-stranded RNA-binding domain capable of interacting with poly(I-C). Expression of MERS-CoV 4a protein suppressed the interferon production induced by poly(I-C) or Sendai virus. RNA binding of MERS-CoV 4a protein was required for IFN antagonism, a property shared by 4a protein of bat coronavirus HKU5 but not by the counterpart in bat coronavirus HKU4. MERS-CoV 4a protein interacted with PACT in an RNA-dependent manner but not with RIG-I or MDA5. It inhibited PACT-induced activation of RIG-I and MDA5 but did not affect the activity of downstream effectors such as RIG-I, MDA5, MAVS, TBK1, and IRF3. Taken together, our findings suggest a new mechanism through which MERS-CoV employs a viral double-stranded RNA-binding protein to circumvent the innate antiviral response by perturbing the function of cellular double-stranded RNA-binding protein PACT. PACT targeting might be a common strategy used by different viruses, including Ebola virus and herpes simplex virus 1, to counteract innate immunity.

IMPORTANCE

Middle East respiratory syndrome coronavirus (MERS-CoV) is an emerging and highly lethal human pathogen. Why MERS-CoV causes severe disease in human is unclear, and one possibility is that MERS-CoV is particularly efficient in counteracting host immunity, including the sensing of virus invasion. It will therefore be critical to clarify how MERS-CoV cripples the host proteins that sense viruses and to compare MERS-CoV with its ancestral viruses in bats in the counteraction of virus sensing. This work not only provides a new understanding of the abilities of MERS-CoV and closely related bat viruses to subvert virus sensing but also might prove useful in revealing new strategies for the development of vaccines and antivirals.

Middle East respiratory syndrome coronavirus (MERS-CoV) is a newly identified human pathogen associated with severe acute respiratory disease occasionally accompanied by renal failure. MERS-CoV has claimed 77 lives (42.8%) among the 180 laboratory-confirmed cases reported to the World Health Organization as of 27 January 2014. Although most cases originated from the Middle East, human-to-human transmission, hospital outbreaks, and family clusters have been found (1–3), and the virus has emerged as a threat to public health worldwide.

Coronaviruses are enveloped viruses with a large single-stranded and positive-sense RNA genome of about 30 kb. Like other coronaviruses, the long genomic mRNA of MERS-CoV encodes replicase polyproteins that are further processed into multiple nonstructural proteins. In contrast, conserved spike (S), envelope (E), membrane (M), and nucleocapsid structural proteins are translated from subgenomic mRNAs. In addition, subgenomic mRNAs of MERS-CoV also encode five unique accessory proteins, designated 3, 4a, 4b, 5, and 8b, which are found only in the same lineage of viruses (4). Phylogenetically, MERS-CoV belongs to lineage C, previously known as group 2c, of the genus *Betacoronavirus* in the family *Coronaviridae* (5–7). In the same

lineage, there are two bat coronaviruses (bCoVs), HKU4 and HKU5, which have been isolated, respectively, from lesser bamboo bats (*Tylonycteris* spp.) and Japanese pipistrelle bats (*Pipistrellus* spp.) captured in Hong Kong (8, 9). MERS-CoV is closely related to these two bat coronaviruses, and a bat origin of the virus was suspected (10–12), but the intermediate host and the proximate animal source of human infection remain to be convincingly established, despite the high prevalence of MERS-CoV neutralizing antibodies in dromedary camels (13, 14).

Infection with MERS-CoV causes severe disease in human through an unknown mechanism. The pathogenic course of

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MERS-CoV resembles that of severe acute respiratory syndrome coronavirus (SARS-CoV), another coronavirus in lineage B of the same genus and the only other coronavirus commonly associated with severe respiratory disease in human (15, 16). Viral evasion of innate immunity is pivotally involved in SARS-CoV pathogenesis (16, 17). By analogy, MERS-CoV might also suppress the innate antiviral response. Indeed, although MERS-CoV replicated well in various types of cells, including human monocyte-derived macrophages, and its replication was susceptible to alpha interferon (IFN- α) (18–23), its induction of type I interferons (IFNs) was severely impaired and delayed (20–25). Similar to SARS-CoV, MERS-CoV might encode multiple immunoregulatory proteins that antagonize type I IFN production. However, many of the IFN-antagonizing accessory proteins in SARS-CoV are not found in MERS-CoV (4). Thus, it is of interest to see whether the unique accessory proteins in MERS-CoV are capable of suppressing type I IFN production.

Production of type I IFNs is induced when host pattern recognition receptors, such as Toll-like receptors and RIG-I-like receptors, are activated by pathogen-associated molecular patterns, such as viral double-stranded RNA (dsRNA). Through an intracellular signaling pathway that involves mitochondrial adaptor protein MAVS as well as protein kinases TBK1 and IKK ϵ , IRF3 and IRF7 transcription factors are translocated to the nucleus and recruited to the IFN promoters to activate transcription (26, 27). Specifically, RIG-I-like receptors RIG-I and MDA5 are known to be critically involved in the sensing of coronavirus infection (28). RIG-I and MDA5 are cytoplasmic sensors of virus-derived RNAs (27). Optimal activity of RIG-I and MDA5 also requires PACT, a cellular dsRNA-binding protein which binds to RIG-I and MDA5 to activate IFN production (29). As such, PACT is also the cellular target of viral IFN antagonists, including Ebola virus VP35 protein, herpes simplex virus 1 (HSV-1) Us11 protein, and probably, influenza A virus NS1 protein (30–32). Both VP35 and Us11 are dsRNA-binding proteins. They physically interact with PACT and prevent it from binding to and activating RIG-I (30, 31). This perturbation of a cellular dsRNA-binding protein by viral dsRNA-binding and IFN-antagonizing proteins represents a new mechanism for viral evasion of innate immunity (32).

MERS-CoV 4a, 4b, and M proteins were recently found to antagonize type I IFN production (33, 34). Particularly, MERS-CoV 4a protein was shown to be capable of binding to dsRNA and inhibiting the activity of MDA5 (33). In this study, we characterized MERS-CoV 4a protein as a dsRNA-binding and IFN-antagonizing protein that targets PACT. MERS-CoV 4a protein was compared with the 4a proteins of bCoV-HKU4 and bCoV-HKU5. The interaction of MERS-CoV 4a protein with and its impact on dsRNA-binding sensor proteins RIG-I, MDA5, and PACT were examined. Our findings provide further support to the notion that PACT targeting by a viral dsRNA-binding and IFN-antagonizing protein is a common countermeasure used by different types of viruses in the suppression of the innate antiviral response.

MATERIALS AND METHODS

Cells and viruses. HEK293, HEK293T, and Calu3 cells were grown and propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies) and antibiotics. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂ and transfected with the Gene-Juice transfection reagent (Novagen) as described previously (29, 35). Sendai virus was purchased from the American Type Cul-

ture Collection. MERS-CoV isolate hCoV-EMC/2012 was kindly provided by Ron Fouchier (5). Viral infection was carried out as described previously (19, 23, 29).

