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A single amino acid mutation in the spike protein of coronavirus infectious bronchitis virus hampers its maturation and incorporation into virions at the nonpermissive temperature

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Abstract

The spike (S) glycoprotein of coronavirus is responsible for receptor binding and membrane fusion. A number of variants with deletions and mutations in the S protein have been isolated from naturally and persistently infected animals and tissue cultures. Here, we report the emergence and isolation of two temperature sensitive (ts) mutants and a revertant in the process of cold-adaptation of coronavirus infectious bronchitis virus (IBV) to a monkey kidney cell line. The complete sequences of wild type (wt) virus, two ts mutants, and the revertant were compared and variations linked to phenotypes were mapped. A single amino acid reversion (L^{294}-to-Q) in the S protein is sufficient to abrogate the ts phenotype. Interestingly, unlike wt virus, the revertant grows well at and below 32 °C, the permissive temperature, as it carries other mutations in multiple genes that might be associated with the cold-adaptation phenotype. If the two ts mutants were allowed to enter cells at 32 °C, the S protein was synthesized, core-glycosylated and at least partially modified at 40 °C. However, compared with wt virus and the revertant, no infectious particles of these ts mutants were assembled and released from the ts mutant-infected cells at 40 °C. Evidence presented demonstrated that the Q^{294}-to-L^{294} mutation, located at a highly conserved domain of the S1 subunit, might hamper processing of the S protein to a matured 180-kDa, endo-glycosidase H-resistant glycoprotein and the translocation of the protein to the cell surface. Consequently, some essential functions of the S protein, including mediation of cell-to-cell fusion and its incorporation into virions, were completely abolished.

Keywords: Glycoprotein; Coronavirus; Virion

Introduction

The Coronaviridae family contains causative agents of a wide spectrum of diseases affecting humans, mammals, and birds. It is the etiologic agent of severe acute respiratory syndrome (Drosten et al., 2003; Fouchier et al., 2003). The molecular basis of host specificity and tissue or cell tropism of coronavirus partially resides in the specific interaction between the surface spike protein and cellular receptor(s). This interaction would determine the outcome of infection: whether it is acute or chronic and persistent (Rowe et al., 1997a, 1997b), which organs or tissues are targeted, what symptoms are shown (Ballesteros et al., 1997; Leparc-Goffart et al., 1997; Navas et al., 2001), and even whether host specificity is changed (Kuo et al., 2000).

Coronaviruses are a group of enveloped RNA viruses with positive-sense, single-stranded genomes of 27 to 32 kb that are packaged into helical structures by the nucleocapsid protein (N). The viruses acquire their envelopes by budding of the structural components into the intermediate compartment (IC) between the endoplasmic reticulum (ER) and the Golgi apparatus (Krijnse-Locker et al., 1994; Lai and Cavanagh, 1998; Tooze et al., 1984). The membrane glycoprotein (M) and the envelope protein (E) mediate the formation of the envelope, as they are involved in the induction of virion assembly and incorporation of other structural components into virus particles (Vennema et al., 1996). The spike (S) glycoproteins incorporate into the envelope (de Haan et al., 1999) in the form of homo-oligomeric, 20 nm projections, giving the distinctive morphology of the coronavirus virion. Some coronaviruses also

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contain another envelope protein, the hemagglutinin esterase (HE).

The largest structural S protein is a type I integral membrane glycoprotein and spans the viral envelope once. In some cases, the S protein is proteolytically cleaved into the N-terminal S1 and the C-terminal S2 subunits of equal size by a host proteinase (Sturman et al., 1985). The S1 and S2 subunits are noncovalently associated to each other. A hydrophobic transmembrane domain (TM) of S2 anchors on the envelope with a short cytoplasmic-tail inside. One Leucine-zipper (heptad repeats) domain overlaps with the TM domain and another extends outside the envelope, folding into α-helices and coiled-coil structures involved in oligomerization (de Groot et al., 1987). It was found that the TM domain and endodomain mediated the incorporation of S into virions but the details of the S/M interaction remain to be elucidated.

Although S protein is dispensable for the formation of virus-like-particles (VLPs), its incorporation into virion is critical to the assembly of infectious virus particles. The S protein is cotranslationally N-glycosylated in the ER and also forms stable complex with M in the pre-Golgi membrane (Holmes et al., 1981; Opstelten et al., 1995; Sturman et al., 1985). It was then transported to the Golgi apparatus where its high mannose side chains are subsequently trimmed and further modified. The matured S protein is an Endo-glycosidase-H-resistant (Endo-H), 180-kDa form (Luytjes et al., 1997). At the trans-Golgi, the matured virions are encapsulated into vesicles of the constitutive secretory pathway and released from infected cells. A portion of S protein might be transported to the plasma membrane and responsible for the cell-to-cell membrane fusion, resulting in the formation of typical syncycia and spread of virus infection to neighboring cells. The TM, Cys-rich and Leucine-zipper domains of S2 and the disparate region of S1 are associated with the membrane fusion activity of the protein (Chang et al., 2000; Gallagher et al., 1991; Krueger et al., 2001; Luo et al., 1999; Tsai et al., 1999). Cleavage of the S protein enhances the membrane fusion, though not necessarily required. The S protein contains receptor-binding domains in the S1 subunit (Kubo et al., 1994; Godet et al., 1994; Saeki et al., 1997; Suzuki and Taguchi, 1996). Upon binding of the S protein to the receptor(s), conformation changes induce the virus–cell membrane fusion and subsequently unloading of the ribonucleocapsid inside the cell. It has been demonstrated that the cell-to-cell spread of MHV infection does not need the presence of the receptor, but the S protein is required to mediate the cell-to-cell fusion (Gallagher et al., 1992; Nash and Buchmeier, 1996). For coronaviruses, however, the detail of molecular mechanisms of membrane fusion, especially those linked to the S1 region, is yet to be fully elucidated.

In this study, ts mutants were generated by growing the Beaudette strain of IBV at progressively lower temperatures from 35 to 28 °C in Vero cells. Two ts mutants were isolated from passages grown at 29 °C (ts291602) and 28 °C (ts282902). A revertant was also obtained by growing the ts mutant at the nonpermissive temperature (40 °C). Sequence comparison revealed that mutations in the S1 subunit were responsible for the ts phenotype. Comparative studies of viral protein synthesis and growth properties of the wt, ts mutant, and revertant viruses demonstrated that the ts mutants were extremely unstable at the nonpermissive temperature. If they were allowed to enter cells by absorption for 1 h at the permissive temperature (32 °C), the S protein would be core-glycosylated and partially modified at the nonpermissive temperature, but was unable to assemble into virions. The membrane fusion activities of the ts mutants were totally abolished in virus-infected cells and in cells overexpressing the mutant S protein at the nonpermissive temperature.

