Organization of Germiston bunyavirus M open reading frame and physicochemical properties of the envelope glycoproteins

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We describe the construction of plasmids which express fusion proteins representing various regions of Germiston virus M polyprotein. The fusion proteins were purified and inoculated into rabbits to produce antisera. The N- and C-terminal regions of the polyprotein induced specific antibodies which reacted with glycoproteins G2 and G1, respectively, and the intermediate region induced antibodies against the NSM polypeptide. This enabled us to determine the gene order: G2–NSM–G1. Glycoproteins G1 and G2 form the spikes on the surface of the virion. We attempted to determine the structural organization of the glycoproteins by using a membrane-permeable cross-linking reagent, dimethyl suberimidate, but were unable to demonstrate that G1 and/or G2 form oligomeric structures. We analysed the glycoproteins further and showed that, like peripheral membrane proteins, the G2 and NSM proteins are almost completely extracted into the aqueous phase of detergent Triton X114-treated cellular extracts, whereas glycoprotein G1 is distributed in almost equal proportions between the aqueous and the detergent fractions. This indicates that G1 is a membrane-associated protein, but its presence in the aqueous phase suggests that it is less hydrophobic than a typical membrane protein. We have also characterized the intracellular transport of the envelope glycoproteins from the endoplasmic reticulum to the Golgi complex. Pulse-chase labelling followed by immunoprecipitation and treatment with endoglycosidase H (endo H) showed that both G1 and G2 are transported from the endoplasmic reticulum to the Golgi complex. Conversion to the endo H-resistant form is a rather slow process which takes more than 2 h. The mature G1 and G2 proteins present in the virion particle contain almost completely endo-H-resistant glycans.

Introduction

The Bunyaviridae family comprises enveloped tripartite RNA viruses classified into five genera (Calisher, 1991): Bunyavirus, Nairovirus, Phlebovirus, Hantavirus and Tospovirus, a newly created genus which includes viruses of plants.

Our laboratory is involved in studying Germiston virus, a member of the Bunyamwera serogroup in the Bunyavirus genus. Like other Bunyaviridae, Germiston virus possesses a three segment ssRNA genome of negative polarity (Ozden & Hannoun, 1980; Pardigon et al., 1982). The large RNA segment (L, approximately 8000 bases) encodes the L protein, the virion-associated transcriptase. The small RNA segment (S, 980 bases) encodes the nucleocapsid (N) protein and a non-structural protein NSs, and contains a third open reading frame (ORF) which could encode an 8.5K polypeptide, but this gene product has not been identified in cellular extracts (Gerbaud et al., 1987). Genetic and molecular analyses have demonstrated that the bunyavirus M RNA encodes the envelope glycoproteins G1 and G2, and a nonstructural polypeptide, NSM (references cited in Bishop, 1986; Bouloy et al., 1984; Elliott, 1985). By cloning and sequencing the Germiston virus M RNA segment (4534 bases), we have shown that the M mRNA has a single large ORF which encodes a polypeptide of 1437 amino acids, equivalent to 162K. This polypeptide is sufficiently large to encode proteins G1 (98K), G2 (37K) and NSM (16K) (Pardigon et al., 1988), but such a precursor has never been identified during bunyavirus infection (Lees et al., 1986; Pardigon et al., 1988), suggesting that its processing is cotranslational. The cleavage and the maturation of the M polypeptide as well as the order of the proteins within the polyprotein are not known. Probably some, if not all, of the cleavages must be performed by cellular enzymes (signalases).

To determine the location of proteins G1, G2 and NSM within the polyprotein, we expressed several regions of the M ORF as fusion proteins, prepared antisera against them and tested the reactivity of the antibodies toward the viral proteins. We show that the N- and C-terminal regions of the polyprotein induce specific antibodies...
which react with glycoproteins G2 and G1, respectively, and that the intermediate region induces antibodies against the NSM polypeptide. This has enabled us to determine the gene order G2–NSM–G1. In addition, to understand better the molecular basis of Germiston virus morphogenesis, we determined the physicochemical properties of the viral glycoproteins and analysed the kinetics of synthesis and maturation by analyses of sensitivity or resistance of the glycoproteins to endoglycosidase treatments, an assay routinely used to study the processing of glycoproteins in the Golgi apparatus.

