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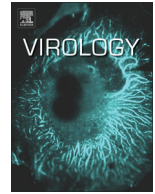
Mapping of the receptor-binding domain and amino acids critical for attachment in the spike protein of avian coronavirus infectious bronchitis virus

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ABSTRACT

The infection of the avian coronavirus infectious bronchitis virus (IBV) is initiated by the binding of the spike glycoprotein S to sialic acids on the chicken host cell. In this study we identified the receptor-binding domain (RBD) of the spike of the prototype IBV strain M41. By analyzing the ability of recombinantly expressed chimeric and truncated spike proteins to bind to chicken tissues, we demonstrate that the N-terminal 253 amino acids of the spike are both required and sufficient for binding to chicken respiratory tract in an α-2,3-sialic acid-dependent manner. Critical amino acids for attachment of M41 spike are present within the N-terminal residues 19–69, which overlap with a hypervariable region in the S1 gene. Our results may help to understand the differences between IBV S1 genotypes and the ultimate pathogenesis of IBV in chickens.

Introduction

Coronaviruses are enveloped, positive-strand RNA viruses of both human and veterinary importance. They cause a variety of diseases in many different species, thereby affecting the respiratory, gastrointestinal, hepatic and central nervous systems (Holmes and Lai, 1996). Infectious bronchitis virus (IBV) is the prototype avian coronavirus, belonging to the genus *Gammacoronaviridae* and is an economically very important pathogen for poultry. Many different IBV strains have been reported in chickens worldwide (Jackwood, 2012), with pathology ranging from mild respiratory symptoms to severe kidney and oviduct disease (Cavanagh, 2007).

Coronavirus binding to and entry into susceptible host cells requires the interaction between cell surface receptors and the viral attachment protein spike (S). The S protein is a type I glyco-protein and contains a signal sequence, ectodomain, transmembrane domain and endodomain. As for some, but not all, coronaviruses the spike is cleaved by host proteases (de Haan et al., 2004; Yamada and Liu, 2009), its ectodomain is further divided into S1 and S2 subunit or domain. Previous studies indicated that the so-called receptor-binding domain (RBD) is located in S1, while the S2 subunit or domain is required for fusion of the virus membrane with that of the host cell. RBDs are usually regions of several hundred amino acids that can fold independently and that are sufficient for binding to the host cell receptor. For a wide variety of coronaviruses the RBD has been mapped, even for the very recently identified MERS-CoV (Mou et al., 2013; Du et al., 2013). They are located in either the N-terminal (NTD) or the C-terminal (CTD) domain of the S1 protein and are required for the interaction with either protein receptors or sugars (reviewed in (Belouzard et al., 2012; Graham and Baric, 2010)). Despite IBV being the first coronavirus ever isolated (Beaudette and Hudson, 1937), its RBD, including that from the best studied IBV strain Beaudette, has not yet been mapped. Elucidation of such critical determinant for binding has partly been hampered by the lack of cell culture systems for avian coronavirus field isolates. In addition, and in contrast to many other coronaviruses, the protein receptor for IBV has yet to be elucidated and only alpha-2,3-linked sialic acids have been shown to be essential for spike attachment (Wickramasinghe et al., 2011) and subsequent infection of host cells (Winter et al., 2008; Winter et al., 2006; Abd El Rahman et al., 2009).

Here we aimed at mapping the RBD of the prototype IBV strain M41. M41 is a virulent respiratory IBV strain, whose S1 protein has affinity for chicken trachea, while the S1 of the embryo- and cell culture adapted IBV strain Beaudette has lost its binding capacity as assessed by spike histochemistry (Wickramasinghe et al., 2011). In this study, we used these differences to identify the RBD of M41 and to elucidate amino acids that are critical for the interaction with host tissues. Our data show that the NTD of S1 (aa 19–272,

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most N-terminal 253 aa of the mature S1) is both required and sufficient for binding to the respiratory tract. In particular, amino acids N38, H43, P63 and T69 of M41 S1 appear to be critical to establish binding, and might therefore be located at the virus-host interface.

