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# Carbohydrate-induced conformational changes strongly modulate the antigenicity of coronavirus TGEV glycoproteins S and M

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## Summary

The carbohydrate composition and the immunoreactivity of the S and M glycoproteins of the coronavirus TGEV were studied at different stages of their maturation. The biosynthesis of S and M was analyzed in the presence of tunicamycin and monensin. The effect of treatment with endoglycosidases H and F and glycopeptidase F on the precursors and mature forms of S and M were also examined. Species 175K and 29K were characterized as high mannose forms of S and M, respectively, and species 220K and 30-36K as complex type glycosylated forms of these two proteins. M was present mainly as a 29K species in mature virions whereas the 175K form of S was not detected, thus implying that the two proteins undergo Golgi modifications at a far different efficiency. Anti-S and -M monoclonal antibodies were examined for their reactivity towards polypeptide species either treated with endo H or produced in the presence of tunicamycin. It was found that (i) among the four major antigenic sites previously defined (Delmas et al., 1986), only site C (amino acids 363 to 371) was notably expressed by the unglycosylated S polypeptide 155K, whereas the three other sites were dependent upon core-glycosylation, (ii) three of the four anti-M mAbs tested did not recognize the unglycosylated M polypeptide 26K. These data led us to conclude that co-translational, but not terminal glycosylation is an essential requirement for both acquisition and maintenance of the antigenicity of TGEV glycoproteins.

Coronavirus; Glycoprotein; Glycosylation; Transmissible gastroenteritis virus; TGEV; Antigenicity; Epitope

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## Introduction

Coronaviruses are enveloped viruses with a large positive-stranded RNA genome. The virions contain basically two envelope proteins: a transmembrane glycoprotein, M and a large glycoprotein, S, which forms the 200 Å petal-shaped peplomers (or spikes) protruding from the virion envelope (see Spaan et al., 1988 for a review).

The S protein is assumed to mediate attachment of virions to the cell, to induce cell fusion and is the major target for virus-neutralizing antibodies. S is a type I glycoprotein with a largely predominant ectodomain. It is co-translationally glycosylated and many potential acceptor sites for N-linked glycosylation (21–35) are found in its sequence. During virus maturation, S carbohydrates undergo Golgi modifications (Niemann et al., 1982; Sturman and Holmes, 1985). S may be cleaved into two subunits of nearly equivalent size, depending on the virus and on the cell system. The amino-subunit S1 and the carboxy-subunit S2 correspond approximately to the globular domain and to the stalk of the spike, respectively. The spike is formed of an oligomer of S which was recently proposed to be a trimer (Delmas and Laude, 1990). An interchain, elongated coiled-coil structure predicted in the carboxy-domain, as described for influenza virus, may contribute to the stabilization of the S oligomer.

M protein accumulates in the Golgi apparatus where it is postulated to have a role in virus maturation and in determining the site for budding of the virion. The M protein of TGEV has also been reported to be an interferogenic glycoprotein, able to induce interferon alpha synthesis by leucocytes (Charley and Laude, 1988). Unlike S, M protein is buried mainly in the viral particle with less than 30 N-terminal residues exposed at the outer surface of the virus membrane. The next 80 residues form three hydrophobic  $\alpha$ -helices, which span the membrane three times. The rest of the molecule is located in the interior of the virus particle. The ectodomain carries few glycosylation sites (1–6) with different types of linkages of the glycans (see Sturman and Holmes, 1985 for a review): O-linked for murine hepatitis virus (MHV) and bovine coronavirus (BCV), N-linked for infectious bronchitis virus (IBV) and TGEV. With the exception of TGEV, translocation of M within the reticular endoplasmic membrane occurs in the absence of a cleavable signal sequence.

One of the particularities of the coronaviruses is that virion budding occurs in the pre-Golgi compartment (Tooze et al., 1988). As a consequence, the addition of complex type chains within the Golgi compartment may take place either on free or virion-associated glycoproteins. The effect of glycosylation inhibitors on virion formation was investigated in earlier studies on MHV and IBV. Viral particles lacking the S protein could be observed, thus indicating that glycosylation of M

protein is not required and that S protein is dispensible for virion formation (Rottier et al., 1981; Holmes et al., 1981; Stern and Sefton, 1982; Niemann et al., 1982).