Methods

Preparation and purification of virus. Germiston virus was grown and purified as described by Pardigon et al. (1982). Labelled virus was prepared by adding 50 μCi/ml [35S]methionine to the medium, which was collected at 24 h post-infection (p.i.).

Preparation of cell lysates. BHK 21 cells infected with virus at a multiplicity of 10 were labelled for 2 h or 4 h p.i. with 50 μCi/ml [35S]methionine, and collected 5 or 6 h p.i. Cells were lysed in CDB buffer (250 mM-NaCl, 10 mM-Tris–HCl pH 8.0, 0.5% Triton X-100 and 0.5% sodium deoxycholate). Nuclei and cellular membranes were eliminated by centrifugation. Proteins were analysed by electrophoresis in an SDS–polyacrylamide gel (Laemmli, 1970).

For pulse-chase experiments, Germiston virus-infected cells were pulse-labelled at 5 h p.i. for 20 min with 70 μCi/ml [35S]methionine. After a 0 to 110 min period of incubation in the presence of an excess of unlabelled methionine, cell extracts were prepared in CDB buffer.

Construction of bacterial expression vectors to produce hybrid proteins. Plasmids pEX (Stanley & Luzio, 1984) or pATH (Dieckmann & Tzagoloff, 1985) were purchased from Genofit and kindly provided by Dr P. W. Mason, respectively. The choice of vector (pEX 1, 2 or 3 and pATH 10 or 11) and site for insertion (Smal or EcoRI) were chosen so that the ORFs of the foreign and bacterial genes were conserved.

Various M cDNA restriction fragments digested from M cDNA-specific plasmids described by Pardigon et al. (1988) were ligated into the dephosphorylated pEX or pATH plasmids digested with Smal or EcoRI. When necessary, the fragments and the vectors were made blunt-ended after filling-in with the Klenow fragment of DNA polymerase I. Hybrid plasmids were used to transform Escherichia coli pop 2136 cells (pEX plasmids) or RR1 cells (pATH plasmids), and ampicillin-resistant colonies were selected. Recombinant plasmids prepared by the method of Birnboim & Doly (1979) were initially analysed using DNA restriction enzymes, and then for protein induction. Conservation of the reading frame in the region of the insertion site was verified by sequencing using the dyeodeoxyxynucleotide chain termination method of Sanger et al. (1977). Synthesis was primed by synthetic oligonucleotides complementary to the plasmid sequence positioned just upstream of the site of insertion.

Analysis and purification of hybrid proteins

(i) pEX proteins. The pEX plasmids possess a cro–lacZ gene under the control of the Pk promoter of bacteriophage λ. Plasmids without an insert express large quantities of a fusion cro–β-galactosidase protein of 117K. Expression of the lacZ gene is controlled in E. coli strain pop 2136, which carries the gene encoding the cts 857 repressor. Thus, transient expression is induced by shifting the culture from 30°C to 42°C, the non-permissive temperature. Cells were harvested by centrifugation, resuspended in Laemmli buffer (60 mM-Tris–HCl pH 6.8, 4% SDS, 100 mM-DTT, 10% glycerol) and the samples were loaded onto a 7.5% polyacrylamide gel. When hybrid proteins were prepared in large quantities, cells from a 200 ml culture were pelleted and resuspended in 3 ml buffer containing 50 mM-Tris–HCl pH 8.0, 1 mM-EDTA and 50 mM-NaCl. Cells and DNA were broken by sonication, and then the hybrid proteins were pelleted and resuspended in 2 ml of Laemmli buffer. After electrophoresis, the band corresponding to the fusion protein was identified by staining the two external sides of the gel with Coomassie blue. The protein band was then cut out, eluted overnight and electroeluted for 4 h at 5 V/cm in a sample concentrator cup containing 100 mM-glycine, 20 mM-Tris base and 0.01% SDS. Protein concentrations were estimated by the Folin–Ciocalteu method.

