Polypeptides of Pneumonia Virus of Mice. II. Characterization of the Glycoproteins

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SUMMARY

The kinetics of synthesis and the nature of the oligosaccharides of the glycoproteins of pneumonia virus of mice (PVM) were studied. Tryptic peptide mapping showed that the two major glycosylated polypeptides G1 and G2 were different forms of the same protein. G2 was derived from G1 which in turn appeared to be derived from an unidentified precursor. The G1/G2 protein of PVM is probably a haemagglutinin since a monoclonal antibody directed against it has a high haemagglutination inhibition titre. On the basis of experiments with inhibitors and glycosidases it was deduced that G1 and G2 have both N-linked and O-linked oligosaccharides. The putative fusion protein-equivalent of PVM was shown to possess N-linked oligosaccharides. In the presence of tunicamycin a high mobility form (F1t) appeared to be derived from a precursor (F0t) with the same mobility as the fully glycosylated protein. If by analogy with other paramyxoviruses this represents a cleavage event, the difference in mobility of the precursor and product suggests that the putative F2 product is smaller than the corresponding F2 protein of other paramyxoviruses. However, no F2 candidate protein was detected and evidence for an F1,2 dimer was inconclusive. The glycoproteins of PVM resemble those of respiratory syncytial virus in terms of their pattern of glycosylation, but differ in their processing.

INTRODUCTION

Pneumonia virus of mice (PVM) is classified along with the human and bovine respiratory syncytial (RS) viruses and turkey rhinotracheitis (TRT) virus in the genus Pneumovirus of the family Paramyxoviridae. The accompanying paper (Ling & Pringle, 1989) describes the polypeptides identified in PVM-infected cells. The kinetics of synthesis and nature of the oligosaccharides of RS virus glycoproteins have been described in some detail by various authors (e.g. Fernie et al., 1985; Gruber & Levine, 1985a, b).

This paper describes the characterization of the oligosaccharides of the corresponding glycoproteins of PVM using tunicamycin to inhibit N-linked glycosylation and glycosidases to remove specifically N-linked and O-linked oligosaccharides. The analysis of glycoprotein processing by pulse-chase experiments in combination with immunoprecipitation by an anti-glycoprotein (anti-G) monoclonal antibody (MAb) or polyclonal anti-PVM serum in the presence or absence of tunicamycin is also described.

METHODS

Propagation of cells and viruses. The cells and virus strains used and the methods used for their growth are as described in the preceding paper (Ling & Pringle, 1989).

MAbs. The anti-G MAb 19/1/C9 is described in the preceding paper (Ling & Pringle, 1989). The anti-RS virus fusion (F) protein MAb 43.1 was kindly provided by Dr B. Fernie.

Radiolabelling of cells and immunoprecipitation. PVM-infected cells were labelled with [35S]methionine or [3H]glucosamine as described previously (Ling & Pringle, 1989). For immunoprecipitation of polypeptides from the surface of infected cells, labelled cells were incubated on ice with a 1/5 dilution of serum in phosphate-buffered
saline (PBS) for 30 min. The cells were then washed five times with PBS containing 1 mM-phenylmethylsulphonyl fluoride and lysed either in high salt RIP buffer (Ling & Pringle, 1989) or in low salt RIP buffer (Ling & Pringle, 1988).

**Tryptic peptide mapping.** Polypeptides labelled with $[^{35}S]$methionine (500 $\mu$Ci/ml) and $[^3H]$leucine (750 $\mu$Ci/ml) were excised from dried gels using autoradiographs as templates. Tryptic peptide mapping was carried out as described by Elder et al. (1977). The bands were washed four times for 30 min each with 25% propan-2-ol and left overnight in 10% methanol. The gel slices were lyophilized and incubated for 24 h at 37 °C in ammonium hydrogen carbonate buffer (0.05 M at pH 8) containing trypsin (50 $\mu$g/ml). The supernatant was lyophilized and dissolved in thin-layer electrophoresis buffer (1.5% acetic acid, 0.5% formic acid).

**Digestion of glycoproteins with glycopeptidase F and endo-$\alpha$-N-acetylgalactosaminidase.** Polypeptides labelled with $[^3H]$glucosamine or $[^{35}S]$methionine were excised from gels and eluted electrophoretically through stacking gels in 10 × 0.5 cm tubes sealed at the bottom with dialysis tubing. The gel slices were rehydrated in sample buffer (Laemmli, 1970) before elution. When the bromophenol blue entered the dialysis tubing the tubing was tied off at the top and the samples were dialysed against five changes of ammonium hydrogen carbonate (0.1 M). Samples to be digested with glycopeptidase F were incubated for 17 h at 37 °C with 20 $\mu$l glycopeptidase F (60 units/ml in 20 mM-EDTA, 2% 2-mercaptoethanol, 0.5% NP40, 0.1% SDS, 200 mM-phosphate buffer pH 8.5).