The main objective of the present study was to analyse the contribution of glycosylation to the antigenic make-up of these two proteins. The carbohydrate moiety of viral or non-viral glycoproteins generally has a strong influence on their antigenic reactivity, transport and their functional activities (Niman and Elder, 1982; Alexander and Elder, 1984; Skehel et al., 1984; Machamer et al., 1985; see Klenk, 1990 for a review). During the last few years, information about the monoclonal antibody binding sites of the S and M proteins of coronaviruses TGEV, MHV and IBV has been obtained and this has led to the localization of epitopes or groups of epitopes (called sites) on the primary structure (Tooze and Stanley, 1986; Cavanagh et al., 1988; Lenstra et al., 1989; Luytjes et al., 1989; Correa et al., 1990; Delmas et al., 1990; Posthumus et al., 1990; Routledge et al., 1991). However, the contribution of carbohydrates to the expression of epitopes has yet to be examined. In this report, the carbohydrate composition and the immunoreactivity of the S and M proteins at different stages of maturation of TGEV were studied. The results demonstrated that the expression of antigenic sites on S and M TGEV proteins has strong site-specific requirements with respect to glycosylation. The co-translational addition of carbohydrates was found to be crucial for both the acquisition and the maintenance of most antigenic sites on TGEV glycoproteins.

## **Materials and Methods**

### *Viruses and cells*

The origin of the high passage Purdue-115 strain of TGEV and the pig kidney cell line PD5 used for propagation of the virus has been described (Laude et al., 1986).

### *Monoclonal and polyclonal antibodies*

The characteristics of the anti-S and anti-M monoclonal antibodies (mAbs) used in this study have also been described (Laude et al., 1986; see Table 1). Ascites fluid from feline infectious peritonitis virus (AFip) was employed as a polyclonal reagent against TGEV polypeptides.

### *Labeling of intracellular S and M molecules and radioimmunoprecipitation assays*

The procedure for metabolic labeling of viral polypeptides has been described elsewhere (Delmas et al., 1990). Briefly, PD5 cell confluent monolayers were infected at a multiplicity of 50 p.f.u. per cell. At 3.5 h post infection (p.i.), cells were labeled with [<sup>35</sup>S]methionine or [<sup>35</sup>S]cysteine (300  $\mu$ Ci per  $10^7$  cells in 3 ml

TABLE 1

Characteristics of the anti TGEV-mAbs used in this study <sup>a</sup>

| Antibody                | Specificity | Antigenic site | Location<br>(mature protein) | Characteristics            |
|-------------------------|-------------|----------------|------------------------------|----------------------------|
| 51.13, 20.9, 12.18      | S/S'        | A              | 506-718                      |                            |
| 48.1                    | S/S'        | A-B            | 506-718                      |                            |
| 8.8, 25b.21             | S/S'        | B              | 506-718                      |                            |
| 3b.5, 10.4, 11.20       | S/S'        | C              | 363-371                      |                            |
| 40.1, 78.17, 5.2, 69.21 | S/S'        | D              | 82-210                       |                            |
| 6.179, 44.4, 76.2       | S/S'        |                |                              |                            |
| 67.9                    | S/S'        |                |                              | Trimer-restricted epitope  |
| 39.1, 13.4, 31.191      | S'          |                |                              | Trimer-restricted epitopes |
| 25.8, 4.3, 61.142       | S'          |                |                              |                            |
| 25.22, 9.34, 49.22      | M           | 1              |                              | External                   |
| 3.60                    | M           | 2              |                              | Internal                   |
| 22.6                    | N           |                |                              |                            |

<sup>a</sup> According to Laude et al., 1986; Delmas et al., 1986; 1990; Delmas and Laude, 1990 and unpublished results.

methionine or cystine-depleted Eagle's MEM supplemented with 2% calf serum). At 8-9 h p.i., the monolayers were rinsed and cell extracts prepared in 4 ml RIPA lysis buffer (2% Triton X-100, 0.15 M NaCl, 0.6 M KCl, 0.5 mM MgCl<sub>2</sub>, 10<sup>3</sup> kall. units/ml Aprotinin). Resulting cytosols were ultracentrifuged for 1 h at 30,000 rpm in a Beckman 50Ti rotor and supernatants were stored in aliquots at -70 °C. Immunoprecipitation assays were performed as previously described (Laude et al., 1986).