Plasmids. Reporter plasmids pIFN β -Luc and pIRF3-Luc, as well as expression plasmids for RIG-I, MDA5, PACT, MAVS, TBK1, IRF3, HSV-1 Us11, influenza A virus NS1, and SARS-CoV M protein, were described elsewhere (29, 31, 35, 36). pIFN β -Luc, provided by Takashi Fujita, contains the entire promoter region of the IFN- β gene (37, 38). In pIRF3-Luc, the expression of firefly luciferase is driven by three tandem IRF3-binding sites derived from the human IFN- β promoter (29). The expression plasmid for VP35 was a gift from Heinz Feldmann (National Institute of Allergy and Infectious Diseases, Bethesda, MD) (39).

Viral RNA was extracted from MERS-CoV-infected Calu3 cells, bCoV-HKU4-infected alimentary specimens of lesser bamboo bats, and bCoV-HKU5-infected alimentary samples of Japanese pipistrelle bats using a QIAamp viral RNA minikit (Qiagen) as described previously (8, 40). Protein 4a cDNAs of MERS-CoV, bCoV-HKU4, and bCoV-HKU5 were generated by reverse transcription of viral RNA templates (GenBank accession numbers JX869059.2, EF065505.1, and EF065510.1, respectively) using random hexamers. The V5-tagged 4a protein clones were then PCR amplified using the following primers: 5'-CCGGAATTCATGGATTACG TGTCTCTGCTTAATC-3' (MERS-CoV 4a sense), 5'-CCGCTCGAGTACCGTAGAATCGAGACCGAGGAGGGTTAGGGATAGCCTTACC GTTGGAGAATGGCTCCTCTTCC-3' (MERS-CoV 4a antisense), 5'-CCGGAATTCATGGACTACGTCTCTTGTCTTAACC-3' (bCoV-HKU4 4a sense), 5'-CCGCTCGAGTACCGTAGAATCGAGACCGAGGAGGGTTAGGGATAGCCTTACC TCGGATAAGGCTTACCTGCCGTTTCGAGAAGAAGTAG-3' (bCoV-HKU4 4a antisense), 5'-CCGGAATTCATGGATTACGTGTCTGCTGCTCA ACC-3' (bCoV-HKU5 4a sense), and 5'-CCGCTCGAGTACCGTAGAATCGAGACCGAGGAGGGTTAGGGATAGCCTTACC TCGGATAAGGCTTACCTGCCGTTTCGAGAAGAAGTAG-3' (bCoV-HKU5 4a antisense). The EcoRI and XhoI restriction sites in the sense and antisense primers are underlined, and the V5 tag sequence is italicized. The expression vector for 4a proteins was based on pCAGGS. The same vector has successfully been used to express other coronavirus proteins (35, 41, 42).

Site-directed mutagenesis of MERS-CoV 4a protein was carried out by overlap extension PCR (43). The primers used to create the dsRNA-binding-defective (dsm) mutant were 5'-ACAGCAGCTTTGGCCGCACAGGACGC AGCTCAGCGAATCG-3' (forward), 5'-TGTGCGGCCAAAGCTGCTGTGA GAATTAACAGCAGATTCAG-3' (reverse), 5'-ACAGCAGCTTTGGCCG CACAGGACGCAGCTCAGCGAATCG-3' (internal mutagenic forward), and 5'-TGTGCGGCCAAAGCTGCTGTAGAATTAACAGCAGATTC AG-3' (internal mutagenic reverse). The dsm mutant was verified by DNA sequencing of the entire coding region.

Protein and RNA analysis. The poly(I-C) pulldown assay, dual-luciferase assay, quantitative reverse transcription-PCR (RT-PCR), Western blot analysis, and coimmunoprecipitation were carried out as previously described (38, 44–46). Cells were cultured in 12-well plates for the dual-luciferase assay and in 60-mm dishes for coimmunoprecipitation. Mouse anti-Flag (M2 and M5) and anti- α -tubulin (DM1A) antibodies from Sigma-Aldrich were used at a 1:10,000 dilution for Western blotting. Mouse anti-V5 from Life Technologies was used at a 1:5,000 dilution for Western blotting.

For the poly(I-C) pulldown assay, poly(I-C)-coated agarose (Sigma-Aldrich) was incubated for 4 h with cell lysates as described previously (38). Poly(C)-coated agarose was used as a negative control. The binding buffer contained 1 mM EDTA, 100 mM NaCl, 1% sodium deoxycholate, 1% NP-40, and protease inhibitors. Beads were collected by centrifugation, washed four times with binding buffer, and collected in SDS-PAGE loading buffer for subsequent Western blot analysis as described previously (47, 48).

For quantitative RT-PCR, total RNAs were isolated from cultured cells using the TRIzol reagent (Life Technologies) as previously described (42, 44). RNA (1 μ g) was reverse transcribed into cDNA using random hexamers. The level of IFN- β mRNA was calculated from 2^{- Δ CT} by the com-

