

Expression, post-translational modification and biochemical characterization of proteins encoded by subgenomic mRNA8 of the severe acute respiratory syndrome coronavirus

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Keywords

glycosylation; mRNA8; rapid degradation; SARS-CoV; ubiquitination

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(Received 22 March 2007, revised 15 June 2007, accepted 19 June 2007)

doi:10.1111/j.1742-4658.2007.05947.x

The most striking difference between the subgenomic mRNA8 of severe acute respiratory syndrome coronavirus isolated from human and some animal species is the deletion of 29 nucleotides, resulting in splitting of a single ORF (ORF8) into two ORFs (ORF8a and ORF8b). ORF8a and ORF8b are predicted to encode two small proteins, 8a and 8b, and ORF8 a single protein, 8ab (a fusion form of 8a and 8b). To understand the functions of these proteins, we cloned cDNA fragments covering these ORFs into expression plasmids, and expressed the constructs in both *in vitro* and *in vivo* systems. Expression of a construct containing ORF8a and ORF8b generated only a single protein, 8a; no 8b protein expression was obtained. Expression of a construct containing ORF8 generated the 8ab fusion protein. Site-directed mutagenesis and enzymatic treatment revealed that protein 8ab is modified by N-linked glycosylation on the N81 residue and by ubiquitination. In the absence of the 8a region, protein 8b undergoes rapid degradation by proteasomes, and addition of proteasome inhibitors inhibits the degradation of protein 8b as well as the protein 8b-induced rapid degradation of the severe acute respiratory syndrome coronavirus E protein. Glycosylation could also stabilize protein 8ab. More interestingly, the two proteins could bind to monoubiquitin and polyubiquitin, suggesting the potential involvement of these proteins in the pathogenesis of severe acute respiratory syndrome coronavirus.

Coronaviruses are important pathogens of human and other animal species. In 2003, a novel coronavirus [severe acute respiratory syndrome coronavirus (SARS-CoV)] was discovered to be the etiologic agent of severe acute respiratory syndrome [1]. In SARS-CoV-infected cells, nine mRNA species are produced, which encode four typical structural proteins, spike (S), membrane (M), envelope (E) and nucleocapsid (N) proteins. In addition, protein 3a, encoded by the first ORF of the subgenomic mRNA3, was recently found to be a minor

structural protein [2]. Among other mRNA species, the genome-length mRNA1 codes for approximately 16 functional proteins involved in viral RNA replication, and subgenomic mRNA3, mRNA6, mRNA7, mRNA8 and mRNA9 code for five to eight accessory proteins that share little sequence homology with proteins of other known coronaviruses [1]. Among the accessory proteins, proteins 3a, 7a and 9b have been shown to be expressed, and some of them can elicit antibody responses in patients [3–5]. Although most of these

Abbreviations

ER, endoplasmic reticulum; GST, glutathione S-transferase; IBV, infectious bronchitis virus; SARS-CoV, severe acute respiratory syndrome coronavirus; UBD, ubiquitin-binding domain.

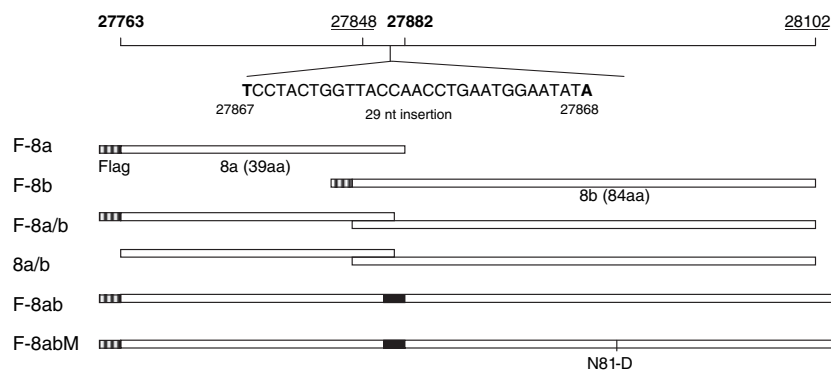


Fig. 1. Diagram showing the six constructs, pF-8a, pF-8b, pF-8a/8b, p8a/b, pF-8ab and pF-8abM, used in this study. The nucleotide positions of ORF8a are shown in bold, and the positions of ORF8b are underlined. Also shown are the nucleotide sequence of the 29 nucleotide insertion, the position of the insertion, the numbers of amino acids for the putative proteins 8a and 8b, and the Flag tag at the N-terminus of each construct.

accessory proteins are dispensable for viral replication in cultured cells [6], their exact roles in the pathogenesis and virulence of SARS-CoV in its natural hosts are yet to be established.

Two such accessory proteins, proteins 8a and 8b, are encoded by two overlapping ORFs (8a/b) present in subgenomic mRNA8 (Fig. 1). Subgenomic mRNA8 from most SARS-CoV isolates obtained from animals and patients at early stages of the SARS epidemic contains a single ORF8 with potential to encode a single protein, 8ab [7]. A 29 nucleotide deletion between T27867 and A27868 for strain SG2774 (accession number AY283798) was found in strains collected from human isolates at late stages of the outbreak, resulting in the splitting of the single ORF8 into two overlapping ORFs, ORF8a and ORF8b (Fig. 1) [1,8]. The deletion of the 29 nucleotides and the formation of two overlapping ORFs were predicted to be the consequence of adaptation of SARS-CoV from animals to humans [7,9]. Experimental infection of civets with SARS-CoV isolates BJ01 (without the 29 nucleotides) and GZ01 (with the 29 nucleotides) showed that animals infected with strain BJ01 tend to have a higher average temperature and slightly stronger antibody responses [10]. Two recent studies have shown that the putative protein 8b can downregulate the SARS-CoV E protein in virus-infected cells and induce DNA synthesis in cells expressing the protein [11,12]. However, more detailed characterization of the expression, biochemical properties and functions of the products encoded by these two mRNA isoforms has yet to be done.

In this study, ORF8a was expressed *in vitro* in both wheat germ extracts and rabbit reticulocyte lysates as a 5.3 kDa protein. Expression of the protein was also observed in Cos-7 cells, as a Flag-tagged protein. Similarly, translation of ORF8ab was observed in all the translation systems used. In contrast, protein 8b was expressed only when ORF8b was separately cloned. When constructs containing the two overlapping ORFs

ORF8a/ORF8b and the single ORF8ab, respectively, were expressed, expression of proteins 8a and 8ab were observed. Protein 8ab was shown to be an N-linked glycosylated protein, and the glycosylation site was identified to be the N81 residue. Proteins 8b and 8ab could be modified by ubiquitination, and in the absence of the 8a region, protein 8b undergoes rapid degradation by proteasome. Addition of the proteasome inhibitors inhibits the degradation of protein 8b as well as the protein 8b-induced rapid degradation of the SARS-CoV E protein. In addition, glycosylation could also stabilize protein 8ab. Furthermore, proteins 8b and 8ab could bind covalently and noncovalently to monoubiquitin and polyubiquitin. As no homology with any known ubiquitin-binding domains (UBDs) was found, they may represent a novel group of ubiquitin-binding proteins.