#### *Preparation of purified labeled virus*

The procedure for purification of labeled virus has been described (Delmas and Laude, 1990). The supernatants of cell cultures labeled as above and collected at 8-9 h p.i. were centrifuged for 10 min at 13,000 × g. Resulting supernatants were ultracentrifuged for 10 min at 100,000 rpm in a TLA 100.2 rotor (Beckman). The pellets were resuspended in distilled water and stored at -70 °C.

#### *Tunicamycin and monensin experiments*

Tunicamycin or monensin (Serva) were added to the cell medium at 1 h p.i. At 2.5 h after addition of the inhibitor, the cultures were labeled with [<sup>35</sup>S]methionine or with [<sup>35</sup>S]cysteine and at 9 h p.i., RIPA cell extracts were prepared as described above.

#### *Treatments with glycosidases*

Endo-β-N-acetylglucosaminidase H (endo H), endo-β-N-acetylglucosaminidase F (endo F) and peptide-N-glycohydrolase F (glyco F) were obtained from

Boehringer. Aliquots of labeled virion suspensions were diluted 7-fold with 0.1% SDS, 1% Triton X-100, 1%  $\beta$ -mercaptoethanol, 50 mM EDTA, 70 mM sodium acetate pH 5.5 (endo H digestion) or pH 7.5 (endo F and glyco F digestions). Samples were treated overnight at 37°C with the following enzyme concentrations: endo H, 0.1 U/ml; endo F, 4 U/ml; glyco F, 25 U/ml. The resulting material was precipitated with 5 volumes of acetone at -70°C and pelleted by centrifugation at  $13,000 \times g$  for 10 min. The pellet was washed in 60% acetone in water and solubilized in Laemmli's buffer. Endo H digestion of cellular extracts was performed as follows: 30  $\mu$ l of about  $6 \times 10^4$   $^{35}\text{S}$ -labeled cells solubilized in RIPA buffer were diluted in 60  $\mu$ l of 0.05 M Tris pH 6.8, 0.1% SDS, 1 mM PMSF and treated with 3 mU endo H overnight at 37°C. Then, 100  $\mu$ l of 0.1 M Tris pH8, 0.5 M NaCl were added and the proteins were recovered by immunoprecipitation.

## Results

### *Biosynthesis of S and M proteins in the presence of inhibitors of glycoprotein processing*

Infected cells were treated or mock-treated in the presence of one of the following drugs: tunicamycin, an antibiotic which inhibits the transfer "en bloc" of Asn-linked side chains; monensin, an ionophore which interferes with the transport of proteins in the Golgi apparatus and thus non-specifically affects the addition of complex type sugar side chains (Elbein, 1987). Both treatments had only a slight effect on the overall cell protein synthesis, as judged by the level of incorporation of [ $^{35}\text{S}$ ]methionine. The virus-specific polypeptides present in the resulting cell extracts were analyzed by immunoprecipitation using a polyclonal reagent and SDS-PAGE (Fig. 1). The patterns from control samples were similar to those previously described and showed the presence of two high  $M_r$  species 220K and 175K, representing the mature S protein and its intracellular precursor S', respectively, and of M protein with a 29K band as a major species (Laude et al., 1986). The above polypeptides were poorly or no longer detected in extracts from cells treated by tunicamycin at  $> 0.5 \mu\text{g/ml}$  (Fig. 1a). Instead, two polypeptides with  $M_r$  155K and 26K became apparent. Their  $M_r$  was close to that predicted for the apoproteins S and M, respectively (Laude et al., 1987; Rasschaert and Laude, 1987). It is remarkable that the 155K species was poorly immunoprecipitated when compared to the other viral polypeptides present in tunicamycin treated cells. The lack of cleaved or additional non-dissociated material indicated that the intrinsic immunoreactivity of the 155K species was markedly decreased. Treatment with 1  $\mu\text{M}$  monensin blocked the synthesis of the mature S protein and yielded the synthesis of a 175K species of which the mobility and immunoreactivity was equivalent to that of S' species (Fig. 1b). The synthesis of the minor M species 30-36K in the presence of monensin was markedly affected, whereas that of the major component 29K was not.