Results

Cold-adaptation of wt virus, and isolation of two ts mutants and a revertant

The Beaudette strain of IBV, grown in Vero cells at 37 °C, was plaque-purified and was initially adapted to grow at 35 °C. After 4 passages, the virus was subsequently adapted to grow at progressively lower temperatures by culturing at 34 °C for 5 passages, 33 °C for 7 passages, 32 °C for 44 passages, 30 °C for 12 passages, 29 °C for 21 passages, and 28 °C for 29 passages. When the virus was initially adapted to a lower temperature, no CPE was observed within 72–96 h postinfection. Apparent CPE appeared usually after 2 to 5 passages. The virus was continuously passaged at the same temperature until CPE became visible within 24–48 h postinfection, and then shifted to a lower temperature.

Plaque assays of different passages were performed at both 32 and 40 °C. It was found that viruses from early passages grown at 32 °C could form plaques at both temperatures at similar levels. But passages grown at 29 °C produced much fewer plaques at 40 °C than at 32 °C, suggesting the emergence of ts mutants during cold-adaptation. These ts mutants dominated quickly at 28 °C.

Two ts mutants, designated ts291602 and ts282902, were plaque-purified from passage 16 grown at 29 °C and passage 29 grown at 28 °C, respectively. Plaque assays showed that both ts291602 and ts282902 formed plaques only at the permissive temperature, but not at all at the nonpermissive temperature. By growing the ts mutant at 37 °C overnight and then at 40 °C for 2 days, a revertant, rev-1, was obtained which produced plaques at both 32 and 40 °C at a similar level (Table 1). The titers of rev-1 were much higher than that of the wild-type virus wt6501 (isolated from passage 65 at 37 °C) at 32 °C, as it was derived from the cold-adapted virus (Table 1).

Table 1
Titers of wild type, mutant and revertant viruses at 32 °C and 40 °C (PFU/ml)

	Titer	
	32 °C	40 °C
wt6501	8.5×10^3	5.5×10^6
ts291602	2.0×10^6	0
ts282902	3.2×10^6	0
rev-1	7.0×10^6	5.5×10^6

Sequence analysis of the ts mutant and revertant viruses

To map the defects responsible for the ts phenotype described above, the complete nucleotide sequences of wt6501, ts291602, ts282902, and rev-1 were determined using RT-PCR products and cDNA clones from each virus. Comparison of the sequence of wt6501 with ts291602 revealed 22 point mutations, a 5-base-deletion, a 1-base-, 3-base- and a 9-base-insertion (Table 2). These alterations caused 15 amino acid substitutions in eight mature viral proteins, a single amino acid insertion in the 3a protein, a truncated 3b (compared with early passages of wt virus) caused by a single base insertion in the 3b of ts291602, and a 3-amino acid insertion in the N protein. In ts282902, similar point mutations, deletion and insertion were found (Table 2). The most different feature of the two ts mutant is the insertion in the 3b gene. In ts291602, a single A insertion was found at a poly-A stretch at positions 24075 to 24080, causing frameshift of the 3b gene and resulting in a C-terminally truncated 3b protein (Shen and Liu, 2003). However, a three-A-insertion at the same poly-A stretch was found in ts282902, which does not affect the open reading frame of the 3b gene. Comparison of the sequence of rev-1 with the two ts mutants showed only one amino acid change (Q^{294}-to-L^{294}) in the S protein and one amino acid deletion in the nonstructural protein 3a (I^{28}), which exists in both ts291602 and ts 282902 but not in rev-1 and wt virus. The genotype of the rev-1 at these two positions (Q^{294} and I^{28}) are therefore the same as that of wt6501, but different from the two ts mutants. As both wt virus and the revertant could form plaques at 32 and 40 °C, these two mutations might be responsible for the ts phenotype. A Blast search of GenBank clearly showed that the amino acid at position 294 of the S protein was a conserved glutamine. The Leu^{294} residue in the two ts mutants is located immediately downstream of a hypervariable region and the upstream of a conserved region of the S1 subunit. One additional mutation, D^{709}–N^{709}, was found in ts291602 only, and an I^{769}–M^{769} mutation in the two ts mutants and rev-1.

Synthesis of the S protein

After mapping the potential determinants responsible for the ts phenotype, we would like to explore the mechanisms by which these mutations affect the propagation of the ts mutants at the nonpermissive temperature. Synthesis of the S protein in cells infected with wt virus, ts mutants, and revertant was analyzed. Two sets of experiments were carried out. First, Vero cells were infected with wt6501, ts291602, ts282902, and rev-1 at 32 and 40 °C, respectively, and lysates were immunoprecipitated with anti-IBV antibodies. It was obvious that synthesis of the ts mutant S

Table 2
Summary of nucleotide (nt) and amino acid (aa) changes in the wild type, mutant and revertant viruses

Nt (aa) position	wt6501		ts291602		ts282902		rev-1	
	nt	aa	nt	aa	nt	aa	nt	aa
195 kDa								
5899	T	Y	C	H	C	H	C	H
41 kDa								
7472	C	T	T	I	T	I	T	I
8865	A	Q	C	H	C	H	C	H
34 kDa								
10,253	G	R	A	N	A	N	A	N
10,494	G	K	T	N	T	N	T	N
Pol								
13,032	C	P	T	S	T	S	T	S
Hel								
16286	T	F	C	L	C	L	C	L
58 kDa								
18195	T	Y	C	H	C	H	C	H
S								
21247/8(294)	CA	Q	TT	L	TT	L	CA	Q
22,492(709)	G	D	A	N	G	D	G	D
22,674(769)	A	I	G	M	G	M	G	M
23,659(1098)	G	V	T	L	G	V	T	L
23,841	C	/	T	/	T	/	T	/
3a								
23939/41(28)	–	–I	TAT	I	TAT	I	–	–I
3b								
24074/5	+1A	(FS)	+1A	(FS)	+3A	+K	+1A	(FS)
E								
24,285	C	L	T	F	C	L	T	F
24,420	C	L	A	I	A	I	A	I
24,508	A	D	T	V	A	D	T	V
Intergenic region between M and 5a								
25,346	T	/	A	/	A	/	A	/
25,413	TTAGA	/	–	/	–	/	–	/
N								
25902/3	/	/	+9	RTL	+9	RTL	+9	RTL
26,385	T	/	C	/	T	/	T	/
3′-UTR								
27,385	T	/	C	/	C	/	C	/
27,461	T	/	C	/	T	/	C	/
27,527	G	/	A	/	A	/	A	/

protein was detectable at 32 °C but not at 40 °C (Fig. 1a, lanes 3, 4, 5, and 6) under these conditions. In contrast, expression of the S protein was observed in cells infected with wt6501 and rev-1 at both temperatures (Fig. 1a, lanes 1, 2, 7, and 8), though the level of the proteins detected in cells infected with wt6501 was much lower at 32 °C. This was consistent with the lower titers of wt virus at 32 °C.