Digestion with endo-$\alpha$-N-acetylgalactosaminidase (Boehringer) was carried out for 17 h at 37 °C using 1 $\mu$l each of endo-$\alpha$-N-acetylgalactosaminidase, neuraminidase (1 unit/ml) and mixed glycosidases from *Turbo cornutus* (0.1 mg/ml). The enzymes were diluted to 10 $\mu$l with 20 mM-phosphate buffer pH 6.0.

Laemmli sample buffer (20 $\mu$l) was added to the samples which were then boiled for 2 min before analysis by SDS–PAGE.

**Synthesis of PVM polypeptides in the presence of tunicamycin.** PVM-infected cells were incubated in the presence of tunicamycin (2.5 $\mu$g/ml) at all stages following adsorption of the virus up to harvesting of the cells and supernatant samples. This concentration inhibited more than 95% of the virus yield and less than 50% of $[^{35}S]$methionine incorporation.

**Haemagglutination inhibition assay.** PVM was diluted so that a further eight-fold dilution would just cause haemagglutination of murine red blood cells. Equal volumes (50 $\mu$l) of this suspension were added to twofold antibody dilutions and 10 $\mu$l of murine erythrocytes (10$^9$ cells) was added to each dilution. The mixtures were incubated for 2 h at room temperature and the dilution of antibody that just inhibited haemagglutination was taken as the haemagglutination inhibition titre.

**RESULTS**

**Synthesis of the G1/G2 protein of PVM**

**Evidence of a relationship between the G1 and G2 glycoproteins**

The G1 and G2 glycoproteins of PVM were coprecipitated by MAb 19/1/C9 (Ling & Pringle, 1989). However, the N and/or 39K protein(s) were also sometimes coprecipitated by this antibody and the antibody did not bind in Western blots. Tryptic peptide mapping was therefore carried out to confirm a relationship between G1 and G2. The peptides showed a similar pattern (Fig. 1) and it was therefore concluded that the G1 and G2 polypeptides were related in some way.

The N and/or 39K polypeptides were the most heavily labelled polypeptides in infected cells which may explain their frequent appearance in immunoprecipitates of poorly labelled polypeptides. In addition, more non-specific binding of these polypeptides to immunoglobulin appeared to occur under the high salt conditions used in many of the experiments (e.g. compare Fig. 7a low salt buffer and Fig. 7b, high salt buffer).

G1 is a precursor to G2 but is not the primary translation product

Radioimmunoprecipitation using MAb 19/1/C9 was used to study the synthesis of the G1 and G2 proteins after a short pulse label followed by incubation in medium containing no radiolabel (Fig. 2). G1 was not detected in abundance until after 60 min of the chase period and G2 was not detected until after 360 min of the chase period. The appearance of G2 was accompanied by a reduction in the intensity of the G1 band so it appears that G2 is derived from G1. No precursor to G1 was clearly identified. The region of the gel corresponding to polypeptides with estimated $M_r$s of 40K to 45K was masked by coprecipitating N and/or 39K protein so that a precursor to the G protein migrating in this region of the gel would have been obscured. None of the other
Fig. 1. PVM glycoproteins labelled with [\textsuperscript{35}S]methionine and [\textsuperscript{3}H]leucine were digested with trypsin and the peptides separated by two-dimensional thin layer electrophoresis/chromatography. Electrophoresis was carried out towards the cathode (on the right) and chromatography was carried out with the solvent front moving upwards. (a) Peptides of G\textsubscript{1}, (b) peptides of G\textsubscript{2}, (c) peptides of G\textsubscript{1} and G\textsubscript{2} on the same chromatograph.

polypeptides observed in Fig. 2 was regularly precipitated by the 19/1/C9 MAb and therefore they were not considered to be specifically precipitated.

