

Severe Acute Respiratory Syndrome Coronavirus M Protein Inhibits Type I Interferon Production by Impeding the Formation of TRAF3·TANK·TBK1/IKK ϵ Complex*

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Severe acute respiratory syndrome (SARS) coronavirus is highly pathogenic in humans and evades innate immunity at multiple levels. It has evolved various strategies to counteract the production and action of type I interferons, which mobilize the front-line defense against viral infection. In this study we demonstrate that SARS coronavirus M protein inhibits gene transcription of type I interferons. M protein potently antagonizes the activation of interferon-stimulated response element-dependent transcription by double-stranded RNA, RIG-I, MDA5, TBK1, IKK ϵ , and virus-induced signaling adaptor (VISA) but has no influence on the transcriptional activity of this element when IRF3 or IRF7 is overexpressed. M protein physically associates with RIG-I, TBK1, IKK ϵ , and TRAF3 and likely sequesters some of them in membrane-associated cytoplasmic compartments. Consequently, the expression of M protein prevents the formation of TRAF3·TANK·TBK1/IKK ϵ complex and thereby inhibits TBK1/IKK ϵ -dependent activation of IRF3/IRF7 transcription factors. Taken together, our findings reveal a new mechanism by which SARS coronavirus circumvents the production of type I interferons.

Severe acute respiratory syndrome (SARS)² coronavirus causes a highly lethal infectious disease in humans characterized by an aberrant immune response (1). The production and action of type I interferons, which are major components of antiviral innate immunity (2, 3), are inhibited at multiple levels by SARS coronavirus (4, 5). This inhibition is thought to be mediated through viral structural and non-structural proteins N, ORF3b, ORF6, nsp1, and papain-like protease (6–12).

The signaling pathways through which viruses induce the production of type I interferons have been well characterized (13–15). In response to double-stranded RNA (dsRNA) produced during viral replication, endosomal Toll-like receptor 3

(TLR3) and cytoplasmic retinoic acid-inducible gene I (RIG-I) trigger the activation of two different pathways adapted to downstream kinases through TRIF (TLR adaptor inducing interferon β) and VISA, respectively. These pathways converge on the formation of TRAF3·TANK·TBK1/IKK ϵ complex, which catalyzes the phosphorylation of IRF3 and IRF7 transcription factors, leading to the activation of type I interferon promoters (15–17).

SARS coronaviral proteins counteract the production of type I interferons at multiple steps. Although IRF3 phosphorylation was inhibited in cells expressing ORF3b, ORF6, or N protein (7), papain-like protease could physically interact with IRF3 and prevent its phosphorylation and nuclear translocation in a protease-independent manner (11). In addition, nsp1 suppressed the synthesis of host proteins including interferons by inducing mRNA degradation (6, 12). Meanwhile, viral proteins such as nsp1 and ORF6 were multifunctional (10, 18, 19) and could also inhibit interferon signaling. For example, both nsp1 and ORF6 inhibited the activity of STAT1, a key regulator of interferon-responsive genes (8, 9). Whereas nsp1 attenuated phosphorylation of STAT1 (9), ORF6 modulated nuclear import and blocked nuclear translocation of STAT1 by sequestering nuclear import factors in the endoplasmic reticulum and Golgi apparatus (8, 19).

SARS coronavirus M protein is a glycosylated structural protein with three membrane-spanning domains (20–23). M protein predominantly localizes to the Golgi complex and is essential for the assembly of viral particles (24, 25). Whereas mutations in M protein of an animal coronavirus named transmissible gastroenteritis virus led to significantly reduced induction of type I interferons (26), an adaptive mutation in SARS coronavirus M protein was found to enhance viral replication and/or infectivity in cultured human cells (27). Additionally, SARS coronavirus M protein was also suggested to modulate apoptosis and the expression or activity of cellular proteins such as NF κ B and Akt (28–30).

In our study of the modulation of cellular function by SARS coronavirus structural proteins (31, 32), we found that M protein suppressed type I interferon production more potently than did N protein or influenza A virus NS1 protein. We went on to dissect the signaling pathway targeted by M protein and demonstrated its inhibition of the formation of TRAF3·TANK·TBK1/IKK ϵ complex. Our work reveals a new coronaviral countermeasure against host innate immunity.

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² The abbreviations used are: SARS, severe acute respiratory syndrome; dsRNA, double-stranded RNA; IKK, I κ B kinase; IRF, interferon regulatory factor; ISRE, interferon-stimulated response element; MDA, melanoma differentiation-associated protein; ORF, open reading frame; RIG, retinoic acid-inducible gene; TANK, TRAF-associated NF κ B activator; TBK, TANK binding kinase; TLR, Toll-like receptor; TRAF, tumor necrosis factor receptor-associated factor; VISA, virus-induced signaling adaptor.