Results

Cloning, expression and post-translational modification of proteins encoded by the SARS-CoV mRNA8

In some animal and early human isolates, the subgenomic mRNA8 of SARS-CoV was predicted to encode a single ORF (ORF8). Owing to the deletion of 29 nucleotides [between T27867 and A27868 for strain SG2774 (accession number AY283798)], two overlapping ORFs (ORF8a/ORF8b) were found in most human isolates (Fig. 1). ORF8a and ORF8b are predicted to encode two small proteins, 8a and 8b, whereas ORF8 encodes a single protein, 8ab, representing a fused form of proteins 8a and 8b. To understand the functions of these proteins, cDNA fragments covering these ORFs were cloned into pFlag, giving rise to five constructs either with or without a Flag-tag at the N-terminus (Fig. 1). These constructs were then expressed in both *in vitro* expression systems and in intact cells.

When constructs p8a/b, pF-8a/b, pF-8b and pF-8ab were expressed in TnT transcription-coupled translation wheat germ extracts in the presence of [³⁵S]methionine, single protein bands of approximately 5.3, 6.5, 10.2 and 14.4 kDa, representing untagged protein 8a, Flag-tagged protein 8a, Flag-tagged protein 8b and Flag-tagged protein 8ab, respectively, were detected (Fig. 2A, lanes 1–4). Expression of the same four constructs in rabbit reticulocyte lysates in the presence of [³⁵S]methionine gave rise to the same four products (Fig. 2B, lanes 1–4). In addition, a series of bands (ladder bands) with increases of approximately 10 kDa were detected when pF-8b and pF-8ab were expressed in the system (Fig. 2B, lanes 3 and 4). The patterns of these bands suggest that they may represent ubiquitinated forms of proteins 8b and 8ab. To determine whether these bands are related to the 8b region, immunoprecipitation was carried out using rabbit polyclonal antibodies to protein 8b. As shown in Fig. 2B, the 10.2 kDa protein 8b and the 14.4 kDa protein 8ab, together with their corresponding ladder bands, were precipitated with the antibodies (lanes 7 and 8). The fact that these bands were efficiently immunoprecipitated by the antibodies to protein 8b suggests that proteins 8b and 8ab may be modified by ubiquitination.

Expression of these constructs was then carried out in HeLa cells using the vaccinia/T7 expression system. As shown in Fig. 2C, Flag-tagged proteins 8a, 8b and 8ab were detected in cells transfected with the corresponding constructs (lanes 2–4). Interestingly, a prominent band (8ab*), migrating more slowly than the 14.4 kDa protein 8ab, was detected in cells expressing pF-8ab (Fig. 2C, lane 4). This may represent a post-translationally modified protein 8ab; characterization of this modification was conducted, and the results are presented in a later section. In addition, multiple species of more slowly migrating bands were also detected from cells expressing F-8b and F-8ab (Fig. 2C, lanes 3 and 4).

Ubiquitination and binding of proteins 8b and 8ab to monoubiquitin and polyubiquitin

To test further whether proteins 8b and 8ab are post-translationally modified by ubiquitination, coexpression of these proteins with a Myc-tagged ubiquitin in HeLa cells was carried out, and the expression and interaction of the proteins were determined by western blot and coimmunoprecipitation assays. As shown in Fig. 3A, western blot analysis using antibody to Myc resulted in the detection of monoubiquitin and

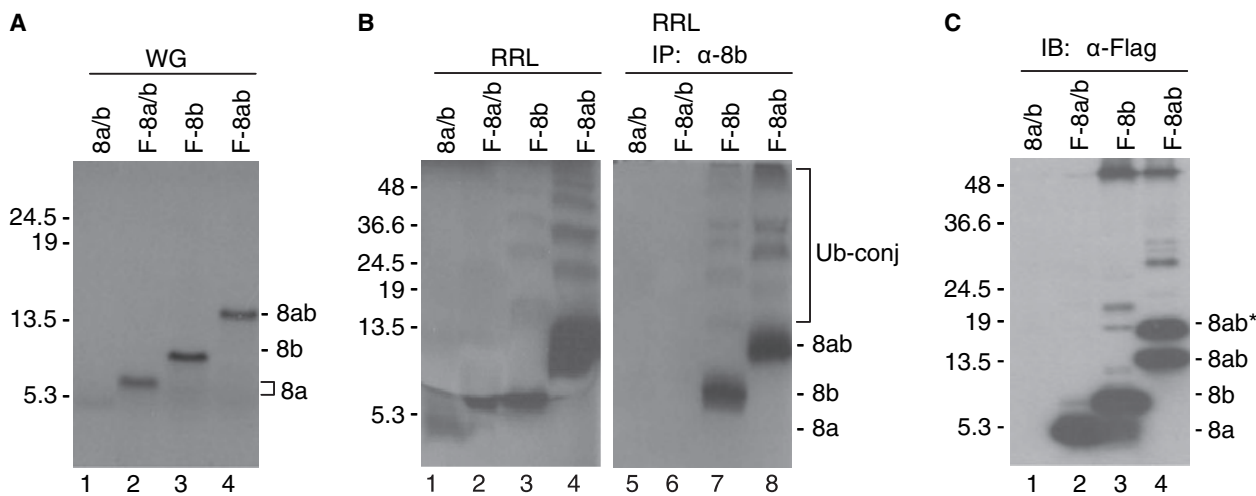


Fig. 2. Expression of constructs covering the 5'-unique ORFs of the subgenomic mRNA8 of SARS-CoV. (A) Expression of p8a/b (lane 1), pF-8a/b (lane 2), pF-8b (lane 3) and pF-8ab (lane 4) in wheat germ extracts in the presence of [³⁵S]methionine. The *in vitro*-synthesized products were resolved on SDS/20% polyacrylamide gel and detected by autoradiography. The numbers on the left indicate molecular masses in kilodaltons. (B) Expression of p8a/b (lanes 1 and 5), pF-8a/b (lanes 2 and 6), pF-8b (lanes 3 and 7) and pF-8ab (lanes 4 and 8) in rabbit reticulocyte lysates in the presence of [³⁵S]methionine. The *in vitro*-synthesized products were resolved on SDS/20% polyacrylamide gel either directly (lanes 1–4) or after immunoprecipitation with polyclonal antibodies to protein 8b (lanes 5–8). Polypeptides were detected by autoradiography. The numbers on the left indicate molecular masses in kilodaltons. (C) Expression of p8a/b (lane 1), pF-8a/b (lane 2), pF-8b (lane 3) and pF-8ab (lane 4) in Cos-7 cells. Cells were infected with the recombinant vaccinia/T7 virus, transfected with each of the four constructs, and harvested at 18 h post-transfection. Polypeptides were resolved on SDS/20% polyacrylamide gel, and analyzed by western blot with antibody to Flag. The numbers on the left indicate molecular masses in kilodaltons.

