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Synthesis and Processing of the Bovine Enteric Coronavirus Haemagglutinin Protein

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SUMMARY

The haemagglutinin molecule on the bovine enteric coronavirus has been identified as a glycoprotein of 140K composed of disulphide-linked subunits of 65K. In this study, we have shown the subunits to be identical by demonstrating an unambiguous amino-terminal amino acid sequence. The unglycosylated subunit was found to have an M_r of 42·5K and to undergo rapid disulphide linkage and glycosylation. Glycosylation was found to be of the asparagine-linked type and some of the oligosaccharides underwent processing to complex forms. Studies with inhibitors of glycosylation suggested that a processing of the haemagglutinin oligosaccharide takes place on the virion whilst it is in the Golgi apparatus. Each haemagglutinin subunit on the mature virion was estimated to possess six or seven carbohydrate chains of either the high-mannose or hybrid type, and three or four chains of the complex type.

INTRODUCTION

Coronaviruses have been generally described as possessing three structural proteins: a phosphorylated nucleocapsid protein (N) of 50K to 60K, a glycosylated matrix-like protein (M or E1) of 23K to 29K, and a glycosylated peplomeric protein (P or E2) of 150K to 200K, that in many cases is proteolytically cleaved into two subunits of roughly equal size (for review, see Siddell *et al.*, 1982). Recently, a fourth structural protein has been described as a part of the mammalian coronaviruses that haemagglutinate. These coronaviruses include the bovine enteric coronavirus (BCV) (King & Brian, 1982), the human respiratory coronavirus OC43 (Hogue & Brian, 1986) and the porcine haemagglutinating encephalomyelitis virus (Callebaut & Pensaert, 1980). The fourth structural protein in BCV has been characterized as a glycoprotein of 140K that comprises disulphide-linked subunits of 65K (King & Brian, 1982). It is this protein that appears to be the virion haemagglutinin (H) (King *et al.*, 1985). Consistent with this picture is the finding that the non-haemagglutinating mouse hepatitis coronavirus strain A59, which is otherwise antigenically closely related to the haemagglutinating mammalian coronaviruses, has no molecular counterpart to the 140K glycoprotein (Hogue *et al.*, 1984).

The present investigation was undertaken to characterize the structure and synthesis of the 140K H glycoprotein on BCV. We have shown it to be an unusual viral glycoprotein in that it is assembled by disulphide linkage from separately synthesized, identical subunits rather than from a cleaved precursor. We have further characterized the type and extent of its glycosylation.

METHODS

Virus and cells. The Mebus strain of BCV was grown on the human rectal carcinoma cell line, HRT 18 (Tompkins *et al.*, 1974), as previously described (King & Brian, 1982; Hogue *et al.*, 1984). Cells were grown as monolayers in Dulbecco's modified Eagle's medium containing 50 μg/ml gentamicin (Sigma) and 10% foetal bovine serum (Sterile Systems). Virus stocks were prepared as previously described (Hogue *et al.*, 1984).

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Antiserum. Preparation and characterization of monospecific rabbit antiserum against the BCV H subunit (gp65) was as previously described (Hogue *et al.*, 1984).

Analysis of proteins. Virion proteins were analysed by fluorography of metabolically radiolabelled and electrophoretically separated proteins, or by immunoblotting unlabelled proteins that had been electrophoretically separated and transferred to nitrocellulose. For radiolabelling virion structural proteins, virus was grown in the presence of 400 μCi per 150 cm^2 flask of either ^3H-labelled essential amino acids (150 to 200 mCi/mg; ICN Pharmaceuticals), [^3H]glucosamine (40 Ci/mmol; Amersham), or [^3H]mannose (25 Ci/mmol; ICN Pharmaceuticals). Virus was purified from clarified supernatant fluids at 48 h post-infection by isopycnic sedimentation in sucrose gradients as previously described (Hogue *et al.*, 1984). For electrophoresis, pelleted virus was suspended in sample treatment buffer (0·0625 M-Tris–HCl pH 6·8, 2% SDS, 5 M-urea, 10% glycerol, bromophenol blue) and heated at 100 °C for 2 min. Where indicated, 2-mercaptoethanol was added to the sample at a final concentration of 5% before heating. Electrophoresis was done on 8% polyacrylamide gels unless otherwise indicated, using the discontinuous buffer system of Laemmli (1970). The immunoblotting protocol was the same as that described previously (Burnette, 1981; Hogue *et al.*, 1984) and quantification of autoradiograms was done by densitometric tracing using an LKB Ultrascan XL laser densitometer. Rabbit polyclonal antiserum specific for the BCV H subunit (gp65) was used in all immunoblotting experiments.

