Coronavirus Endoribonuclease Ensures Efficient Viral Replication and Prevents Protein Kinase R Activation

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ABSTRACT Coronavirus (CoV) nsp15 is an endoribonuclease conserved throughout the CoV family. The enzymatic activity and crystal structure of infectious bronchitis virus (IBV) nsp15 are undefined, and the protein’s role in replication remains unclear. We verified the uridylate-specific endoribonuclease (EndoU) activity of IBV and found that the EndoU active sites were located in the C terminus of nsp15 and included His223, His238, Lys278, and Tyr334. We further constructed an infectious clone of the IBV-rSD strain (rSD-wild type [WT]) and EndoU-deficient IBVs by changing the codon for the EndoU catalytic residues to alanine. Both the rSD-WT and EndoU-deficient viruses propagated efficiently in embryonated chicken eggs. Conversely, EndoU-deficient viral propagation was severely impaired in chicken embryonic kidney cells, which was reflected in the lower viral mRNA accumulation and protein synthesis. After infecting chickens with the parental rSD-WT strain and EndoU-deficient viruses, the EndoU-deficient virus-infected chickens presented reduced mortality, tissue injury, and viral shedding.

IMPORTANCE Coronavirus can emerge from animal reservoirs into naive host species to cause pandemic respiratory and gastrointestinal diseases with significant mortality in humans and domestic animals. Infectious bronchitis virus (IBV), a γ-coronavirus, infects respiratory, renal, and reproductive systems, causing millions of dollars in lost revenue worldwide annually. Mutating the viral endoribonuclease resulted in an attenuated virus and prevented protein kinase R activation. Therefore, EndoU activity is a virulence factor in IBV infections, providing an approach for generating live attenuated vaccine candidates for emerging coronaviruses.

KEYWORDS γ-coronavirus, infectious bronchitis virus, nonstructural protein 15 (nsp15), uridylate-specific endoribonuclease (EndoU), protein kinase R
CoV-2 nsp15 comprises three domains: a small N-terminal domain, a middle domain, and a large C-terminal domain. EndoU is located in the C-terminal domain, consistent with other coronaviruses (11–13). The crystal structure of avian γ-CoV, as well as its EndoU activity, remain unanalyzed. Because of its special enzymatic activity and localization with the replicating viral RNA, nsp15 was initially thought to play a key role in viral replication. However, mouse hepatitis virus (MHV) encoding catalytic-defective EndoU exhibited only a subtle defect in RNA synthesis and slightly reduced viral titers (~1 log) compared with those of the parental virus (14). SARS-CoV and human CoV 229E nsp15 mutants yielded similar results (15). Thus, nsp15 EndoU activity is not essential for CoV replication, as was initially proposed (16–18).

The host innate immune system is the first line of defense against viral infection. Innate immunity involves complex cellular pathways to rapidly respond to infection. The recognition of pathogen-associated molecular patterns by pattern recognition receptors induces a highly sophisticated innate immune system response to inhibit viral protein synthesis and, ultimately, viral replication (19–21). To ensure efficient viral replication, many viruses encode proteins with specialized functions to evade innate antiviral responses, although their modes of action and the points of interference may differ. Viruses usually interfere in several antiviral pathways and can disrupt pathways at multiple levels to ensure efficient suppression of the host innate antiviral response. One interferon-stimulated protein, protein kinase R (PKR), detects viral RNA in the cytoplasm, which induces its autophosphorylation and subsequent phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α) (22, 23). PKR-mediated phosphorylation of eIF2α inactivates protein synthesis, thereby affecting viral propagation (24, 25). The influenza A virus NS1 protein is a well-studied example; this protein shields viral double-stranded RNA (dsRNA) from detection by pattern recognition receptors and PKR (26–28).

Here, we addressed a possible role of the highly conserved coronavirus EndoU activity in viral replication, pathogenicity, and innate immune evasion. We verified the EndoU activity of infectious bronchitis virus (IBV) and found the EndoU active site and then evaluated the IBV EndoU-deficient viral replication. The mutants replicated differently under different culture conditions, and the nsp15 EndoU activity is an important PKR antagonist.