**Forms of the PVM G glycoprotein synthesized in the presence of tunicamycin**

Polypeptides synthesized in tunicamycin-treated, PVM-infected cells and those released into the supernatant were detected by radioimmunoprecipitation. The result of [\textsuperscript{3}H]glucosamine labelling and immunoprecipitation with murine anti-PVM antibody is shown in Fig. 3. The G\textsubscript{1} and G\textsubscript{2} polypeptides were not detected in tunicamycin-treated cells and two different polypeptides (G\textsubscript{1t} and G\textsubscript{2t}) were observed. G\textsubscript{2t} could be detected in cell lysates only when a long exposure was used (Fig. 3). The G-related 42K polypeptide precipitated from [\textsuperscript{3}H]glucosamine-labelled samples would have comigrated with the leading edge of G\textsubscript{2t} so it is not clear whether it was present in tunicamycin-treated cells. The G\textsubscript{1t} and G\textsubscript{2t} polypeptides were also immunoprecipitated from polyethylene glycol (PEG) precipitates of the supernatants of tunicamycin-treated [\textsuperscript{35}S]methionine-labelled cells using MAb 19/1/C9 (data not shown).

The broad smear of label centred around 31K observed on prolonged exposure of the gels was not observed with tunicamycin-treated cells (Fig. 3). The nature of the 31K material is not known since no amino acid-labelled protein can be associated with it, but this result suggests that it has N-linked carbohydrate.

Removal of N-linked oligosaccharides from G\textsubscript{1} and G\textsubscript{2} using glycopeptidase F gave polypeptides with the same mobilities as G\textsubscript{1t} and G\textsubscript{2t} respectively (Fig. 4). G\textsubscript{2t} was not susceptible to digestion by glycopeptidase F confirming the absence of N-linked oligosaccharides from this polypeptide. G\textsubscript{1t} also appeared to be resistant to digestion by glycopeptidase F (data not shown). The nature of the higher mobility bands observed in each lane in Fig. 4 is not known.

**N-linked oligosaccharides are not required for further processing of the PVM G protein**

Addition of non-N-linked sugars to the G protein of PVM appears to occur since G\textsubscript{1t} and G\textsubscript{2t} were labelled with [\textsuperscript{3}H]glucosamine as described in the previous section. In order to determine whether the kinetics of synthesis of G\textsubscript{1t} and G\textsubscript{2t} are similar to those of G\textsubscript{1} and G\textsubscript{2} a pulse–chase experiment with tunicamycin was carried out. Fig. 5 shows that the two forms of the G protein did appear to be processed normally, showing that N-linked oligosaccharides were not required for this processing.
Evidence for the presence of O-linked oligosaccharides on the PVM G1 and G2 proteins

Monensin has been widely used as an inhibitor of O-linked glycosylation (e.g. Gruber & Levine, 1985a) but proved unsatisfactory for studies with PVM glycoproteins since it either did not affect or completely inhibited G-related polypeptide synthesis. G1, G2, G1t and G2t were digested with endo-α-N-acetylgalactosaminidase to identify O-linked oligosaccharides. This enzyme will only remove unsubstituted disaccharides so exoglycosidases were also included in the digestion mixtures. The glycoproteins digested with the exoglycosidases alone are shown in Fig. 6 as controls. It can be seen that each polypeptide showed a higher mobility when digested with endo-α-N-acetylgalactosaminidase and exoglycosidases than when digested with exoglycosidases alone. Digestion products are indicated by open circles, unmarked bands are breakdown products observed even in the absence of the enzymes. The estimated Mr’s of the unglycosylated polypeptides are given in Table 1.
Evidence that the G1/G2 protein of PVM is the haemagglutinin

Antibodies 26/1/A2, 26/1/E11 and 26/4/C4 directed against the N or 39K, M, and 39K proteins, respectively along with three additional anti-PVM MAbs of unknown specificity did not inhibit haemagglutination (titre <20). In contrast the anti-G1/G2 MAb 19/1/C9 strongly inhibited haemagglutination (titre 20480). This result indicates that the G1/G2 protein is the viral haemagglutinin.

Synthesis of the F1 protein of PVM

Synthesis of the F1 protein in the presence of tunicamycin

The F1 protein described here has a mobility similar to that of the F1 cleavage product of the fusion proteins of other paramyxoviruses and like them is glycosylated and expressed at the cell surface. The protein designated F1 was the major polypeptide detected on the surface of
Fig. 4. [35S]Methionine-labelled PVM polypeptides digested with glycopeptidase F (lanes 2) or undigested (lanes 1). Positions of the $M_r$ markers are shown on the right. The circles represent the digested or undigested polypeptides.