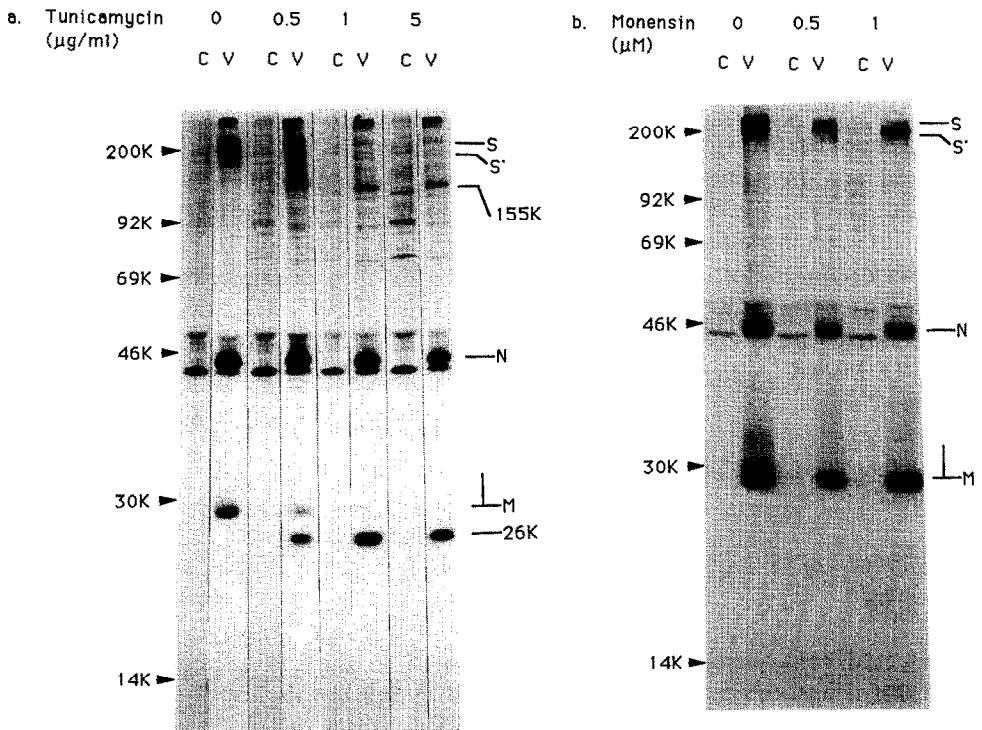


Fig. 1. Effects of tunicamycin and monensin on the biosynthesis of TGEV-specific proteins. Cells were labeled with [ $^{35}\text{S}$ ]methionine in the presence of one of these drugs at the indicated concentration. Immunoprecipitates were prepared from these cultures by using a polyclonal reagent and were analysed by SDS-PAGE and autoradiography. Estimates of the  $M_r$  (in thousands [K]) were determined from the indicated marker proteins. c: mock-infected cells; v: infected cells.

The polypeptide composition of the virus particles produced in the presence of one of these two drugs was examined. Virions produced in tunicamycin-treated cells sedimented in sucrose gradients as a broader band compared to control virions and lacked all forms of the S species, whereas M protein was present in the 26K form only. Virions purified from monensin-treated cultures exhibited a polypeptide pattern similar to that of control virions, indicating that the inhibition was incomplete; however, a 175K species appeared to be incorporated to some extent into the virions (data not shown).

#### *Digestion by glycosidases of virion-associated and intracellular S and M proteins*

To further characterize the oligosaccharide moieties of the different forms of S and M proteins, we examined their susceptibility to different endoglycosidases which cleave N-linked oligosaccharides. Endo H cleaves predominantly high mannose oligosaccharide chains. Glyco F cleaves both high mannose and complex type oligosaccharides whereas endo F cleaves high mannose, hybrid and biantennary complex glycans (Tarentino et al., 1989).

Fig. 2a shows the effect of these enzymes on purified virion polypeptides. Incubation with endo H and endo F caused little or no shift of the mobility of 220K mature S protein, whereas incubation with glyco F generated a product with  $M_r$  160–190K, thus indicating that a notable proportion of the sugar chains was removed. The 29K M component was found to be sensitive to the three N-glycanases, leading to the appearance of a 26K species. The latter was assumed to correspond to the unglycosylated polypeptide since it comigrated with the backbone product synthesized in the presence of tunicamycin (not shown). The M species 30–36K were sensitive to glyco F only and were converted into 26K species.

The effect of endo H on intracellular virus-specific polypeptides was also examined (Fig. 2b). The species S' 175K and M 29K were found to be markedly sensitive to endo H digestion and being converted to 155K and 26K products, respectively, whereas the S 220K species was essentially resistant as was its virion-associated homolog.