RESULTS

Endoribonuclease activity and catalytic residues. Sequence alignment of IBV nsp15 with CoV nsp15s demonstrated that His223, His238, Lys278, and Tyr334 were conserved between different CoV genera (Fig. 1A), indicating relatively conserved key active-site residues and similar endoribonuclease cleavage mechanisms among the Coronaviridae. To verify the endoribonuclease activity of the wild-type (WT) and mutant nsp15 proteins, we first expressed and purified the recombinant protein in Escherichia coli. The purified recombinant protein concentrations were nsp15-WT (0.356 μg/μl), nsp15-H223A (1.380 μg/μl), nsp15-H238A (1.256 μg/μl), nsp15-K278A (0.709 μg/μl), and nsp15-Y334A (1.393 μg/μl) (Fig. 1B). Notably, WT nsp15 expression was toxic to E. coli, as indicated by slower cell growth and low yields. Four mutants, H223A, H238A, K278A, and Y334A, significantly improved expression under the same conditions as those of the WT.

Next, we performed enzyme activity assays for WT nsp15 and the H223A, H238A, K278A, and Y334A mutants under identical conditions. Using 0.2 μM enzyme and 1.2 μM substrate (5′-6-carboxyfluorescein [FAM]-dA-rU-dA-dA-3′-6-carboxytetramethylrhodamine [TAMRA]), the mutant activity was at least 10-fold less than that of the WT, which was monitored using a LightCycler480 II at 465 to 510 nm (Fig. 1C). Thus, the H223A, H238A, K278A, and Y334A mutations severely diminished the enzyme activity, revealing that the 4 residues are important for catalytic activity.

Coronaviruses encoding nsp15 mutants exhibited impaired replication in kidney cells. The parental WT rSD (rSD-WT) and all endoribonuclease activity-deficient viruses (EndoU-deficient mutants) were generated using reverse genetics (29). Figure 2A
FIG 1 Mutagenesis studies of IBV nsp15 endoribonuclease activity. (A) Sequence alignment of IBV-nsp15 with CoV nsp15s. Putative EndoU activity catalytic sites are marked by black arrowheads; strictly conserved residues are depicted in white characters on a red background. Representatives of the three CoV genera were included: IBV (γ-coronavirus); HCoV-229E and PEDV (α-coronavirus); and SARS-CoV, SARS-CoV-2, MERS-CoV, and MHV-A59 (β-coronavirus). (B) SDS-PAGE analysis of WT and mutant (H223A, H238A, K278A, and Y334A) nsp15 purified proteins. Each lane represents the order in which the elution buffer was eluted. (C) FRET-based enzyme activity assay. WT and mutant (H223A, H238A, K278A, and Y334A) nsp15 endoribonucleases were evaluated by cleaving the substrate (5’-6-FAM-dArUdAdA-6-TAMRA-3’). The values ± SD of the results from triplicate experiments are shown.
summarizes their viral viability. For each mutant, we used 3 independent clones for viral recovery, which were deep sequenced to validate the genotypes. The EndoU-deficient mutants contained an alanine substitution in the nsp15 catalytic residue, which was previously shown to inactivate endoribonuclease activity (Fig. 1C). However, we could not rescue the viral nsp15 gene deletion, indicating that nsp15 contains functional regions other than those involved in endoribonuclease activity, which play fatal roles in CoV replication.

We next investigated the mutational effects on viral replication in embryonated chicken eggs (ECEs) and chicken embryonic kidney (CEK) cells. rSD-WT and the five mutant viruses replicated efficiently in the ECEs, and EndoU deficiency slightly influenced the replication compared with that of the WT virus (Fig. 2B), indicating that WT nsp15 was not required for viral replication in ECEs. We further explored viral reproduction in CEK cells and found that rSD-WT caused severe cytopathic effects on the CEK cells, but the EndoU-deficient mutants did not (Fig. 2C). Additionally, the CEK cells containing EndoU-deficient mutants exhibited significant growth defects, with viral copies being decreased by 3.5 to 5.5 log units (Fig. 2D).