Results and discussion

Previously, we have shown that recombinantly produced spike S1 proteins of Beaudette lack the ability to bind chicken respiratory tract tissue, in contrast to the M41 S1 protein. M41 when compared to Beaudette has 26 amino acid differences in the S1 protein (shown in Fig. 1A). One of these amino acid differences is located in the signal sequence (position 14), while the others are scattered throughout the mature S1 protein (aa 19–532). To elucidate the amino acids important for binding of M41 S1 to the respiratory tract we constructed chimeric S1 proteins by swapping coding regions for the mature S1 protein of Beaudette with that of M41. At the protein level, this resulted in introduction of the 15 most N-terminal unique amino acids of M41 and the 10 most C-terminal unique amino acids of M41 into Beaudette S1, resulting in respectively MB.S1 and BM.S1 (Fig. 1B). Chimeric S1 and control M.S1 and B.S1 proteins were produced in HEK293T cells and analyzed on Western blot using Strep-Tactin. All S1 proteins were expressed and appeared to be glycosylated to a similar extent (Fig. 1C). Spike histochemistry revealed that MB.S1, but not BM.S1, had the ability to bind to chicken trachea (Fig. 1D) and lung (Fig. 1E), indicating that amino acids in the N-terminal part of M41 S1 are essential for binding to chicken respiratory tract.

To pinpoint specific residues in M41 S1 that are important for binding to chicken tissues, we next introduced fewer unique amino acids of M41 into the Beaudette S1 background by swapping smaller regions, resulting in MBB.S1 and BMB.S1, having the first 7 or second 8 unique amino acids of M41 respectively (Fig. 2A). Again, soluble chimeric proteins were expressed and appeared to be similar to M.S1 and B.S1 with respect to size and glycosylation (results not shown). Spike histochemistry revealed that only MBB, and not BMB, had the ability to bind to chicken trachea (Fig. 2B), indicating that the M41 S1 region comprising aa 19–69 contains an important determinant for binding. Further elucidation of particular S1 amino acids present at the interface with the host cell surface focused on the single amino acids 19, 38, 43, 56, 63, 66 or 69, by individually introducing the M41 amino acids at those positions into Beaudette S1 (expression cassettes depicted in Fig. 2A). While all proteins were expressed, none of them had the ability to bind to chicken respiratory tract tissues (results not shown). Presumably, while gain of binding might require more than one substitution, mutation of one critical amino acid in M41 S1 would directly result in loss of binding, making it easier to identify critical residues. We therefore decided to try the reciprocal approach, by performing site-directed mutagenesis of M41 S1 and introducing the particular amino acids of Beaudette at each of those 7 positions individually (schematically depicted in Fig. 2A). Indeed, spike histochemistry results showed that S1 mutants N38S, H43Q, P63S, and T69I lost their ability to bind to the trachea (Fig. 2C), indicating that these amino acids are critical for M41 spike attachment. Introducing these 4 amino acids of M41 into the Beaudette S1 background, however, did not result in gain of binding (Fig. 2D). These results suggest that while these 4 amino acids are situated at the virus–host interface, the previously identified residues A19, S56, and I66 also contribute to host attachment. Of course, other amino acids present in these constructs that are identical between Beaudette and M41 also very likely contribute to the binding of the spike.