In the second set of experiments shown in Fig. 1b, cells were absorbed with each virus at 32 °C for 1 h and one of the duplicates was shifted to 40 °C. Under these conditions, the two ts mutant S proteins were synthesized at both 32 and 40 °C (Fig. 1b, lanes 3, 4, 5, and 6), like those of the wt virus and revertant (Fig. 1b, lanes 1, 2, 7 and 8). These results indicated that the viral structural protein could be synthesized at nonpermissive temperature if the mutants were allowed to enter cells. The viral subgenomic RNA and one of the processed proteins, the 3C-like proteinase, were also detected in the same ways (data not shown), and the results were consistent with those described above.

Fusogenic properties of the ts mutant S protein

Comparison of the nucleotide sequences and the S protein synthesis of wt, ts mutants and revertant indicate that the single amino acid mutation (Q^{294}–L^{294}) in the S protein may be responsible for the temperature sensitivity of the mutant virus. This possibility was studied by biochemical and functional characterization of the S protein from the revertant and a ts mutant.

The S gene of rev-1 and ts291602 was cloned under the control of a T7 promoter to investigate whether membrane-fusion activity was affected by the mutations in the S protein. Cells were infected with recombinant vaccinia/T7 virus and were transfected with plasmids containing the S gene from either ts291602 or rev-1. The expression of the S proteins was analyzed by Western blotting using anti-IBV antibodies. As shown in Fig. 2a, the S protein of both revertant and ts mutant was expressed at 32 and 40 °C (lanes 3, 4, 5, and 6), though the S protein of ts291602 was expressed at a relatively lower level than the rev-1 at 40 °C (compare lanes 4 and 6).

The fusion activity of the S protein derived from the revertant and ts291602, respectively, was then examined. As shown in Fig. 2b, membrane fusion was observed in cells expressing the S gene of ts291602 at 32 °C (panel C) but not at 40 °C (panel F), at 48 h posttransfection. In contrast, membrane fusion was observed in cells expressing the S gene of revertant at both 32 and 40 °C (panels B and E). Interestingly, membrane fusion appeared at 72 h posttransfection, after the cells expressing the S gene of ts291602 were shifted from 40 to 32 °C at 48 h posttransfection (panel I).

Sensitivity of the ts mutant S protein to Endo-H digestion

The recombinant S genes of the ts mutant and revertant were transfected into cells in 35 mm dishes at 32 °C and one of the duplicates was shifted to 40 °C at 4 h posttransfection. Radiolabeled proteins were immunoprecipitated and treated with Endo-H at 37 °C. As shown in Fig. 3a, the ts mutant S protein, synthesized at 40 °C, was sensitive to Endo-H digestion, as no 180-kDa form was observed (lane 12). In contrast, a portion of the ts mutant S protein, synthesized at 32 °C (lanes 10), was resistant to Endo-H digestion. The revertant S protein, synthesized at both 32 and 40 °C, was also resistant to Endo-H digestion (lanes 6 and 8). These

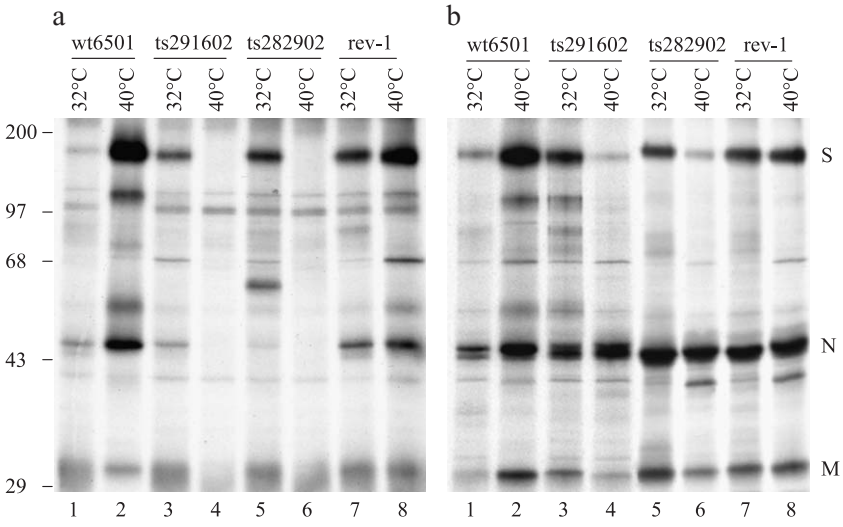

Fig. 1. Analysis of the expression of the S protein from wt (wt6501), ts mutants (ts291602 and 282902) and revertant (rev-1) viruses. (a) Cells were infected with each virus at 32 °C and 40 °C as indicated on the top for 1 h and were maintained at the same temperatures. (b) Two dishes of cells were infected with each virus at 32 °C for 1 h. One of the duplicates was maintained at 32 °C (lanes 1, 3, 5, and 7) and the other one was shifted to 40 °C (lanes 2, 4, 6, and 8). Radiolabeled cell lysates were immunoprecipitated with anti-IBV antibodies. The proteins were separated on 12.5% polyacrylamide gels and detected by autoradiography. Numbers on the left indicate molecular mass in kilodalton and the position of the S protein is indicated on the right.