EndoU-deficient mutants presented defective viral protein synthesis in infected CEK cells. To analyze the mutational block in the IBV life cycle, we examined synthesis of the viral structural protein (nucleocapsid protein) during infection. rSD-H238A and rSD-Y334A were selected for the further investigation, because the phenotypes of those EndoU-deficient mutants are identical. CEK cells were infected with rSD-WT or EndoU-deficient viruses at low doses (10⁷ viral copies). The cells were collected at different time points postinfection, and the intracellular viral protein expression was analyzed via Western blotting. For the WT virus, N proteins were readily detectable around 24 h postinfection (hpi). Conversely, no N protein expression was detected in the EndoU-deficient virus-infected cells (Fig. 3A). Interestingly, increasing the infection dose to 10⁹ viral copies yielded different results (Fig. 3B). In the early stages of infection (1 to 12 hpi), differences in the N protein levels were minimal, but later in the viral infection (24 to 60 hpi), the viral protein accumulation differed greatly, especially at 36, 48, and 60 hpi, when many proteins were detected in the infected group. EndoU-
deicient viruses maintained lower N protein expression levels than did the WT viruses, and the protein content did not accumulate over time.

We also evaluated the infection dynamics via immunofluorescence assay (IFA) (Fig. 4), in which the cells were infected at 10⁹ viral copies. The rSD-WT and EndoU-decient virus-infected groups did not differ at 6 hpi, and only sporadic cells were positive for IBV. However, IFA signals increased gradually as the rSD-WT infection proceeded; at 24 hpi, most cells were infected, and N proteins were evenly distributed throughout the infected cells. Conversely, the proteins did not increase significantly with infection time for the EndoU-deficient mutants, and the protein distribution and morphology differed significantly from that of the rSD-WT group. The N proteins were distributed, aggregated, and punctated at 12 and 24 hpi, and the positive signals were much weaker than those of the parental group. Thus, the defect for the EndoU-deficient mutants was either at the same level or upstream of the viral protein synthesis.

**EndoU-deficient mutants led to fewer viral mRNA species and dsRNA in CEK cells.** CoV replication produces subgenomic RNA (sgmRNA) and double-stranded RNA (dsRNA) intermediates, while EndoU-deficient viruses more severely affect replication in CEK cells. We next determined whether CoV endoribonuclease activity affects sgmRNA and dsRNA synthesis. We investigated the EndoU-deficient virus effects on viral mRNA and dsRNA synthesis in CEK cells. For expression profiling of individual IBV mRNA species, we measured the relative abundances of genomic RNA (gmRNA) and structural protein sgmRNAs via quantitative PCR (qPCR) with specific primers targeting the same leader-body junctions and the structural protein genes for the spike (S), nucleocapsid (N), membrane (M), and envelope (E) proteins. The respective RNA levels were analyzed from the sgmRNA (sgmRNA-S, -N, -M, and -E)/gmRNA ratios. All EndoU-deficient viruses exhibited dramatically reduced sgmRNA abundances at 24 hpi compared with those of the parental virus (Fig. 5A to D). This reduction was particularly noticeable in the early stages (24 hpi) of infection. N and M protein sgmRNA abundances also decreased significantly in the late stages (72 hpi) of infection, although the reduction in sgmRNA-S was not obvious. sgmRNA-E exceeded the test minimum in the EndoU-deficient virus-infected groups, while a small amount was detected in the WT group, because the sgmRNA-E proportion in the viral transcription process was low; hence, the detection abundance was low. We also measured and compared the positive and negative RNAs during the infection. Compared with the rSD-WT, EndoU-deficient viruses showed significantly lower abundances of positive (Fig. 5E) and negative (Fig. 5F) RNA.
FIG 4 CEK cells infected at 10⁹ viral copies were fixed at the indicated time points postinfection and subjected to IFA with antibody to N protein. Nuclear DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). The images were captured with a Nikon confocal microscope and processed using ImageJ.
To measure the dsRNA abundance during viral infection, we performed an IFA to observe the dsRNA formation of two representative EndoU-deficient viruses (rSD-H238A and rSD-Y334A) at different time points postinfection. Positive signals were detected for both the WT and EndoU-deficient viruses at 12 and 24 hpi but not at 6 hpi. The EndoU-deficient virus groups presented significantly fewer positive signals at both time points than did the WT virus groups (Fig. 6).