For analysis of intracellular viral proteins, whole cell lysates were prepared at 24 h post-infection by sonication. Briefly, infected cell monolayers in 60 mm Petri dishes were washed twice with cold phosphate-buffered saline (PBS), scraped into cold PBS, pelleted, resuspended in 100 μl sterile distilled water and sonicated at 50% full power for 10 s in a model W-220 F Heat Systems bath sonicator (Ultrasonics). Equal volumes of whole cell lysate and double concentration sample treatment buffer were mixed and heated at 100 °C for 2 min before electrophoresis.

M_r markers were ^{14}C-methylated myosin (200 000), ^{14}C-methylated phosphorylase b (92500), ^{14}C-methylated bovine serum albumin (69000) and ^{14}C-methylated lysozyme (14300), all obtained from Amersham.

Inhibitors and enzymes. Tunicamycin (Sigma) was used at a final concentration of 1 μg/ml in all experiments described below. Swainsonine (Calbiochem) was used at a final concentration of 500 μg/ml in the experiments described below, although concentrations of 5 to 500 μg/ml were found to behave in the same way with regard to inhibition of intracellular H processing. All inhibitors were included in the medium used to re-feed the infected or uninfected control cells, and cells were maintained in this medium until harvested. Endoglycosidase H (Division of Laboratories and Research, New York State Department of Health) was used at a final concentration of 50 milliunits/ml in a sample mixture containing the protein, 0·1% SDS and 50 mM-sodium citrate (pH 5·7). Before digestion, proteins were solubilized in 0·8% (w/v) SDS, heated 2 min at 100 °C, then diluted for digestion. Digestions were carried out overnight at 37 °C and proteins were recovered by precipitation with 10 volumes of acetone. *N*-glycanase (peptide : *N*-glycosidase F; Genzyme) was used at final concentrations of 0·87 to 35 units/ml. Samples were prepared for *N*-glycanase treatment by boiling for 3 min in 0·5% SDS, 0·1 M-2-mercaptoethanol, and then diluting the mixture to a final concentration of 0·1% SDS with a buffer containing 0·68 M-sodium phosphate buffer pH 8·6, 0·3 mM-EDTA and 1·25% NP40. Samples were incubated with *N*-glycanase overnight at 37 °C then diluted with an equal volume of double concentration sample treatment buffer for electrophoresis.

Amino acid sequencing. The amino-terminal end of the reduced H subunit was sequenced by the method of Matsudaira (1987) in his laboratory. Unlabelled BCV was purified by isopycnic sedimentation in sucrose gradients and the proteins were electrophoretically separated after reduction in 2-mercaptoethanol and electroblotted (Hogue *et al.*, 1984) onto polyvinylidene difluoride membrane (Matsudaira, 1987). The proteins were visualized by staining with Coomassie Brilliant Blue and the reduced H band was excised and analysed.

RESULTS

The 140K H protein is assembled from separately synthesized subunits and each subunit of the completed molecule is comprised of a 42·5K polypeptide and asparagine-linked oligosaccharides

The virion form of the 140K H glycoprotein (gp140) has been shown to be completely reducible with 2-mercaptoethanol to glycosylated subunits of 65K (gp65) regardless of the age of the virion (King & Brian, 1982; King *et al.*, 1985; Hogue *et al.*, 1984). Preliminary experiments have also demonstrated that the intracellular form of the H, the vast majority of which is 140K and the minority of which is 65K, is likewise completely reducible to 65K subunits, regardless of the time post-synthesis (Fig. 1, lanes 2 and 5; and data not shown). The times at which 2-mercaptoethanol was added for these experiments ranged from 1 to 24 h post-infection. These data suggest that H is synthesized as separate subunits which undergo rapid disulphide linkage and glycosylation. To characterize this mode of synthesis more fully, the size of the unglycosylated protein and the mode of glycosylation were examined using an inhibitor of asparagine(*N*)-linked glycosylation. Earlier experiments demonstrated that tunicamycin, an