Table 1. Calculated $M_r$ of G glycoproteins without N-linked and/or O-linked carbohydrate

<table>
<thead>
<tr>
<th>Protein</th>
<th>Undigested protein ($M_r$)</th>
<th>N-linked carbohydrate</th>
<th>O-linked carbohydrate</th>
<th>N- and O-linked carbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>76.4K</td>
<td>58.4K</td>
<td>57.6K</td>
<td>39.6K</td>
</tr>
<tr>
<td>G2</td>
<td>62.0K</td>
<td>48.2K</td>
<td>51K–58.6K*</td>
<td>37.2K–44.8K*</td>
</tr>
<tr>
<td>G1t</td>
<td>60.9K</td>
<td>60.9K</td>
<td>44.3K</td>
<td>44.3K</td>
</tr>
<tr>
<td>G2t</td>
<td>49.8K</td>
<td>49.8K</td>
<td>41.3K</td>
<td>41.3K</td>
</tr>
</tbody>
</table>

*The mobility difference due to O-linked oligosaccharides could not be determined precisely because the exoglycosidases used with the endo-N-acetylgalactosaminidase would have removed residues from both N-linked and O-linked oligosaccharides. The limits assume that all of the carbohydrate removed by exoglycosidases was N-linked or it was all O-linked.

[35S]Methionine-labelled PVM-infected cells and this aided its identification (Fig. 7a). The 12K polypeptide also appeared to be expressed on the cell surface, but was not considered to be an F2-like protein because it appeared after a chase period when no change in the mobility of the F1 protein was observed (Ling & Pringle, 1989).

The RS virus F1 protein together with polypeptides with $M_r$ values of 22K and 12K were expressed on the surface of RS virus-infected cells. The 22K protein may represent a breakdown product of F1 (Norrby et al., 1986) or the 22K non-glycosylated membrane protein (Routledge et
Fig. 5. Pulse-chase experiment carried out as in Fig. 2 but with tunicamycin in the medium. PVM polypeptides were immunoprecipitated with MAb 19/1/C9 following a 10 min pulse (lanes 1 and 2) and various chase periods (20 min, lanes 3; 60 min, lane 4; 360 min, lane 5; 22 h, lanes 6 and 7). Samples immunoprecipitated from cell lysates (CL) and polypeptides immunoprecipitated from PEG precipitates of culture supernatants (PEG) are shown. Lanes 1 and 7 show polypeptides precipitated from mock-infected culture samples at 10 min and 22 h of the chase period, respectively. Positions of M. markers are shown in the centre.

The 22K protein is not F2 because the F2 protein is most readily identified by [3H]glucosamine labelling and has an estimated Mr of 18K in our gel system.

The PVM N and/or 39K polypeptide was also observed in some immunoprecipitations but this was found to be due to non-specific precipitation when high salt buffers were used (compare the result in Fig. 7a obtained using low salt conditions with that in Fig. 7b obtained using high salt conditions). Fig. 7(b) shows the polypeptides observed on the surface of PVM-infected cells labelled in the presence and absence of tunicamycin. It can be seen that a polypeptide designated Flt was expressed on the surface of PVM-infected cells labelled with [35S]methionine in the presence of tunicamycin and that the F1 polypeptide was absent.

Flt is derived from a precursor

A pulse–chase experiment was carried out using tunicamycin (Fig. 8). It appears that Flt was derived from a precursor (F0t) since these bands have similar intensities and Flt appears as F0t disappears. There are no other polypeptides recognized by the antiserum that could be precursors for Flt or account for the disappearance of label from F0t. F0t may represent an unglycosylated form of an F0 protein that is cleaved to give Flt and an unidentified F2t protein. However, since no F0 protein has been identified following pulse-labelling in the absence of
tunicamycin, it must be assumed if this interpretation is correct that cleavage of the glycosylated form occurs much more rapidly.

Since F0t and F1 have similar mobilities, an alternative explanation might be that F0t represents the unglycosylated form of F1 as no mobility change was observed on digestion of F1 with glycopeptidase F (Fig. 4). This unglycosylated polypeptide would then undergo some modification resulting in a polypeptide (F1t) with an increased mobility.

In Fig. 8 the 12K polypeptide (and possibly the 20K polypeptide) show increased label incorporation during the chase periods. However, this does not correlate with the shift from F0t to F1t. The 12K polypeptide shows a similar increase in label incorporation during chase periods in the absence of tunicamycin when there is no change in the mobility of the F1 protein. The 12K protein therefore does not behave as an F2-like protein.
**PVM glycoproteins**

Fig. 7. (a) Polypeptides immunoprecipitated from the surface of PVM- (lane 1), RS virus- (lane 2) or mock-infected cells (lane 3) with homologous antisera under low salt conditions. (b) PVM polypeptides precipitated from the surface of PVM-infected cells using anti-PVM serum (lanes 1 and 2) or without antibody (lanes 3 and 4). Cells were labelled in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of tunicamycin and the immunoprecipitations were carried out under high salt conditions.