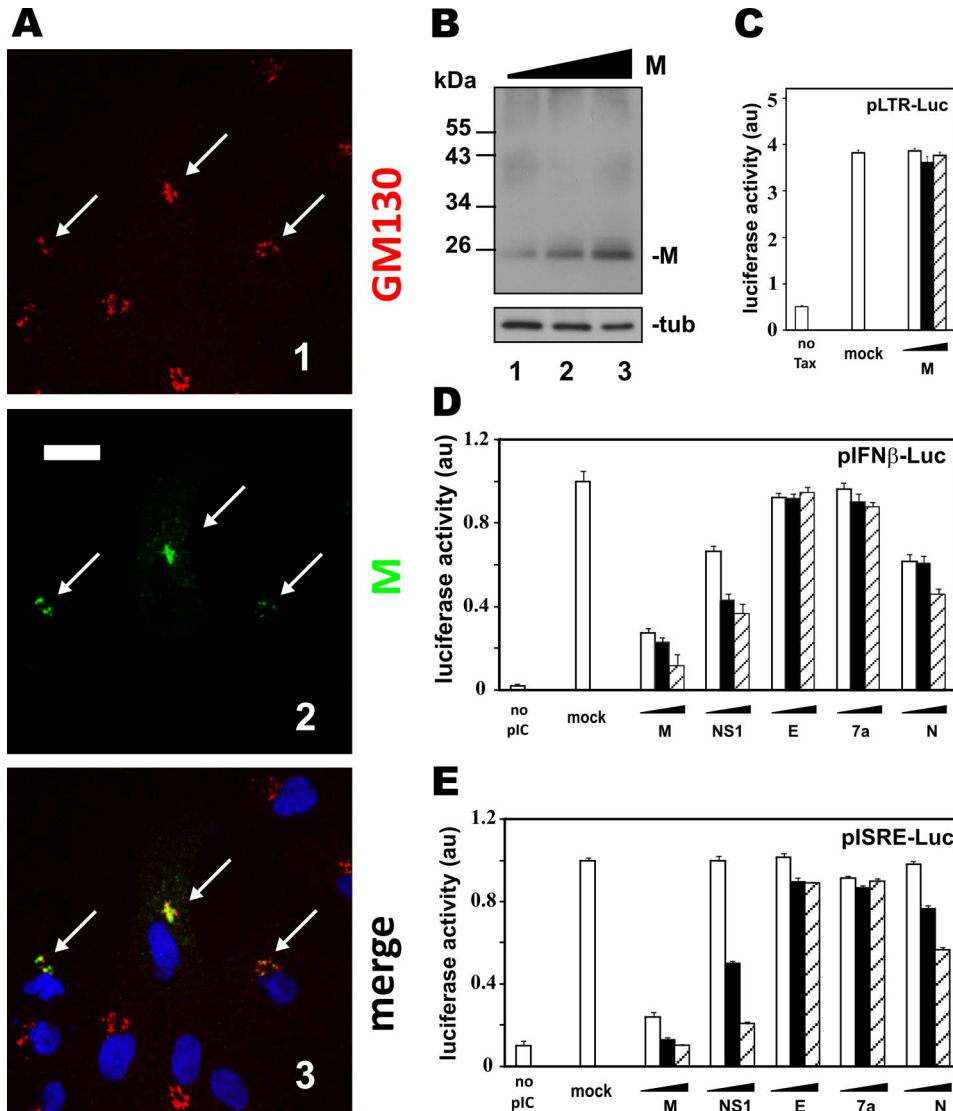


FIGURE 1. SARS coronavirus M protein inhibits dsRNA-induced production of type I interferons. *A*, Golgi localization of M protein. HeLa cells were transfected with an expression vector for Myc-tagged M protein and then stained for GM130 and Myc-tagged M. The GM130- (green) and M-specific (red) fluorescent signals were then merged. Nuclear morphology (blue) was visualized with 4',6-diamidino-2-phenylindole. Colocalization appeared yellow. Bar, 20 μ m. *B*, expression of M protein in cultured cells. HEK293 cells were transfected with increasing amounts of an expression vector for Myc-tagged M protein, and cell lysates were analyzed by Western blotting with anti-Myc and anti- α -tubulin (*tub*). *C*, M protein does not inhibit the activity of human T-cell leukemia virus type 1 long terminal repeats. HEK293/TLR3 cells were transfected with pLTR-Luc, pLEX, and increasing amounts of an expression plasmid for M protein. Cells in one control group (*mock*) did not receive the expression plasmid for M protein, whereas cells in another control group (*no Tax*) received neither pLEX nor the expression plasmid for M protein. Relative luciferase activity was expressed in arbitrary units (*au*), and the results represent the mean \pm S.D. from three independent experiments. *D* and *E*, suppression of type I interferon production by M protein. HEK293/TLR3 cells were transfected with pIFN β -Luc or pISRE-Luc and increasing amounts of plasmids expressing the indicated viral proteins (influenza A virus NS1, SARS coronavirus M, E, ORF7a, and N). At 24 h after transfection, cells were stimulated with 1 μ g/ml poly(I:C) (*pI:C*) for 12 h. Cells in one control group (*no pI:C*) were not treated with poly(I:C).

MATERIALS AND METHODS

Plasmids—pIFN β -Luc and RIG-I expression vector (33) were kind gifts from Dr. Takashi Fujita (Kyoto University, Kyoto, Japan). TBK1 and IRF3 expression plasmids (34, 35) were provided by Dr. Genhong Cheng (University of California, Los Angeles, CA). IRF7 plasmid (36) was supplied by Dr. Luwen Zhang (University of Nebraska, Lincoln, NE). TRAF6 expression vector (37) was obtained from Dr. Tohru Ishitani (Kyushu University, Kyushu, Japan). TRAF3 cDNA (38) was kindly

provided by Drs. Liusheng He and Peter Lipsky (NIAMS, National Institutes of Health, Bethesda, MD). pISRE-Luc was from Clontech. cDNA clones for IKK ϵ , MDA5, VISA, and TANK were obtained from imaGenes GmbH (Berlin, Germany). TLR3 plasmid used in the construction of HEK293/TLR3 stable cell line has been described (39). pLTR-Luc reporter plasmid driven by the long terminal repeats of human T-cell leukemia virus type 1 and Tax expression vector pLEX have been described (40).