Finally we aimed at identifying the minimal RBD of M41. As folding is a major concern when generating truncated proteins, we first analyzed the predicted folding of the S1 protein. The S1 proteins of coronaviruses contain two domains that can fold independently (Li 2012) and indeed, for IBV two such distinct domains, separated by a short disordered domain, can be distinguished using FoldIndex (Fig. 3A). Sequences encoding these domains, comprising residues 19-272 (M.NTD) and 273-532 (M.CTD), were cloned and expressed in mammalian HEK293T cells (Fig. 3B). Both truncated proteins could be isolated, indicating that they indeed could fold independently. After PNGaseF treatment, the NTD migrated according to the predicted 33 kDa of its backbone, while the CTD moved slightly slower than its expected 35 kDa (Fig. 3C). Both proteins are highly glycosylated, resulting in migration patterns of around 60 and 55 kDa for NTD and CTD, respectively. Since N-linked glycosylation adds 2.5 kDa of molecular mass to a protein (Kornfeld and Kornfeld, 1985), this suggests that all predicted N-glycosylation sites (8 and 5 in NTD and CTD, respectively) may be used for glycosylation of these truncated proteins. Spike histochemistry revealed that M.NTD, but not M.CTD could bind to chicken respiratory tract, including trachea (Fig. 3D) and lung (not shown). In addition, M.NTD is also sufficient to bind to other chicken tissues, in a similar manner as M.S1, as exemplified by staining of the oviduct (Fig. 3E). An attempt to further truncate the minimal RBD was not successful (data not shown), which might be due to the inability of smaller domains to fold correctly or because multiple domains in the NTD might be involved in binding to host tissues. The binding of NTD appeared to be completely dependent on sialic acids, as binding was lost upon pretreatment of the slides with VCNA to cleave off sialic acids (Fig. 3F). No indication of binding to a protein (co) receptor was observed, but it might be that downstream entry events do require such (host specific) factor for productive infection. Sialic acid binding was previously observed for complete M41 S1 (Wickramasinghe et al., 2011), and also recently for the S1 of the nephropathogenic strain B1648 (Shahwan et al., 2013). M.NTD preferred the same sugar substrate as complete M.S1 (Wickramasinghe et al., 2011), as binding to several Neu5Aca2-3Gal-containing sugars was observed in glycan array (data not shown). Taken together, our results show that the N-terminal 253 amino acids of M41 S1 are both required and sufficient for binding to host tissues, in an alpha-2,3-sialic acid-dependent way.

Sugar-binding has also been observed for several other coronaviruses, including bovine coronavirus BCoV (Schultze et al., 1991), human coronavirus HCoV-OC43 (Kunkel and Herrler, 1993), and porcine coronavirus TGEV (Shahwan et al., 2013; Schultze et al., 1996; Krempl et al., 1997). Of these, only the sugar-binding domain of BCoV S1 has been mapped (Peng et al., 2012), and is like that of IBV located in the NTD of S1. Interestingly, the only other coronavirus in which the S1 NTD contains the RBD is murine coronavirus MHV, with binding of a 330-amino acid domain (Kubo et al., 1994) to the protein receptor CEACAM1a (Williams et al., 1991; Dveksler et al., 1991). Crystal structures of the RBDs of BCoV NTD (Peng et al., 2012) and MHV NTD (Peng et al., 2011) revealed that both NTDs have structural topology similar to that of human galactin. For IBV the tertiary structure of the NTD remains to be determined, but it might well share a similar galactin fold as MHV NTD and BCoV NTD, despite of their sequence diversity. Importantly, and in contrast to the sugar-binding coronaviruses BCoV and HCoV-OC43, IBV does not have hemagglutinin–esterase activity to detach virus from sugars on host cells. This discrepancy, and how IBV overcomes being trapped in the sialic acid-rich mucus layer of the respiratory tract, is currently not understood.