**EndoU-deficient mutants profoundly attenuated avian CoV infection in vivo.**

We infected chickens with the virus to investigate the replication and pathogenic ability of the EndoU-deficient mutants in vivo. Chickens infected with rSD-WT began showing clinical signs, including ruffled feathers, gasping, sneezing, tracheal rales, and depression, at 4 days postinfection (dpi). Three of 10 rSD-WT-challenged chickens died during the 14-day observation period, yielding a 30% mortality rate. No clinical signs or death were observed in the EndoU-deficient or mock groups (Fig. 7A). Ciliary activity was inhibited to evaluate the degree of damage to the upper respiratory system. The EndoU-deficient-virus-infected chickens showed less severe tracheal ciliary injury (40% to 60%) than did the rSD-WT chickens (100%) at 7 hpi (Fig. 7B). Next, we used qPCR to investigate how endoribonuclease activity contributed to IBV virulence by analyzing the viral infection efficiency from the viral tissue distribution. The EndoU-deficient groups showed lower viral loads in the trachea, lungs, kidneys, and bursa at 7 hpi (Fig. 7C). The rSD-WT-challenged chickens presented swollen and pale kidneys with urate deposition in the kidneys and ureters, whereas chickens in the EndoU-deficient groups...
CEK cells infected at 10^9 viral copies were fixed at the indicated time points postinfection and subjected to IFA with antibody to dsRNA. Nuclear DNA was stained with DAPI. The images were captured with a Nikon confocal microscope and processed using ImageJ.

**FIG 6** CEK cells infected at 10^9 viral copies were fixed at the indicated time points postinfection and subjected to IFA with antibody to dsRNA. Nuclear DNA was stained with DAPI. The images were captured with a Nikon confocal microscope and processed using ImageJ.
showed no lesions (Fig. 7D). The microscopic tracheal, lung, and kidney lesions from all groups were also analyzed histopathologically, and the hematoxylin and eosin staining results were consistent with those of the gross lesions described above. The rSD-WT-challenged group presented tracheal epithelial damage, mucosal injury, and cilia shedding with severe lymphocyte infiltration, whereas the EndoU-deficient and control groups presented no obvious lesions (Fig. 8A). Immunohistochemistry results revealed viral presence in the susceptible tracheal, lung and kidney tissues in the rSD-WT group. The positive signals were significantly lower in the EndoU-deficient groups, which was consistent with the qPCR results (Fig. 8B). These data demonstrate that endoribonuclease activity defects were strongly attenuated in vivo, indicating that nsp15 is necessary for virulence.

**IBV nsp15 endoribonuclease activity prevented PKR activation.** To further explore why IBV nsp15 endoribonuclease activity affected viral replication in CEKs, we investigated whether IBV nsp15 is associated with PKR activation. We expressed nsp15-WT, nsp15-H223A, nsp15-H238A, nsp15-K278A, and nsp15-Y334A individually as myc tag fusion proteins and monitored the PKR phosphorylation in the transfected cells. We used this approach because transfection of plasmid DNA can activate PKR, most likely because the dsRNA production forms from positive- and negative-sense mRNA transcription from cryptic promoters (30). Transfecting the myc tag plasmid DNA in HEK-293T cells triggered PKR phosphorylation. We set the influenza viral NS1 protein, which inhibits PKR phosphorylation, as the positive control (31, 32). No phosphorylated PKR was detected in cells transfected with WT nsp15 or avian influenza virus (AIV)-NS1 (Fig. 9A, lanes 3 and 6). This phenomenon depended on the nsp15 endoribonuclease activity, because PKR was strongly activated in cells transfected with the EndoU-deficient form of nsp15 (lanes 4 and 5). To further confirm these results, we used poly(I:C), a dsRNA mimic that can activate PKR, to stimulate cells after 24 h of transfection (Fig. 9B). Compared with that of the control group, poly(I:C) upregulated PKR phosphorylation (lanes 1 and 2), whereas nsp15-WT and AIV-NS1 inhibited the poly(I:C)-induced PKR phosphorylation (lanes 4 and 7). Conversely, p-PKR inhibition disappeared with the absence of endoribonuclease activity (lanes 5 and 6), inducing more intense p-PKR levels. We further verified the effect of the other two enzyme activity sites (H223A and K278A) on PKR phosphorylation and obtained consistent results (Fig. 9C). Thus, IBV nsp15 endoribonuclease activity inhibited PKR phosphorylation, and when the endoribonuclease function was absent, p-PKR inhibition was also relieved.