(ii) pATH protein. The pATH vectors contain the trpE promoter and the N-terminal sequence (969 nucleotides) of the trpE structural gene, followed immediately by a multiple cloning site. Upon induction with an artificial inducer of the trp operon, indoleacrylic acid (IAA), cells containing the pATH vector without an insert express a truncated trpE protein of an approximate size of 37K .

The conditions used were those described by Spindler et al. (1984), except that cells were grown at 30°C and induction with IAA was for 2 or 4 h (for analytical or preparative purposes, respectively). For analytical purposes, bacteria were lysed by incubation for 30 min at 37°C in 50 µl of cracking buffer (0.01 M-sodium phosphate pH 7.2, 1% 2-mercaptoethanol, 1% SDS, 6 M-urea). For preparative purposes, bacteria from a 100 ml culture were centrifuged, treated with lysozyme, lysed with NP40 in high salt buffer (0.3 M-NaCl) and sonicated. Soluble and insoluble protein fractions were separated by centrifugation. Hybrid proteins usually present in the pellet were purified on 10% SDS–polyacrylamide gels and eluted as described above.

Immunization. Approximately 250 µg of the fusion proteins (or 150 µg of a peptide corresponding to the sequence of residues 287 to 306 of the M RNA ORF synthesized by Neosystem) was inoculated into each of two rabbits according to the protocol described by Akkina et al. (1987). The first two inoculations were intradermal into multiple shaved sites at 21 day intervals; rabbits were then boosted intramuscularly with the same amount of fusion protein or peptide at 2-week intervals and bled from the ears 14 days post-boost. The sera were tested by immunoprecipitation and Western blotting.

Immunoprecipitation. In many experiments, the cytoplasmic extracts prepared in CDB buffer were treated with RNase (0.1 µg/ml), SDS (1%), 2-mercaptoethanol (0.7 M), and centrifuged for 10 min at 10000 g after being diluted 10-fold in solubilizing buffer (50 mM-Tris–HCl pH 7.5, 150 mM-NaCl, 1% NP40) to eliminate insoluble material or aggregates. The supernatant was collected and used for immunoprecipitation.

Samples in a 50 µl volume were first pre-incubated for 30 min at 4°C with 10 µl of non-immune serum and 35 µl of a slurry preparation of Protein A-Sepharose. Non-specific complexes were eliminated by centrifugation. The supernatant was incubated at 4°C for 60 min with 10 µl of antiserum, and then Protein A-Sepharose was added and incubated at 4°C for 1 h or overnight. Immunoprecipitates were washed three times with buffer A (10 mM-Tris–HCl pH 7.5, 150 mM-NaCl, 0.2% NP40, 2 mM-EDTA), twice in buffer B containing 500 mM-NaCl and finally once in 10 mM-Tris–HCl pH 7.5. Proteins were eluted from the beads in 25 µl of denaturing buffer and analysed in a 14% or 17.5% SDS-containing polyacrylamide gel. Proteins were then fixed, and the gel was impregnated with AmphiLyse (Amersham), dried and exposed to X-ray film using intensifying screens.