Viruses—The GZ50 strain of SARS coronavirus was propagated in Vero cells in a Biosafety Level 3 laboratory as described (31, 32). Sendai virus was obtained from American Type Culture Collection. HEK293/ACE2 cells infected with 5 multiplicity of infection of SARS coronavirus or Sendai virus were harvested to 1 \times SDS gel loading buffer (50 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 1% β -mercaptoethanol, and 0.02% bromophenol blue) for protein analysis or to cell lysis buffer (50 mM Tris-Cl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl $_2$, 0.5% Nonidet P-40, and 1 mM dithiothreitol) for RNA analysis.

Antibodies—Anti-FLAG and anti- α -tubulin antibodies were purchased from Sigma. Anti-V5 was from Invitrogen. Anti-IFR3, anti-TRAF3, anti-IKK ϵ , and anti-Myc were from Santa Cruz. Anti-phospho-IRF3 was from Cell Signaling. Anti-GM130 was from BD Transduction Laboratories. Anti-N was from Imgenex.

Protein Analysis and Reporter Assay—Immunoprecipitation, Western blotting, and dual luciferase assay were carried out as described (41, 42). Relative luciferase activity in arbitrary units was calculated by normalizing firefly luciferase activity to *Renilla* luciferase activity.

Confocal Microscopy—Confocal immunofluorescence microscopy was performed as described (43, 44). Cells were fixed in 1:1 (v:v) acetone/methanol for 10 min and imaged using LSM510 system (Carl Zeiss).

In Vitro Kinase Assay—TBK1/IKK ϵ kinase activity was assayed *in vitro* as described (42, 43). Briefly, TBK1/IKK ϵ -containing protein complex was immunoprecipitated from transfected HEK293 cells. The precipitates were suspended in kinase

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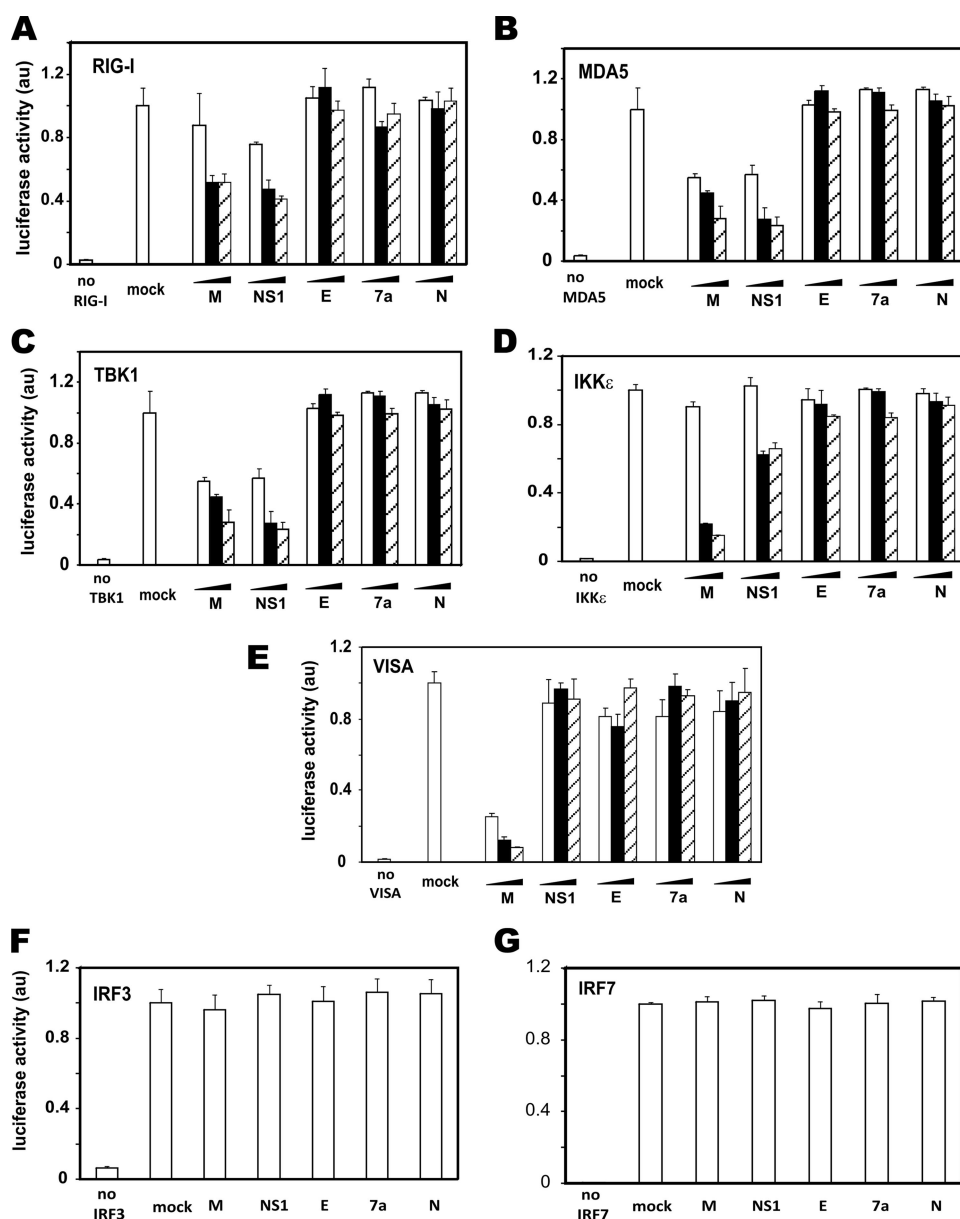


FIGURE 2. SARS coronavirus M protein inhibits interferon-inducing activity of RIG-I/MDA5, VISA, and TBK1/IKK ϵ . Experiments were carried out as in Fig. 1C except that HEK293 cells were not stimulated with poly(I:C) but co-transfected with an expression vector for RIG-I (A), MDA5 (B), TBK1 (C), IKK ϵ (D), VISA (E), IRF3 (F), or IRF7 (G). au, arbitrary units.

buffer (25 mM Tris-Cl, pH 7.5, 5 mM β -glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, 10 mM MgCl₂) and incubated with [γ -³²P]ATP and recombinant I κ B α (Santa Cruz) or IRF3 (Abnova). Samples were analyzed by SDS-PAGE and autoradiography.