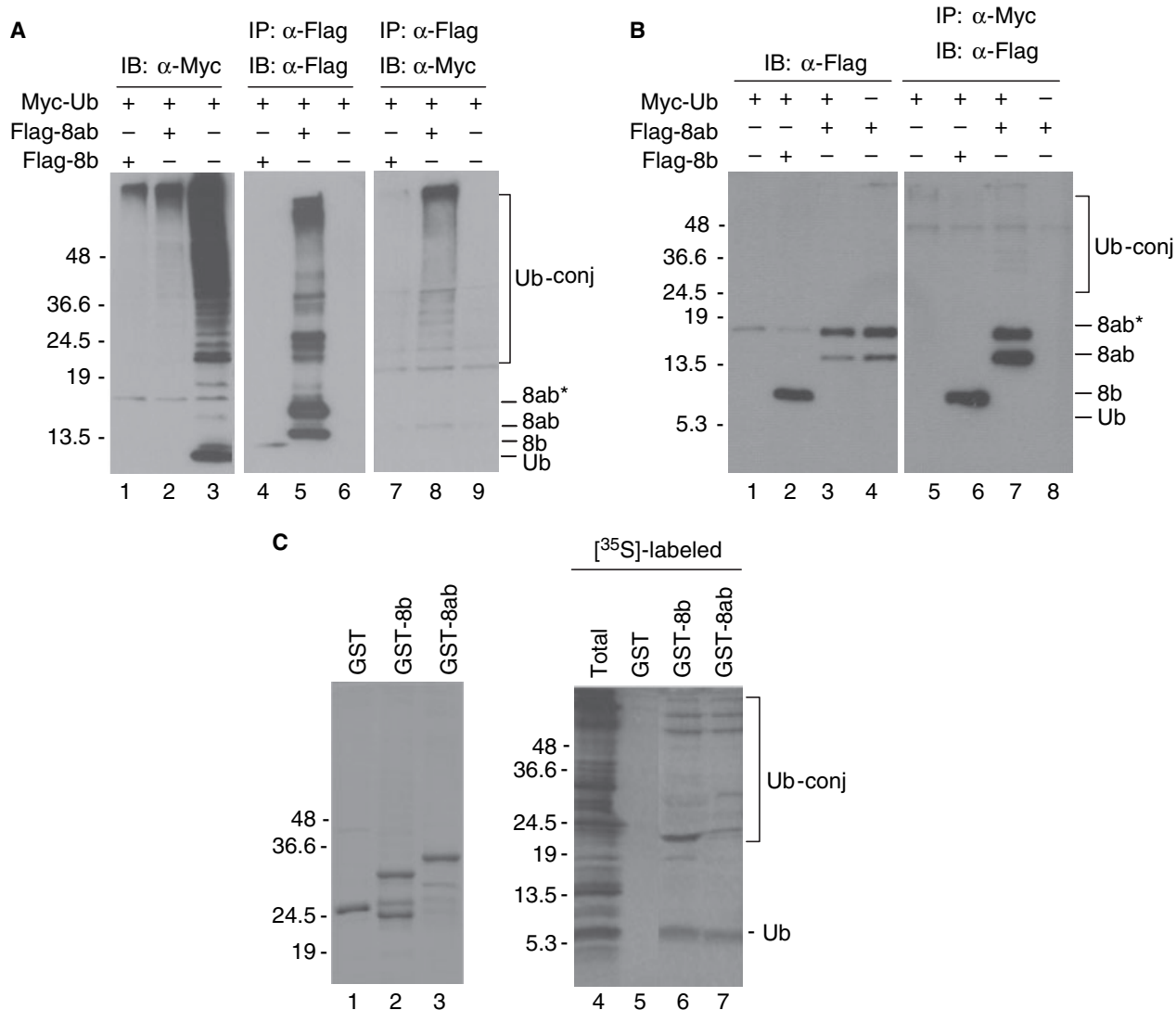


Fig. 3. Binding of proteins 8b and 8ab to monoubiquitin and polyubiquitin. (A) Cos-7 cells were transfected with the Myc-tagged ubiquitin (lanes 3, 6 and 9), the Myc-tagged ubiquitin together with the Flag-tagged protein 8b (lanes 1, 4 and 7), and the Myc-tagged ubiquitin together with the Flag-tagged protein 8ab (lanes 2, 5 and 8). Total cell lysates were prepared, separated on SDS/20% polyacrylamide gel, and analyzed either directly by western blot (lanes 1–3) or after coimmunoprecipitation with antibody to Flag (lanes 4–9). The numbers on the left indicate molecular masses in kilodaltons. (B) Cos-7 cells were transfected with the Myc-tagged ubiquitin (lanes 1 and 5), the Flag-tagged protein 8ab (lanes 4 and 8), the Myc-tagged ubiquitin together with the Flag-tagged protein 8b (lanes 2 and 6), and the Myc-tagged ubiquitin together with the Flag-tagged protein 8ab (lanes 3 and 7). Total cell lysates were prepared, separated on SDS/20% polyacrylamide gel, and analyzed either directly by western blot (lanes 1–4) or after coimmunoprecipitation with antibody to Myc (lanes 5–8). The numbers on the left indicate molecular masses in kilodaltons. (C) Pull-down of monoubiquitin and polyubiquitin by GST-8b and GST-8ab fusion proteins. GST, GST-8b and GST-8ab were purified from bacteria, separated on SDS/12% polyacrylamide gel, and visualized by staining with Comassie blue (lanes 1–3). Cos-7 cells expressing the Myc-tagged ubiquitin were labeled with [³⁵S]methionine. Total cell lysates were prepared and precleared by incubation with GST beads at 4 °C for 2 h, and then incubated with beads prebound with GST, GST-8b, and GST-8ab, respectively, at 4 °C for 2 h. After washing three times with RIPA buffer, polypeptides were eluted from the beads by adding 30 μ L of the loading dye and boiling for 5 min. The total cell lysates (lane 4) and the eluted polypeptides (lanes 5–7) were separated on 20% SDS/20% polyacrylamide gel and visualized by autoradiography. The numbers on the left of each panel indicate molecular masses in kilodaltons.

polyubiquitin in cells expressing the Myc-tagged ubiquitin alone (Fig. 3A, lane 3). The monoubiquitin was not detected in cells coexpressing the Myc-tagged ubiquitin

with either protein 8b (Fig. 3A, lane 1) or protein 8ab (Fig. 3A, lane 2). Instead, weak polyubiquitin bands were observed (Fig. 3A, lanes 1 and 2). Immunoprecip-

itation of cell lysates prepared from these transfected cells with antibody to Flag led to the detection of massive free and ubiquitin-conjugated protein 8ab by western blot with antibody to Flag (Fig. 3A, lane 5). However, much lower amounts of protein 8b were detected in cells coexpressing ubiquitin and protein 8b (Fig. 3A, lane 4). Western blot analysis of the same precipitates with antibody to Myc resulted in efficient detection of polyubiquitin bands in cells coexpressing the Myc-tagged ubiquitin and the Flag-tagged protein 8ab (Fig. 3A, lane 8). Much weaker detection of polyubiquitin bands, however, was obtained with cells coexpressing the Myc-tagged ubiquitin and the Flag-tagged protein 8b by western blot analysis with antibody to Myc (Fig. 3A, lane 7). These results strongly suggest that proteins 8b and 8ab are modified by ubiquitination.