**DISCUSSION**

nsp15 is completely conserved among CoVs and has endoribonuclease activity in vitro. Previous studies on the structural analysis and endoribonuclease activity of nsp15 focused on α-, β-, and δ-CoVs (11, 33–36); however, those for γ-CoVs remain unreported. Here, we verified the nsp15 endoribonuclease activity of the representative γ-CoV, IBV, by expressing the purified protein and determined the amino acid sites affecting its catalytic activity. Consistent with that of other CoVs, IBV nsp15 is a uridylate-specific endoribonuclease, and its catalytic amino acid sites are concentrated on the C terminus.

Because of its unique enzymatic activity and colocalization with the replicating viral RNA, nsp15/EndoU was initially thought to play an important role in viral replication. Results vary among studies on EndoU-defective mutants of different CoVs. For example, MHVs encoding catalytic-defective EndoU showed only a subtle defect in RNA synthesis and slightly reduced viral titers compared with those of the WT virus when evaluated in fibroblasts (12). However, a study on macrophages revealed that MHV containing nsp15 mutants exhibited impaired replication in macrophages (37). Thus, nsp15 EndoU activity is not essential for CoV replication, and its effect on viral replication varies among cell types. IBV yielded results similar to those of MHV; the viral replication of EndoU-deficient IBV only minimally affected ECEs (Fig. 2B) but greatly affected CEKs (Fig. 2D). Notably, we did not rescue the nsp15-deficient recombinant
virus, suggesting that in addition to its endonuclease function, it is also critical for viral replication. Because nsp15 colocalizes with RTCs and is necessary for efficient viral RNA replication in cell cultures, the proteins adjacent to nsp15 are critical replicative components, processing the neighboring components and, thus, leading to a nonviable phenotype.

Our findings also provide insight into IBV gmRNA and sgmRNA synthesis. Similar to other positive-strand RNA viruses, CoV replication involves the synthesis of the complementary full-length negative-strand RNA, which serves as a template for generating positive-strand progeny genomes (38). The negative-strand templates are turned over via unknown mechanisms (39), and the positive-strand genomes are packaged into
FIG 8  Histology and immunohistochemical staining of the uninfected control, rSD-WT-, rSD-H238A-, and rSD-Y334A-infected chicken tissues. Chickens were euthanized on day 7. Images show representative histological slides of the trachea, lungs, and kidneys. Images were stained with hematoxylin and eosin (A) and mouse anti-IBV-N antibody (B). In panel A, the black arrow indicates extensive dropout, degeneration, and necrosis of the ciliated epithelial cells; the black triangle indicates hemorrhaging in the bronchial lumen; the open triangles indicate exfoliated fibroblasts and mucus generated from the accessory bronchial lumen; the open arrow indicates renal tubulointerstitial lymphocyte infiltration.
virions. In addition to genomic replication, the RTCs also synthesize sgmRNAs, which encode the open reading frames located in the 3'-proximal one-third of the genome (40). Here, we provide genetic evidence for the involvement of nsp15 in IBV sgmRNA, positive-strand RNA, negative-strand RNA (Fig. 5), and dsRNA (Fig. 6) synthesis. The results indicated that IBV nsp15 is directly involved in viral mRNA synthesis and that endoribonuclease activity plays a regulatory role in this process. Low N protein levels were detected in the EndoU-deficient mutants owing to decreased viral sgmRNA synthesis (Fig. 3A and B). In vivo, EndoU-deficient mutants revealed lower pathogenicity and viral loads in the trachea, lungs, and kidneys. These results showed that IBV endoribonuclease activity affected the viral pathogenicity and tissue tropism.