*Reactivity of unglycosylated forms of S and M proteins towards monoclonal antibodies*

The above experiments revealed that the reactivity of S protein produced in tunicamycin-treated cells towards polyclonal antibodies was markedly altered. To

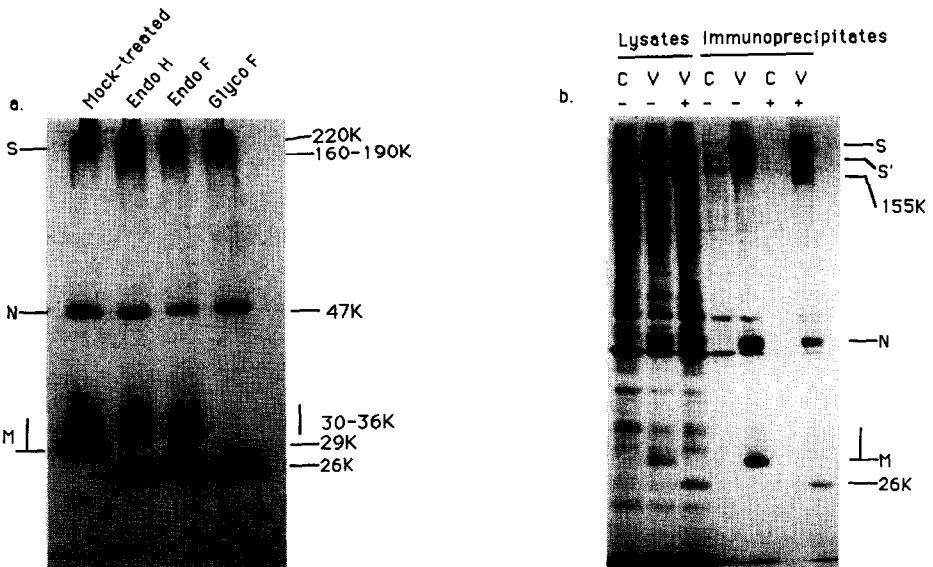


Fig. 2. Digestion of viral proteins with glycosidases. Panel a: [ $^{35}$ S]cysteine-labeled virions were purified and incubated with endo H, endo F, glyco F or mock-treated. Samples were analyzed by PAGE as in Fig. 1. Panel b: [ $^{35}$ S]cysteine-labeled intracellular proteins were subjected to endo H digestion. Samples were subjected to analysis in SDS-PAGE either directly or after immunoprecipitation as specified at the top of the figure. c: mock-infected cells; v: infected cells. Estimates of the  $M_r$  determined from marker proteins are given at the left (in thousands [K]).



investigate in more detail the influence of the glycosylation on the antigenicity of the S protein, we analyzed the immunoreactivity of the 155K S species produced in tunicamycin-treated cells towards a panel of 23 mAbs. Among the mAbs directed against both S and S' species, 13 defined one of the major antigenic sites A, B, C and D, all located in the amino-half of the polypeptide chain, and 4 defined topologically unrelated epitopes; six mAbs recognized epitopes specific to the precursor species S' (Table 1). It was found that all the mAbs exhibited a low or very low residual reactivity towards the 155K species produced in the presence of tunicamycin, with the notable exception of the 3 site C-specific mAbs, which showed a substantial glycosylation independence. Partial data obtained with 4 mAbs representative of the major sites are presented in Fig. 3. In order to preserve possibly fragile antigen-antibody complexes, the carrier beads were washed only two times in these experiments, which explains the presence of non-specific bands. Only site C-specific mAb 3b.5 efficiently immunoprecipitated the 155K species.

The effect of endo H treatment on the immunoreactivity of the intracellular S' species towards anti-S/S' mAbs was also examined (Fig. 4). As already shown, of the two species S and S', only the latter was susceptible to endo H. The deglycosylated S' species (155K) reacted with the site C mAb 3b.5 as efficiently as with the polyclonal reagent, whereas no or little reactivity towards site A, B and D mAbs could be evidenced.