Spike genes of IBV strains are highly diverse, with nucleotide sequence identities in S1 ranging from 50% to 99%. The S1 gene, or parts thereof, is therefore a common target for genotyping viral
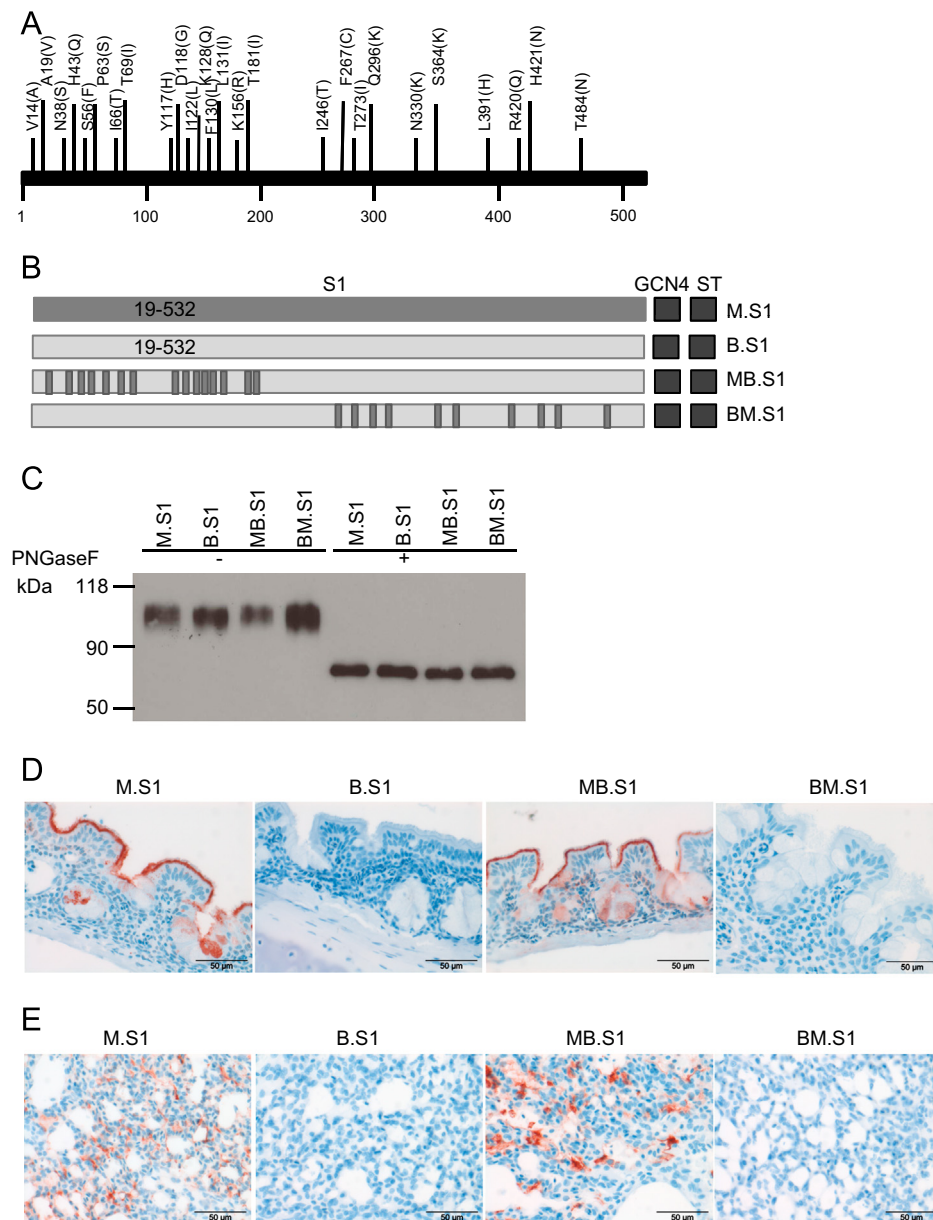
Fig. 1. Recombinant chimeric IBV-S1 proteins. (A) Schematic representation of M41 spike protein (S1 region) in which the amino acids of M41 different from Beaudette S1 and their positions are indicated; amino acid residues of Beaudette are shown between brackets; (B) schematic representation of the chimeric S1 proteins. Amino acids unique for M41 are in dark gray; light gray are amino acids similar between M41 and Beaudette, or unique for Beaudette. The S1 proteins are in frame with a GCN4 isoleucine zipper trimerization motif (GCN4) and the Strep-tag II (ST); (C) S1 proteins expressed in HEK293T cells and purified from the culture media were analyzed by SDS-PAGE followed by Western blot using Strep-Tactin; when indicated the samples were treated with PNGaseF prior to electrophoresis; (D/E) Spike histochemistry was performed by incubating chicken trachea (D) and lung (E) tissues with Strep-Tactin-precomplexed IBV-S1 (5 μg) as described in Section 3.