**FIG 9** IBV nsp15 endoribonuclease activity prevented PKR activation. (A) Western blot analysis of PKR and phospho-PKR in HEK-293T cell lysates at 36 h after pMYC plasmid transfection. Actin expression was detected as the loading control. An avian influenza virus NS1 protein plasmid was set as the positive control. (B) HEK-293T cells were transfected with an empty vector, nsp15-WT, nsp15-H238A, nsp15-Y334A, or IAV-NS1. After 24 h, the cells were treated with poly(I·C) for 12 h, and the cell lysates were collected to analyze PKR and phospho-PKR. (C) HEK-293T cell were transfected with empty vector, nsp15-WT, nsp15-H238A, nsp15-H278A, or nsp15-Y334A. After 24 h, the cells were treated with poly(I·C) for 12 h, and the cell lysates were collected to analyze PKR and phospho-PKR.
Studies on overexpression of IBV nsp15 indicate that the endoribonuclease activity prevents PKR phosphorylation (Fig. 9). Previous studies revealed that Middle East respiratory syndrome (MERS)-CoV NS4a encodes a dsRNA-binding protein that limits PKR activation. However, Rabouw et al. reported that NS4a deletion alone did not activate PKR, suggesting that MERS-CoV encodes redundant mechanisms to suppress PKR recognition and activation and dsRNA sensors (41). Therefore, we assumed that nsp15 plays a dominant role over NS4a activity in antagonizing host dsRNA sensors, such that loss of NS4a alone is insufficient to activate these sensors in all cell types.

In our study, no additional dsRNA was produced during EndoU-deficient viral infection, indicating that PKR activation is not caused by increases in total dsRNA but possibly by increased dsRNA that can be recognized by PKR. Previous studies suggest that viral dsRNA can be sequestered within membrane-associated RTCs to protect it from detection by host sensors (42), and nsp15 may sequester viral dsRNA within RTCs and away from host dsRNA sensors. Further studies are needed to clarify the mechanisms of nsp15 to potentially hide or degrade viral RNA and ultimately prevent activation of host dsRNA sensors. PKR is an interferon-stimulated gene product that restricts viral translation/replication via eIF2 phosphorylation and consequent inhibition of the cellular translation machinery and SG formation. In our previous study, IBV-infected cells failed to induce SG formation (data not shown), indicating that the virus evolved redundant mechanisms to suppress the stress response pathway, which may relate to nsp15 endoribonuclease activity.

In conclusion, our study on IBV, a CoV that causes lethal disease in chickens, revealed that inactivation of EndoU activity is associated with disease attenuation. We believe the information provided here can be applied to the recently emerged novel SARS-CoV-2. More research is needed to determine whether inactivating multiple enzymatic proteins, including EndoU, is an effective approach for generating safe and protective live attenuated CoV vaccines.

MATERIALS AND METHODS

Animals and ethics statement. All specific-pathogen-free (SPF) chickens and SPF embryonated eggs were purchased from Beijing Boehringer Ingelheim Vital Biotechnology Co., Ltd. (Beijing, China). The Beijing Administration Committee of Laboratory Animals approved the animal experimental protocols under the auspices of the Beijing Association for Science and Technology (approval ID SYXK [Jing] 2018-0038) and Ethical Censor Committee at China Agricultural University (CAU approval no. 20180).

Viruses and cells. Volker Thiel (University of Bern, Switzerland) kindly provided the vaccinia virus vNott/HK, CV-1, D980R, and BHK-21 cells, which were used to assemble the full-length mutant IBV genome. CV-1 and D980R were cultured in minimum essential medium (Thermo Fisher, Waltham, MA, USA) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). BHK cells were electroporated to rescue the rSD strain with the EndoU defects. CEK cells were prepared from 18-day-old SPF chicken embryos and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (HyClone, Logan, UT, USA) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/ml streptomycin.

Protein expression and purification. The complete gene fragment encoding IBV-rSD nsp15 was cloned from the viral RNA genome via reverse transcription PCR into the pGEX6p-1 vector (Beyotime Technology, Beijing, China), and the resulting plasmid was transformed into E. coli BL21(DE3) cells (TransGen Biotech, Beijing, China). The recombinant glutathione S-transferase (GST) fusion protein GST-nsp15 was purified using glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ, USA). The GST tag was removed via PreScission protease (Beyotime Technology, Beijing, China), yielding two additional residues (GP) at the N terminus.