In a similar way, western blot analysis using antibody to Flag resulted in the detection of Flag-tagged protein 8b (Fig. 3B, lane 2) and protein 8ab (Fig. 3B, lanes 3 and 4), either expressed alone or coexpressed with the Myc-tagged ubiquitin. The same cell lysates were immunoprecipitated with antibody to Myc and subsequently analyzed by western blot with antibody to Flag. In addition to the detection of several more slowly migrating bands, the Flag-tagged protein 8b (lane 6) and protein 8ab (lane 7) were efficiently detected when they were coexpressed with the Myc-tagged ubiquitin. Protein 8ab was not detected when it was expressed on its own (Fig. 3B, lane 8). The reason for preferential precipitation of the nonubiquitinated forms of proteins 8b and 8ab by antibody to Myc (Fig. 3B, lanes 9–12) is not clear. However, it may indicate that the unmodified proteins 8b and 8ab might have higher binding affinity to the monoubiquitin and polyubiquitin molecules than their ubiquitinated counterparts.

The coimmunoprecipitation results shown above demonstrated that proteins 8b and 8ab could interact with monoubiquitin and polyubiquitin. To further confirm these results, proteins 8ab and 8b were expressed in *Escherichia coli* as glutathione *S*-transferase (GST) fusion proteins and purified. Relatively pure GST, GST-8b and GST-8ab were obtained (Fig. 3C, lanes 1–3). Equal amounts of GST, GST-8b and GST-8ab bound to GST beads were then used in a pull-down assay by mixing them with [³⁵S]methionine-labeled lysates prepared from cells overexpressing the Myc-tagged ubiquitin. Efficient pull-down of monoubiquitin and polyubiquitin by GST-8b and GST-8ab was observed (Fig. 3C, lanes 6 and 7). However, no monoubiquitin or polyubiquitin bands were detected when GST protein was used in the same experiment

(Fig. 3C, lane 5). These results confirm that proteins 8b and 8ab can efficiently bind to the monoubiquitin and polyubiquitin molecules.

Inhibition of the rapid degradation of protein 8b by proteasome inhibitors

To study the effect of ubiquitin binding and ubiquitination on the degradation and accumulation of proteins 8b and 8ab, a system that mimics the expression level of the proteins in SARS-CoV-infected cells was developed on the basis of an infectious clone system derived from the coronavirus infectious bronchitis virus (IBV) [13]. As shown in Fig. 4A, the 8b and 8ab genes were cloned into the IBV genome between the N gene and the 3'-UTR under the control of a transcriptional regulatory sequence for the subgenomic mRNA5 of IBV. Electroporation of the *in vitro*-synthesized full-length RNAs containing the 8b and 8ab genes, respectively, into Vero cells resulted in the recovery of infectious viruses, which were stable during propagation in cells (data not shown).

Northern blot analysis was then carried out to determine whether an additional mRNA species deriving from the inserted 8b and 8ab genes was produced in cells infected with the recombinant viruses. The results showed that additional mRNA species with sizes corresponding to the inserted 8b and 8ab genes, respectively, were produced in cells infected with the rIBV/8b and rIBV/8ab viruses (Fig. 4B, lanes 2 and 3). Analysis of cells infected with the recombinant viruses by western blot with antibodies to protein 8b resulted in efficient detection of proteins 8ab and 8ab* in cells infected with the rIBV/8ab virus (Fig. 4C, lane 3). However, no protein 8b was detected in cells infected with the rIBV/8b virus (Fig. 4C, lane 2). Considering that similar amounts of mRNAs were detected in cells infected with the two recombinant viruses, and that the two proteins could bind to ubiquitin and be modified by ubiquitination, the failure to detect 8b expression at the protein level in this system suggests that protein 8b may undergo rapid degradation via the proteasome pathway.

To confirm this possibility, high concentrations of two proteasome inhibitors, lactacystin (8 μM) and trileucine vinyl sulfone (NLVS; 32 μM), were added to cells infected with rIBV/8b virus at a multiplicity of infection of approximately 1. Western blot analysis with antibodies to IBV N protein resulted in efficient detection of the N protein from cells infected with the virus in the presence of dimethylsulfoxide (Fig. 4D, upper panel, lane 1). In rIBV/8b-infected cells treated