isolates (Lee et al., 2003; Wang and Huang, 2000; Jackwood et al., 2005). In Fig. 4 an alignment of residues 19–69 of S1 proteins of various IBV field and vaccine strains is presented, showing considerable difference in this entire region. However, the consensus at position 38 and 43 are asparagine and histidine, respectively, both amino acids that appear critical for attachment of M41. The two other amino acids important for binding (P63 and T69) are located in the previously recognized hypervariable region 1 (HVR1; residues 56–69 with numbering based on spike-coding region including signal sequence; 34). HVR1 has been associated with neutralization escape mutants (Cavanagh et al., 1988), and variants of M41 that resisted neutralization had a substitution of aa 63 from proline to histidine (selection variants) or serine (Beaudette; 35). Sequence alignment shows that, in addition to Beaudette, other strains that are passaged in embryonated eggs and are derivatives of M41, including H52 and H120 (Bijlenga et al., 2004),

also have mutations at both positions 63 and 69. As Beaudette not only has extended host tropism for mammalian cells (Fang et al., 2005; Otsuki et al., 1979), but also lost its ability to cause disease in chickens (Geilhausen et al., 1973), these amino acids in the spike might contribute to determine host tropism and pathogenesis. The consequences of the S1 sequence, including the RBD, on the outcome of IBV infection has previously been suggested previously when comparing the binding of M41 S1 with that of H120 and Beaudette to chicken respiratory tract (Wickramasinghe et al., 2011). In particular, the vaccine H120 and the non-virulent Beaudette strain had reduced or no binding to chicken tissues, respectively, in agreement with the reduced ability of these viruses to replicate in vivo. Also for other coronaviruses the RBDs and neutralization determinants are often located in the same domain (Mou et al., 2013; Du et al., 2013; Godet et al., 1994; Liu et al., 2007; Breslin et al., 2003), stressing that key spike–receptor interactions determine the outcome in vivo.
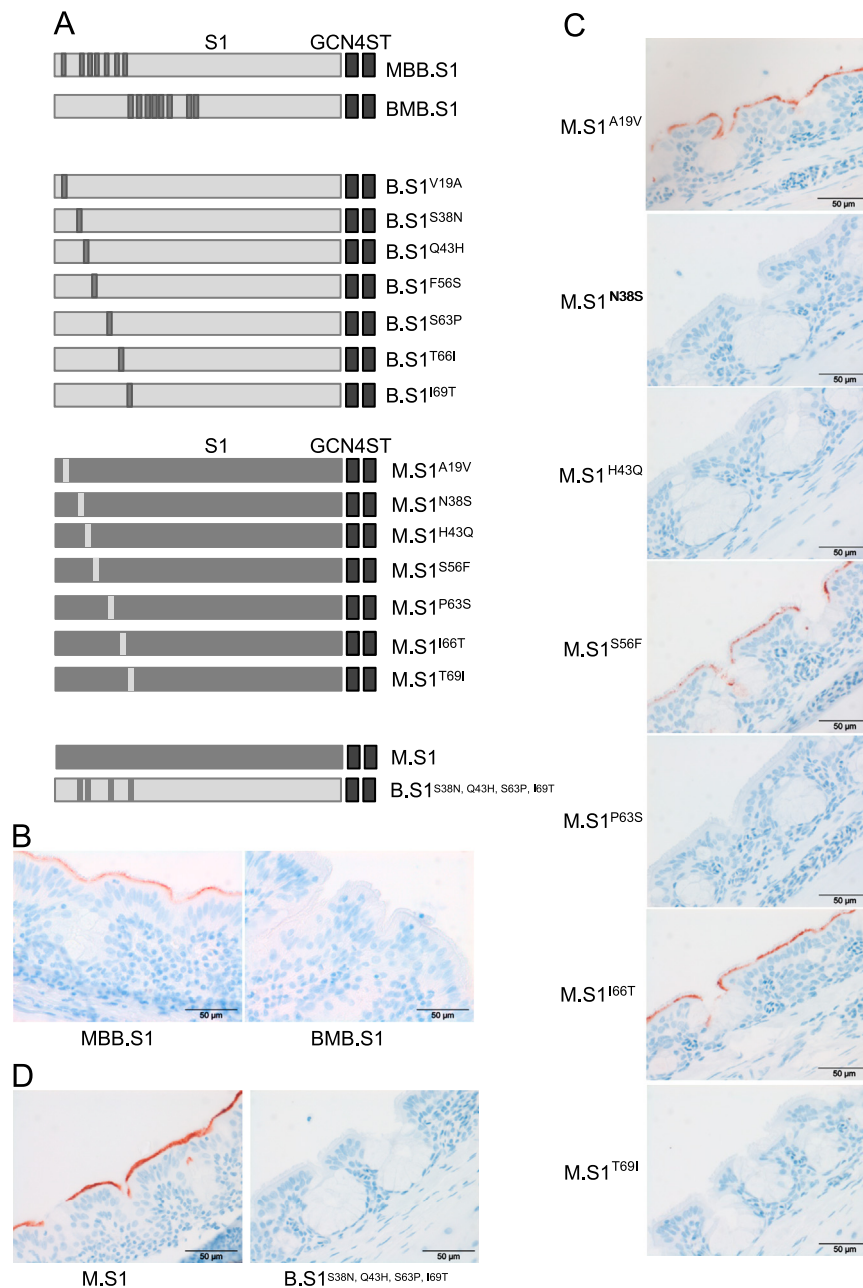
Fig. 2. Critical residues in M41 S1. (A) Schematic representation of the chimeric S1 proteins; the backbone of Beaudette and M41 are shown in light and dark gray, respectively. Unique amino acids for M41 and Beaudette are depicted with dark and light gray boxes, respectively; (B) spike histochemistry of MBB and BMB on chicken trachea tissue; (C/D) spike histochemistry of S1 mutant proteins containing single (C) or 4 (D) amino acid mutations.