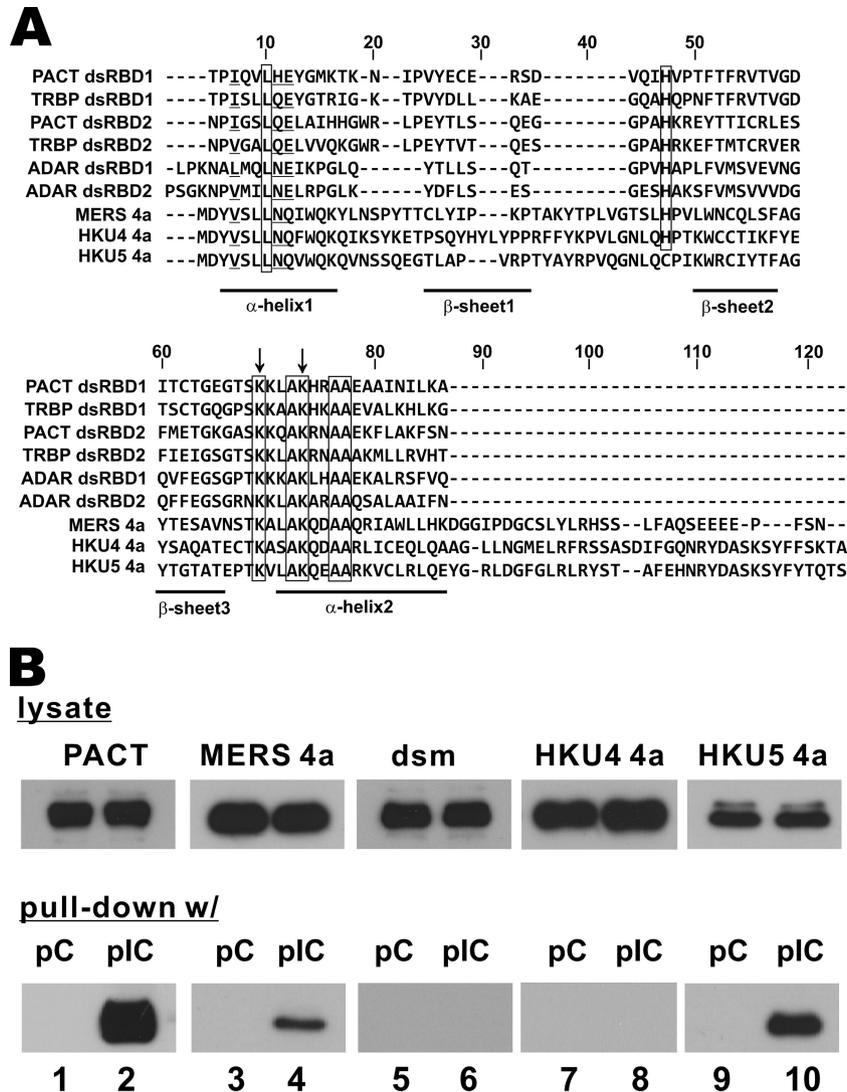


FIG 1 MERS-CoV 4a protein is a dsRNA-binding protein. (A) Sequence alignment. The alignment was generated with the ClustalW program. Identical residues are boxed. Highly similar residues are underlined. The most conservative K63 and K67 residues mutated in the dsm mutant are identified with arrows. Predicted secondary structures are indicated. Classical dsRNA-binding domains are composed of an α - β - β - α architecture. dsRBD1 and dsRBD2, dsRNA-binding domains 1 and 2, respectively. (B) Poly(I-C) pull-down assay. Plasmids (1.5 μ g each) expressing the indicated V5-tagged proteins were individually transfected into HEK293T cells for 30 h. Cell lysates were incubated with poly(C)- or poly(I-C)-coated agarose for pull-down assay. Lysates and proteins retained in the agarose beads were analyzed by Western blotting. dsm, 4a protein mutant in which the dsRNA-binding domain is disrupted by replacing K63 and K67 with A; pC, poly(C); pIC, poly(I-C).

parative threshold cycle (C_T) method. Primers for IFN- β mRNA were 5'-GCACTGGCTGGAATGAGACTA-3' (forward) and 5'-CTCCTTGG CCTTCAGGTAAT-3' (reverse). Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were described elsewhere (44).

RESULTS

MERS-CoV 4a protein is a dsRNA-binding protein. Accessory proteins in SARS-CoV are not absolutely required for viral replication but have an immunoregulatory function (17, 49). Since SARS-CoV and MERS-CoV are betacoronaviruses of lineages B and C, respectively, they encode completely different sets of accessory proteins (4). In the search for potential IFN-antagonizing and immunoregulatory proteins encoded by MERS-CoV, we focused on its accessory proteins, proteins 3, 4a, 4b, 5, and 8b. Interestingly, a single dsRNA-binding domain, as defined by Pfam

(<http://pfam.sanger.ac.uk/>) on the basis of multiple-sequence alignments and hidden Markov models, was found in 4a protein. The 4a protein has 109 amino acid residues, and the dsRNA-binding domain is in residues 3 to 83. We observed a high degree of conservation when the amino acid sequences of 4a proteins encoded by MERS-CoV and its closest relatives, bCoV-HKU4 and bCoV-HKU5, were aligned (Fig. 1A). Other well-defined dsRNA-binding domains were also included for comparison.

To experimentally validate whether 4a proteins of the three coronaviruses indeed bind to dsRNA, we performed a pull-down assay with poly(I-C), a synthetic mimic of dsRNA. Cellular dsRNA-binding protein PACT was included as a positive control in this assay. Consistent with its ability to bind to dsRNA with a high affinity, a substantial amount of PACT in the cell lysate was

found to be bound to poly(I-C) but not to poly(C) (Fig. 1B, lane 2 compared to lane 1). MERS-CoV 4a protein was abundantly expressed in cells and was also bound to poly(I-C) (Fig. 1B, lane 4 compared to lane 3). When the two highly conserved lysine residues in the dsRNA-binding domain of MERS-CoV 4a protein were replaced by alanine (i.e., K63A/K67A), the resulting dsRNA-binding-defective (dsm) mutant was no longer capable of binding to poly(I-C) (Fig. 1B, lane 6 compared to lane 5). Interestingly, whereas bCoV-HKU5 4a protein exhibited poly(I-C)-binding activity (Fig. 1B, lane 10 compared to lane 9), its counterpart in bCoV-HKU4 was not detected in the poly(I-C)-coated agarose beads (Fig. 1B, lane 8 compared to lane 7). Thus, the 4a proteins of MERS-CoV and bCoV-HKU5, but not the 4a protein of bCoV-HKU4, are dsRNA-binding proteins. Our findings are consistent with the recent report that MERS-CoV 4a protein is a dsRNA-binding protein (33).

MERS-CoV 4a protein suppresses IFN production. The dsRNA-binding activity of MERS-CoV 4a protein prompted us to examine whether it might perturb the sensing of dsRNA by the innate immune system. To address this, poly(I-C) was used to stimulate IFN- β mRNA transcription in cultured HEK293T cells (Fig. 2A, bar 2 compared to bar 1). Although the basal level of IFN- β mRNA transcription was unaffected by MERS-CoV 4a protein (Fig. 2A, bar 3), it suppressed poly(I-C)-induced activation of IFN- β expression in a dose-dependent manner (Fig. 2A, bars 4 to 6). Similar results were obtained with a luciferase reporter construct driven by the human IFN- β promoter (Fig. 2B). Since the reporter activity correlated well with the level of the IFN- β transcript, it was used throughout this study to reflect IFN- β production in cells.

To verify the ability of MERS-CoV 4a protein to suppress IFN production in the context of viral infection, we assessed the impact of MERS-CoV 4a protein on the induction of IFN- β by Sendai virus. When MERS-CoV 4a protein was expressed in HEK293T cells, the Sendai virus-induced activation of IFN- β mRNA transcription or IFN- β promoter-dependent luciferase activity was less pronounced (Fig. 2C and D, bar 4 compared to bar 2). Similar results were also obtained in HeLa cells (Fig. 2E and F, bar 4 compared to bar 2), suggesting that the suppressive effect of MERS-CoV 4a protein was not cell type specific. Thus, MERS-CoV 4a protein sufficiently antagonized the IFN production induced by Sendai virus. These results are in keeping with the recent findings on the IFN antagonism of MERS-CoV 4a protein (33, 34).

Virus specificity of IFN antagonism. All three betacoronaviruses of lineage C encode 4a proteins. In sharp contrast to MERS-CoV and bCoV-HKU5 4a proteins, the counterpart in bCoV-HKU4 does not bind to dsRNA (Fig. 1B). It is therefore of interest to see whether these proteins behave differently in the suppression of type I IFN production. Three well-characterized viral IFN antagonists and the dsRNA-binding-defective (dsm) mutant of MERS-CoV 4a protein were also included for comparison. These three proteins, Us11 of HSV-1, NS1 of influenza A virus, and Ebola virus VP35, potently inhibited IFN- β promoter activity activated by poly(I-C) or Sendai virus (Fig. 3A and B, bars 2 to 5). Under the same conditions, 4a proteins of MERS-CoV and bCoV-HKU5 significantly mitigated the poly(I-C)- and Sendai virus-induced activation of the IFN- β promoter, but to a lesser extent (Fig. 3A, bars 7 to 9 and 19 to 21 compared to bar 2; Fig. 3B, bars 7 and 13 compared to bar 2). However, neither the dsm mutant of MERS-CoV 4a nor the bCoV-HKU4 4a protein defective in

dsRNA binding influenced the IFN- β promoter activity activated by poly(I-C) or Sendai virus (Fig. 3A, bars 11 to 13 and 15 to 17 compared to bar 2; Fig. 3B, bars 9 and 11 compared to bar 2). Hence, IFN antagonism might require dsRNA binding and was specific to MERS-CoV and bCoV-HKU5 4a proteins.