Western blotting. After electrophoresis, proteins were electrophoretically transferred onto nitrocellulose paper (Schleicher & Schuell). Non-specific sites were blocked by incubation in a solution (20 mM-Tris–HCl pH 7.5, 500 mM-NaCl containing 3% foetal calf serum, 3% non-fat dry milk and 0.05% Triton X-100). A 1:100 dilution of the appropriate rabbit antiserum was utilized. The bands were visualized as described by Desprès et al. (1991).
**Results**

*Expression of Germiston virus M cDNA sequences in E. coli*

To produce large quantities of Germiston virus M proteins for use as immunogens, cDNA fragments corresponding to various regions of the Germiston virus M RNA coding sequence were joined in-phase to the bacterial lacZ-pEX or trpE-pATH vectors. Fig. 1 shows the regions of the M cDNA subcloned into these vectors and expressed as fusion proteins. Three restriction fragments were cloned into the pEX plasmid: a SacI–Hinfl fragment containing nucleotides 175 to 466 (fragment A), a TaqI–AatII fragment containing nucleotides 1157 to 1368 (fragment B), and an EcoRI–EcoRI fragment containing nucleotides 1961 to 2425 (fragment C). Five restriction fragments were fused in-frame to the N-terminal half of the *trpE* gene: a TaqI–TaqI fragment containing nucleotides 1056 to 1157 (fragment B1), an Rsal–Rsai fragment containing nucleotides 1235 to 1407 (fragment B2), an XbaI–AccI fragment containing nucleotides 3061 to 3329 (fragment D), an AccI–AccI fragment containing nucleotides 3329 to 3511 (fragment E) and a SfaNI–TaqI fragment containing nucleotides 4031 to 4174 (fragment F).

Upon induction, all the hybrid plasmids with Germiston virus M RNA sequences were able to produce a fusion protein of the expected size (data not shown). Large amounts (approximately 5 or 10 μg/ml of original volume of bacterial culture harbouring the pEX or pATH vector, respectively) of the fusion protein were produced from recombinant plasmids. The cro–β-galactosidase fusion proteins were insoluble and accumulated inside the cells. Similarly, most of the trpE fusion proteins were insoluble, except polypeptides B1 and B2 which remained soluble.

**Immunization of rabbits with Germiston virus fusion proteins: specificity of antisera**

The different fusion proteins were purified and prepared in sufficient quantities to immunize rabbits. The specificity of each antiserum raised was determined by Western blotting and immunoprecipitation. The results obtained when the proteins were probed by Western blotting are shown in Fig. 2. The antibodies raised against polypeptide A reacted specifically with glycoprotein G2 (a), those directed against polypeptides C to F recognized glycoprotein G1 (d to g). The slight difference in migration between G1 and G2 from a virus-infected cellular extract (lanes 2) and from purified virus (lanes 1) was probably due to an artefact during electrophoresis or transfer onto nitrocellulose. Polypeptide B was not found to induce Germiston virus-specific antibodies when tested by Western blotting or by immunoprecipitation (not shown).

As determined from the hydropathy profile (Fig. 1), polypeptide B contains a large hydrophobic domain. This might explain its lack of immunogenicity. For this reason, we selected two other sequences located in the vicinity of fragment B lacking the hydrophobic domain.
We expressed them as trpE fusion proteins B1 and B2 (Fig. 1a). The antisera directed against polypeptide B1 or B2 reacted to a significant extent with NSM (Fig. 2b, c).

The specificity of the antisera was confirmed by immunoprecipitation experiments (Fig. 3). Whereas the serum against fragment A reacted specifically with G2 by Western blot analysis (Fig. 2a), it immunoprecipitated G1 as well as G2. This surprising observation, which occurred with proteins from infected cells and with those from purified virus, as well as with two independent sera, will be discussed below. It should also be noted that the N protein and, in some cases, a cellular protein of approximately 45K were non-specifically immunoprecipitated with all the rabbit sera that we tested, even with those from non-immunized animals. Together, these results indicate that the order of the proteins in the M polyprotein is G2–NSM–G1, from the N to the C terminus of the precursor protein (Fig. 1).

Structural organization of G1 and G2 proteins

Oligomerization of the spike protein(s) has been demonstrated for many enveloped viruses, especially for Uukunenemi and Punta Toro viruses, members of the Phlebovirus genus in the Bunyaviridae family (Persson & Pettersson, 1991; Chen et al., 1991). The structural organization of the bunyavirus glycoproteins on the surface of the virion is unknown. Although they belong to the same family, electron microscopic studies have revealed a different organization of the surface of uuku-, phlebo- and hantavirus virions (reviewed in Bishop, 1990; Schmaljohn & Patterson, 1990; Elliott, 1990; Bouloy, 1991).