In conclusion, we mapped the minimal RBD of the IBV M41 to the most N-terminal 19-272 residues of its spike protein. Moreover, we found that amino acids N38, H43, P63, and T69 appear to be critical for binding of the spike to the chicken respiratory tract. These data point toward important virus–host interactions ultimately affecting the outcome of the infection in chickens.

Materials and methods

Cells

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified eagle medium (DMEM) (BioWhittaker) supplemented with 2% glutamine, 10% fetal calf serum (FCS) (BioWhittaker) and 0.1 mg/ml gentamicin (Gibco Invitrogen).

Construction of expression plasmids

The expression vectors pCD5-M.S1 and pCD5-B.S1, encoding codon-optimized S1 genes of M41 (M.S1; accession number AY851295) or Beaudette (B.S1; accession number AJ311317) respectively, preceded by the CD5 signal peptide and followed by a GCN4 trimerization motif and Strep-tag II were previously described (Wickramasinghe et al., 2011). Constructs coding for chimeric S1 proteins were generated by splice overlap extension PCR on pCD5-B.S1 and pCD5-M.S1 using the outer forward primer IBV-S1-dom#1-FW (GTCGCTTCCGTGCTAGCA) and outer reversed primer IBV-S1-dom#1 RV (ACTGACAGGCCAGCAGCT). Specific primers to make chimeras and to introduce point mutations were generated based upon codon-optimized sequences and sequences are available upon request. The resulting fragments were cloned using NheI and BamHI into the pCD5 vector and the sequences were verified by