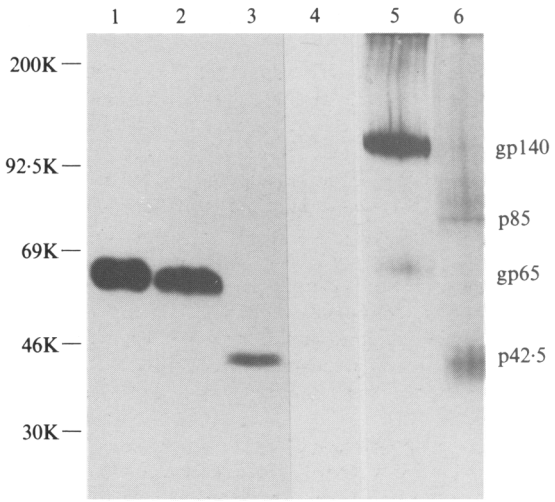

Fig. 1. Effect of tunicamycin on synthesis and processing of BCV H. Whole cell lysates from infected cells maintained in the presence of tunicamycin (lanes 3 and 6) were electrophoresed and immunoblotted with H-specific antiserum (anti-gp65). Lysate from tunicamycin-treated uninfected cells is shown in lane 4, and an immunoblot of purified virus is shown in lane 1. Samples in lanes 1 to 4 were treated with 2-mercaptoethanol. M_r markers are indicated on the left.

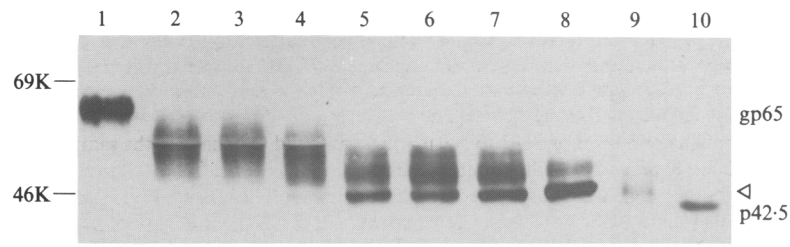

Fig. 2. Removal of *N*-linked oligosaccharides from the H subunit with *N*-glycanase. Purified virus was treated with 0 (lane 1), 0·87 (lane 2), 1·7 (lane 3), 2·6 (lane 4), 8·7 (lane 5), 17 (lane 6), 26 (lane 7) or 35 units/ml (lanes 8 and 9) of *N*-glycanase, electrophoresed and immunoblotted. A whole cell lysate from infected cells maintained in the presence of tunicamycin was likewise analysed (lane 10). All samples were treated with 2-mercaptoethanol. M_r markers are indicated on the left. Open arrow indicates the 45K digestion product.

inhibitor of *N*-linked glycosylation (Takasuki *et al.*, 1975), inhibited the glycosylation of the peplomeric protein and H, but not the matrix protein (Hogue, 1986; Lapps *et al.*, 1987). Tunicamycin was therefore used in widely differing concentrations (0·5 to 10 μg/ml) and its effects on intracellular H were examined by immunoblotting. Only one protein band with an M_r of 42·5K was identified under reducing conditions with H-specific polyclonal antiserum and this was observed at all concentrations of tunicamycin used (Fig. 1, lane 3; data not shown). When no reducing agent was included, a faint band of unglycosylated protein with an M_r of approx. 85K was observed in addition to the 42·5K species (Fig. 1, lane 6). The 85K species was never observed under reducing conditions. These results, therefore, also suggest that H is synthesized as separate identical subunits that become joined by disulphide linkage, and furthermore that the subunits are highly glycosylated with *N*-linked carbohydrates. Glycosylation is apparently not a prerequisite for disulphide linkage since some of the unglycosylated subunits can form dimers (e.g. the 85K species).