Suppression of PACT by MERS-CoV 4a protein. RIG-I and MDA5 are RNA sensors activated by their respective RNA ligands. Whereas MDA5 prefers long dsRNA, RIG-I also recognizes dsRNA of intermediate length (27). MERS-CoV 4a protein has recently been shown to antagonize the function of MDA5 (33). To verify the action point of MERS-CoV 4a protein in its suppression of IFN production, we stimulated IFN- β promoter activity using RIG-I, MDA5, and three downstream effector proteins, MAVS, TBK1, and IRF3. If MERS-CoV 4a protein counteracts IFN induction by any of these activators, it should act at or downstream of that activation point in the signaling pathway that leads to IFN production. On the contrary, if MERS-CoV 4a protein fails to suppress its activity, it should affect an upstream inducer. As a positive control, we also analyzed the suppressive activity of SARS-CoV M protein, which has been shown to perturb type I IFN production at a step upstream of IRF3 phosphorylation (35). Surprisingly, whereas SARS-CoV M protein was fully capable of suppressing the activity of RIG-I, MDA5, MAVS, and TBK1 (Fig. 4A to D), as anticipated, MERS-CoV 4a protein did not affect the activation of the IFN- β promoter by any of the five stimulators tested (Fig. 4A to E). That is to say, MERS-CoV 4a protein might antagonize an activator that acts upstream of RIG-I and MDA5. One such activator that stimulates the activity of RIG-I and MDA5 is PACT (29).

We next investigated whether the suppressive effect of MERS-CoV 4a protein on IFN production is PACT dependent or not. The suppressive activity of MERS-CoV 4a protein was assessed in the presence of PACT and either RIG-I or MDA5. Generally consistent with previous findings (29, 30), PACT remarkably augmented the activation of the IFN- β promoter by RIG-I and MDA5 (Fig. 5A and B, bars 4 compared to bars 2). This PACT-dependent activation of RIG-I was reversed by HSV-1 Us11 (Fig. 5A, bars 5 and 6), which has recently been shown to suppress IFN production by targeting PACT (31). The same trend of inhibition was also observed for MERS-CoV and bCoV-HKU5 4a proteins (Fig. 5A, bars 7 and 8 and bars 13 and 14), suggesting that they also counteracted the activity of PACT. Notably, neither the dsm mutant of MERS-CoV 4a protein nor bCoV-HKU4 4a protein was capable of suppressing PACT (Fig. 5A, bars 9 to 12). This indicates the requirement of dsRNA binding for the suppressive effect of MERS-CoV 4a protein on IFN production. Similar results were also obtained when we repeated the experiments using a reporter construct driven by IRF3-binding enhancer elements (Fig. 5B, bars 6 to 13), indicating the suppression of IRF3 activity by MERS-CoV 4a protein. Likewise, MERS-CoV and bCoV-HKU5 4a proteins but neither the dsm mutant of MERS-CoV 4a protein nor bCoV-HKU4 4a protein dampened the PACT-induced activation of MDA5 activity on the IFN- β promoter (Fig. 5C, bars 7 to 14) and IRF3-binding enhancer elements (Fig. 5D, bars 6 to 13). Considered together with its influence on the activity of RIG-I or MDA5 alone (Fig. 4A and B), MERS-CoV 4a protein apparently antagonized IFN production by targeting PACT but not RIG-I or MDA5 directly.

RNA-dependent interaction of MERS-CoV 4a protein with PACT but not with RIG-I or MDA5. If MERS-CoV 4a protein