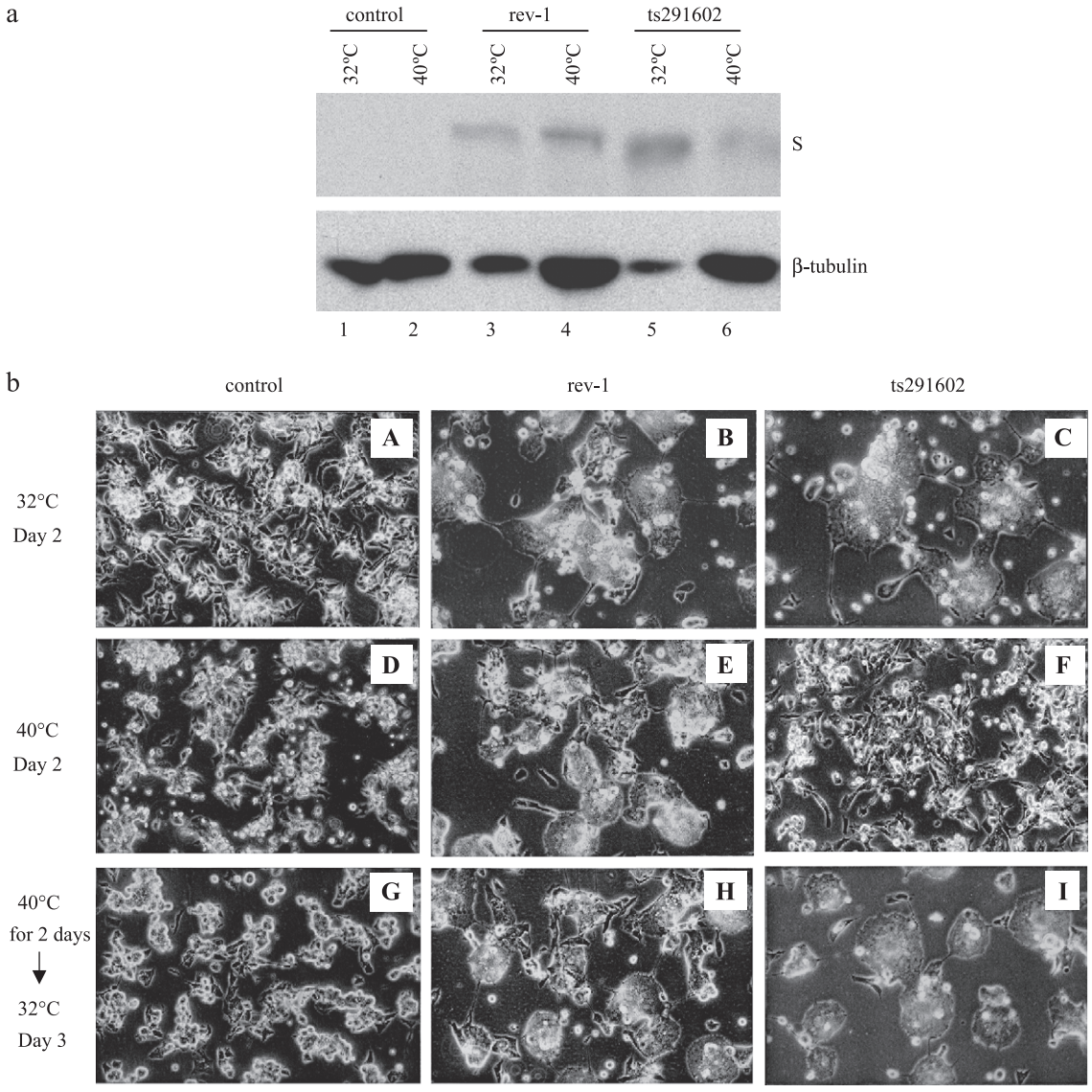

Fig. 2. Membrane fusion activity of the S protein expressed from wt and ts291602. (a) Vero cells were transfected with plasmids without insert (lanes 1 and 2) or with the S gene from the wt (lanes 3 and 4) and ts291602 (lanes 5 and 6), and were cultured at 32 °C (lanes 1, 3, and 5) or 40 °C (lanes 2, 4, and 6). The expression of the S protein was examined in Western blot using anti-IBV antibodies, and β-tubulin was immunostained as loading controls. (b) The membrane-fusion activity of the S protein from the wt (panels B, E, and H) and ts mutant (panels C, F, and I) at 32 °C (panels B and C) and 40 °C (panels C and F) 2 days post-transfection was compared. Panels H and I show induction of membrane fusion on cells transfected with the wt and ts291602 by shifting cells shown in panels E and F to 32 °C for 1 day. Panels A, D, and G show cells transfected with empty plasmids.

results indicate that the ts mutant S protein synthesized at 40 °C was not a mature form of the glycoprotein.

In addition, a novel glycosylated form of the S protein, migrating between the Endo-H treated 130-kDa and the matured 180-kDa forms, was observed. It might represent trimming of an initial, core-glycosylated form of the S protein in the ER and the cis-Golgi. Interestingly, this band was also observed in ts291602-infected cells (Fig. 3b, lane 2), when the incubation time at 32 °C was extended before the culture was shifted to 40 °C. This band is much stronger than the 180-kDa form (lane 2), giving direct evidence that the mutations in the S protein of ts291602 dramatically hampered the maturation process of the S glycoprotein. Compared with ts282902, the S protein of ts291602 contains another amino acid substitution, D^{709}–N^{709} (Table 1), which is not present in the S protein of rev-1 and wt6501. As this band was not observed in ts281602-infected cells (data not shown), it is not clear at this point if this additional mutation may also play a role in the maturation of the S protein.

It was also noted that, at 40 °C for all viruses, the S1 and S2 subunits were hardly detected in cell lysates but abundant in the supernatants (Fig. 3b). At the nonpermissive temperature, the S protein was not cleaved at all (lanes 2 and 6). These results may suggest that only the mature form of the S glycoprotein could be cleaved into S1 and S2 and

