NOTES

Severe Acute Respiratory Syndrome Coronavirus Protein 6 Is Required for Optimal Replication[▽]

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Severe acute respiratory syndrome coronavirus (SARS-CoV) encodes several accessory proteins of unknown function. One of these proteins, protein 6 (p6), which is encoded by ORF6, enhances virus replication when introduced into a heterologous murine coronavirus (mouse hepatitis virus [MHV]) but is not essential for optimal SARS-CoV replication after infection at a relatively high multiplicity of infection (MOI). Here, we reconcile these apparently conflicting results by showing that p6 enhances SARS-CoV replication to nearly the same extent as when expressed in the context of MHV if cells are infected at a low MOI and accelerates disease in mice transgenic for the human SARS-CoV receptor.

The genome of severe acute respiratory syndrome coronavirus (SARS-CoV) encodes several structural proteins, including the spike, nucleocapsid, membrane, and envelope proteins (13). Integrated between and within these structural proteins are eight accessory proteins (6, 8, 10, 15, 16, 18, 21–27). Our laboratory showed previously that one of these SARS-CoV-specific accessory proteins, encoded by ORF6, showed a clearly recognizable phenotype when introduced into a heterologous attenuated murine coronavirus, mouse hepatitis virus (MHV) strain J2.2-V-1 (rJ2.2.6). rJ2.2.6 grew more rapidly and to higher titers in tissue culture cells and in the murine central nervous system than control viruses, and the presence of p6 increased mortality in mice from 10 to 20% to 80% (7, 19, 20). However, the absence of p6 did not diminish SARS-CoV growth in tissue culture cells when cells were infected with 1 PFU/cell (31). In addition to a role in enhancing virus replication, when expressed in the context of a SARS-CoV infection or by transfection, p6 blocked interferon (IFN)-induced STAT1 nuclear translocation by retention of the nuclear import adaptor molecule karyopherin alpha 2 in the cytoplasm, indicating a role in thwarting innate immune effectors (5, 11). In contrast, p6 did not significantly diminish IFN sensitivity when expressed in the context of rJ2.2 (20).

The results described above were puzzling, because p6 seemed to be required for the optimal replication of a heterologous coronavirus but not for that of SARS-CoV. Thus, the objective of this study was to determine whether p6 could enhance SARS-CoV replication in tissue culture cells under

any conditions. For this purpose, we examined its function by comparing the growth of a recombinant SARS-CoV (rSARS-CoV) in which p6 was deleted (rSARS-CoVΔ6) with that of wild-type rSARS-CoV at a range of multiplicities of infection (MOIs). Normal mice infected with SARS-CoV readily cleared the infection, making it difficult to detect a role for p6 in vivo. However, mice that are transgenic for expression of the human receptor angiotensin-converting enzyme 2 (hACE2) are exquisitely sensitive to infection with SARS-CoV and are useful for identifying an in vivo role for p6 (14).

p6 enhances growth of rSARS-CoV. Infection with MHV recombinant rJ2.2.6, which encodes SARS-CoV p6, yielded titers of infectious virus that were 0.5 to 1 log₁₀ higher than those detected in cells infected with the control virus (rJ2.2.6^{KO}, in which p6 expression was disabled) (20, 28). To assess whether p6 had a similar effect in the context of SARS-CoV, we constructed rSARS-CoVΔ6 by deleting amino acids 13 to 43 and mutating the start codon of p6, using previously published methods (1, 3). We confirmed the lack of p6 expression by rSARS-CoVΔ6 by using Western blot analysis (Fig. 1A). To compare growth kinetics, we infected Vero E6 cells with rSARS-CoV and rSARS-CoVΔ6 at low MOIs (0.01) in order to magnify any differences in viral replication conferred by p6. We used Vero E6 cells for these assays because p6 inhibits IFN signaling and because these cells lack type 1 IFN expression (4). rSARS-CoVΔ6 grew to titers that were 3 to 5 times lower than those of rSARS-CoV, although these differences disappeared by 24 h postinfection (p.i.) (Fig. 1B). In confirmation of these results, we also showed that rSARS-CoVΔ6 grew more slowly and to lower titers than rSARS-CoV in a second cell line, Huh-7 (data not shown).