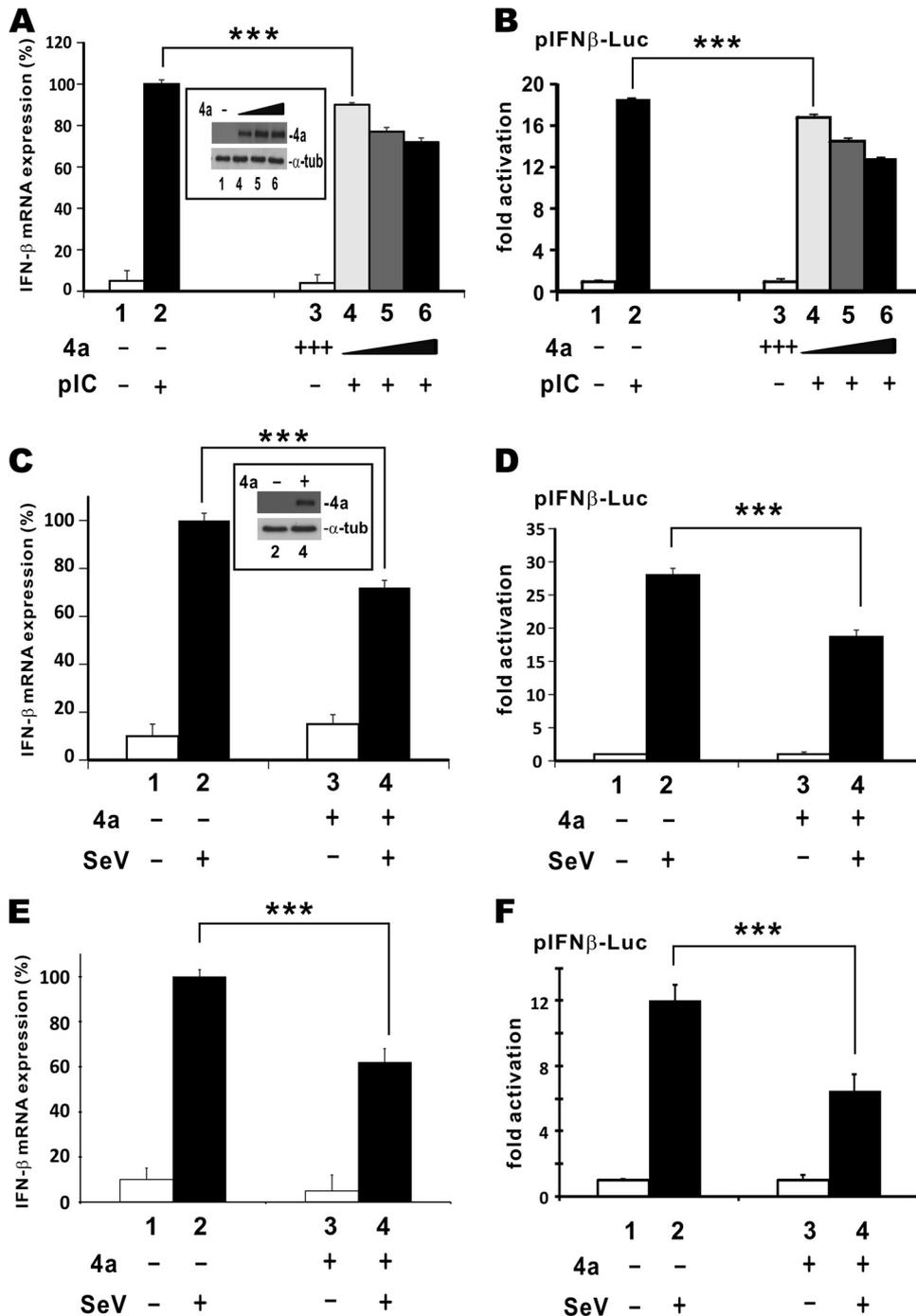


FIG 2 MERS-CoV 4a inhibits type I IFN production induced by poly(I:C) or Sendai virus. (A and B) Poly(I:C)-induced IFN production. HEK293T cells grown in 12-well plates were transfected with escalating doses (200, 400, and 600 ng) of a 4a protein expression plasmid. Empty vector was added as appropriate to ensure that cells in each well received the same amount of plasmids. At 24 h posttransfection, cells were further transfected with 1 μ g/ml of poly(I:C). Samples were collected after an additional 12 h. Similar results were also obtained from HeLa cells. pIC, poly(I:C). (C to F) Sendai virus-induced IFN production. HEK293T (C and D) and HeLa (E and F) cells grown in 12-well plates were transfected with 100 ng of pIFN β -Luc, 5 ng of pTK-RLuc, and escalating doses (200, 400, and 600 ng) of 4a protein expression plasmid. At 24 h posttransfection, cells were infected with Sendai virus (100 hemagglutinating units/ml). Cells were harvested at 12 h postinfection. Relative expression of IFN- β mRNA was analyzed by quantitative RT-PCR and normalized to the level of GAPDH mRNA expression (A, C, and E). Results from the dual-luciferase assay are expressed as the fold activation calculated from the pIFN β -Luc activity normalized to that of pTK-RLuc (B, D, and F). Expression of 4a protein in selected groups was verified by Western blotting (insets in panels A and C). SeV, Sendai virus; α -tub, α -tubulin. Data are means of triplicate groups in one transfection, and error bars indicate SDs. Two-tailed Student's *t* test was performed, and the differences between the selected groups were statistically significant with the following *P* values which were less than 0.001 (***): 0.00052 (bars 2 and 4 in panel A), 0.00062 (bars 2 and 4 in panel B), 0.00039 (bars 2 and 4 in panel C), 0.00027 (bars 2 and 4 in panel D), 0.00062 (bars 2 and 4 in panel E), and 0.00062 (bars 2 and 4 in panel F). Results are representative of those from three independent transfections.

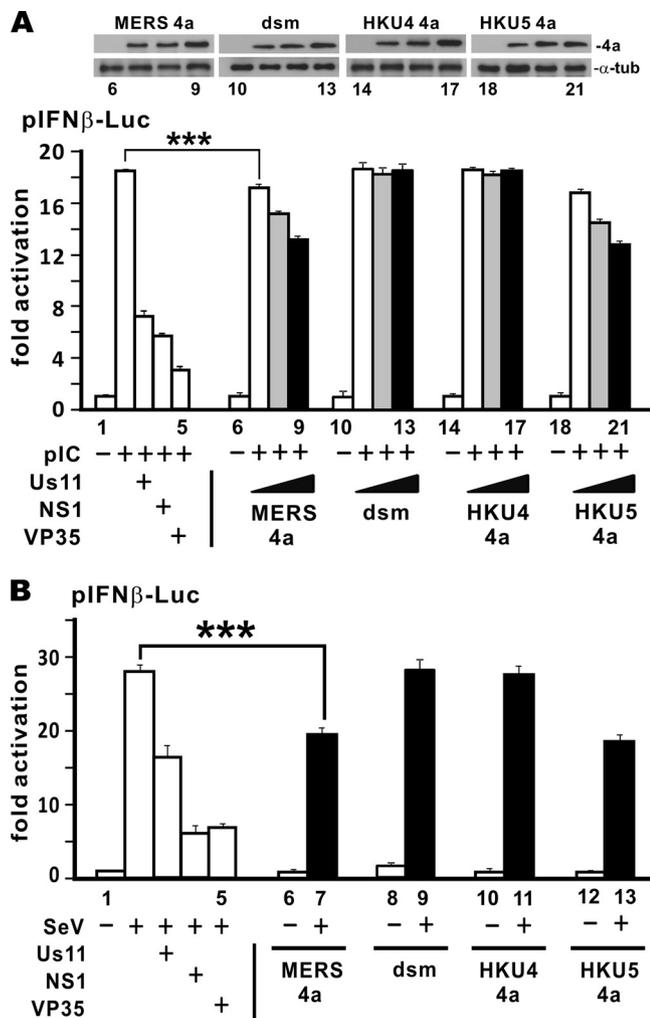


FIG 3 Comparative analysis of 4a proteins. (A) poly(I:C)-induced IFN production. Escalating doses (200, 400, and 600 ng) of a 4a protein plasmid were transfected into HEK293T cells grown in 12-well plates. Empty vectors were used to adjust the total amounts of plasmids in the transfection so that the cells in each well received the same dose of plasmids. Expression of 4a proteins in selected groups was verified by Western blotting (top). α -tub, α -tubulin; dsm, K63A/K67A mutant of MERS-CoV 4a protein. (B) Sendai virus-induced IFN production. Data presented are means from triplicate groups in one transfection, and error bars indicate SDs. Statistical analysis was performed on selected groups by two-tailed Student's *t* test. The *P* value (***) for bars 2 and 7 in panel A was 0.00066, and the one for bars 2 and 7 in panel B was 0.00033; both were less than 0.001. Results are representative of those from three independent transfections.

targets PACT, the two proteins should associate with each other in cells, probably through dsRNA. To test this association, we performed reciprocal coimmunoprecipitation and immunoblotting experiments. Because PACT was found in the MERS-CoV 4a protein precipitate and, vice versa, MERS-CoV 4a protein was present in the PACT precipitate (Fig. 6A, lane 1), these two proteins were in the same complex that might also contain dsRNA. Likewise, bCoV-HKU5 4a protein, which migrated more slowly in the SDS-polyacrylamide gel for an unknown reason, also associated with PACT (Fig. 6A, lane 4). In contrast, neither the dsm mutant, the dsRNA-binding-defective mutant of MERS-CoV 4a protein, nor bCoV-HKU4 4a protein was capable of interacting with PACT (Fig. 6A, lanes 2 and 3).

Since both MERS-CoV 4a protein and PACT are dsRNA-binding proteins, we asked whether their interaction might be mediated by RNA. The coimmunoprecipitation experiments were repeated in the presence of RNase A. The amount of MERS-CoV 4a protein detected in the PACT precipitate was diminished when 1 U of RNase A was added, and MERS-CoV 4a protein completely disappeared from the PACT precipitate when the dose of RNase A was increased to 5 U (Fig. 6B, bottom two panels, lane 2 compared to lane 1). We verified that dsRNA was effectively degraded by 1 U of RNase A in our experimental setting (Fig. 6C). Thus, the interaction between MERS-CoV 4a protein and PACT is RNA dependent. Because MERS-CoV was thought to suppress the activity of MDA5 but not that of RIG-I (33), we performed further coimmunoprecipitation experiments to determine whether MERS-CoV 4a protein might also interact with RIG-I and MDA5, both of which are also dsRNA-binding proteins. MERS-CoV 4a protein was not detected in either the RIG-I or the MDA5 precipitate. Reciprocally, RIG-I or MDA5 was not found in the precipitate that contained MERS-CoV 4a protein (Fig. 6D, lanes 1 and 2). In the same setting, both PACT and MERS-CoV 4a were present in the precipitates (Fig. 6D, lane 3). Hence, MERS-CoV 4a protein specifically interacted with PACT in an RNA-dependent manner, and in our experimental setting it was not found in the same complex that contains RIG-I or MDA5.