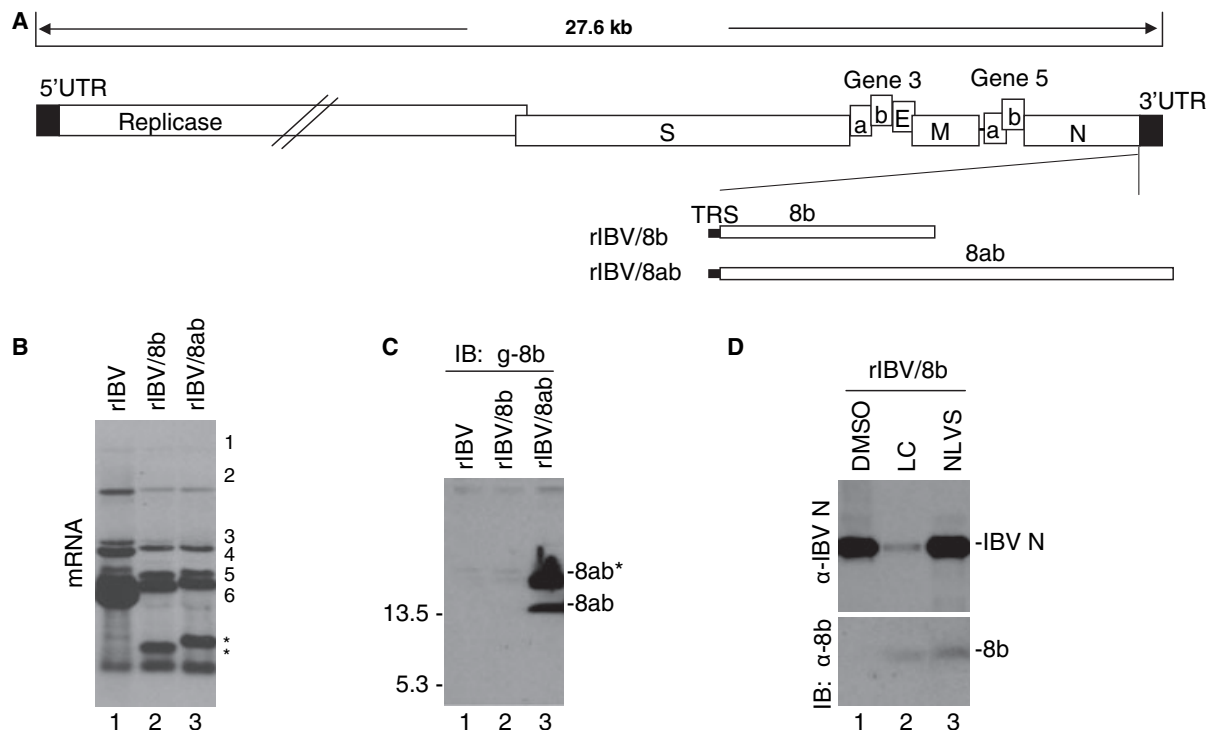


Fig. 4. Expression of proteins 8b and 8ab using an infectious clone system based on coronavirus IBV, and inhibition of the rapid degradation of protein 8b by the proteasome inhibitors lactacystin and NLVS. (A) Diagram showing the genome organization of the IBV genome and the position of the inserted 8b or 8ab gene. (B) Northern blot analysis of the genomic and subgenomic RNAs in cells infected with rIBV (wild type), rIBV/8b, and rIBV/8ab, respectively. Ten micrograms of total RNA extracted from Vero cells infected with rIBV and the fourth passage (p4) of rIBV/8b and rIBV/8ab, respectively, were separated on 1% agarose gel and transferred to a Hybond N+ membrane. Viral RNAs were probed with a Dig-labeled DNA probe corresponding to the 3'-UTR of the IBV genome. The numbers on the right indicate the genomic and subgenomic RNA species of IBV, and the additional mRNA species derived from the inserted 8b and 8ab genes are indicated by asterisks. (C) Western blot analysis of proteins 8b and 8ab in cells infected with rIBV, rIBV/8b, and rIBV/8ab. Vero cells infected with rIBV, rIBV/8b and rIBV/8ab were harvested at 24 h postinfection, and lysates prepared and separated on SDS/20% polyacrylamide gel. Expression of proteins 8b and 8ab was analyzed by western blot with polyclonal antibodies to protein 8b. The numbers on the left indicate molecular masses in kilodaltons. (D) Inhibition of the rapid degradation of protein 8b by lactacystin and NLVS. Vero cells infected with rIBV/8b at a multiplicity of infection of approximately 1 were treated with dimethylsulfoxide alone (lane 1), 8 μ M lactacystin (lane 2) and 32 μ M NLVS (lane 3), respectively, and harvested at 24 h postinfection. Total cell lysates were separated on SDS/17.5% polyacrylamide gel and analyzed by western blot with antibodies to IBV N (upper panel) and protein 8b (lower panel).

with NLVS, approximately the same amounts of N protein were detected (Fig. 4D, upper panel, lane 3). However, in cells treated with lactacystin, much less N protein was detected (Fig. 4D, upper panel, lane 2), suggesting that this inhibitor may have some inhibitory effect on viral replication. Analysis of the same cell lysates by western blot with antibodies to protein 8b resulted in no detection of the protein in cells treated with dimethylsulfoxide alone (Fig. 4D, lower panel, lane 1). However, in rIBV/8b-infected cells treated with the two inhibitors, protein 8b was clearly detectable (Fig. 4D, lower panel, lanes 2 and 3), confirming that the two proteasome inhibitors can indeed stabilize protein 8b and increase the accumulation of the protein in infected cells.

Inhibition of the protein 8b-mediated rapid degradation of the SARS-CoV E protein by proteasome inhibitors

In a recent report, protein 8b was shown to mediate rapid degradation of the SARS-CoV E protein [11]. To determine whether the two proteasome inhibitors could also inhibit the protein 8b-mediated rapid degradation of the SARS-CoV E protein, coexpression of protein 8b with E protein in the presence or absence of the inhibitors was carried out. Analysis of the E protein expression by western blot with antibodies to E protein showed that addition of the two inhibitors blocked the rapid degradation of the SARS-CoV E protein (Fig. 5, top panel). Similar results were

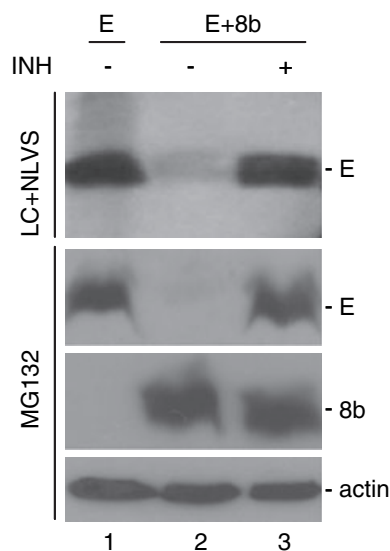


Fig. 5. Inhibition of protein 8b-mediated rapid degradation of the SARS-CoV E protein by the proteasome inhibitors lactacystin, NLVS, and MG132. Cos-7 (top panel) and 293T (other panels) cells expressing the SARS-CoV E protein Alone (lane 1) or coexpressing with protein 8b (lanes 2 and 3) were incubated either with dimethylsulfoxide (lanes 1 and 2), with 8 μ M lactacystin plus 32 μ M NLVS (top panel, lane 3), or with 50 μ M MG132 (other panels, lane 3), and harvested at 18 h post-transfection. Total cell lysates were separated on SDS/17.5% polyacrylamide gel and analyzed by western blot with appropriate antibodies.

obtained when the transfected cells were incubated in the presence of another proteasome inhibitor, MG132 (50 μ M) (Fig. 5, second panel). However, addition of 50 μ M MG132 did not significantly affect the accumulation of protein 8b when the protein was overexpressed in cells (Fig. 5, third panel).

Stabilization of protein 8ab by glycosylation on the N81 residue

As mentioned, detection of protein 8ab* and its migration properties on SDS/PAGE suggested that the protein may undergo some post-translational modification in addition to ubiquitination. ORF8b was predicted to encode a potential N-linked glycosylation site on asparagine 43 (N43, equivalent to N81 in protein 8ab). To test the possibility that proteins 8b and 8ab may be modified by N-linked glycosylation, total cell lysates prepared from cells expressing the Flag-tagged proteins 8b and 8ab were treated with peptide N-glycosidase F (PNGaseF). The protein 8ab* band disappeared after treatment with the enzyme (Fig. 6A, lanes 2 and 4), but the treatment did not affect the migration of protein 8b (Fig. 6A, lanes 1 and 3). This suggests that protein 8b is not modified by N-linked glycosylation. During the

course of this study, it was also noted that variable amounts of the N-linked glycosylated protein 8ab* were detected when pF-8ab was expressed in different cells. As can be seen in Fig. 6A, similar amounts of the modified and unmodified protein 8ab were detected when the construct was expressed in Cos-7 cells (lanes 5 and 6). In HeLa cells, however, much more glycosylated protein 8ab was detected under the same expression and detection conditions (Fig. 6A, lanes 5 and 6).

To further determine whether N81 is the site for the N-linked glycosylation of protein 8ab, mutation of the residue to an aspartic acid was carried out. Expression of the mutant construct resulted in detection of the 14.4 kDa protein 8ab but not the more slowly migrating glycosylated form (Fig. 6B, lanes 1 and 2), confirming that N81 is the site for N-linked glycosylation of protein 8ab. In addition to the 14.4 kDa protein 8ab, several more rapidly migrating bands were observed in cells expressing pF-8abM (Fig. 6B, lane 1). Western blot analysis using antibody to Flag resulted in the detection of two bands of approximately 10 and 5 kDa (Fig. 6B, lane 1). They represent the N-terminal portions of protein 8ab (Fig. 6B, upper diagram). Western blot analysis of the same lysates using polyclonal antibodies to protein 8b resulted in the detection of two bands of approximately 7 and 3 kDa (Fig. 6B, lane 3), representing the C-terminal portions of protein 8ab (Fig. 6B, upper diagram). The detection of multiple degraded products in cells expressing the glycosylation-defective mutant protein 8ab suggests that, like protein 8b, the unglycosylated protein 8ab is unstable.