To use a second approach to determine the polypeptide size of the unglycosylated H subunit, *N*-glycanase, an enzyme that hydrolyses the glucosylamine linkage of all forms of *N*-linked oligosaccharides to yield a carbohydrate-free polypeptide (Plummer *et al.*, 1984), was used to deglycosylate the virion protein, and the size of the product was examined by electrophoresis and immunoblotting. Fig. 2 shows that enzyme concentrations as low as 0·87 units/ml reduced the size of the H subunit, but the smallest digestion product of approx. 45K became apparent only after 10 times this amount of enzyme was used (8·7 units/ml; Fig. 2, lane 5). Digestion with

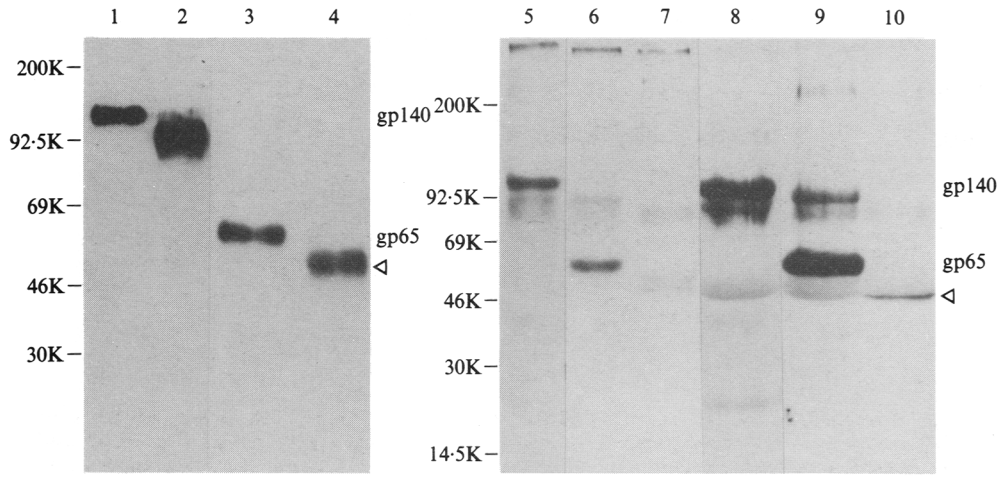

Fig. 3. Endoglycosidase H susceptibility of the virion H subunit. Purified virus was either digested with endoglycosidase H (lanes 2 and 4) or not digested (lanes 1 and 3), electrophoresed on an 8% polyacrylamide gel, and immunoblotted using anti-gp65 antiserum (lanes 1 to 4). Virus metabolically labelled with [^{3}H]glucosamine (lanes 5 to 7), or [^{3}H]mannose (lanes 8 to 10) was likewise either treated with endoglycosidase H (lanes 7 and 10) or not (lanes 5, 6, 8, 9), and electrophoresed on a gradient gel of 5 to 15% polyacrylamide, and fluorographed. Samples in lanes 3, 4, 6, 7, 9 and 10 were treated with 2-mercaptoethanol. M_r markers are on the left. Open arrow indicates the comigrating 52K nucleocapsid protein and endoglycosidase H-treated 65K monomer.

35 units/ml, which is over three times the maximum concentration previously used (Plummer *et al.*, 1984), or recommended by the supplier, did not reduce the size of the protein any further (Fig. 2, lanes 8 and 9) suggesting that removal of carbohydrates had become complete. These results clearly confirm that essentially all oligosaccharides on the H subunit are *N*-linked, but they also suggest that there may have been an additional modification since the product of *N*-glycanase digestion (45K) is clearly larger than the polypeptide identified following tunicamycin treatment (42·5K) (Fig. 2, lanes 8, 9, 10). Theoretically, two kinds of modification could have taken place. First, while it is unlikely that addition of palmitic acid occurs on any but the peplomer (P or E2) protein of BCV (Schmidt, 1982), another fatty acid modification (Sefton & Buss, 1987) might have occurred to alter the electrophoretic migration of the protein. Second, although we know of no precedent, there might have been *O*-linked glycosylation that was somehow strictly dependent upon an earlier *N*-linked glycosylation event. Finally, there may have been no additional modification at all, but rather an incomplete removal of *N*-linked oligosaccharides by *N*-glycanase. It has recently been discovered that extremely high concentrations of *N*-glycanase (200 units/ml) are required for complete removal of *N*-linked oligosaccharides from certain rare glycoproteins (T. H. Plummer, personal communication) and the BCV H may be one of these. Our results with swainsonine (described below) strongly support this last possibility.