In contrast to S apoprotein, M apoprotein reacted efficiently with polyclonal

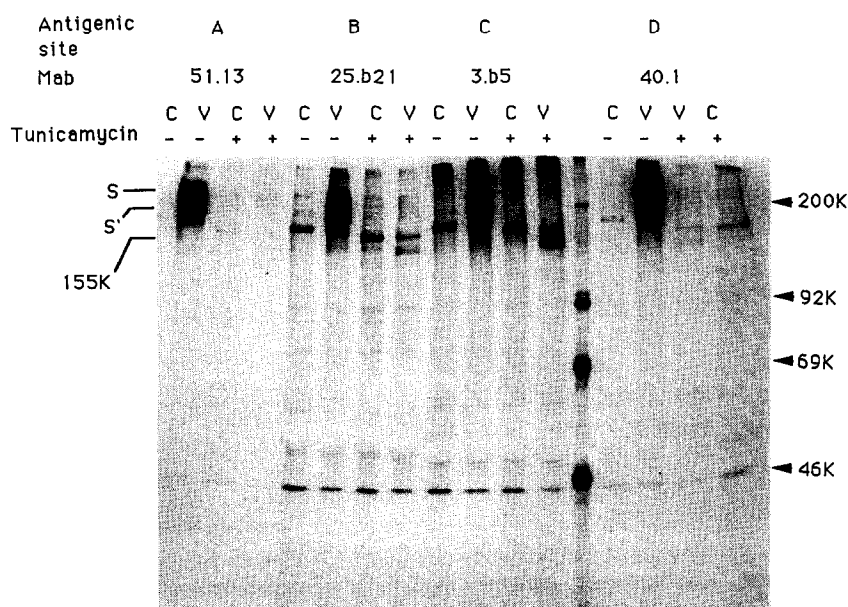


Fig. 3. Antigenicity of S species synthesized in the presence of tunicamycin. [<sup>35</sup>S]cysteine-labeled S polypeptides were immunoprecipitated with the indicated mAbs, then analyzed by SDS-PAGE and autoradiography. c: mock-infected cells; v: infected cells; +: 4 μg/ml tunicamycin.

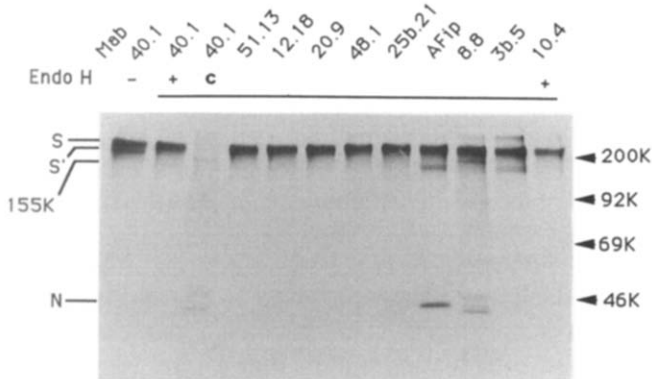


Fig. 4. Antigenicity of endo H-deglycosylated S species. Immunoprecipitates with the indicated mAb were subjected to analysis by SDS-PAGE and autoradiography. AFip: polyclonal antibodies; c: mock-infected cells.

antibodies. Nevertheless, the glycosylation state appeared to influence expression of certain epitopes as shown by the dramatic alteration in the reactivity of 3 of the 4 mAbs tested towards the 26K species produced in tunicamycin-treated cells (Fig. 5). Only the mAb 3.60, which defined an epitope spatially unrelated to the 3 others

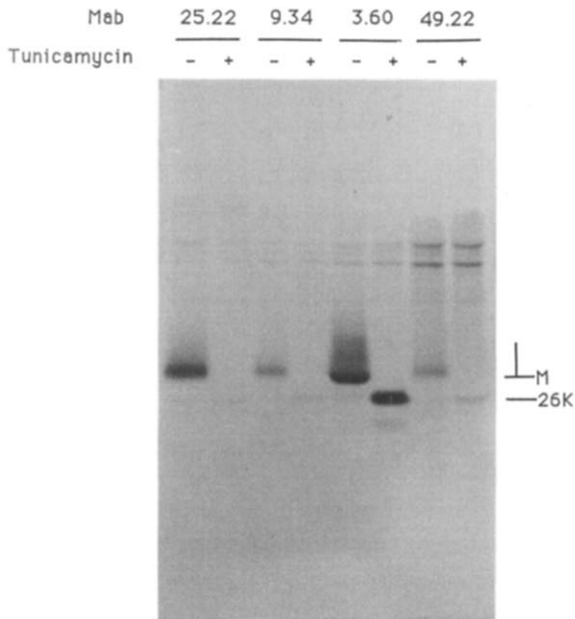


Fig. 5. Antigenicity of M species synthesized in the presence of tunicamycin. [ $^{35}$ S]cysteine-labeled M polypeptides were immunoprecipitated with the indicated mAbs and analyzed by SDS-PAGE. +: 4  $\mu$ g/ml tunicamycin.