Studies of glycoproteins G1 and G2 from several viruses of the Bunyavirus genus have been complicated by the instability of G2 (Madoff & Lenard, 1982; Elliott, 1985). During analyses of Germiston virus we made a similar observation; glycoprotein G2 is extremely heat-sensitive because the corresponding band disappears in SDS–polyacrylamide gels when the sample is boiled for 3 to 5 min (not shown). Once it is denatured, the protein is probably degraded. Nevertheless, it has been possible to study this glycoprotein by avoiding the heat denaturation step and merely denaturing it at room temperature in the presence of SDS and 2-mercaptoethanol (except when stated).

The observation that the antiserum against fragment A immunoprecipitated not only G2 but also G1 prompted us to address the question whether the two glycoproteins are highly linked in the virus particle or in the cell extract to form oligomeric structures. If such structures exist in the particle, a polyclonal serum directed against G1 or G2 would be expected to immunoprecipitate both glycoproteins. Although we observed the phenomenon only with the antiserum against G2 and not with any of the antisera directed against G1, we considered further the possibility that G1 and G2 form complexes. The sequences of glycoproteins G1 and G2 contain many cysteine residues which are
conserved in several bunyaviruses, e.g. La Crosse (Grady et al., 1987), snowshoe hare (Eshita & Bishop, 1984) and Bunyamwera (Lees et al., 1986) viruses. To determine whether these glycoproteins form disulphide bonds involved in complex structures, we analysed the viral proteins under non-reducing conditions. The pattern of migration in SDS–polyacrylamide gels was the same as that obtained with the sample denatured in the presence of 2-mercaptoethanol (not shown). This indicated that no disulphide bridge exists between structural proteins G1 and G2.

In another approach, we studied the distribution of the glycoproteins during sedimentation in sucrose gradients after dissociation of purified [35S]methionine-labelled Germiston virus in the presence of Triton X-100. Whereas ribonucleoproteins pelleted at the bottom, where the N and L proteins were detected, glycoproteins G1 and G2 cosedimented as a single peak in the middle of the gradient but did not form hetero-oligomers, as demonstrated by the fact that G1-specific antibodies immunoprecipitated exclusively the corresponding protein (not shown).

If the putative complexes (homo- or hetero-oligomers) are weakly associated, it might be necessary to stabilize them before analysis, as demonstrated for rabies virus (Whitt et al., 1991), coronavirus (Delmas & Laude, 1990) and Punta Toro virus (Chen & Compans, 1991). DMS is a membrane-penetrating reagent which covalently cross-links proteins via lysine amino groups, and has been successfully applied to the study of the structural details and spatial relationships in protein complexes and viruses (Davies & Stark, 1970). However, in this study, treatment of Germiston virus with DMS did not help to demonstrate the presence of oligomeric structures (not shown).

Thus, except for the results repeatedly obtained and presented in Fig. 3 which indicated that antibodies raised against G2 immunoprecipitate G1, we did not obtain any evidence that G1 and G2 form a complex structure. The reactivity of the anti-G2 antiserum most probably can be explained by the presence of epitopes common to both G1 and G2. It would have been of interest to test other anti-G2 antibodies. Unfortunately, this has not been possible because this region of the M
approximately equal proportions in the detergent and aqueous fractions. Although the presence of a large proportion of the protein in the detergent phase indicates that it is a membrane-associated protein, this protein seems less hydrophobic than a typical membrane protein. The NS1 protein of several flaviviruses exhibits a similar partitioning in Triton X-114 (Winkler et al., 1988; Mason, 1989; Després et al., 1991) but, unlike the bunyavirus G1 glycoprotein, NS1 lacks a C-terminal anchoring domain.