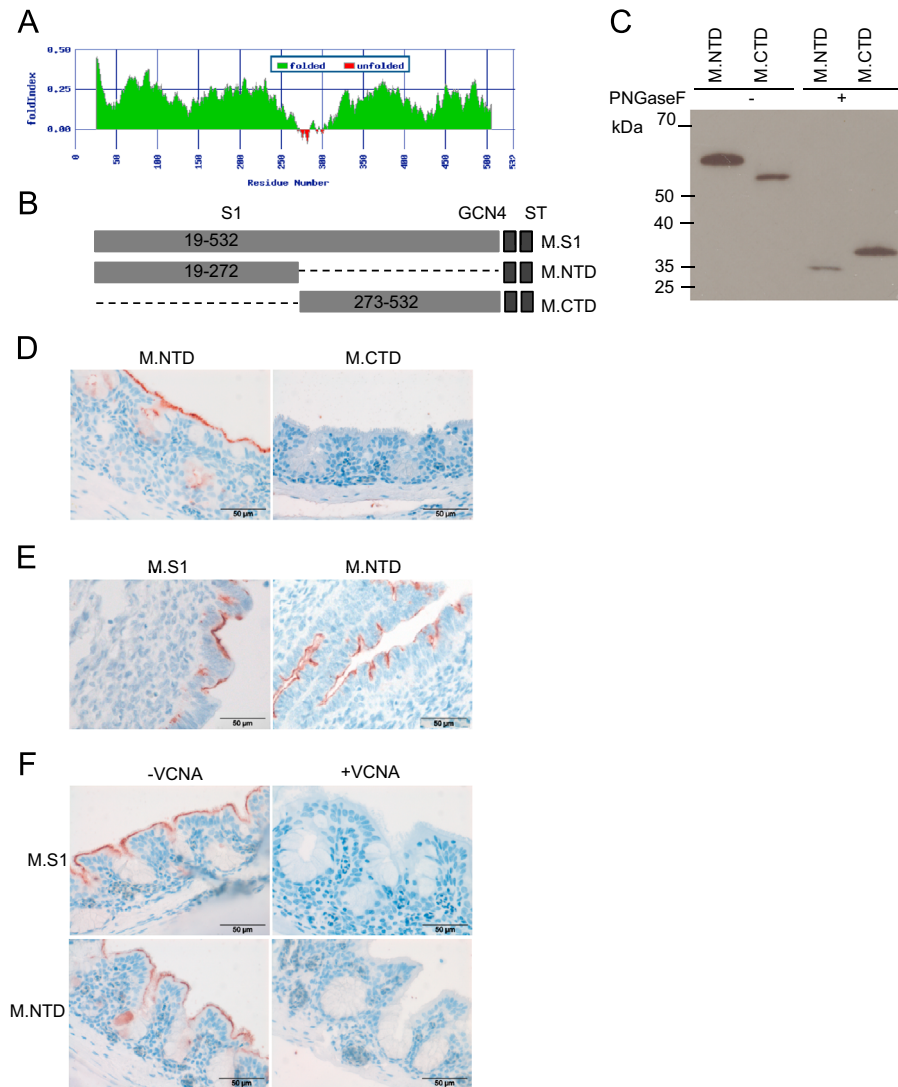
Fig. 3. The minimal RBD of M41 spike. (A) Prediction of the folding of M41 spike protein using FoldIndex (http://bip.weizmann.ac.il/fldbin/findex/); (B) schematic representation of the expression cassettes of truncated S1 genes; (C) Western blot analysis of proteins using Strep-Tactin; when indicated the samples were treated with PNGaseF prior to electrophoresis; (D/E) spike histochemistry on chicken trachea (D/F) and oviduct before (D/E) and after (F) pretreatment of slides with neuraminidase. Equimolar concentrations of truncated M.NTD and M.CTD compared to M.S1 were used (0.06 mg/ml and 0.1 mg/ml, respectively).

automated nucleotide sequencing. Constructs encoding the N-terminal (NTD) or C-terminal (CTD) domain were generated after verifying the predicted folding using FoldIndex (http://bip.weiz-mann.ac.il/fldbin/findex/) using primer set IBV-S1-dom#1 FW and IBV-S1-RBdom#1 RV (GCGCTTAATTAATGGGAAGTTGTGCAGGGT) and the set IBV-S1-RB#2 FW (CCGTGCTAGCAACATTTCATAATGA-GACCGGAG) and IBV-S1-dom#3 RV (ATGGATCCGTACCCCTCAA), respectively. Restriction sites *Nhe*I–*Pac*I and *Nhe*I–*Bam*HI (underlined) respectively were used to introduce these S1 domains into pCD5.