Tangudu et al. previously found that both viral RNA and protein syntheses were detected at earlier times and at higher levels in rJ2.2.6-infected cells than in rJ2.2.6^{KO}-infected cells but that p6

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FIG. 1. Time course of SARS-CoV infection and viral RNA and protein accumulations. (A) To assess p6 expression by SARS-CoV $\Delta 6$, Vero E6 cells were infected with wild-type rSARS-CoV (wt) or rSARS-CoV $\Delta 6$ ($\Delta 6$) at an MOI of 1 and analyzed by Western blot assay, using mouse anti-N monoclonal antibody (MAb; kindly provided by John Nicholls, University of Hong Kong) (N) and rat anti-p6 polyclonal antibody (P6). The latter was produced in 6-week-old female Wistar rats by intraperitoneal inoculation with 40 μ g of high-pressure liquid chromatography-purified peptide 36-IVRQLFKPLTKKNYSELDDDEPM-58 coupled to *Limulus polyphemus* hemocyanin, followed by two boosts with peptide delivered intraperitoneally. (B to D) Vero E6 cells were infected with rSARS-CoV or rSARS-CoV $\Delta 6$ at an MOI of 0.01. (B) Cells were harvested at the indicated times, and titers on Vero E6 cells were determined. The increase in virus titers mediated by p6 is shown at each time point. (C) Total cellular RNAs were harvested from individual cultures at 5, 6, 7, and 9 h p.i. SARS-CoV N gene-specific RNA was quantified by real-time PCR, normalizing the level of N gene amplicons to that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) amplicons, as described previously (20). *, $P < 0.05$ by Student's t test. (D) Infected cell cultures were harvested and analyzed by Western blot assay, using anti-N antibody. Twenty-four-hour samples were diluted eightfold to avoid overexposure (indicated by "1/8" in the figure). Data shown are representative of three independent experiments.

did not enhance virus entry (28). These results suggested that p6 functioned by augmenting virus RNA synthesis or translation during the eclipse phase of MHV infection. To determine if p6 also accelerated the early appearance of viral products in the context of SARS-CoV, we quantified N gene-specific RNA accumulation at 5, 6, 7, and 9 h p.i. by real-time reverse transcriptase PCR. Viral RNA levels were four- to fivefold higher at 5 h p.i. in cells infected with the wild-type virus than in those infected with rSARS-CoV $\Delta 6$, but these differences diminished as the infection progressed (Fig. 1C). After 9 h p.i., there was no significant difference in the levels of viral RNA in rSARS-CoV- and rSARS-CoV $\Delta 6$ -infected cells. Similarly, viral N protein was detectable as early as 8 h p.i. in both wild-type virus- and rSARS-CoV $\Delta 6$ -infected cells as measured by Western blot assay, but greater amounts were detected in cells expressing p6 (Fig. 1D). Differences in viral protein levels diminished at later time points, and

there were no significant differences after 24 h p.i. Similar to results observed in rJ2.2-infected cells (28), these results suggest that small quantities of p6 synthesized at early times p.i. function to augment viral replication.

Plasmid DNA encoding p6 enhances growth of rSARS-CoV $\Delta 6$. To confirm that the delay in virus growth that we observed in rSARS-CoV $\Delta 6$ -infected cells was due to the absence of p6 expression, we transfected cells with cDNA encoding p6 prior to infecting them with rSARS-CoV $\Delta 6$. Viral titers were measured at 8 h p.i. More than 70% of the cells were positive for p6 expression after being transfected (Fig. 2A). Compared to cells transfected with empty vector, approximately fourfold-more virus was produced in cells expressing p6 (Fig. 2B). These data collectively indicate that p6 creates a cellular environment that is more optimal for SARS-CoV replication.