**Endoglycosidase treatment**

Seven potential glycosylation sites were identified in the Germiston virus M polyprotein. At least one of them, at position 897, is probably not recognized because it contains the sequence Asn–Pro–Thr. Based on the proposed localization of G1, G2 and NSM, two sites lie in G1, three in G2 and one in the hydrophobic region preceding G1 (position 474), which is probably not utilized. Some of these sites are recognized by glycosylating enzymes because synthesis of both G1 and G2 envelope proteins is sensitive to tunicamycin (Pardigon et al., 1988). We estimated that the difference in apparent Mr between the unglycosylated and glycosylated forms of G1 and G2 is 4K to 5K, consistent with the utilization of two sites in each protein. It is possible that these sites are those which are strictly conserved in their positions in the different bunyaviruses (Elliott, 1990), i.e. positions 65 and 252 in G2 and positions 627 and 1173 in G1.

Since budding and bunyavirus particle formation occur in the Golgi complex, it is of interest to study how glycoproteins are transported to the Golgi apparatus. Indeed, to some extent, it is possible to gain insight into the mode of virus maturation by analysing the type and amount of oligosaccharides attached to viral proteins (for reviews see Klenk & Rott, 1980; Kornfeld & Kornfeld, 1985). Most of the envelope proteins examined possess N-linked oligosaccharides. Generally, two classes are found on mature glycoproteins: high mannose and complex types. The high mannose type is added in the endoplasmic reticulum and such oligosaccharides are sensitive to endoglycosidase H (endo H). Later, during transport of the glycoproteins through the Golgi complex (of most eukaryotic but not insect cells), mannose residues are trimmed to a trimannosyl core and complex sugars are added. At this step, the complex oligosaccharides become resistant to treatment with endo H.

[35S]Methionine-labelled Germiston virus-infected cells were lysed at different times after pulse–chase labelling. Cell lysates were then subjected to immunoprecipitation with G1- or G2-specific antibodies, and the immunoprecipitated polypeptide was treated with either
Fig. 5. Endo F or endo H treatment of [35S]methionine-labelled Germiston virus-infected cells after immunoprecipitation with anti-G1 antibodies. Proteins from infected cells pulse-labelled and chased for 0 (a), 50 (b), 80 (c) or 110 (d) min or from purified virus (e) were immunoprecipitated with anti-G1 antibodies and treated with endo H (lanes 2), endo F (lanes 3) or left untreated (lanes 1). Proteins were analysed in polyacrylamide gels. Proteins synthesized in the presence of tunicamycin were used as a reference for unglycosylated polypeptides (lane 4). Positions of Mr markers are indicated to the left.

endo H or F, or left untreated. Labelled proteins from purified virus were also analysed. As shown in Fig. 5, at any time of the chase, G1 glycoprotein was sensitive to endo H treatment (lanes 3), and the resulting polypeptide comigrated with the non-glycosylated polypeptide G1 glycoprotein exhibited in the presence of tunicamycin, a well established inhibitor of all N-glycosylation (lane 4).

Early after its synthesis, G1 glycoprotein was completely sensitive to endo H (a, lane 2), and the treated polypeptide comigrated with the endo F-treated form (a, lane 3) or the unglycosylated product from tunicamycin-treated cells (lane 4). After a 50 and an 80 min period of chase, the endo H-treated G1 glycoprotein exhibited a different aspect in the gel, appearing as a diffuse thick band (b and c, lanes 2), which suggests the presence of both sensitive and resistant forms. After a chase of 110 min, G1 was still incompletely resistant (d, lane 2). Finally, we analysed the extracellular protein present in the virion which could be considered as the final stage of maturation; it was almost completely resistant to endo H (e, lane 2). During the virus cycle as well as in virus particles, the G2 glycoprotein is always less abundant than G1, so that analysis of G2 was more difficult. However, it was possible to show that the kinetics of acquisition of endo H resistance were similar to those observed for G1 (data not shown).