RESULTS

Inhibition of Type I Interferon Production by SARS Coronavirus M Protein—SARS coronavirus N protein has been shown to antagonize the production of type I interferons (7). In our study of the gene regulatory function of N protein (32), we included M protein as a control and found serendipitously that M protein was a more potent inhibitor of interferon β promoter when compared with N protein (Fig. 1). Considered together with the ability of M protein to interact with host factors and to modu-

late expression of cellular genes (25–29), we set out to characterize M protein-mediated inhibition of type I interferon production in detail.

As a first step, we expressed M protein transiently in HEK293 and HeLa cells. The M protein-expressing cells did not show any growth defect, and no signs of apoptosis were observed (Fig. 1A). M protein was abundantly found in transfected cells (Fig. 1B). Consistent with a predominant Golgi localization shown in previous reports (21, 23), M protein was found to co-localize substantially with GM130 (Fig. 1A), a marker of the Golgi apparatus (45). Expression of M protein did not suppress transcription driven by the long terminal repeats of human T-cell leukemia virus type 1, which was stimulated by viral transactivator Tax (Fig. 1C). Hence, M protein is unlikely a general inhibitor of RNA polymerase II-dependent transcription. Next, we assessed the influence of M protein expression on the induction of interferon β gene transcription using pIFN β -Luc, a luciferase reporter construct driven by interferon β promoter (33). Notably, expression of M protein led to significant and dose-dependent suppression of dsRNA-induced activation of interferon β promoter (Fig. 1D). This suppression by M protein was more pronounced than the effect of two previously reported viral antagonists of interferon production, SARS coronavirus N protein and influenza A virus NS1

protein (7, 39). In contrast, E or ORF7a protein of SARS coronavirus did not modulate the activation of interferon β promoter by dsRNA (Fig. 1D). When we repeated the luciferase assay with a reporter construct driven by canonical ISRE enhancer elements, which were bound to IRF3 and IRF7 (36, 46, 47), the same pattern of inhibitory activity mediated by M, N, and influenza A virus NS1 proteins was observed (Fig. 1E). These results suggested that SARS coronavirus M protein is a potent inhibitor of the activity of IRF-responsive elements in the interferon β promoter.

SARS Coronavirus M Protein Inhibits Interferon-inducing Activity of TBK1/IKK ϵ —We next addressed at which step of the signaling pathway M protein might inhibit dsRNA-induced production of type I interferons. Various transducer proteins that are previously known to stimulate interferon production

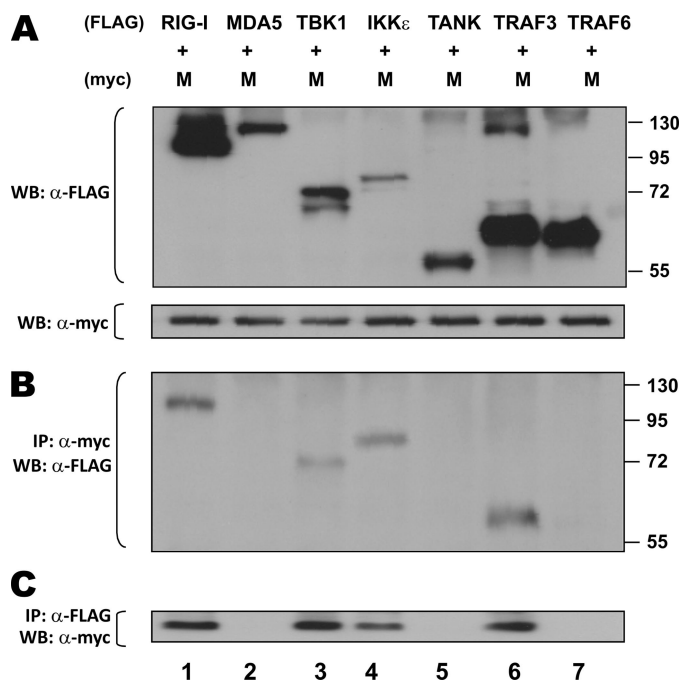


FIGURE 3. Association of SARS coronavirus M protein with RIG-I, TBK1, IKK ϵ , and TRAF3. HEK293 cells were co-transfected with expression plasmids for Myc-tagged M protein and the indicated FLAG-tagged transducer proteins. Input cell lysates (*A*) and immunoprecipitates (*IP*, *B* and *C*) were analyzed by Western blotting (*WB*) with anti-myc (α -Myc) and anti-FLAG (α -FLAG) antibodies.