Further confirming that the unglycosylated protein 8ab is less stable than the glycosylated protein 8ab*, addition of high concentrations of the two proteasome inhibitors lactacystin (8 μ M) and NLVS (32 μ M) to cells expressing F-8ab showed that the two inhibitors significantly enhanced the accumulation of the unglycosylated protein 8ab (Fig. 6C). However, a much lower enhancement effect was observed for accumulation of the glycosylated form (Fig. 6C).

Discussion

The subgenomic mRNA8 of SARS-CoV isolated from humans at late stages of the epidemic contains a deletion of 29 nucleotides, resulting in splitting of a single ORF, ORF8, into ORF8a and ORF8b [7]. In this study, ORF8a was expressed *in vitro* in both wheat germ extracts and rabbit reticulocyte lysates as a 5.3 kDa protein. Owing to the lack of a specific antibody, the protein was not detected in cells. In the genomic RNA of SARS-CoV, ORF8a is located immediately downstream of a strong body transcriptional

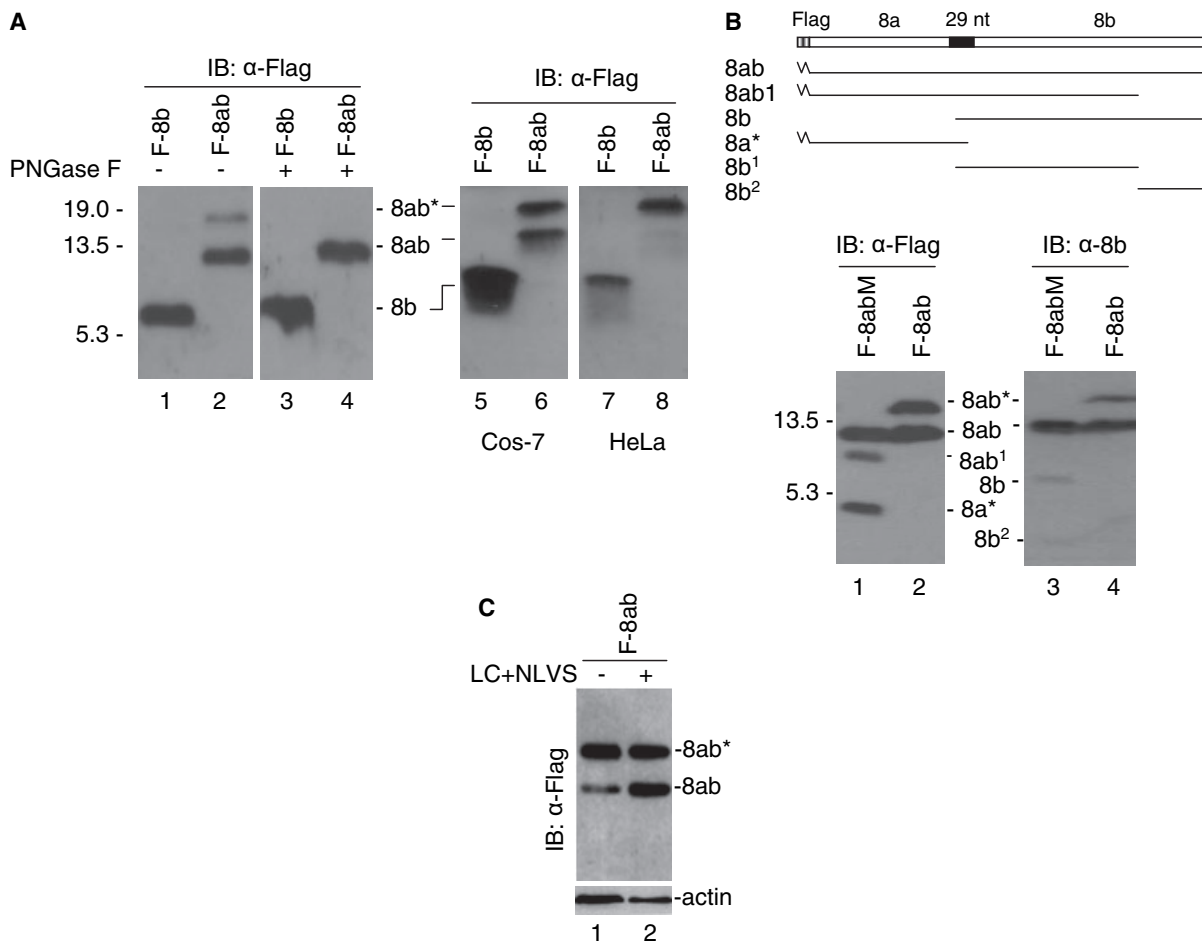


Fig. 6. N-linked glycosylation of protein 8ab. (A) Western blot analysis of proteins 8b and 8ab treated with PNGaseF (lanes 1–4), and detection of variable amounts of the N-linked glycosylated protein 8ab in Cos-7 and HeLa cells. Cos-7 (lanes 1–6) and HeLa (lanes 7–8) cells were transfected with pF-8b (lanes 1, 3, 5 and 7) and pF-8ab (lanes 2, 4, 6 and 8), and harvested at 18 h post-transfection. Total cell lysates were prepared and treated with (lanes 3 and 4) or without (lanes 1, 2, 5–8) 4 μL of PNGase. Polypeptides were resolved on SDS/20% polyacrylamide gel and analyzed by western blot with antibody to Flag. The numbers on the left indicate molecular masses in kilodaltons. (B) Mutational analysis of the N-linked glycosylation site and rapid degradation of the N81D mutant protein 8ab. Total cell lysates prepared from Cos-7 cells expressing the wild-type (lanes 2 and 4) and the mutant (lanes 1 and 2) protein 8ab were analyzed by western blot with either antibodies to Flag (lanes 1 and 2) or antibodies to protein 8b (lanes 3 and 4). In cells expressing the mutant protein 8ab, smaller bands representing degraded products of the mutant protein were detected (lanes 1 and 3). The upper diagram illustrates the regions that these fragments may be derived from. The numbers on the left indicate molecular masses in kilodaltons. (C) Inhibition of the degradation of the unglycosylated protein 8ab by lactacystin and NLVS. Cos-7 cells expressing protein 8ab were incubated either with dimethylsulfoxide (lane 1) or with 8 μM lactacystin plus 32 μM NLVS (lane 2), and harvested at 18 h post-transfection. Total cell lysates were separated on SDS/20% polyacrylamide gel and analyzed by western blot with antibodies to Flag. The same membrane was also probed with antibodies to actin as a control.