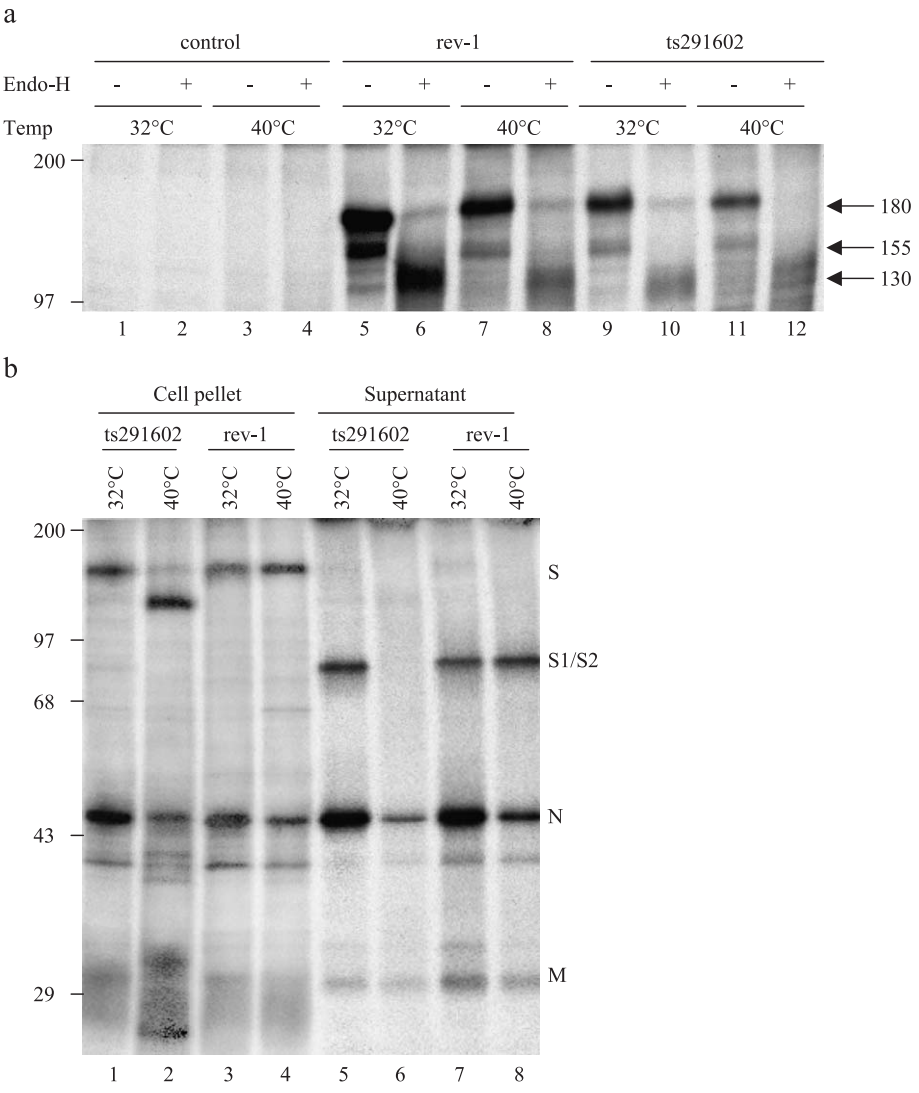

Fig. 3. Maturation defects of the S protein from the ts mutant. (a) Endo-H treatment of the S protein from rev-1 and ts291602. The S protein derived from rev-1 (lanes 5, 6, 7, and 8) and ts291602 (lanes 9, 10, 11, and 12) were expressed in Vero cells at 32 °C (lanes 5, 6, 9, and 10) and 40 °C (lanes 7, 8, 11, and 12), using a T7-vaccinia expression system. The radiolabeled proteins were immunoprecipitated with anti-IBV antibodies, the eluted proteins were Endo-H- (lanes 6, 8, 10, and 12) or mock-treated (lanes 5, 7, 9, and 11) and analyzed by SDS-PAGE. (b) Detection of the less matured S protein from ts291602 at 40 °C and the defect in cleavage of the mutant S protein. Cells were infected with ts291602 and rev-1 for 5 h at 32 °C, one of the duplicates was maintained at 32 °C (lanes 1, 3, 5, and 7), and the other one was shifted to 40 °C (lanes 2, 4, 6, and 8). Cells were radiolabeled and viral proteins were immunoprecipitated with anti-IBV antibodies and analyzed by SDS-PAGE. Lanes 1, 2, 3, and 4 refer to viral products detected from cell lysates and lanes 5, 6, 7, and 8 refer to viral proteins detected from virus particles released to the cultured media.

reinforce the conclusion that the majority of the S protein of the ts mutants synthesized at the nonpermissive temperature may represent a pre-mature form of the S glycoprotein.

Cell surface expression of the S protein in transfected Vero cells

To detect whether the ts mutant S protein would be transported to the plasma membrane at the nonpermissive temperature, the S protein was transiently expressed in Vero cells using the vaccinia virus-T7 expression system. The transfected cells were treated with cycloheximide at 3.5 h posttransfection for 30 min to inhibit further protein synthesis. The cells were then incubated at 32 and 40 °C, respectively. The subcellular localization of the S protein was analyzed by indirect immunofluorescent staining at 4, 8, 16, and 24 h postinfection, respectively. The confocal microscopy images of transfected cells are shown in Fig. 4. Plasma membrane staining of the ts mutant S protein was not observed at any time points posttransfection at 40 °C, but was clearly seen at 32 °C at 16 and 24 h postinfection (Fig. 4). The revertant S protein was expressed on the cell surface at both temperatures at 16 and 24 h posttransfection. These results may explain the lack of membrane fusion activity of the ts mutant S protein in both infected and transfected cells.

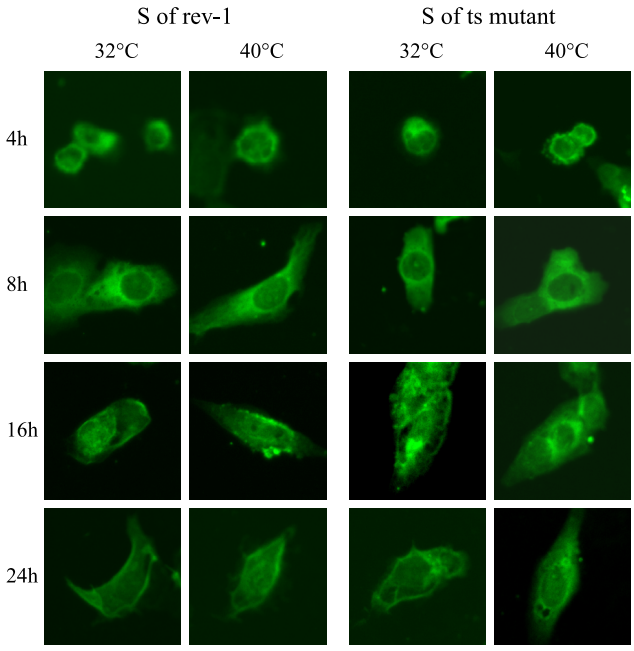

Fig. 4. Subcellular localization and translocation of the wt and ts mutant S protein in Vero cells at the permissive- and nonpermissive temperatures. Vero cells transiently expressing the wt and ts mutant S protein (as indicated on the top) were treated with cycloheximide at 3.5 h posttransfection for 30 min and were cultured at either 32 °C or 40 °C as indicated on the top. Cells were incubated with anti-IBV antibodies and then with the FITC-conjugated secondary antibodies at different time points as indicated on the left. The fluorescence was viewed using a confocal scanning Zeiss microscope.