(13–17) were used to activate ISRE-dependent expression of luciferase reporter (Fig. 2). These include cytoplasmic dsRNA sensors RIG-I (Fig. 2*A*) and MDA5 (Fig. 2*B*), adaptor protein VISA/IPS1/MAVS/Cardif (Fig. 2*E*), and protein kinases TBK1 (Fig. 2*C*) and IKK ϵ (Fig. 2*D*) as well as ISRE binding transcription factors IRF3 (Fig. 2*F*) and IRF7 (Fig. 2*G*). All of these transducer proteins tested strongly activated the enhancer activity of ISRE (Fig. 2).

Interestingly, expression of M protein suppressed ISRE-driven transcriptional activity induced by RIG-I, MDA5, VISA, TBK1, and IKK ϵ but not by IRF3 or IRF7 (Fig. 2). In these assays, SARS coronavirus N, E, or ORF7a protein was unable to counteract any ISRE activator. Thus, although both N and M proteins inhibited interferon production (7), they plausibly acted through different mechanisms. Consistent with previous findings (48–50), influenza A virus NS1 protein inhibited the stimulatory activity of RIG-I, MDA5, TBK1, and IKK ϵ (Fig. 2, *A*–*D*). However, unlike SARS coronavirus M protein, NS1 had no influence on the activity of VISA (Fig. 2*E*). These results suggested that M protein suppressed interferon production in a manner distinct from that of NS1.

Association of SARS Coronavirus M Protein with TBK1/IKK ϵ —M protein inhibited TBK1/IKK ϵ -dependent activation of interferon production but was unable to counteract the action of transcription factors IRF3 and IRF7 (Fig. 2). This pattern of inhibitory activity suggested that M protein might function at a step before nuclear activation of IRF3 and IRF7. In light of this, we next investigated whether M protein physically interacted with the cytoplasmic transducer proteins. We overexpressed M and individual transducer proteins RIG-I, MDA5, TBK1, IKK ϵ , TANK, TRAF3, and TRAF6 in HEK293 cells and performed

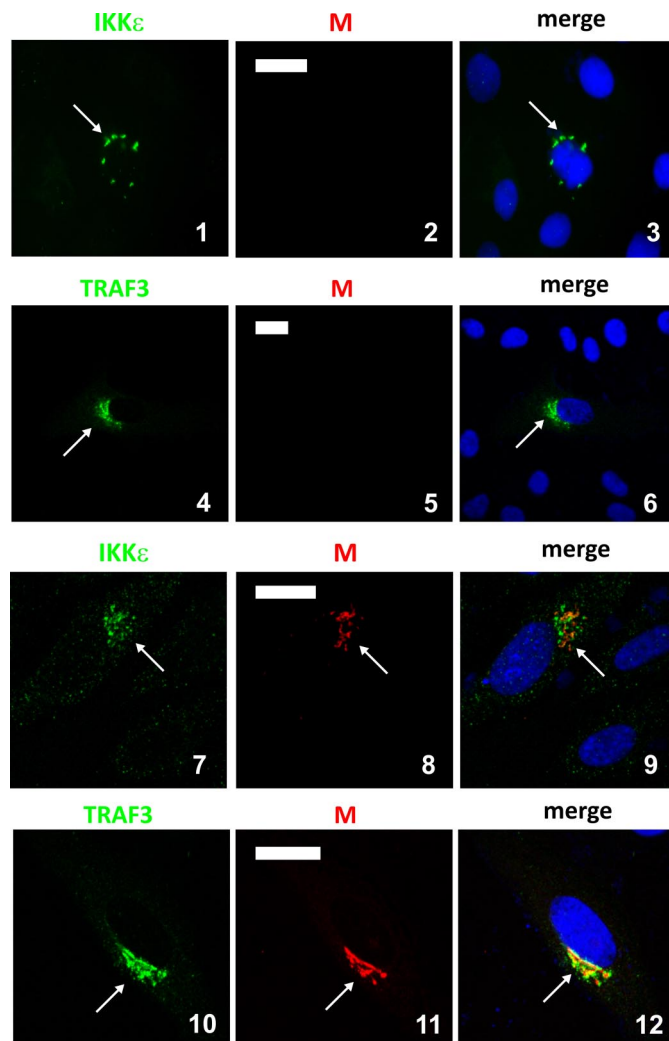


FIGURE 4. Colocalization of SARS coronavirus M protein with IKK ϵ and TRAF3. HeLa cells were transfected individually with expression plasmids for IKK ϵ (panels 1–3), TRAF3 (panels 4–6), IKK ϵ + M (panels 7–9), and TRAF3 + M (panels 10–12). Cells were then stained for M and IKK ϵ /TRAF3 with anti-Myc and anti-FLAG, respectively. The green and red fluorescent signals were merged, and nuclear morphology (blue) was visualized with 4',6-diamidino-2-phenylindole. Colocalization appeared yellow. Bar, 20 μ m.

reciprocal co-immunoprecipitation to assess their interactions (Fig. 3). Although both M protein and the transducer proteins were expressed abundantly in HEK293 cells (Fig. 3*A*), M protein was found to co-precipitate with RIG-I, TBK1, IKK ϵ , and TRAF3 but not with MDA5, TANK, or TRAF6 (Fig. 3, *B* and *C*). Notably, precipitations with two different antibodies yielded similar results (Fig. 3*B* compared with Fig. 3*C*), suggesting that M protein consistently formed a complex with RIG-I, TBK1, IKK ϵ , and TRAF3. In keeping with this notion, M protein was found to co-localize substantially with IKK ϵ and TRAF3 in HeLa cells to discrete cytoplasmic subdomains that were compatible with the Golgi complex (Fig. 4). Particularly, IKK ϵ was sporadically found in multiple cytoplasmic dots in the absence of M protein (Fig. 4, panels 1–3). In contrast, IKK ϵ was more concentrated to the M protein-containing region in cells expressing M protein (Fig. 4, panels 7–9).