FIG. 2. Complementation of rSARS-CoVΔ6 with plasmid DNA encoding p6. Vero E6 cells were grown in 24- or 6-well plates and transfected for 16 h with 1.0 or 4.0 μ g pCAGGS-ORF6-HA plasmid, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. As controls, cells were transfected with the same amount of empty vector. (A) Transfection efficiency was determined by an immunofluorescence assay using mouse anti-hemagglutinin antibody (Covance, Berkeley, CA), followed by Cy3-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) (red). Nuclei were stained with Topro-3 (blue). (B) Sixteen hours after transfection, cells were infected with rSARS-CoVΔ6 at an MOI of 0.01 in serum-free Dulbecco's modified Eagle's medium. Viral titers were determined at 8 h p.i. by plaque assay on Vero E6 cells. *, $P < 0.05$ by Student's t test.

p6 modestly enhances virulence of rSARS-CoV in hACE2 transgenic mice. Mice that are transgenic for the expression of the SARS-CoV receptor hACE2 are very susceptible to infection with SARS-CoV. We previously showed that the deletion of accessory genes 6, 7a, 7b, 8, and 9b slightly diminished the rate of weight loss and the time to death compared to those of mice infected with rSARS-CoV (3). To determine whether this difference in clinical outcome could be attributed to p6 expres-

sion, we infected hACE2 transgenic mice with 24,000 PFU of rSARS-CoV or rSARS-CoVΔ6 by intranasal inoculation. Mice infected with rSARS-CoV developed clinical disease and lost weight at earlier times p.i. (day 3) than did those infected with rSARS-CoVΔ6 (day 4) (Fig. 3A). Further, 75% of rSARS-CoV-infected mice died by day 5 p.i.; in contrast, mice infected with rSARS-CoVΔ6 survived for an additional day (Fig. 3B), although all the mice eventually died. Consistent with these

FIG. 3. Weight loss, mortality, and viral titers in hACE2 transgenic mice infected with rSARS-CoV and rSARS-CoVΔ6. Mice were infected with 2.4×10^4 PFU rSARS-CoV or rSARS-CoVΔ6 and monitored for weight loss (A) and mortality (B). Groups of four infected mice were analyzed in two independent experiments, and the data were combined. Virus titers in the lung (C) and brain (D) were determined on days 1 (D1) to 4 p.i. as previously described (2). Each group includes eight infected mice from two independent experiments. *, $P < 0.05$; **, $P < 0.001$ by Student's t test.

FIG. 4. Effects of p6 on IFN-induced STAT1 nuclear translocation in SARS-CoV- or MHV-infected cells and on susceptibility to IFN. (A) Vero E6 cells were infected with rSARS-CoV or rSARS-CoV $\Delta 6$ at an MOI of 0.1 and then treated with 100 U/ml of human IFN- γ for 30 min at 12 h p.i. Cells were fixed with methanol and stained with anti-p6 followed by Cy5-conjugated donkey anti-rat antibody (Jackson ImmunoResearch) (blue), anti-SARS-CoV N followed by fluorescein isothiocyanate-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch) (green), and rabbit anti-phospho-STAT1 (Tyr701) (p-STAT1) antibody (Cell Signaling, Danvers, MA) followed by Cy3-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch) (red). (B) HeLa-MHVR cells were infected with rJ2.2 or rJ2.2.6 at an MOI of 0.5 and then treated with human IFN- γ for 30 min at 14 h p.i. Cells were stained with fluorescein isothiocyanate-conjugated anti-hemagglutinin (HA) MAb (Roche, Switzerland) (green), anti-MHV N (MAb 5B188.2, kindly provided by M. Buchmeier, University of California, Irvine) followed by Cy5-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch) (blue), and anti-p-STAT1 followed by Cy3-conjugated donkey anti-rabbit antibody (red). Original magnification, $\times 40$. (C) Vero E6 cells in triplicate were treated with the indicated concentrations of human IFN- β (PBL Biomedical Laboratories, Piscataway, NJ) 24 h prior to being infected with rSARS-CoV or rSARS-CoV $\Delta 6$ at 0.01 or 0.001 PFU/cell. Cells were then incubated for another 24 h in the presence of the same concentration of IFN- β . Samples were harvested, and virus titers were determined.