Endoribonuclease assay. Real-time endoribonuclease assays were performed using fluorescent resonance energy transfer (FRET) as described previously (13). The substrate (5′-FAM-dArUdAdA-TAMRA-3′) was purchased from Takara (Tokyo, Japan). The substrate had FAM at the 5′ end and TAMRA at the 3′ end, which quenched the FAM fluorescent emission at 510 nm. Cleavage of the substrate leads to increasing fluorescence emissions at 510 nm. The cleavage reaction was performed at room temperature, and the mixture contained 0.2 μM protein and 1.2 μM RNA substrate in a final volume of 50 μL. The fluorescence intensity over time was monitored with a LightCycler480 II at 465 to 510 nm. The endoribonuclease assays of WT nsp15 and its mutants were performed in buffer containing 50 mM HEPES (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, and 5 mM MnCl2.

Virus rescue. EndoU-deficient mutants were constructed as follows. The pGPT-nsp15-positive plasmids were integrated into the vaccinia virus genome by homologous recombination, to which only recombinant vaccinia viruses with the Ecogpt gene show resistance. The verified viruses were reconstructed with the pGPT-EndoU-deficient-negative plasmids. The amino acid mutations were introduced into the rSD CDNA by homologous recombination using the transient dominant selection system (30).
TABLE 1 PCR amplification primers used

| Name       | Sequence               | Use                            |
|------------|------------------------|-------------------------------|
| gRNA-F     | 5′-TTGGGCTACGTCTCAGC-3′ | gmRNA quantification          |
| gRNA-R     | 5′-CTCCTTATTAGCTGAAGC-3′ |                               |
| sgRNA-F    | 5′-CTATATACTACGCTGCTG-3′ | sgRNA quantification          |
| sgRNA-S-R  | 5′-CTGGAACTGACTGACTGAT-3′ | sgRNA-S quantification        |
| sgRNA-N-R  | 5′-CTATCACGGAACACACTA-3′ | sgRNA-N quantification        |
| sgRNA-M-R  | 5′-CTGCTGATCGATCGATCC-3′ | sgRNA-M quantification        |
| sgRNA-E-R  | 5′-CACAACGTTTCTAAGCCTC-3′ | sgRNA-E quantification        |
| 18sRNA-F   | 5′-AGACCTAACTATCGCGAC-3′ | Relative quantification as control |
| 18sRNA-R   | 5′-GCGCTGCGTCTCCGC-3′   |                               |
| cDNA primer| 5′-CGAGGTGCGAGGAACCTCTT-3′ | Negative-stranded RNA reverse transcription |

After screening and transcription in vitro, the full-length RNAs of rSD-H223A, rSD-H238A, rSD-K278A, rSD-Y334A, and rSD-H223A/H238A/K278A/Y334A were electroporated into BHK cells. The cells and supernatant were frozen and thawed three times and then inoculated into 10-day-old SPF ECEs. The successfully rescued viruses were passaged in 10-day-old ECEs until the stable appearance of dwarf embryos, and then the recombinant viruses were sequenced to ensure no extra mutations existed.

Growth kinetics of rescued viruses in ECEs and primary CEK cells. Serum-free medium (200 μl) containing 100 50% egg infectious dose (EID50) of rSD-WT, rSD-H238A, rSD-H238A, rSD-K278A, rSD-Y334A, or rSD-H223A/H238A/K278A/Y334A virus was inoculated into the allantoic cavities of 10-day-old ECEs, and the allantoic fluid of five eggs per group was harvested at 12, 24, 36, 48, 60, and 72 h and pooled for real-time PCR detection. Next, 103 viral copies of the rSD-WT and EndoU-deficient mutants were inoculated onto CEK cells in 24-well plates. After 1 to 2 h of incubation at 37°C, CEK cells were washed three times with phosphate-buffered saline (PBS) and finally overlaid with DMEM containing 2% FBS. Cell culture supernatants were collected at 1, 24, 48, 72, 96, 120, and 144 h for real-time PCR detection assays for the IBV genome. Viral copy numbers at each time point were detected in triplicate and calculated according to a standard curve.

Animal experiments. One-day-old SPF chickens were randomly divided into groups A, B, C, and D and inoculated via eye dropper with 100 μl of 103 EID50 of IBV rSD-WT (A), rSD-H238A (B), rSD-Y334A (C), or PBS (D) as the negative control. Chickens were observed daily, and their clinical signs and mortality were recorded. At 7 and 14 dpi, three birds per group were euthanized and necropsied. Gross lesions were noted, and tissue samples from the trachea, kidneys, and lungs were collected for viral detection via real-time PCR and immunohistochemical assay or histopathological examination. Tracheas of the necropsied chickens were evaluated for tracheal ciliary activity as previously described (29).