Fig. 8. Pulse–chase experiment carried out in the presence of tunicamycin using anti-PVM serum to precipitate PVM polypeptides from infected cell lysates. Cells were pulse-labelled with [35S]methionine for 10 min (lane 1) or labelled and then chased for various periods of time (20 min, lane 2; 60 min, lane 3; 360 min, lane 4; 22 h, lane 5).

**F1-related polypeptides identified under non-reducing conditions**

The behaviour of F1-related proteins under non-reducing conditions has been studied to identify a homologue of the F1,2 polypeptide of other paramyxoviruses. This consists of the disulphide-bonded F1 and F2 proteins and has a similar mobility to the uncleaved F0 protein. [3H]Glucosamine labelling and immunoprecipitation of polypeptides expressed on the cell surface were used to identify the F-related proteins.
The RS virus F protein was detected mainly as F1 and F2 under reducing conditions (Fig. 9a). A small amount of F0 was precipitated from RS virus-infected cell lysates. Under non-reducing conditions most of the label was present in F1,2 with some in higher mobility bands.

Under non-reducing conditions the PVM F1 protein band was slightly reduced in intensity (Fig. 9a and b). An additional band (F1,2) is observed under non-reducing conditions, which may represent F1 disulphide-bonded to an F2 protein. If this interpretation is correct the F2 protein would have an estimated Mr of only about 5000. However, F1,2 could represent a form of F1 with different internal disulphide bonding.

**DISCUSSION**

The glycoproteins of PVM appear to differ from those of other paramyxoviruses and resemble those of the pneumovirus RS virus in terms of their pattern of glycosylation. They differ, however, in terms of their processing. The large G1 and G2 glycoproteins of PVM have both N-linked and O-linked oligosaccharides like the G protein of RS virus, although the proportion of O-linked oligosaccharides may be lower (Table 1). Unlike RS virus but like the other paramyxoviruses, PVM has haemagglutinating activity with murine erythrocytes and the G1/G2 glycoprotein appears to be the haemagglutinin since a MAb to this protein inhibits haemagglutination. The antibody does not neutralize PVM so if the large glycoprotein of PVM is the attachment protein as is the case for other paramyxoviruses, attachment to productively
infected cells may involve a site on the protein different to that involved in attachment to erythrocytes.

The kinetics of synthesis of the G1/G2 protein appear to differ from those of all the other paramyxoviruses that have been described. Differences between our results and those reported for RS virus do not appear to be due to different experimental systems since the kinetics of synthesis of the RS virus G protein under our conditions are similar to those reported by Fernie et al. (1985) and Gruber & Levine (1985) (C. Caravokyri, personal communication).

There are two forms of the PVM G glycoprotein, G2 probably being derived from the lower mobility G1 polypeptide. This conversion appears to be a late event in processing, since G2 was not detected until several hours after pulse-labelling. Alternatively G1 and G2 may arise independently, G1 appearing to decrease in intensity due to competition with G2 for the antibody used to immunoprecipitate the samples. G1 may be derived from a precursor that has not been identified possibly due to failure of antibodies to bind to it, since a change in the reactivity with MAbs of the HN glycoprotein of mumps virus, Newcastle disease virus and Sendai virus has been observed during HN maturation (Mottet et al., 1986; Nishikawa et al., 1986; Yamada et al., 1988). G2 may have a lesser degree of O-linked glycosylation than G1. The RS virus G protein exists in two forms but the smaller form is soluble (Hendricks et al., 1988) whereas G1 and G2 of PVM are virion proteins (Cash et al., 1979).

It is not clear whether the PVM F protein is processed in the same way as that of other paramyxoviruses. The results of pulse–chase experiments and experiments carried out under reducing and non-reducing conditions may be interpreted in two ways. There may be an F0 protein that is cleaved too rapidly to allow its detection in pulse–chase experiments except when glycosylation is inhibited by tunicamycin (Fig. 8). By analogy with other paramyxoviruses F0 would be the same size as the F1,2 protein detected under non-reducing conditions, the latter would comprise the disulphide-bonded F1 and F2 proteins. No F2 protein has been detected, but since it would have an $M_r$ of only 5000 its detection would be difficult. Alternatively F1 may be the only F-related protein synthesized in infected cells and may be unstable in the absence of N-linked glycosylation. The two forms of F1 observed under non-reducing conditions (Fig. 9) could be due to different arrangements of intramolecular disulphide bonds. The function of the F1 protein is unknown since syncytium formation is rarely observed during infection of cell monolayers with PVM, although it is possible that fusion of infected cells occurs in vivo.

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