(see Table 1), showed a reactivity equivalent to that of the glycosylated forms of M protein.

## Discussion

It has been shown for a number of viruses that the carbohydrate moiety of the viral glycoproteins may have a strong influence on antigenic as well as functional activities. The present study addresses the nature of the carbohydrate's side chains and their contribution to the antigenic make-up of the two major envelope glycoproteins (the spike protein S and the membrane protein M) of the enteropathogenic porcine coronavirus TGEV. Both S and M of TGEV have been shown to contain Asn-N-linked sugars (Garwes et al., 1984; Jacobs et al., 1986).

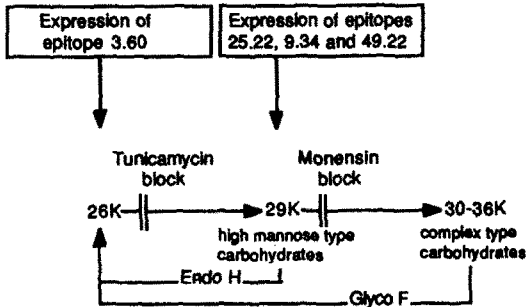
The S protein has been found to appear in infected cells as a 175K species, designated S', which remains present in substantial amounts throughout the viral cycle (Laude et al., 1986). In this study, the S' species was shown to be converted by endo H to a species with  $M_r$  155K (Fig. 2b), consistent with both the  $M_r$  of the S unglycosylated species produced in tunicamycin-treated cells (Fig. 1a) and the predicted  $M_r$  of the polypeptide backbone (Rasschaert and Laude, 1987). These results led us to conclude that S' represents a glycopolypeptide bearing rich mannose side chains. In contrast, the S species 220K was shown to be essentially resistant to endo H digestion, thus establishing that it bears predominantly complex type side chains. Moreover, the S species was no longer detected in the presence of monensin (Fig. 1b), indicating that the transition of S' to S is the result of Golgi specific modifications. The oligosaccharides present on S species are likely to represent tri- and tetra-antennary complex type carbohydrates since they were only sensitive to glyco F treatment (Fig. 2a).

The M protein was detected in tunicamycin-treated cells as a unique species with  $M_r$  26K equivalent to the predicted  $M_r$  of the apoprotein (Laude et al., 1987). This result confirms that the M protein is N-glycosylated and establishes that the observed heterogeneity in  $M_r$  (29 to 36K) of both intracellular and virion-associated M protein essentially represents different states of glycosylation. The major M species 29K would therefore be a high mannose form, as it was sensitive to treatment with endo H, whereas the 30–36K species would bear complex type carbohydrates since they were shown to be sensitive to glyco F only (Fig. 2a). The process of glycosylation for TGEV and IBV M proteins thus appears to be similar in that both endo H sensitive and endo H resistant M species have been found in IBV virions (Stern and Sefton, 1982). Finally, it is worth mentioning that M protein found in TGEV virions was predominantly of high mannose type, whereas only S species bearing essentially complex type carbohydrates (220K) were found in TGEV virions. This finding implies that the Golgi-specific modifications are poorly efficient in the case of M protein.

A major objective of this study was to investigate the contribution of the carbohydrates to the expression of different epitopes identified on the S and M proteins. The information obtained about the acquisition of immunoreactivity by

M protein in relation with carbohydrate processing is summarized in Fig. 6a. The expression of 3 out of the 4 epitopes defined on the M protein was greatly altered when addition of carbohydrates was blocked with tunicamycin (Fig. 5). This strongly suggests that these epitopes are located in the vicinity of the unique glycosylation site predicted in the hydrophilic N-terminus and assumed to be exposed at the external side of the membrane (Laude et al., 1987). The epitope defined by mAb 3.60 was shown to be glycosylation independent, which would be consistent with a location in the intracytoplasmic domain of the polypeptide chain. The non-reactivity of this mAb in membrane immunofluorescence experiments (Delmas and Laude, unpublished data) supports this view. The finding that both unglycosylated (Fig. 1a) and deglycosylated M proteins (Fig. 2b) retained a strong reactivity against polyclonal sera implies that at least one glycosylation independent epitope, putatively located in the endodomain of the M protein, is a potent B cell inducer.

a.



b.