Quantitative analysis of surface expression of the wild type and ts mutant S protein was carried out by immunofluorescent staining with anti-S protein antiserum and the positive staining cells were sorted by flow cytometry. As shown in Fig. 5, 5.5% of nonpermeabilizing (panel A) and 0.7% of permeabilizing (panel F) cells expressing the empty plasmid showed background staining. When the cells were incubated at the permissive temperature, 3.8% (9.2−5.5) of HeLa cells expressing the wt S protein (panel B) and 4.1% (9.6−5.5) of cells expressing the ts mutant S protein (panel C) displayed surface staining. After permeabilizing with 0.1% saponin, 11.8% (12.5−0.7) of cells expressing the wt S protein (panel G) and 10.9% (11.6−0.7) of cells expressing the ts mutant S protein (panel H) showed positive staining. When the cells were incubated at the nonpermissive temperature, 10.4% (15.9−5.5) of cells expressing the wt S protein (panel D) and 2.3% (7.8−5.5) of cells expressing the ts mutant S protein (panel E) exhibited surface staining. After permeabilizing with 0.1% saponin, 9.3% (10−0.7) of cells expressing the wt S protein (panel I) and 9.7% (10.4−0.7) of cells expressing the ts mutant S protein (panel J) showed positive staining. These results confirm that the ts mutant S protein could not be efficiently translocated to the cell surface at the nonpermissive temperature.

Absence of the S protein in virions purified from ts291602-infected cells at the nonpermissive temperature

To investigate whether the S protein of ts mutant was assembled into virion or not, ts291602 and rev-1-infected cells were radiolabeled and the virus particles were purified through sucrose gradients twice. Immunoprecipitation of the purified virions using anti-IBV antibodies showed that the S protein of ts291602 was not detected at 40 °C (Fig. 6, lane 2), but was observed at 32 °C (Fig. 6, lane 1). The S protein was detected at both temperatures for rev-1 (Fig. 6, lanes 3 and 4). These results render support that the spike

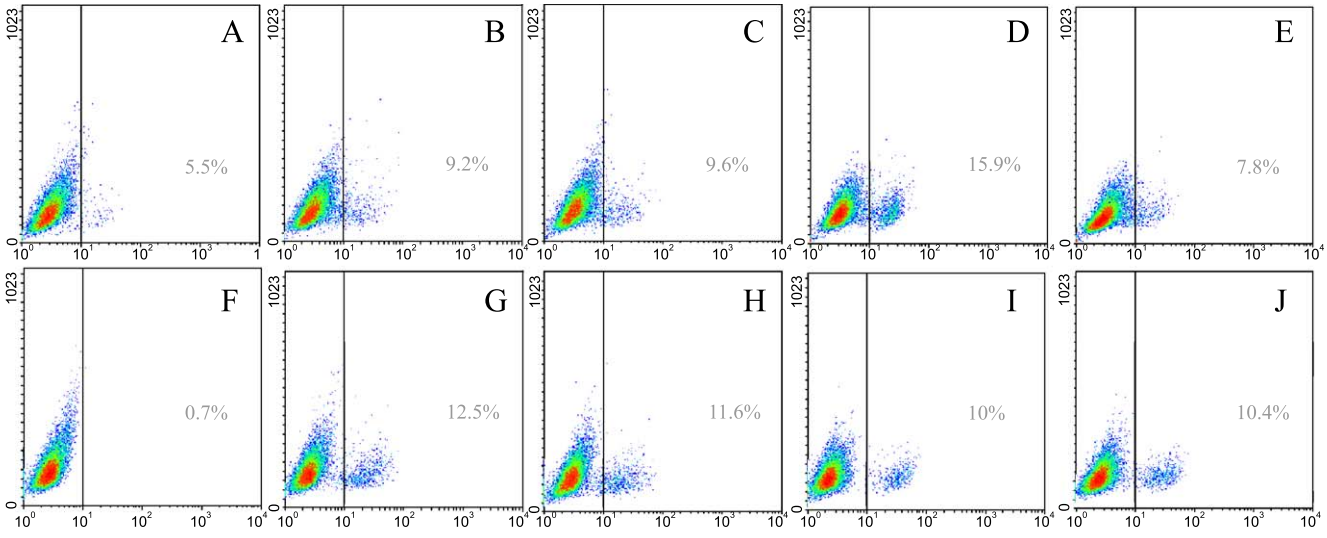

Fig. 5. Quantitative analysis of cell surface expression of the wt and ts mutant S protein. HeLa cells expressing the empty plasmid (panels A and F), wt (panels B, D, G, and I) and ts mutant (panels C, E, H, and J) were stained directly with 1:100 diluted rabbit anti-IBV S protein polyclonal antibodies (panels A–E). The cells were also permeabilized with 0.1% saponin and stained with the same primary antiserum (panels F–J). Cells were then stained with 1:20 diluted FITC-conjugated swine anti-rabbit antibody (DAKO), fixed with 1% ice cold paraformaldehyde and analyzed by flow cytometry. Percentages indicate positive staining cells.