Western blotting. Total cell protein lysates were extracted from the transfected or infected cells using the ProteinExt mammalian total protein extraction kit (TransGen). The total protein concentration was determined with a bicinchoninic acid protein assay kit (CW8Bio, Beijing, China). The cellular proteins were separated with 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Freiburg, Germany). Each PVDF membrane was blocked with 5% (wt/vol) skim milk and 0.1% Tween 20 in Tris-buffered saline (TBST) for 2 h at room temperature and then incubated overnight at 4°C with a primary antibody. The following antibodies were used in the experiments: anti-IBV-N (mouse; 3BN1; HyTest, Turku, Finland), anti-PKR (rabbit; Ab32506; Abcam, Cambridge, UK), anti-phospho-T446 PKR (rabbit; Ab32036; Abcam), and anti-actin (mouse; 3700S; Cell Signaling Technology, Danvers, MA, USA). The membranes were washed three times with TBST and then incubated with the corresponding horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibody and diluted at 1:10,000 for 1 h at room temperature. HRP was detected with Western lightning chemiluminescence reagent (CW8Bio).

Poly(I·C) transfection. 293T-HEK cells were transfected with each plasmid, and poly(I·C) was used for stimulation 24 h after transfection. Poly(I·C) was transfected by dropwise deposition of a mix containing 1 μg of poly(I·C) and StarFect high-efficiency transfection reagent (Genstar, Beijing, China). Transfected cells were harvested 12 h after transfection.

Analysis of the relative abundances of sgRNA species via qPCR. CEK cells were infected with the IBV rSD-WT and mutant strains. At 24 and 72 hpi, the total RNA was extracted with TRIzol reagent (product no. 15596026; Thermo Fisher). cDNA of positive-sense genome RNA and subgenomic RNA was synthesized with replicase transcriptase using oligo(dT), and cDNA of negative-sense RNA was synthesized with replicase transcriptase using the cDNA primer 5′-CGAGTGCGAGGAACCTCTT-3′. Primers gRNA-F and gRNA-R were used for amplification of plus-sense RNA and minus-sense RNA. The sgRNA-F and individual primers (sgRNA-S-R, sgRNA-N-R, sgRNA-M-R, or sgRNA-E-R) were used for qPCR of each structural protein subgenomic RNA (Table 1). The relative qPCR was performed with the 2−ΔΔCt method using SYBR premix Ex Taq (with Tli RNaseH) (TaKaRa, Japan) as the control and relative abundance was calculated.
Immunostaining. CEK cells were grown on 12-mm cover slips and infected with rSD-WT and EndoU-deficient mutants. Cells were infected with WT and mutant viruses, and after 1.5 h of incubation, cells were washed three times with PBS to remove unabsorbed virus and finally overlaid with DMEM containing 2% FBS. At the indicated time points after transfection, cells were fixed with 4% paraformaldehyde. After permeabilization using 0.2% Triton X-100 and subsequent blocking with 5% bovine serum albumin, N proteins were detected using the anti-IBV N (mouse; 38N1; HyTest) at 1/500 and J2 (mouse, anti-dsRNA; English and Scientific Consulting Kft, Hungary) at 1/250 and then incubated with secondary antibody conjugated to Alexa 488- or 594-conjugated antibodies (Cell Signaling Technology). Fluorescence signals were captured with a Nikon A1 confocal microscope (Nikon, Tokyo, Japan).

 Statistical analysis. All statistical analyses were performed with GraphPad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was determined by two-way analysis of variance. 

 Data availability. The whole-genome nucleotide sequences of IBV-rSD, IBV-rSD-H223A, IBV-rSD-H238A, IBV-rSD-K278A, IBV-rSD-Y334A, and IBV-rSD-H223A/K238A/K278A/Y334A are available in the GenBank database under the accession numbers MW351623, MW351624, MW351625, MW351626, MW351628, and MW351629, respectively.

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