DISCUSSION

Exactly how MERS-CoV counteracts innate immunity to cause severe disease in human remains elusive. In this study, we used both gain-of-function and loss-of-function approaches to demonstrate the suppression of innate IFN production by MERS-CoV 4a protein. On the one hand, overexpression of MERS-CoV 4a protein dampened type I IFN production induced by poly(I:C) or Sendai virus (Fig. 2). This effect was highly specific, as a closely related 4a protein encoded by bCoV-HKU4 had no IFN-antagonizing activity (Fig. 3). This is generally consistent with two recent reports on IFN antagonism of MERS-CoV 4a protein (33, 34). In those two reports, IFN antagonism was demonstrated with several additional assays, including IRF3 and STAT1 translocation as well as ISG54 induction assays (33, 34). Another recent study demonstrated that replication of a recombinant MERS-CoV with 4a and 4b protein deletions was attenuated (50). Although MERS-CoV 4b protein did not inhibit the induction of IFN- β by viral RNA in one study (33), it was found to antagonize IFN production potentially by another group (34) and us (unpublished data). Exactly how 4a and 4b proteins cooperate to perturb IFN production and to facilitate viral replication remains to be clarified. Nevertheless, emerging evidence from different experimental approaches supports IFN antagonism of MERS-CoV 4a protein. Importantly, our work provided the mechanistic details, including a cellular target and the action point, for this antagonism.

Another salient point that emerged from our study is the targeting of PACT by MERS-CoV 4a protein. We presented several lines of evidence to support this model. First, MERS-CoV 4a protein did not affect the IFN-inducing activity of RIG-I and MDA5 (Fig. 4), suggesting that it acts upstream of these two RNA sensors. Second, MERS-CoV 4a protein counteracted PACT-dependent activation of RIG-I and MDA5 (Fig. 5). Third, MERS-CoV 4a protein associated with PACT in an RNA-dependent manner but not with RIG-I or MDA5 (Fig. 6). Finally, bCoV-HKU4 4a protein and the dsm mutant of MERS-CoV 4a protein, which was defec-

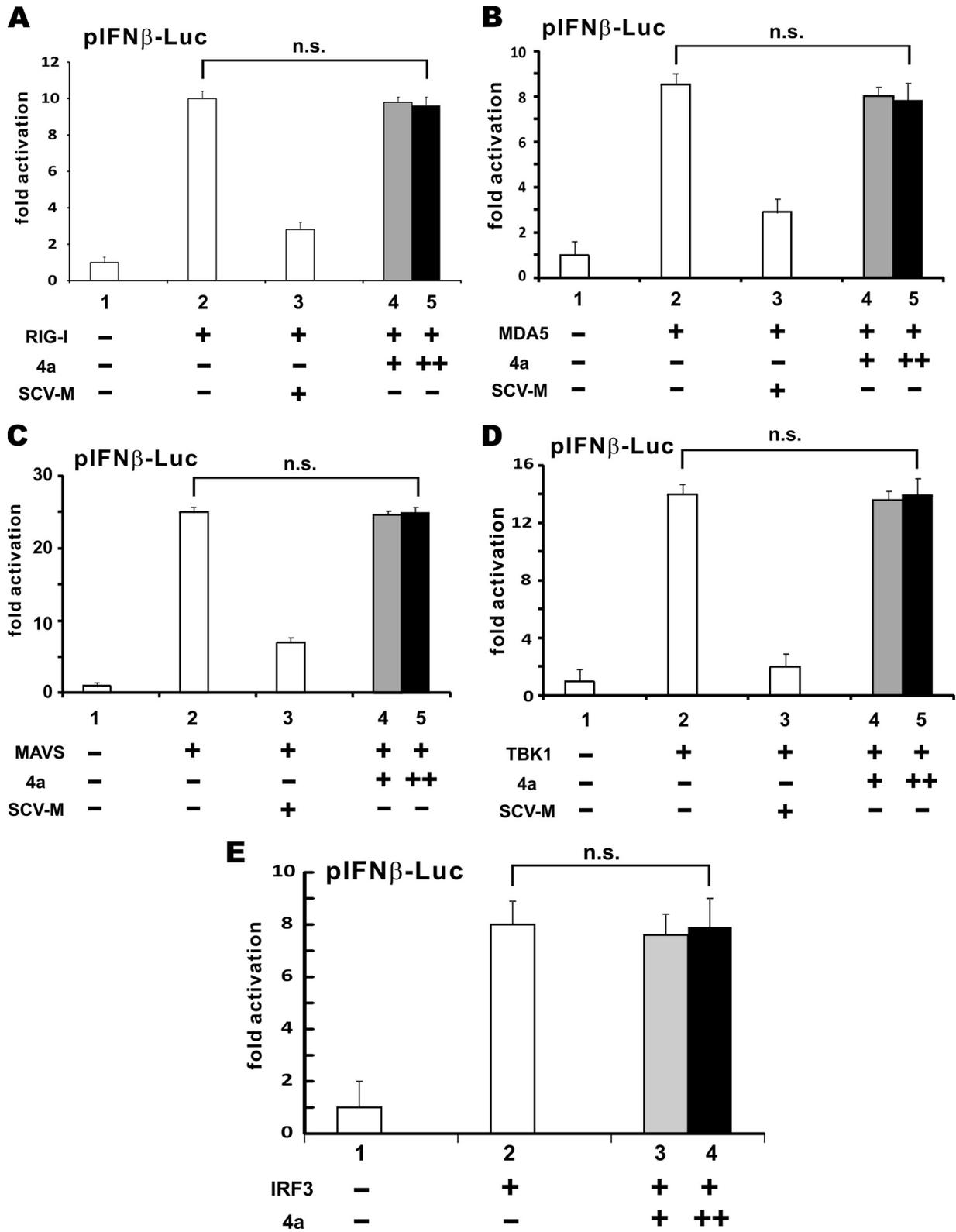


FIG 4 MERS-CoV 4a protein does not affect IFN production induced by RIG-I (A), MDA5 (B), MAVS (C), TBK1 (D), or IRF3 (E). Escalating doses (400 and 600 ng) of a 4a protein plasmid plus a fixed dose (50 ng) of activator plasmid was transfected into HEK293T cells grown in 12-well plates. Cells in the control group received empty vector alone. An expression plasmid (400 ng) for SARS-CoV M protein (SCV-M) was used as a positive control. Data are means of triplicate groups in one transfection, and error bars indicate SDs. A two-tailed Student's *t* test was performed, and no statistically significant difference was found between the tested groups. The *P* values were as follows: 0.34 (bars 2 and 5 in panel A), 0.28 (bars 2 and 5 in panel B), 0.32 (bars 2 and 5 in panel C), 0.42 (bars 2 and 5 in panel D), and 0.59 (bars 2 and 4 in panel E). n.s., not significant. Results are representative of those from three independent transfections.