SARS Coronavirus M Protein Impedes TRAF3·TANK·TBK1/IKK ϵ Complex Formation and IRF3 Phosphorylation—The association of M protein with TBK1/IKK ϵ (Fig. 3) raised two

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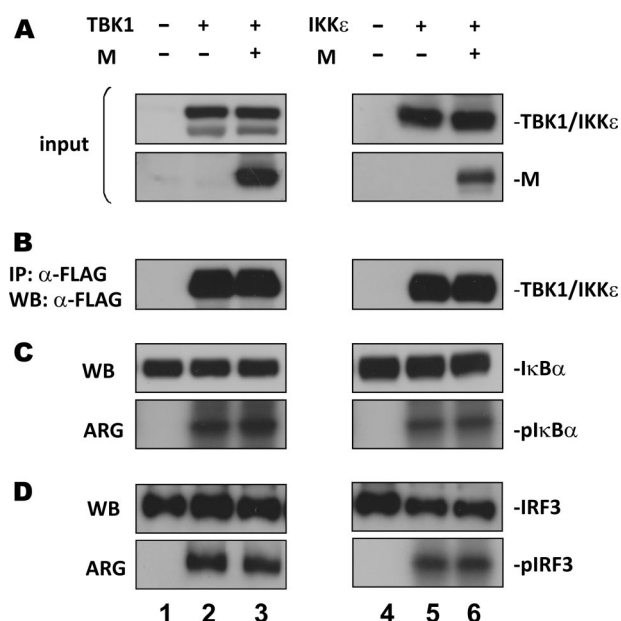


FIGURE 5. M protein does not affect *in vitro* kinase activity of TBK1/IKKε. HEK293 cells were transfected with different combinations of expression plasmids for Myc-tagged M protein and FLAG-tagged TBK1/IKKε. Cell lysates were analyzed by Western blotting (WB) with anti-FLAG and anti-myc (A). TBK1/IKKε kinase was precipitated (IP) with anti-FLAG (B). The precipitates were incubated with recombinant IκBα (C) or IRF3 (D). Phosphorylated IκBα (pIκBα) and IRF3 (pIRF3) were analyzed by autoradiography (ARG).

possibilities that might explain the inhibition of interferon production. First, M protein might inhibit catalytic activity of TBK1/IKKε to phosphorylate IRF3/IRF7. Second, M protein might interfere with the formation of TRAF3·TANK·TBK1/IKKε complex required for phosphorylation of IRF3/IRF7 in the cytoplasm. To test these possibilities, we assessed the impact of M protein on the kinase activity of TBK1/IKKε both *in vitro* and *in vivo*. We expressed M protein and TBK1/IKKε in HEK293 cells (Fig. 5A) and immunoprecipitated TBK1/IKKε-containing protein complex (Fig. 5B). The precipitates were then incubated with recombinant IκBα or recombinant IRF3. Phosphorylated IκBα and IRF3 were analyzed by autoradiography (Fig. 5, C and D). Notably, the TBK1/IKKε complex purified from M protein-expressing cells contained M protein (Fig. 5C). However, the TBK1/IKKε complexes containing or not containing M protein showed similar activity to phosphorylate IκBα or IRF3 (Fig. 5, C and D, lane 3 compared with lane 2 and lane 6 compared with lane 5). Thus, the expression of M protein did not modulate the catalytic activity of TBK1/IKKε kinase.

On the other hand, phosphorylated IRF3 was detected in cultured HEK293 cells expressing TBK1/IKKε alone but not in cells simultaneously expressing TBK1/IKKε and M protein, whereas comparable amounts of IRF3 were found in all different groups of cells (Fig. 6, lane 2 compared with lane 3 and lane 4 compared with lane 5). These results were consistent with the notion that M protein inhibits *in vivo* phosphorylation of IRF3 by TBK1/IKKε.

To verify the relevance of M protein-mediated inhibitory effects to SARS coronavirus infection, we also examined phosphorylation of IRF3 and production of interferon β transcript in cells infected with SARS coronavirus. Both phosphorylated

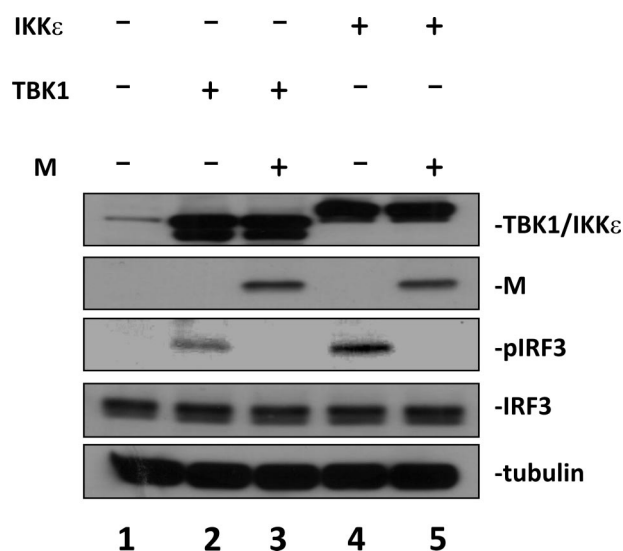


FIGURE 6. Inhibition of IRF3 phosphorylation in cells expressing SARS coronavirus M protein. HEK293 cells were transfected with different combinations of expression plasmids for myc-tagged M protein and FLAG-tagged TBK1/IKKε. Cell lysates were analyzed by Western blotting with anti-FLAG, anti-Myc, anti-phosphoIRF3, anti-IRF3, and anti-α-tubulin antibodies.