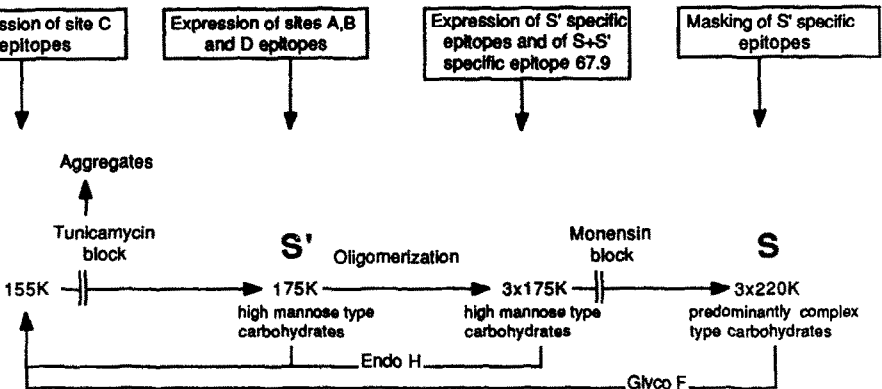


Fig. 6. A model for the biosynthesis and the folding of M (a) and S (b) glycoproteins.

The carbohydrate requirement for the expression of S individual epitopes was investigated using a panel of 23 neutralizing and non-neutralizing mAbs which has been shown to delineate four major antigenic sites (A, B, C and D), all located in the N terminal half of the S molecule and a few unrelated epitopes (Laude et al., 1986; Delmas et al., 1986; 1990; see Table 1). The following results led us to conclude that most of these antigenic sites are highly dependent upon glycosylation for both their expression and maintenance. First, the unglycosylated intracellular species produced in the presence of tunicamycin exhibited little or no reactivity towards mAbs defining the sites A, B and D (Fig. 3). Second, the same mAbs recognized poorly the deglycosylated form of S' obtained by endo H treatment of infected cell extracts (Fig. 4). No definite conclusions can presently be drawn about the way in which the carbohydrate's side chains modulate the antigenicity. However, the observed residual reactivity of non-glycosylated chains indicates that the carbohydrate moieties themselves are not directly involved as recognition structures in the epitopes, but rather are required for proper folding of the nascent glycopeptide chain. Third, by contrast to the other sites, site C was revealed to be largely independent of the core glycosylation for its expression (Figs. 3 and 4). This finding agrees with our earlier observation that only the site C epitopes could be mapped through expression of large fragments of S gene in a bacterial expression system (Delmas et al., 1990). Few bacterially expressible epitopes have been characterized so far on the S1 and S2 subunits of MHV and the S2 subunit of IBV (Lenstra et al., 1989; Luytjes et al., 1989; Routledge et al., 1991). This may suggest that at least part of the major antigenic sites assumed to be located in the globular part S1 of the protein (see Laude, 1990) are carbohydrate-dependent, as found in the S protein of TGEV.

The present state of our knowledge concerning the relationship between expression of S protein epitopes and co-post-translational processing is summarized in Fig. 6b, which combines the present data and those reported in a separate study, dealing with the oligomerization process of the S protein of TGEV (Delmas and Laude, 1990). According to the proposed model, the biosynthesis of mature S protein comprises three main events. First, 175K species bearing high mannose side chains are synthesized in the endoplasmic reticulum (ER). These monomers express the major antigenic sites A, B, C and D localized in the globular part of S molecule. Among these, only site C is largely independent of core-glycosylation for its expression. The next step, which was shown to be rate-limiting and to occur in the ER, consists in the assembly of 175K species into  $3 \times 175K$  oligomers. These structures express new epitopes, in particular S' specific epitopes. Finally, newly assembled trimers are transported in the Golgi compartment, where terminal-glycosylation occurs. S' specific epitopes are lost as a consequence of Golgi-modifications.

In conclusion, co-translational addition of sugars on the nascent polypeptide chain appeared to play a key role in the antigenic make up of the S and M glycoproteins of TGEV. However, the expression of particular epitopes of S protein was shown to be modulated by oligomerisation or terminal glycosylation. These findings suggest that carbohydrates may also modulate the immunogenicity of these proteins.

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