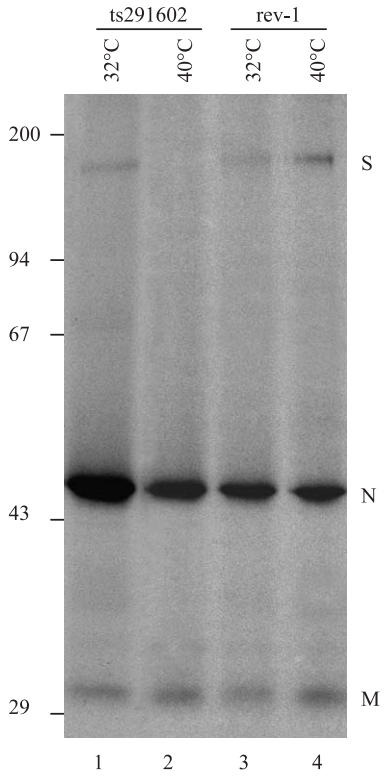

Fig. 6. Analysis of the structural proteins on purified ts291602 and rev-1 virions. Immunoprecipitations were performed using virions, purified from ts291602- (lanes 1 and 2) and rev-1-infected (lanes 3 and 4) Vero cells at 32 °C (lanes 1 and 3) and 40 °C (lanes 2 and 4). The viral proteins were separated on 12.5% polyacrylamide gels and detected by autoradiography. Numbers on the left indicate molecular masses in kilodalton and the positions of the three structural proteins are indicated on the right.

protein of the ts mutant may not be assembled into virus particles at 40 °C.

Discussion

Coronavirus S protein is responsible for receptor binding and membrane fusion. It also induces neutralizing antibodies and bears determinants for virulence. A considerable diversity in S protein among coronaviruses exists, which contributes to host specificity, cell and organ tropisms, and pathogenesis. Characterization of mutants with point or deletion mutations in the S protein has helped to establish links between the variations and the functions or altered antigenicity and virulence of viruses. Through sequence analysis of wt virus, two ts mutants, a revertant and different passages of a cold-adapted IBV, data presented in this study not only mapped the defects of the ts mutants to the S gene, but also revealed the molecular events occurred during evolution of the IBV S gene. Compared with the parental wt virus, mutations that are identical in the two ts mutants and the revertant might be associated with the cold-adaptation phenotype, while those identical only in the two mutants but different from the revertant and wt virus were considered to be linked to the ts phenotype. According to this criterion, the Q^{294}-to-L^{294} mutation in the S protein that exists in the two ts mutants, and interestingly, occurred at a highly conserved domain among IBV viruses immediately downstream a variable domain in the S1 subunit may be responsible for the ts phenotype. The mutants with this mutation accumulated and became dominant under selective pressures, in this case, changes in the hosts and temperatures. The I^{769}–M^{769} mutation emerged earlier than the Q^{294}–L^{294} mutation, which among variations in other gene products may be associated with cold-adaptation, suggesting potential segregation of mutations responsible for either cold-adaptation or ts phenotypes. Further confirmation of the possibility that the Q^{294}-to-L^{294} mutation may cause the ts phenotypic changes is currently being carried out by introducing the mutation to an infectious IBV clones and isolation of a recombinant virus containing this mutation only.

The S protein is co-translationally N-glycosylated in the ER, oligomerized (Luytjes et al., 1997) if folded properly, and associated with the M protein in pre-Golgi membrane (Opstelten et al., 1995). One of the essential steps in N-linked glycosylation is transfer of a preformed, 14-core-unit-oligosaccharide to a specific Asn residue in the sequence Asn-X-Ser/Thr (where X is any residue except Pro, Asp, or Glu). The oligosaccharide chain is donated by dolichol-pyrophosphate-oligosaccharide to the protein chain in the ER. It is trimmed down in the ER and cis-Golgi, and different external sugars are then added to the trimmed chain in the medial- and trans-Golgi. Glycoproteins with high mannose oligosaccharides in the ER and cis-Golgi remain sensitive to Endo-H. They become Endo-H resistant after being processed by the medial- and trans-Golgi resident enzymes to glycoproteins with complex oligosaccharides. The acquisition of Endo-H resistance is therefore an indication that viral N-linked glycoproteins are properly processed and transported to the Golgi apparatus (Luo and Weiss, 1998; Luo et al., 1999; Luytjes et al., 1997). Examination of the amino acid sequence of IBV S protein showed that there are 13 and 9 potential glycosylation sites in the S1 and S2 subunits, respectively. The calculated molecular weight of the S protein is 128 kDa. If all 21 sites are used, the initially transferred oligosaccharides account for 50 kDa. In cells overexpressing S protein, core-glycosylation of the S protein in the ER resulted in a 180-kDa product as expected. Trimming of the S glycoprotein in the ER and cis-Golgi led to a partially processed 155-kDa form, larger than the Endo-H-treated 130-kDa form which only has one sugar residue left at each site. Both the trimmed 155- and core-glycosylated 180-kDa forms were sensitive to Endo-H digestion. At the permissive temperature, further modification of the mutant S protein in the medial- and trans-Golgi by addition of sugars to the trimmed chains resulted in the maturation of S, which co-migrates with the core-glycosylated 180-kDa form but is resistant to Endo-H digestion. At the nonpermissive temperature, however, no mature form of the

mutant S protein was produced. In virus-infected cells, the Endo-H sensitive, 155-kDa form of ts291602 was abundant at the nonpermissive temperature, clearly indicating that the mutant S protein was trimmed in the ER and cis-Golgi but was not transported to the trans-Golgi. Furthermore, the mutant S protein was not cleaved into S1 and S2 at the nonpermissive temperature, which usually occurs in the trans-Golgi shortly before the release of virions from cells (Frana et al., 1985). As only spikeless virions were produced, these results demonstrated that the core-glycosylated or the trimmed forms of S were unable to incorporate into virions. In addition, the mutant S protein fails to induce cell–cell fusion at the nonpermissive temperature. As the Q^{294}-to-L^{294} mutation is not in the fusogenic domain of S, it renders support to the conclusion that the mutation causes the defect in its modification and intracellular transport, instead of having direct impact on the fusion process. Taken together, these studies clearly indicate that the mutant S protein could not reach the site where the S protein is incorporated into virions.

The reasons for the retention of the mutant S protein in the ER and cis-Golgi are yet to be explored. Previous studies show that the retention of monomeric S protein in the ER is due to misfolding induced by disruption of disulfide bonds (Opstelten et al., 1993), and also caused by lack of oligomerization of Endo-H-sensitive S protein of ts mutants though the locations were not determined (Luytjes et al., 1997). It is not clear whether mutations in IBV S protein have an effect on its folding or oligomerization. Nevertheless, in both circumstances, the intracellular transport, modification of the S protein and its incorporation into virion would have been affected. Studies with some of the other enveloped viruses demonstrated that molecular chaperones (like calnexin and calreticulin) in the ER transiently and specifically interact with partially trimmed, monoglycosylated form of viral N-linked glycoproteins (Tatu and Helenius, 1997). For instance, calnexin interacts with the influenza hemagglutinin and vesicular stomatitis virus G protein (Hebert et al., 1996; Hammond and Helenius, 1994). These and other proteins could facilitate proper folding and ensure quality control for viral glycoproteins. Proteins that fail to fold and oligomerize are prevented from transport and ultimately degraded. With the ts mutants, it is possible to explore the interaction of the S protein with molecular chaperones and folding enzymes. This approach would provide new insights into the maturation and assembly of the coronavirus S protein.