Discussion

In this report, we have shown that bacterial expression vectors can be used to prepare relatively large amounts of fusion proteins containing specific portions of Germiston virus M protein from cDNA and that these fusion proteins are able to induce specific antibodies. Analysis of these antisera led us to the conclusion that the gene order of Germiston virus M polyprotein from N to C terminus is G2-NSM-G1. These data confirm those previously reported by Fazakerley et al. (1988) using snowshoe hare virus. To map the three proteins precisely we tried to sequence the N terminus by Edman degradation but were unsuccessful, probably because the proteins have a blocked N-terminal residue. Nevertheless, the following reasoning helped us to localize the extremities of the envelope glycoproteins.

The published sequences of the M polyproteins of four Bunyaviruses, Germiston, Bunyamwera (Bunyamwera serogroup), La Crosse and snowshoe hare viruses (California serogroup), have been compared by Elliott (1990). The amino acid sequences can be aligned and many cysteine residues are conserved. The hydropathy profiles of the four polypeptides determined according to the algorithm of Kyte & Doolittle (1982) are very similar and reveal several hydrophobic regions. Among them is a putative signal peptide of, depending on the virus, 13 to 21 residues at the N terminus of the polyprotein which might be involved in membrane translocation of the G2 polypeptide. The N-terminal sequence of snowshoe hare virus (Fazakerley et al., 1988) confirms that cleavage does occur after an Ala residue at position 13. Thus, if the prediction of the cleavage site after the Ala residue at position 21 in Germiston virus M polyprotein is correct, glycoprotein G2 contains the N-terminal sequence Pro-Ile. Fazakerley et al. (1988) also sequenced the C terminus of snowshoe hare virus glycoprotein G2 and found it to be located at amino acid 299 in the polypeptide. Alignment of the M polyprotein amino acid sequence of Germiston and snowshoe hare viruses has revealed that the C terminus of the snowshoe hare virus G2 protein is located in a region well conserved between these viruses, ...K-S-L-R-(A/V)-A-R. This provides indirect evidence for the location of the C terminus of G2 in the Germiston virus M polypeptide precursor; it would be cleaved after the conserved Arg residue at position 306. In an attempt to confirm this positioning, we tested the specificity of a rabbit antiserum raised against a synthetic peptide representing the sequence from amino acids 287 to 306 but, unfortunately, no reactivity was detected by immunoprecipitation or Western blotting. From this positioning, it is predicted that the G2 protein contains a rather long biphasic hydrophobic domain from residues 200 to 240, followed by a C-terminal region rich in charged amino acids (Fig. 1).

The exact location and function of NSM are still unknown. In all the gel analyses, we found a polypeptide migrating as a doublet of estimated Mr, 16K. We do not
know whether the fast migrating element represents a cleavage or degradation product of the large one. In addition, tunicamycin did not affect the synthesis of NSM, suggesting that the protein is not glycosylated (Pardigon et al., 1988). The proposed location of NSM in the polyprotein is in accordance with these data because this region does not contain potential glycosylation sites (Fig. 1).

The N terminus of snowshoe hare virus G1 glycoprotein has been located at Ala residue 474 in the M polypeptide (Fazakerley et al., 1988). Although the level of similarity between Germiston and snowshoe hare viruses is somewhat low in this region, alignment of the M polypeptides suggests that the N terminus of Germiston virus G1 protein corresponds to the conserved Ala residue at position 481 in the polyprotein. Upstream from this sequence there is a hydrophobic domain which could act as a signal peptide for translocation (Fig. 1). The hydrophyty profile also reveals the presence of a large hydrophobic domain at the C terminus of the polyprotein from residues 1382 to 1412, followed by charged amino acids. This arrangement is typical of C-terminal transmembrane domains of integral membrane proteins. In the case of snowshoe hare virus, it has been shown that an antiserum to a synthetic peptide representing the last 16 residues of the translated ORF reacts with G1, demonstrating that this polypeptide extends to the end of the protein. It can be inferred from this result that the C terminus of the Germiston virus G1 glycoprotein must also extend to the end of the polypeptide. The physicochemical properties of the envelope proteins and the non-structural protein NSM are in good agreement with those determined theoretically from the sequence data and the hydrophyty profile.