To examine whether the subunits are identical, reduced H from purified virus was sequenced from the amino terminus. The terminus was not blocked, and an unambiguous sequence of N-Phe-Asp-Asn-Pro-Pro-Thr-Asn-Val-Val was obtained. These results therefore support the notion that identical subunits are separately synthesized and become assembled, and are not the product of protein cleavage.

H on the mature virion contains oligosaccharides of both the high-mannose and complex types

To determine the nature and extent of oligosaccharide processing that has taken place on the H of the mature (released) virion, purified virus was digested with endoglycosidase H and analysed. Endoglycosidase H removes oligosaccharides of the unprocessed, high-mannose type, and many of the processed, hybrid (partial high-mannose) type by cleaving between the two

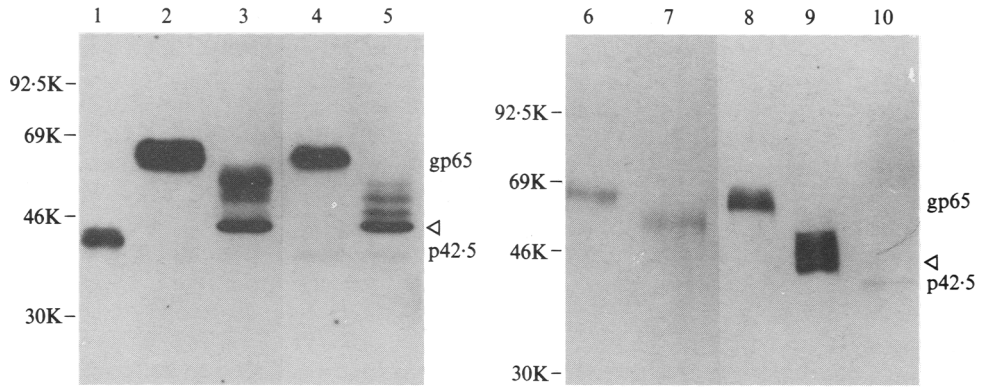

Fig. 4. Immunoblot (anti-gp65) showing the effect of swainsonine on the carbohydrate processing of the H subunit. Cells in lanes 1 and 10 were maintained in tunicamycin and cells in lane 5 were maintained in swainsonine before preparation of the cell lysate. Cell lysates in lanes 3 and 5 were treated with endoglycosidase H before electrophoresis. Virus in lane 9 was purified from cells maintained in swainsonine. Virus in lanes 7 and 9 was treated with endoglycosidase H before electrophoresis. All samples were treated with 2-mercaptoethanol. M_r markers are shown on the left of the gels. Open arrows indicate the 45K product of endoglycosidase H treatment.

proximal *N*-acetylglucosamine residues (Trimble & Maley, 1984). It does not, however, remove carbohydrates that have been modified to complex forms by the processing enzymes found in the Golgi apparatus. When purified virions were treated with endoglycosidase H, electrophoresed under reducing or non-reducing conditions and analysed by immunoblotting, neither the dimeric nor monomeric forms of H were found to be totally resistant to the action of endoglycosidase H as would be expected if all carbohydrates had been processed to complex forms (Fig. 3, lanes 2 and 4). Following endoglycosidase H treatment, the 140K dimer decreased in size by 20K to 40K (Fig. 3, lane 2), and the 65K monomer decreased by 10K to 13K (Fig. 3, lane 4). The digested monomer, as a result, comigrated with the 52K nucleocapsid protein. Because the dimeric H from the virion did not reduce completely to 85K, nor the monomer to 42·5K, we concluded that some of the carbohydrates on the virion H are of the complex type, and some remain as high-mannose unprocessed or possibly hybrid types.