regulatory sequence (AGUCUAAACGAAAUG) [14], and is the 5'-most ORF of the subgenomic mRNA. With the recent detection of proteins 3a and 7a from subgenomic mRNA3 and mRNA7, respectively, the majority of the 5'-most ORFs of genomic and subgenomic mRNAs of SARS-CoV are known to encode proteins [13,15]. In patients infected with SARS-CoV strains carrying the 29 nucleotide deletion, antibodies against the 8a peptide were present, confirming the

expression of the protein [16,17]. ORF8ab was expressed as a 14.4 kDa Flag-tagged protein in this study. Although expression of ORF8ab in virus-infected cells and production of antibodies against this protein in patients are yet to be established, protein 8ab was likely to be expressed by a mechanism similar to ORF8a, as it is the only ORF in the subgenomic mRNA8 encoded by SARS-CoV strains without the 29 nucleotide deletion. ORF8b was expressed as a

10.2 kDa Flag-tagged protein. However, translation of the protein was not detected from expression of the overlapping ORF8a/ORF8b. This ORF was previously speculated to be translationally silent [14], and no antibodies against the protein have been found in patients [16]. Although ORF8b was located downstream of a weak transcriptional regulatory sequence (CUAAUAA ACUCAUG) [1], no additional subgenomic mRNA starting from this position was identified. If protein 8b was synthesized from ORF8a/ORF8b, it would be unlikely to be translated by the conventional ribosomal scanning mechanism.

Protein 8ab was found to be post-translationally modified by N-linked glycosylation on the N81 residue. Although the same residue is present in protein 8b, glycosylation of protein 8b was not detected. Glycosylation plays a fundamental role in the oligomerization, sorting, transport and folding of proteins [18]. During glycosylation, glycan residues are transferred to Asn of the target sequence motif Asn-X-Ser/Thr on a nascent peptide by oligosaccharyltransferase in eukaryotic cells [19]. The glycan is the 'admission ticket' for proteins to undergo folding or oligomerization, sorting and transport [18]. Substitution mutation of the N81 to an Asp in protein 8ab made the protein more susceptible to degradation. In the absence of the 8a region, protein 8b was also rapidly degraded, as the protein was not glycosylated, although it contains the potential target sequence motif for glycosylation. It is therefore speculated that glycosylation may assist in folding of protein 8b. In general, the folding process happens in the endoplasmic reticulum (ER) and the Golgi apparatus [20,21]. In the ER, the calnexin-calreticulin cycle is involved in the folding process of glycosylated proteins [22]. In this process, calnexin and calreticulin bind transiently to glycosylated proteins, and subsequently form a complex with cochaperone ERp57 [23]. The three-protein complex folds glycosylated proteins by forming the appropriate disulfide bonds [24]. Protein 8ab is rich in cysteine residues in the 8a region, and may attain the correct conformation by forming disulfide bonds in this region.

More intriguingly, proteins 8b and 8ab could covalently and noncovalently bind to monoubiquitin and polyubiquitin, and facilitate the binding of the two proteins to cellular ubiquitinated proteins. The non-covalent binding of proteins 8b and 8ab to ubiquitin suggests that the two proteins may contain a ubiquitin-binding domain (UBD). UBDS are able to bind to ubiquitin noncovalently and covalently [25]. So far, nine UBDS, such as the ubiquitin-binding motif and ubiquitin-associated domain, have been identified [26–28]. Many UBDS were initially discovered by homol-

ogy search with the identified UBDS [25,29,30]. However, proteins 8b and 8ab do not share homology with any known UBD, suggesting that they might contain a novel UBD. As proteins 8b and 8ab but not protein 8a are conjugated with polyubiquitin and form complexes with monoubiquitin and polyubiquitin, the novel UBD is probably located in the 8b region. Ubiquitin conjugation of UBDS could facilitate the formation of complexes between a UBD and free mono/polyubiquitin. It is generally believed that UBDS are able to bind to other ubiquitinated proteins [25], disrupting the function or transportation of polyubiquitinated proteins [27,28]. As ubiquitination and degradation of cellular proteins by proteasomes may play an important role in maintaining a constant level of these proteins in cells [30,31], a wider effect of proteins 8b and 8ab on cellular ubiquitinated proteins would be expected.

Another intriguing and essential issue is whether splitting of the single ORF8 into ORF8a and ORF8b by the deletion of 29 nucleotides is evolutionarily advantageous. The following considerations and clues provided by this study would suggest that it is beneficial to the virus. First, as splitting of the single ORF8 results in the formation of a bicistronic mRNA species, expression of the downstream ORF8b would be regulated by various translational control mechanisms. Evidence obtained from *in vitro* and *in vivo* expression of constructs containing the two separate ORFs suggests that the downstream ORF is either silent or expressed at a low level. It is unclear at the moment whether this ORF is expressed in virus-infected cells. One recent study found positive staining in some of the SARS-CoV-infected cells with polyclonal antibodies to protein 8b, suggesting that the protein may be expressed [11]. Second, the data presented in this study show that the 8b region may contain multiple functional domains, such as for ubiquitin binding, ubiquitination and glycosylation, involved in the regulation of the stability, host-virus interaction and biochemical properties of protein 8ab. However, no functional domain has so far identified in the 8a region of the protein. Although protein 8a would certainly be expressed from the bicistronic mRNA8, the absence of the 8b region would result in the loss of the functions assigned to the 8b region of protein 8ab. Finally, as ubiquitination and the binding to mono/polyubiquitins, as well as ubiquitinated cellular proteins, may regulate cellular signaling pathways involved in the host antiviral response, silencing the expression of protein 8ab may constitute a viral strategy to evade the host antiviral defense mechanisms. More systematic studies of the functions of these proteins are required to address this issue further.

Experimental procedures

In vitro transcription and translation

Plasmid DNA was transcribed and translated *in vitro* by using the TNT T7 coupled wheat germ extracts or reticulocyte lysates (Promega, Madison, WI, USA) in a 50 μ L reaction mixture at 30 °C for 1.5 h. [³⁵S]Methionine was used to label the synthesized proteins.

Transient transfection of mammalian cells

Semiconfluent monolayers of mammalian cells grown on 30 mm dishes (Nunc, Roskilde, Denmark) were infected with a recombinant vaccinia/T7 virus that generates bacteriophage T7 RNA polymerase. The cells were transfected with 0.4 μ g of plasmid DNA mixed with effectene reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions. After incubation at 37 °C in 5% CO₂ for 18 h, the cells were washed once with NaCl/P_i and harvested. To label the polypeptides with [³⁵S]methionine, cells were washed twice with methionine-free medium at 4 h post-transfection, starved in methionine-free medium for 30 min, and labeled with 25 mCi·mL⁻¹ [³⁵S]methionine. The radiolabeled cells were washed twice with NaCl/P_i and harvested at 18 h post-transfection. Polypeptides were separated on SDS/PAGE gel, and the labeled proteins were detected by autoradiography.

Purification of GST-fusion proteins and pull-down assay

The expression of GST, GST-8b and GST-8ab was induced in *E. coli* BL21 cells with 1 mM isopropyl thio- β -D-galactoside for 4 h. Cells were washed three times with cold NaCl/P_i, and broken by sonication. Pellets from cells expressing GST-8b and GST-8ab, respectively, were lysed with 8 M urea, and purified with glutathione-Sepharose beads (Amersham, Little Chalfont, UK) after dialysis.