IRF3 and elevated expression of interferon β mRNA were detected in cells infected with Sendai virus (Fig. 7, A and C). In contrast, although IRF3 was abundantly found in cells infected with SARS coronavirus, neither phosphorylation of IRF3 nor induced expression of interferon β transcript was observed. These results were consistent with previous findings (4–12).

The formation of TRAF3·TANK·TBK1/IKKε complex in the cytoplasm is an essential step in the activation of IRF3/IRF7 (15–17, 34). M protein physically interacted with TBK1/IKKε and TRAF3 (Fig. 3). In addition, they also colocalized to the cytoplasmic subdomains that were likely the Golgi apparatus (Fig. 4). Plausibly, M protein might impede the formation of a functional TRAF3·TANK·TBK1/IKKε complex by masking the interaction domains or retaining some of the subunits in the Golgi complex. Indeed, when we co-expressed TBK1, TRAF3, and M in HEK293 cells, the interaction between TBK1 and TRAF3, which was evident in cells expressing TBK1 and TRAF3, was not detected (Fig. 8A, lane 2 compared with lane 1). Likewise, the interaction between IKKε and TRAF3 was not observed in M protein-expressing cells (Fig. 8A, lane 4 compared with lane 3). Furthermore, the formation of TRAF3·TANK complex was also blocked in the presence of M protein (Fig. 8A, lane 6 compared with lane 5). Hence, M protein impeded the formation of a functional TRAF3·TANK·TBK1/IKKε complex. Importantly, the formation of this complex was also impaired in SARS coronavirus-infected cells (Fig. 8B). As such, the interaction of TRAF3 with TBK1, IKKε, and TANK was impeded in infected cells where N protein was expressed (Fig. 8B, lanes 2, 4, and 6 compared with lanes 1, 3, and 5, respectively). Although the roles of other viral proteins could not be excluded, the same pattern for the impeded formation of TRAF3·TANK·TBK1/IKKε complex in cells expressing M protein alone and cells infected with SARS coronavirus supported the notion that M protein contributes significantly to the inhibition of type I interferon production during viral infection.

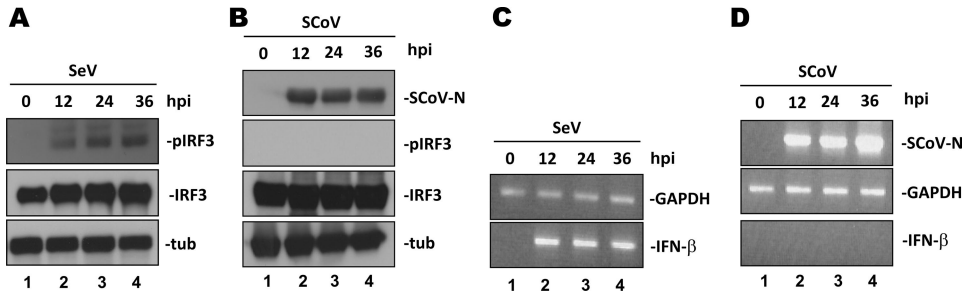


FIGURE 7. Analysis of interferon production during SARS coronavirus infection. HEK293/ACE2 cells were infected with 5 multiplicity of infection of Sendai virus (SeV) or SARS coronavirus (SCoV). A and B, cell lysates were analyzed by Western blotting with anti-phospho-IRF3, anti-IRF3, and anti- α -tubulin (*tub*) or antibodies against SARS coronavirus N protein (SCoV-N). *hpi*, hours post-infection. C and D, transcripts of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), interferon β (*IFN- β*), and SARS coronavirus N gene (SCoV-N) were analyzed by reverse transcription-PCR.