To examine whether the endoglycosidase H-treated subunit in fact does contain carbohydrate, virions were radiolabelled with [^{3}H]mannose or [^{3}H]glucosamine, purified and examined by electrophoresis before and after endoglycosidase treatment. Radiolabelled, endoglycosidase H-treated material was found to comigrate with the nucleocapsid protein in both cases (Fig. 3, lanes 7 and 10) and this was concluded to represent the partially deglycosylated H subunit since the other two viral glycoproteins (the 200K peplomeric protein, observed on the virion primarily as subunits of 100K and 120K, and the 26K matrix protein) have never been found by immunoblotting to migrate in this region of the gel, before or after endoglycosidase H treatment (Hogue *et al.*, 1984; King *et al.*, 1985; unpublished data).

We conclude, therefore, that 10K to 13K of the mass of the mature H subunit is contributed by carbohydrates of the high-mannose unprocessed or hybrid types, and 9K to 12K is contributed by the fully processed complex type. By calculation, if the mannose-rich oligosaccharide contributed 2K to the M_r of a protein and the complex type contributes 2·5K to 3·5K (Klenk & Rott, 1982), then the mature (virion) H possesses approximately six or seven high-mannose and three or four complex chains per subunit.

Processing of H carbohydrates appears to take place in the Golgi apparatus on assembled virions

It has recently been documented that the mouse hepatitis coronavirus buds only into smooth-membraned transitional elements that exist between the rough endoplasmic reticulum and the cis Golgi compartment (Tooze *et al.*, 1984; Tooze *et al.*, 1988). This is presumably the site of budding for the closely related BCV and other coronaviruses that have been shown to bud into

intracytoplasmic vesicles (Mebus *et al.*, 1973; Siddell *et al.*, 1982). It has also been documented for the peplomer (E2) protein of mouse hepatitis virus that initial *N*-linked glycosylation is a cotranslational or very early (pre-Golgi) event, and that processing of the carbohydrate chains to complex forms by Golgi enzymes (Kornfeld & Kornfeld, 1985) occurs on proteins of assembled virions as the virions progress through the Golgi on a secretory pathway to the cell surface (Repp *et al.*, 1985). We would predict, therefore, that processing of the H protein follows a similar pattern since no mechanism other than inclusion at the time of budding is known for the addition of a viral envelope glycoprotein to the virion.

To confirm that H carbohydrate processing depends on Golgi function and hence probably occurs on the assembled virion, virus was grown in the presence of an inhibitor of a Golgi carbohydrate-processing enzyme and the effects on carbohydrate processing were studied. Swainsonine, an inhibitor of Golgi mannosidase II (Tulsiani & Touster, 1983; Kang & Elbein, 1983), was used to determine whether processing of the H carbohydrate to complex forms could be blocked. From these studies two points emerged. First, much of the intracellular H is already in a high-mannose (pre-Golgi) form, even at 24 h post-infection, and second, swainsonine effectively keeps both the intracellular and virion forms of H in the high-mannose structure. The first observation comes from experiments described in Fig. 3 and 4. Whereas all of the H subunit on mature (released) virus decreased in size from 65K to about 52K (a difference of 10K to 13K) after treatment with endoglycosidase H (Fig. 3, lane 4; Fig. 4, lane 7), only 44 to 46% of the intracellular H decreased by this amount (Fig. 4, lane 3; data not shown). Between 54 and 56% decreased to a size of 45K (note arrow in Fig. 4), and products intermediate between 45K and 52K (Fig. 4, lane 3). The 45K product is concluded to be equivalent to the 42·5K polypeptide identified by tunicamycin treatment since endoglycosidase H leaves approx. 10 *N*-acetylglucosamine molecules bound per polypeptide chain, and this would contribute about 2K to the total mass of the polypeptide. These experiments therefore show that whereas none of the virion H has carbohydrate chains entirely of the high-mannose type, approx. 50% of the intracellular H does. With regard to our second observation, swainsonine is shown to have had a dramatic effect on the carbohydrate pattern of the intracellular H (Fig. 4, lane 5). In the presence of swainsonine, essentially all (93%) of the intracellular H became endoglycosidase H-sensitive and digestible to a product of 45K and species intermediate between 45K and 52K. Swainsonine therefore inhibited the processing of carbohydrates on the intracellular H to complex forms. A similar dramatic effect of swainsonine on the processing of the H that becomes assembled into virions was also observed (Fig. 4, lane 9). In this case as well, swainsonine caused conversion of essentially all of the endoglycosidase H-digested H to molecules migrating as a 45K species or species intermediate between 45K and 52K. Swainsonine therefore also inhibited the processing of carbohydrates on molecules that became part of released virions.