We have also studied the intracellular processing of the envelope glycoproteins of Germiston virus. At various times after synthesis, we analysed the glycosylation of G1 and G2 by assaying their sensitivity or resistance to endo F and endo H. Pulse–chase experiments indicated that a significant fraction of G1 polypeptide becomes endo H-resistant as early as 40 min p.i., but more than 2 h are required for G1 to become completely resistant. In purified virion preparations, the majority of the glycoproteins are endo H-resistant. The time required for a glycoprotein to become endo H-resistant is believed to correspond to the time required for the transport of the protein from the endoplasmic reticulum to the medial cisternae of the Golgi complex, where the N-acetylgalcosamine residues are added to the mannose core and the building of the complex type of glycans is initiated (Dunphy et al., 1985). When compared with that of vesicular stomatitis virus G protein, which becomes completely endo H-resistant within 20 min (Strous & Lodish, 1980), the processing of Germiston virus glycoprotein is slow but similar to that determined for La Crosse virus (Madoff & Lenard, 1982) or Uukuniemi virus (Kuismanen, 1984). Moreover, as in the La Crosse virion, mature G1 and G2 of Germiston virus contain mainly oligosaccharides of the complex type. In other representatives of the family, the situation is somewhat different. In Hantaan virus, glycoproteins contain a majority of high mannose-type oligosaccharides (Schmaljohn et al., 1986), and in Uukuniemi and Inkoo viruses, three types of oligosaccharides have been detected: protein G2 contains oligosaccharides of the high mannose type, whereas G1 contains oligosaccharides of the complex type as well as those of an intermediate type which represents a novel class in the biogenesis of oligosaccharides (Pesonen et al., 1982; Kuismanen, 1984).

One of the features of the Bunyaviridae family is maturation by budding at smooth membrane vesicles in the Golgi complex (for a review see Bishop & Shope, 1979). At an early stage, maturation of membrane glycoproteins requires disulphide bond formation, folding and oligomerization in the endoplasmic reticulum. Such phenomena have been described for Uukuniemi virus glycoproteins, which associate with the cellular BiP and PDI proteins and form heterodimers (Persson & Pettersson, 1991), and for Punta Toro virus (Chen & Compans, 1991). At present, little is known about members of the Bunyavirus genus in this regard. Electron microscopic studies of vitrified and hydrated La Crosse virions have revealed the presence of 10 nm long spikes protruding from the membrane bilayer at the surface of the particle (Talmon et al., 1987). In contrast with the lattice observed for Uukuniemi, Punta Toro and Hantaan viruses, there is no periodicity on the surface of bunyaviruses. This might reflect differences in the glycoproteins and peplomer composition between different genera. In the case of bunyaviruses, G1 and G2 play different roles; G1 but not G2 induces neutralizing antibodies and G1 binds to vertebrate and mosquito cells, but not midgut cells, whereas G2 binds to mosquito but not vertebrate cells (Ludwig et al., 1989, 1991).

During the course of our analyses, the existence of hetero-oligomers in the Germiston virion was proposed. So far, we have not obtained any evidence that G1 and/or G2 form complex structures. This is surprising, but we cannot conclude that such structures do not exist because the lack of demonstrable association between G1 and/or G2 might be due to the cross-linking reagent. There are known examples, such as Semliki Forest virus (Ziemiecki et al., 1980), human immunodeficiency virus (Schawaller et al., 1989; Weiss et al., 1990) and, to some extent, lymphocytic choriomeningitis virus (Burns & Buchmeier, 1991), for which the failure of the reagent to
cross-link the components of viral spikes has been reported. Thus, more experiments will be necessary to provide an unambiguous answer.

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References


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