The ts mutant quickly lost its infectivity at the nonpermissive temperature. This low thermostability indicated that even if the mutant S protein was synthesized, fully modified and assembled into virions at the permissive temperature, the resulting particles were relatively unstable and lost infectivity at the nonpermissive temperature. It suggests that the Q^{294} residue in the conserved S1 domain of IBV may also play an essential role in maintaining the thermal stability of the spike in the virion, resembling the S^{287} residue in the S1 region of an MHV ts mutant (Ricard et al., 1995). In addition, if cells were incubated with the ts mutants at the nonpermissive temperature, viral proteins were hardly detected, indicating that at the nonpermissive temperature, the ts mutant S protein may be unable to either bind efficiently to the receptor or induce virus–cell membrane fusion in contrast to the S protein of the revertant and wt virus. This observation suggests that the mutation in the S protein might induce conformation changes of the spikes on virions at the nonpermissive temperature and hamper either process.

Materials and methods

Virus and cells

Vero cells were maintained in complete DMEM medium (GIBCO BRL), supplemented with newborn calf serum (10%), streptomycin (1000 μg) and penicillin (1000 units/ml).

The Beaudette stain of IBV was purchased from ATCC and propagated in chicken embryonated eggs for three passages. The virus was then adapted to grow and passage on Vero cells for 65 times at 37 °C. In this study, the viruses were further passaged on Vero cells at progressively lower temperatures for 121 passages (at 35, 34, 33, 32, 30, 29, and 28 °C for 4, 5, 7, 44, 12, 21, and 29 passages). Virus stocks were used for plaque assays, plaque-to-plaque purification and viral RNA extraction as described previously (Shen et al., 1994). The wild-type virus, wt6501, was plaque-purified from passage 65 grown at 37 °C, while ts mutants, ts291602 and ts282902, were purified from passage 16 grown at 29 °C and passage 29 grown at 28 °C, respectively. The revertant, rev-1, was plaque-purified from cells infected with ts 291602 for 12 h at 37 °C and then shifted to 40 °C for 2 days.

Recombinant vaccinia/T7 (V/T3) virus was propagated and was tittered on Vero cells. Virus stocks were kept at −80 °C until use.

Purification of viruses through sucrose gradient

Confluent monolayers of Vero cells were infected with viruses at a multiplicity of infection (MOI) of 0.1. After 2-h absorption, the viruses were radiolabeled by replacing medium with methionine-free DMEM supplemented with 30 μci/ml [^{35}S]-methionine. After 16-h incubation at temperatures appropriate for each virus, cells were harvested and virus stock was prepared by freezing and thawing three times. Cell debris was removed by centrifugation at 5000 rpm for 15 min (Beckmen, 25.50). The supernatant was centrifuged through a 20% sucrose cushion and the resulting pellet was resuspended in TNE buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA). The viruses were further purified by centrifugation through a 20–55% su-

crose gradient in TNE buffer at 45,000 rpm (Beckmen, SW50) twice. Fractions containing the virus were pooled together and used for protein analysis.

Analysis of viral structural proteins

Viral structural proteins were labeled with [^{35}S]-methionine, immunoprecipitated and separated on 12.5% polyacrylamide gels as described previously (Liu et al., 1998). The anti-IBV serum was raised in rabbit against purified IBV virions and was used in immunoprecipitation and Western blot as previously described (Liu and Inglis, 1991).

Endo-glycosidase (Endo-H) treatment of the S proteins

A portion of cell lysates was immunoprecipitated with anti-IBV serum. The pellets were washed three times with standard RIPA buffer, dissolved in 20 μl of digestion buffer (50 mM Tris, pH 6.8, 0.25% SDS) and incubated at 95 °C for 5 min. Ten microliters of the supernatants were mixed with 10 μl of digestion buffer with or without Endo-H (0.2 mU, Boehringer Mannheim) and incubated for 4 h at 37 °C.

Membrane fusion experiments

Confluent monolayers of Vero cells were infected with recombinant vaccinia/T7 viruses at a MOI of 0.1. After 1-h absorption, cells (2×10^7) were trypsinized, centrifuged at 250 g and resuspended in 2 ml of PBS. The cells (0.3 ml) were mixed with 5 μg plasmid and electroporated (Easyject, EquiBio) at 600 V in a 0.4-cm cuvette (BioRad). The cells were then plated in a 35-mm dish containing 3 ml of DMEM with 2% of newborn calf serum and incubated at temperatures indicated.

RT-PCR and sequencing

Viral RNA was extracted from the purified viruses using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription and polymerase chain reaction (RT-PCR) were performed using the Expand Reverse Transcription and High Fidelity PCR Kits (Boehringer Mannheim). Annealing and extension times of PCR were optimized for amplification of PCR products with different sizes using different primers. More than 100 specific primers were used for amplification, sequencing and cloning. Automated sequencing was carried out using PCR products or cDNA clones and specific primers as previously described (Shen et al., 1999). Sequence analysis was carried out using the GCG and Blast suite of programs as described previously (Shen and Liu, 2000).

Flow cytometry

HeLa cells were infected with vaccinia/T7 virus, transfected with constructs encoding wt and ts mutant S protein using QIAGEN effectene transfection reagent, and incubated at the permissive and nonpermissive temperatures, respectively, for 18 h. Cells were harvested, washed once with PBS, resuspended in blocking buffer (20% FBS and 1% BSA in PBS), incubated on ice for 30 min. A half of the cells were permeabilized with 0.1% saponin in FACS washing buffer (2.5% FBS and 0.05% sodium azide in PBS) incubated for 10 min at room temperature, and stained with 1:100 diluted primary antiserum, the rabbit anti-IBV S protein. The other half was stained directly with the same primary antibody. Cells were then washed two times with the FACS washing buffer, stained with 1:20 diluted FITC conjugated swine anti-rabbit antibody (DAKO). After washing two times with the FACS washing buffer, cells were then fixed with 1% ice cold paraformaldehyde and analyzed by flow cytometry.

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