DISCUSSION

We have shown by two basic approaches that BCV H is assembled from subunits that are synthesized separately and joined by disulphide links. First, all forms of dimeric H, whether they are intracellular or on the mature virion, unglycosylated or glycosylated, are reducible to subunits by the action of a sulphydryl agent. Second, the amino-terminal amino acid sequence derived from the reduced subunit is unambiguous, suggesting that the subunits are identical. A third approach published by Deregt *et al.* (1987) demonstrates that radiolabelled, intracellular subunits of the BCV H, immunoprecipitable with monoclonal antibodies, are first synthesized as subunits that chase into the dimeric form. This makes the BCV H an unusual glycoprotein since few other viral glycoproteins have been shown to be homodimers synthesized in this manner. A majority having disulphide-linked subunits, including the coronavirus peplomer protein (Sturman & Holmes, 1977; Cavanagh, 1983; Stern & Sefton, 1982), are cleavage products of larger precursors. One of the best characterized viral homodimeric glycoproteins is the H–neuraminidase protein of paramyxoviruses which happens also to exist as a homotetramer (Markwell & Fox, 1980). No data to date, however, suggest that multimeric forms other than the dimeric form exist for BCV H.

One outstanding feature of BCV H is its high degree of glycosylation; 35% of its mass is carbohydrate which compares with only 17% for the peplomer protein and 13% for the matrix protein (Hogue, 1986; Lapps *et al.*, 1987). Few other viral glycoproteins are as highly glycosylated. One is the G protein of respiratory syncytial virus which is 30% carbohydrate by mass (Wertz *et al.*, 1985). Like the coronavirus peplomer protein, the H protein apparently contains only *N*-linked carbohydrates, and also like the peplomer protein the intracellular H contains a large number of unprocessed, high-mannose side chains, and processing to complex forms appears to occur on the virion as it is transported through the Golgi on its way to the cell surface.

We therefore predict that BCV H subunits are synthesized independently and undergo membrane translocation, disulphide linkage, and glycosylation either as cotranslational or early post-translational events. We further predict that the H is anchored in the endoplasmic reticulum and pre-Golgi transition bodies where virus budding occurs. We do not yet know the orientation of the protein in the virion envelope, nor whether it goes independently to the cytoplasmic membrane.

Because the primary translation product is approx. 42·5K (it may be slightly larger if there is a cleaved amino-terminal signal peptide), then the polypeptide is at least 425 amino acids in length. This would require an mRNA of 1275 bases. Because the haemagglutinating polypeptide is not found in the antigenically related mouse hepatitis virus A59 which possesses six subgenomic polyadenylated mRNA molecules (Spaan *et al.*, 1981), we would expect to find a seventh subgenomic mRNA in BCV-infected cells. We have recently shown there to be such a molecule in BCV-infected cells, that it migrates between mouse hepatitis virus subgenomic RNA species 2 and 3, and termed it BCV RNA species 2A (Keck *et al.*, 1988). The unique 5′ region of species 2A is approx. 1200 bases and it could thus represent the mRNA for the H subunit. If it does, then the gene for the H subunit will probably reside on the 5′ side of the peplomer gene because of the 3′ nested set arrangement of the coronavirus mRNAs. Studies are currently under way to test this hypothesis.

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