Production and purification of recombinant spike proteins

Spike proteins were produced from mammalian HEK293T cells as described previously (Wickramasinghe et al., 2011). The supernatants were harvested at 6 days post transfection, and the proteins were purified using Strep-Tactin sepharose beads according to the manufacturer's protocols (IBA GmbH). The protein concentration was determined by Qubit® 2.0 fluorometer (Invitrogen) and their presence was confirmed by Western blotting using horseradish peroxidase (HRPO)-conjugated Strep-Tactin (IBA GmbH). Where indicated, the spike proteins were treated with

N-glycosidase F (PNGase F; New England Biolabs Inc.) to remove oligosaccharide side chains prior to electrophoresis according to the manufacturer's procedures.

Spike histochemistry

Sections of formalin-fixed, paraffin-embedded tissues from the trachea and lung of a 6-week-old male broiler chicken were used to study the binding of spike glycoproteins as described previously (Wickramasinghe et al., 2011). Equimolar concentrations of chimeric S1 spike proteins and M.NTD and M.CTD proteins were used, applying 100 μg/ml S1 and 60 μg/ml for M.NTD and M.CTD respectively to each section. To study the dependence of the binding of protein on sialic acids, tissue slides were pre-incubated with 1 mU *Vibrio cholera* neuraminidase (VCNA; Roche) at 37 °C overnight, before performing spike histochemistry.

Glycan array analysis

Ligand profiling of M.NTD was performed in a similar way as previously reported for M.S1 (Wickramasinghe et al., 2011) using printed slides of the CFG glycan library.
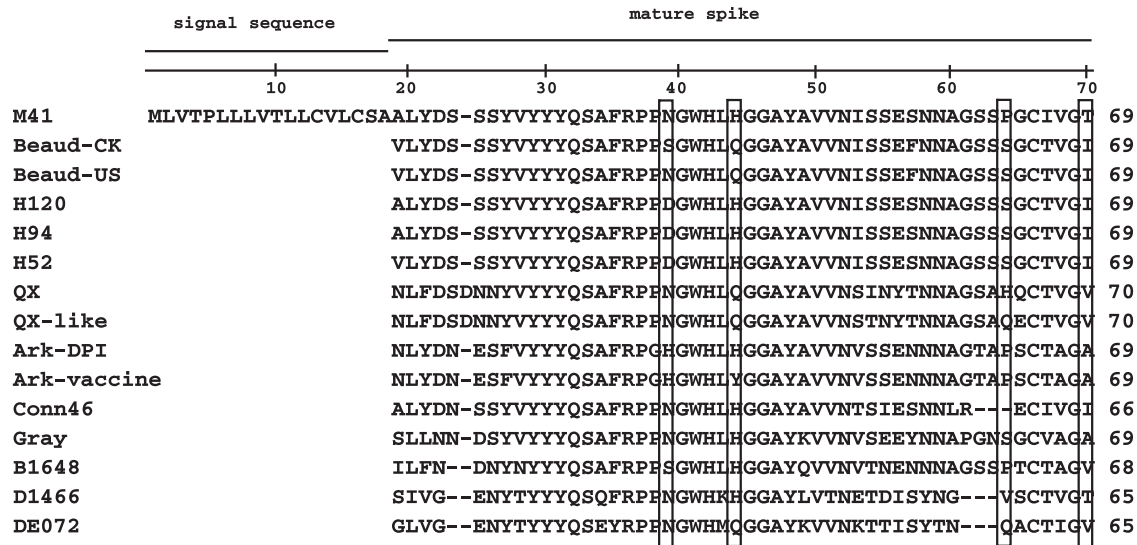
Fig. 4. Sequence alignment of the IBV M41 N-terminal 19–69 amino acids of S1. The critical amino acids 38, 43, 63, and 69 are boxed and the hypervariable region HVR1 is underlined. The signal sequence (aa 1–18) is only indicated for M41.

Acknowledgments

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