Radiolabeled lysates prepared from cells overexpressing ubiquitin were precleared by mixing with GST-prebound beads for 2 h at 4 °C to remove nonspecifically binding proteins. Beads prebound with GST, GST-8b, and GST-8ab, respectively, were mixed with the precleared [³⁵S]methionine-labeled or unlabeled cell lysates at 4 °C for 4 h, washed three times with RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 0.05% SDS), and resolved by SDS/PAGE. The labeled samples were visualized by autoradiography, and the unlabeled samples were analyzed by western blot with antibody to Myc.

Coimmunoprecipitation

Cells transfected or cotransfected with plasmids were washed once with NaCl/P_i, harvested, and lysed with

500 μ L of RIPA buffer in the presence of proteinase inhibitors (Roche Diagnostics AG, Rotkreuz, Switzerland). Cell debris was removed by centrifugation at 15 000 *g* at 4 °C for 30 min using an Eppendorf 5415-R, and the supernatants were immunoprecipitated with 1 μ g of appropriate antibodies at room temperature for 2 h. The mixtures were incubated with 50 μ L of protein A agarose beads for 2 h, and washed three times with RIPA buffer. The immunoprecipitated proteins were separated on SDS/PAGE and analyzed by western blot using appropriate antibodies.

Western blot analysis

Polypeptides were separated by SDS/PAGE, and transferred to poly(vinylidene difluoride) membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 10% nonfat dry milk for 1 h, and this was followed by 1 h of incubation with an appropriate primary antibody. The membrane was then washed five times with PBST (NaCl/P_i containing 1% Tween-20), and this was followed by incubation with an appropriate horseradish peroxidase-conjugated secondary antibody (DAKO) at room temperature for 1 h; detection was with an ECL plus system (Amersham Biosciences, Piscataway, NJ, USA). When necessary, the membrane can be reincubated with another antibody after stripping off the signals with the stripping buffer (2% SDS, 100 mM β -mercaptoethanol, 62.5 mM Tris/HCl, pH 6.8) at 55 °C for 30 min, and washed extensively with PBST.

Northern blot analysis

Vero cells were infected with rIBV, rIBV/8b and rIBV/8ab, respectively, at a multiplicity of approximately one plaque-forming unit per cell. Total RNA was extracted from cells infected with wild-type or mutant viruses. Ten micrograms of RNA was added to a mixture of 1 \times Mops, 37% formaldehyde and formamide, and incubated at 65 °C for 20 min before being subjected to gel electrophoresis. The segregated RNA bands were transferred onto a Hybond N+ membrane (Amersham Biosciences) via capillary action overnight, and fixed by UV crosslinking (Stratalinker TM 2400, Stratagene, La Jolla, CA, USA). Hybridization of Dig-labeled DNA probes was carried out at 50 °C in hybridization oven overnight. Membranes were washed three times for 15 min each with the probe buffer, before proceeding to detection with CDP-Star (Roche) according to the manufacturer's instructions.

Construction of a full-length cDNA clone for IBV, insertion of the 8b and 8ab genes into the clones, and rescue of recombinant viruses

Construction of a full-length cDNA clone for IBV was carried out essentially as described previously [13,32]. Briefly, five fragments spanning the entire IBV genome

were obtained by RT-PCR from Vero cells infected with the Vero cell-adapted IBV p65. The PCR products were purified from agarose gels and cloned into pCR-XL-TOPO (Invitrogen, Carlsbad, CA, USA) or pGEM-T Easy (Promega) vectors. Subsequently, fragment A was removed from pCR-XL-TOPO by digestion with *NheI* and *EcoRI*, and subcloned into the pKT0 vector. Plasmids were digested with either *BsmBI* (fragment A) or *BsaI* (fragments B, C, D and E). The digested plasmids were separated on 0.8% agarose gels containing crystal violet. Bands corresponding to each of the fragments were cut from the gels and purified with a QIAquick gel extraction kit (Qiagen). Fragments A and B, and fragments C, D and E, were first ligated with T4 DNA ligase at 4 °C overnight. The two reaction mixtures were then mixed and further ligated at 4 °C overnight. The final ligation products were extracted with phenol/chloroform/isoamyl alcohol (25 : 24 : 1), precipitated with ethanol, and detected by electrophoresis on 0.4% agarose gels.

Full-length transcripts were generated *in vitro* using the mMessage mMachine T7 kit (Ambion, Austin, TX). The N transcripts were generated by using a linearized pKT0-IBVN containing the IBV N gene and the 3'-UTR region as templates. The *in vitro*-synthesized full-length and N transcripts were treated with DNaseI and purified with phenol/chloroform. Vero cells were grown to 90% confluence, trypsinized, washed twice with cold NaCl/P_i, and resuspended in NaCl/P_i. RNA transcripts were added to 400 µL of Vero cell suspension in an electroporation cuvette, and electroporated with one pulse at 450 V and 50 µF with a Bio-Rad Gene Pulser II electroporator. The transfected Vero cells were cultured overnight in 1% fetal bovine serum-containing MEM in a 60 mm dish or a six-well plate, and further cultured in MEM without fetal bovine serum.

The 8b and 8ab genes were inserted into the IBV genome between the N gene and the 3'-UTR under the control of the TRS for the subgenomic mRNA5 of IBV.

Treatment with PGNaseF

Total lysates (30 µL) prepared from Cos-7 cells expressing protein 8ab were directly treated with 4 µL of PGNaseF (New England Biolabs, Beverly, MA, USA) at 37 °C for 2 h. The samples were then separated on SDS/PAGE and analyzed by western blot with antibody to Flag.

Construction of plasmids

Plasmid p8a/b was constructed by cloning a PCR fragment covering the SARS-CoV sequence from nucleotides 27 763 to 28 102. The PCR fragment was digested with *EcoRV* and *XbaI*, and cloned into *EcoRV*- and *XbaI*-digested pKT0. The sequences of the two primers used were 5'-GCG AATTCAACATGAAATTCTTCTCAT-3' and 5'-GCTCT AGATTAATTTGTTTCGTTTATT-3'. Plasmid pF-8a/b was

created by cloning an *EcoRV*-*XbaI*-digested PCR fragment into *EcoRV*- and *XbaI*-digested pFlag. The two primers used were 5'-GGGATATCCATGAACTTCTC AT-3' and the same reverse primer as used for the construction of p8a/b. Plasmid pF-8a was made by cloning a PCR fragment covering the ORF8a region into pFlag.

Plasmid pF-8ab was cloned by adding the 29 nucleotide insertion into pF-8a/b using two rounds of PCR. The two primers used to introduce the 29 nucleotide insertion were 5'-ATTCAGGTTGGTAACCAGTAGGACAAGGATCTT CAA-3' and 5'-TACCAACCTGAATGGAATATAAGGT ACAACACTAGG-3'.

All constructs were confirmed by automated nucleotide sequencing, and diagrams of these plasmids are shown in Fig. 1A.

Acknowledgements

This work was supported by the Agency for Science Technology and Research, Singapore.

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