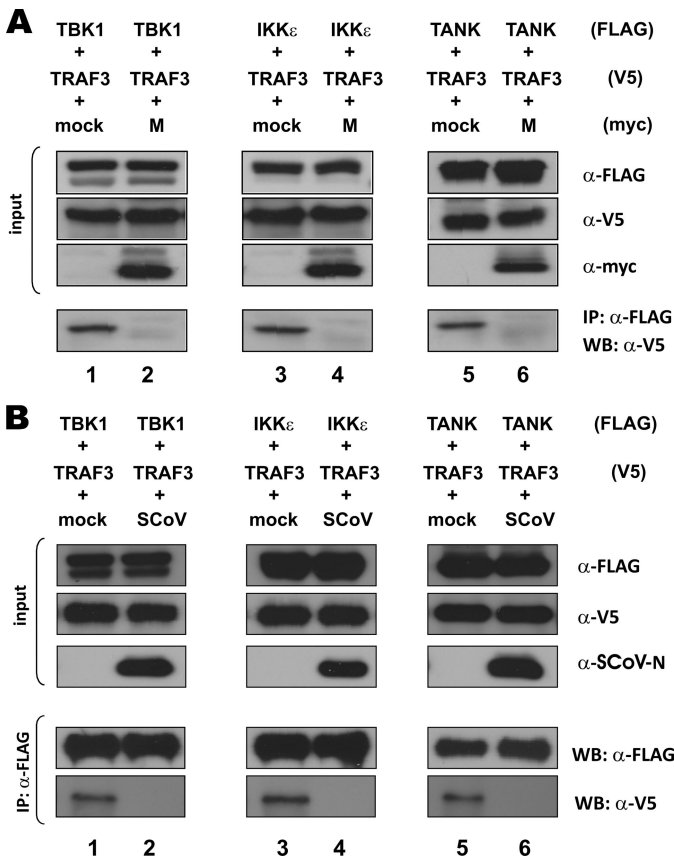


FIGURE 8. Suppression of TRAF3-TANK-TBK1/IKK ϵ complex formation by SARS coronavirus M protein. A, HEK 293 cells were co-transfected with different combinations of expression plasmids for Myc-tagged M protein as well as FLAG- and V5-tagged transducer proteins. B, HEK293/ACE2 cells were co-transfected with plasmids for the indicated transducer proteins and then infected with 5 multiplicity of infection of SARS coronavirus. Cell lysates were collected 48 h post-transfection and 24 h post-infection. Proteins were analyzed by Western blotting (WB) with anti-FLAG (α -FLAG), anti-V5 (α -V5), anti-Myc (α -myc), and antibodies against SARS coronavirus N protein (α -SCoV-N). Co-immunoprecipitation (IP) was performed with anti-FLAG, and the precipitates were probed with anti-FLAG and anti-V5.

DISCUSSION

We demonstrated in this study that SARS coronavirus M protein inhibited the production of type I interferons through a new mechanism. M protein predominantly localized to the Golgi complex and potently suppressed dsRNA-induced expression of type I interferon genes (Fig. 1). M protein exerted

its inhibitory effect on cytoplasmic dsRNA sensors RIG1 and MDA5 as well as other transducer proteins VISA, TBK1, and IKK ϵ , but it could not counteract the activation mediated directly by IRF3 and IRF7 (Fig. 2). M protein interacted with RIG-1, TBK1, IKK ϵ , and TRAF3 in the cytoplasm (Fig. 3) and likely sequestered some of them in discrete compartments that were consistent with the membrane-associated Golgi apparatus (Fig. 4). Although M protein did not directly inhibit the catalytic activity of

TBK1/IKK ϵ kinase (Fig. 5), it impeded the formation of a functional TRAF3-TANK-TBK1/IKK ϵ complex (Fig. 8), thereby inhibiting the phosphorylation of IRF3 (Figs. 6 and 7). Importantly, formation of the TRAF3-TANK-TBK1/IKK ϵ complex, phosphorylation of IRF3, and production of interferon β were also inhibited in SARS coronavirus-infected cells (Figs. 7 and 8).

Our findings suggested a new role for M protein in pathogenesis of SARS coronavirus. This is not surprising since M protein was previously shown to modulate cellular signaling and apoptosis (28–30). Interestingly, an adaptive mutant of M protein was found to enhance viral replication and/or infectivity (27). Because a reduction in interferon-inducing activity was shown to be associated with M protein mutations in transmissible gastroenteritis virus (26), M protein of SARS coronavirus might also influence viral replication through inhibition of interferon production. Thus, it will be of great interest to understand whether the particular mutation in SARS coronavirus M protein might influence its ability to inhibit the induction of type I interferons. A better understanding of the role of M protein in pathogenesis of SARS has important implications in the development of new antivirals and vaccines.

Other SARS coronaviral proteins including ORF3b, ORF6, N, nsp1, and papain-like protease were also known to inhibit interferon production and/or signaling (6–12). However, their modes of action were thought to be different from that of M protein. Particularly, our detailed comparison of M and N proteins in their inhibition of various activators (Figs. 1 and 2) suggested that they targeted different steps. Although the requirement of M protein for virion assembly prevents us from studying interferon production with M protein deletion mutants of SARS coronavirus, the coordinated actions of different interferon-antagonizing viral proteins in infected cells merit further investigations. This also poses major technical challenges for future attempts to verify and distinguish the contributions from different viral proteins during infection. Identification of M protein mutants lacking the interferon-antagonizing activity alone might prove useful in future studies.

Viral countermeasures for interferon production and signaling have been well described (17). Compared with influenza A virus NS1 protein, an extensively studied viral antagonist of interferon (48–51), SARS coronavirus M protein displayed a similar pattern of inhibitory activity on RIG-1, MDA5, TBK1, and IKK ϵ (Fig. 2). However, M protein inhibited the activity of

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VISA, whereas NS1 did not (Fig. 2E). Additional experiments are required to elucidate the underlying cause of this difference between M and NS1 proteins. Both TRAF3 and TANK are known to be important for interferon response (34, 52, 53). Our findings that M protein associated with TRAF3 (Fig. 3) and impeded its interaction with TANK, TBK1, and IKK ϵ (Fig. 7) revealed a new mechanism for modulation of interferon production by SARS coronavirus. In this regard, NY-1 hantavirus Gn cytoplasmic tail was recently shown to co-precipitate with TRAF3 and inhibit the formation of TRAF3-TBK1 complex (54), whereas Ebola virus VP35 was able to impair the function of TBK1/IKK ϵ by preventing their interaction with VISA and IRF3/IRF7 (55). Plausibly, inhibition of the formation of a functional TRAF3-TBK1/IKK ϵ -IRF3/IRF7 complex might be a common mechanism through which viruses inhibit interferon production.

Cytoplasmic modulators of interferon induction have just begun to be understood. Whereas NLRX1 and MULAN are mitochondrial proteins that interact with VISA to inhibit RIG-I activity (56, 57), DDX3X interacts with TBK1 directly (58) and is targeted by vaccinia virus K7 protein (59). In this connection, our demonstration of a coronaviral inhibitor of interferon production not only adds a new member to the group of viral and cellular modulators of interferon response but might also reveal new targets and strategies in the design of new antiviral agents.

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