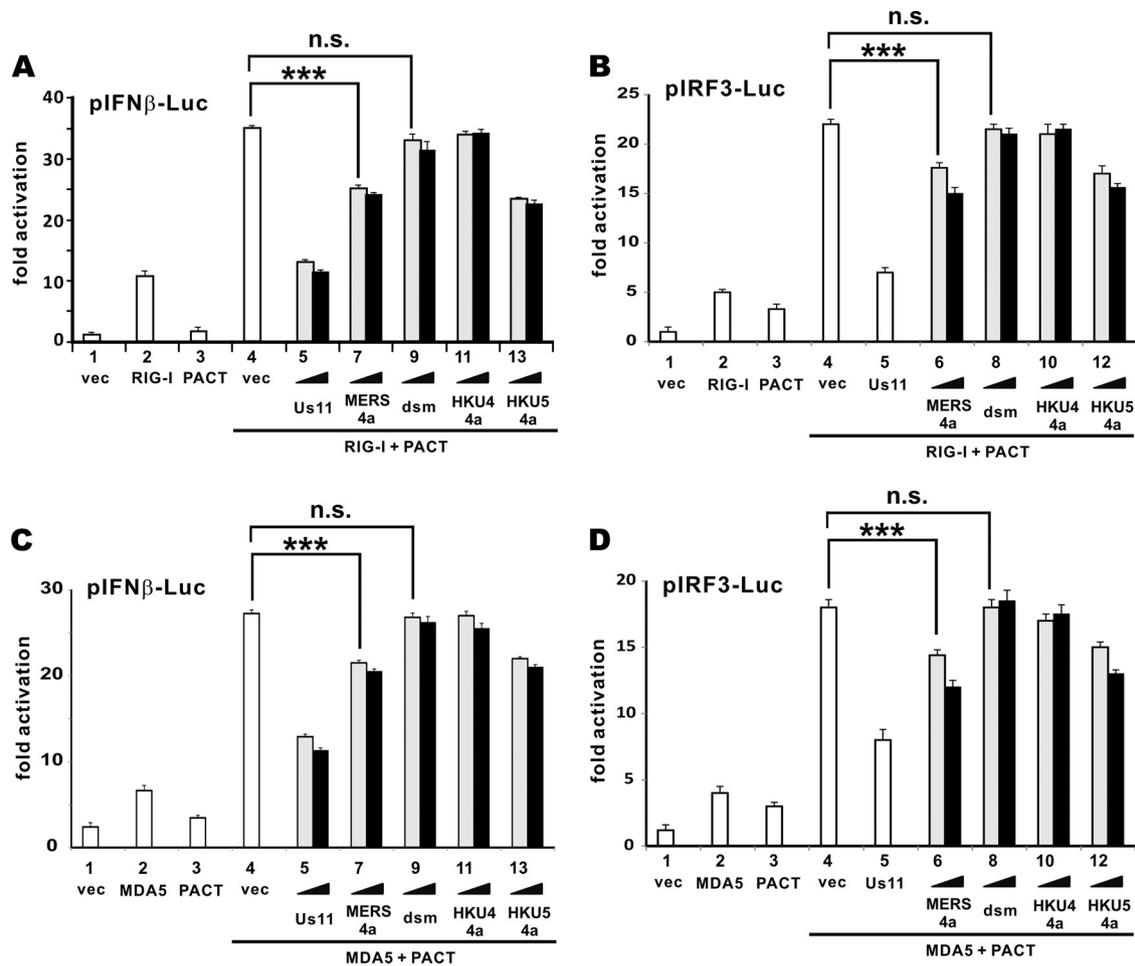


FIG 5 MERS-CoV 4a protein inhibits PACT-induced activation of RIG-I and MDA5. (A and B) Influence of RIG-I on PACT activation. HEK293T cells grown in 12-well plates were transfected with pIFN β -Luc or the pIRF3-Luc reporter as well as expression plasmids for RIG-I, PACT, and 4a proteins from MERS-CoV, bCoV-HKU4, and bCoV-HKU5. dsm is the K63A/K67A mutant of MERS-CoV 4a protein defective in dsRNA binding. HSV-1 Us11 was included as a control. Escalating doses (400 and 600 ng) of viral protein were used. The dual-luciferase assay was carried out at 30 h posttransfection. (C and D) Influence of MDA5 on PACT activation. Data are presented as fold activation and means \pm SDs derived from triplicate groups in one transfection. A two-tailed Student's *t* test was performed for selected groups. The differences between bars 4 and 7 in panel A ($P = 0.00032$), between bars 4 and 6 in panel B ($P = 0.00052$), between bars 4 and 7 in panel C ($P = 0.00041$), as well as between 4 and 6 in panel D ($P = 0.00032$) were statistically significant (***, $P < 0.001$). The differences between bars 4 and 9 in panel A ($P = 0.34$), between bars 4 and 8 in panel B ($P = 0.22$), between bars 4 and 9 in panel C ($P = 0.38$), as well as between bars 4 and 8 in panel D ($P = 0.35$) were statistically not significant (n.s.). Results are representative of those from three independent transfections. vec, vector.

tive in PACT binding, were unable to suppress IFN production (Fig. 3, 5, and 6). Our demonstration of the ability of MERS-CoV 4a protein to perturb PACT function in innate immunity adds MERS-CoV 4a protein to an expanding list of dsRNA-binding, IFN-antagonizing, and PACT-targeting viral proteins, including VP35 of Ebola virus, Us11 of HSV-1, and plausibly, NS1 of influenza A virus (30–32). It is noteworthy that PACT has also been shown to perturb the function of VP35 in viral RNA replication (30). Whether and how PACT might modulate other activities of MERS-CoV 4a protein in the viral life cycle require further investigations. The binding of VP35 and Us11 to PACT impedes the interaction of PACT with RIG-I (30, 31). Whether MERS-CoV 4a protein might also prevent PACT from binding to RIG-I and MDA5 merits clarification. Nevertheless, PACT targeting represents a new and common strategy used by different types of viruses to evade innate immunity. In this regard, our findings have paved the avenue for further analysis of PACT targeting by virus-encoded IFN antagonists.

Our work demonstrated the requirement of dsRNA binding for MERS-CoV 4a protein-mediated suppression of IFN production. The interaction between MERS-CoV 4a protein and PACT was mediated by RNA (Fig. 6B). Both bCoV-HKU4 4a protein and the dsm point mutant of MERS-CoV 4a protein were defective in dsRNA binding. Accordingly, they were also defective in PACT binding (Fig. 6A) and IFN antagonism (Fig. 3). Mechanistically, MERS-CoV 4a protein might compete with PACT for dsRNA or sequester dsRNA agonists of PACT, RIG-I, and MDA5. Alternatively, its binding to dsRNA could render it nonfunctional in the activation of PACT. Likewise, its interaction with PACT might keep it in an inactive conformation, preventing its subsequent activation of RIG-I and MDA5. In one model of PACT action, PACT selects or concentrates RNA agonists and transmits them to RIG-I and MDA5 (29, 32). Plausibly, the interaction of MERS-CoV 4a protein with dsRNA and PACT would perturb this process. To clarify this, biophysical methods such as surface plasmon resonance spectroscopy and fluorescence polarization assay could