results, virus titers were significantly lower at days 1 and 2 p.i. in the lungs and at day 2 in the brains of rSARS-CoVΔ6-infected mice than in rSARS-CoV-infected mice, although these differences disappeared by 4 days p.i. (Fig. 3C and D). Moreover, at 2 days p.i., virus was detected in the brains of only 5 of 8 mice infected with rSARS-CoVΔ6, compared to 8 of 8 mice infected with rSARS-CoV, suggesting that viral entry into the brain was slightly delayed in the absence of p6. Of note, we previously reported that there were no differences in the titers of SARS-CoV and SARS-CoV-Δ6-9b in the brains of infected mice (3). We suspect that this lack of detection of a difference reflected the small numbers of mice used in the previous study and not a biological difference between rSARS-CoVΔ6 and rSARS-CoVΔ6-9b.

p6 inhibits STAT1 nuclear translocation in rJ2.2.6-infected cells but is not required for IFN evasion in either SARS-CoV- or MHV-infected cells. Since p6 inhibits IFN-induced STAT1 nuclear translocation in SARS-CoV-infected cells (5), we next investigated whether p6 could also inhibit STAT1 nuclear translocation in rJ2.2.6-infected cells. Initially, we confirmed the results of Freiman et al. (5) and showed that IFN-induced STAT1 nuclear translocation was inhibited in SARS-CoV-infected Vero E6 cells but not if p6 was genetically deleted (Fig. 4A). We treated cells with 100 U of gamma interferon (IFN-γ) in these assays, because IFN-γ activates p-STAT1 to form a homodimer, resulting in a stronger signal in immunofluorescence assays. We then infected HeLa-MHVR cells (HeLa cells expressing MHV vector) with rJ2.2.6 or rJ2.2 and then treated them with IFN-γ at various times p.i. As in cells infected with rSARS-CoV, expression of p6 by recombinant MHV resulted in inhibition of STAT1 nuclear translocation. Translocation was inhibited only in large virus-induced syncytia, not in single infected cells, suggesting that adequate levels of p6 need to be generated before STAT1 translocation is inhibited (Fig. 4B). Consistent with this finding, IFN-induced STAT1 nuclear transport was not inhibited at 8 h. p.i. in SARS-CoV-infected cells, probably because insufficient amounts of p6 had accumulated by this time p.i. (data not shown).

To begin to determine the relative importance of the virus-enhancing and IFN-inhibiting functions of p6, we examined the role of p6 in diminishing IFN signaling in SARS-CoV-infected cells; previously we reported that p6 did not significantly enhance the resistance of MHV to IFN treatment (20). As shown in Fig. 4C, p6 was not required for resistance to IFN-β treatment in cells infected with SARS-CoV. Cells were treated with IFN-β in these assays because SARS-CoV is more sensitive to treatment with IFN-β than with IFN-γ (2).

Here, we showed that p6 is required for optimal SARS-CoV replication in tissue culture cells and that it augments the early stages of virus replication to nearly the same extent as when expressed heterologously in MHV-infected cells (3- to 10-fold) (20) (Fig. 1). p6 also inhibits STAT1 translocation in response to IFN when expressed in either SARS-CoV- or MHV-infected cells, but its absence does not affect the IFN sensitivity of either virus. Inhibition of STAT1 nuclear translocation by p6 occurs only at later times p.i. in both SARS-CoV- and MHV-infected cells, suggesting that other viral proteins are more important in counteracting the IFN response during the early stages of infection. At least four other SARS-CoV-specific proteins, nsp1, nsp3, ORF3b, and N, and two MHV pro-

teins, nsp1 and N, have been implicated in inhibition of IFN induction or function and may contribute to this early anti-IFN effect (9, 11, 17, 29, 30).

p6 is the only SARS-CoV-specific accessory protein identified thus far that is required for optimal virus replication. The ORF6 gene is intact and highly conserved in SARS-related coronaviruses isolated from species ranging from bats to humans (12). While the effects of p6 on virus growth in tissue culture cells are fairly modest, its expression in MHV-infected cells results in greatly enhanced mortality in mice. It is also possible that p6 has an equally important role when expressed in natural SARS-CoV hosts.

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