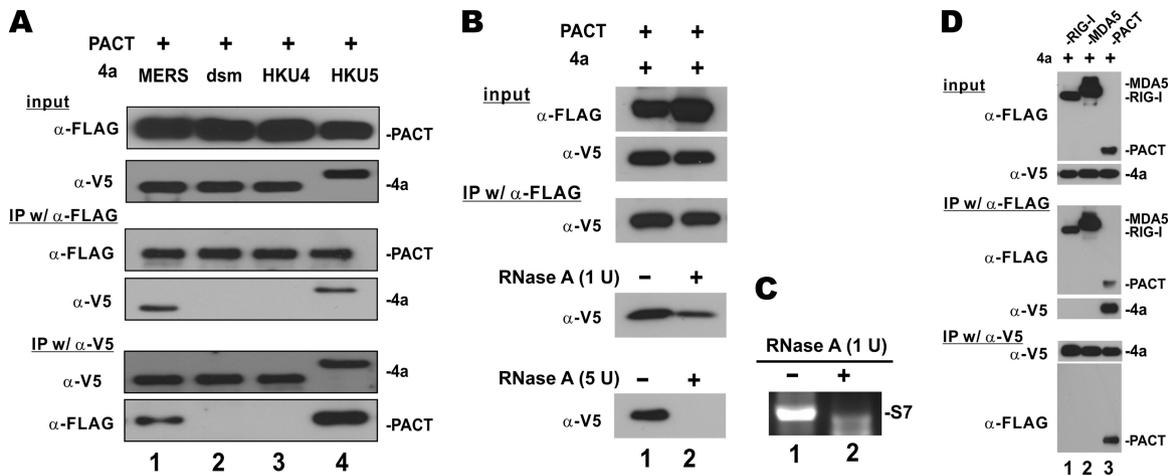


FIG 6 RNA-dependent association of MERS-CoV 4a protein with PACT. (A) Coimmunoprecipitation. HEK293T cells were transfected with plasmids (1.5 μ g each) expressing the indicated proteins. Immunoprecipitation (IP) was carried out at 48 h posttransfection with 0.5 μ g of mouse anti-FLAG (α -FLAG) or anti-V5 (α -V5) and 20 μ l of recombinant protein A-Sepharose fast-flow beads (GE Healthcare Life Sciences). Input cell lysates and precipitates were probed by Western blotting. (B) The interaction between MERS-CoV 4a and PACT is mediated through RNA. Coimmunoprecipitation was carried out in the presence of 1 or 5 U of RNase A. (C) Digestion of dsRNA by RNase A. dsRNA of about 1 kb, the sequence of which was derived from segment 7 (S7) of the influenza A virus WSN strain, was *in vitro* transcribed and annealed. It was then incubated with 1 U RNase A for 15 min. Agarose gel electrophoresis was performed to check for RNA integrity. (D) MERS-CoV 4a protein does not interact with RIG-I or MDA5.

be used to determine the affinity of binding between MERS-CoV 4a protein and dsRNA or between MERS-CoV 4a protein and PACT. It will also be of interest to see whether MERS-CoV 4a protein might preferentially bind to certain types of dsRNA. In addition, structural analysis of dsRNA-bound MERS-CoV 4a protein and MERS-CoV 4a protein-PACT complex bound to dsRNA might shed new mechanistic light on some of the issues mentioned above.

One recent report suggests that MERS-CoV 4a protein antagonizes IFN production by targeting MDA5. In that study, the conclusion for MDA5 was supported by the suppression of MDA5-induced activation of the IFN- β promoter by MERS-CoV 4a protein and the association of both 4a protein and MDA5 with poly(I-C) (33). Although both reports point to IFN antagonism of MERS-CoV 4a protein, our study favors a different mechanism. Our results did not support the suppression of MDA5 by MERS-CoV 4a protein. First, MERS-CoV 4a protein failed to suppress MDA5-dependent activation of the IFN- β promoter (Fig. 4B). Second, MERS-CoV 4a protein did not form a complex with MDA5 in our coimmunoprecipitation experiment (Fig. 6D). Finally, MERS-CoV 4a protein suppressed PACT-mediated activation of MDA5 (Fig. 5C and D). Thus, MERS-CoV 4a protein targets PACT but not MDA5 in our experimental setting. We did not understand whether variations in the expression of endogenous PACT in different cell lines or different variants of the same cell line might account for the different observations in the two studies. Indeed, PACT expression varied significantly in different cells, and its expression level is relatively low in the HEK293T cells used in this study, as previously shown (29). In cells where PACT was more abundantly expressed, inhibition of RIG-I and MDA5 by MERS-CoV 4a protein could be observed. Additional experiments are required to clarify whether MERS-CoV 4a protein might suppress the activity of RIG-I and MDA5 through PACT under certain circumstances. We also noted other technical differences in the analysis of MDA5 activity in the two studies, including the amounts of plasmids used, the magnitude of IFN- β promoter

activation, and the use of a negative control. Whether these differences might explain the different results on the suppression of MDA5 by MERS-CoV 4a protein remains to be determined. To resolve the discrepancies, it will be helpful if both groups can redo the MDA5 experiment in exactly the same cells and in exactly the same way. In addition, it will be of great interest to see whether and how 4a protein might counteract PACT, RIG-I, and MDA5 in MERS-CoV-infected Calu3 and THP-1 cells.

Compared to other virus-encoded IFN-antagonizing proteins, including HSV-1 Us11, influenza A virus NS1, and Ebola virus VP35, MERS-CoV 4a protein is a relatively weak IFN antagonist (Fig. 3 and 5). Plausibly, MERS-CoV 4a protein would cooperate with other structural and nonstructural proteins of MERS-CoV to circumvent innate immunity at multiple levels. In the recent report on the attenuation of MERS-CoV replication caused by the deletion of 4a and 4b proteins (50), the expression of both 4a and 4b proteins was compromised. Both 4b and M proteins of MERS-CoV have recently been shown by another group (34) and us (our unpublished data) to antagonize type I IFN production. Whether and how 4a protein might cooperate with 4b and M proteins in immunosuppression warrant further analysis. Additionally, as a dsRNA-binding protein, MERS-CoV 4a protein could affect other dsRNA-dependent processes in the innate immune response. For instance, it will be intriguing to see whether MERS-CoV 4a protein might modulate the activity of dsRNA-dependent protein kinase and 2'-5' oligoadenylate synthetase.

To our surprise, bCoV-HKU4 4a protein did not bind to poly(I-C) or PACT and did not inhibit type I IFN production (Fig. 3 and 5). PACT is a highly conserved protein that has a bat homolog. It remains to be understood whether bCoV-HKU4 4a protein lost the ability to bind dsRNA and to suppress PACT during evolution. An alternative possibility is that bCoV-HKU5 and MERS-CoV 4a proteins might acquire their dsRNA- and PACT-binding properties at a later stage. The bat coronaviruses are well adapted to bats, the innate immune system of which is unique and probably defective in some aspects (51, 52). This might account

for the asymptomatic infection of coronaviruses in bats. However, when these viruses cross the species barrier to infect human, a strong innate immune response is elicited, leading to severe disease. Whether differential targeting of PACT by lineage C betacoronaviruses might be relevant to host adaptation and immune evasion remains to be understood. Defining the molecular basis of differential PACT targeting and immunosuppression might derive new knowledge about the coronavirus-host interaction and MERS-CoV pathogenesis.

The recent availability of MERS-CoV infectious clones will greatly enhance the reverse genetic study of MERS-CoV 4a protein (50, 53). In particular, the creation and characterization of a recombinant virus in which only 4a protein and not 4b protein is disrupted will provide definite answers to many questions concerning the function of MERS-CoV 4a protein. In addition, the attenuated viruses generated might prove useful in vaccine development.

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