Characterization of the high $M_r$ glycoprotein (gP300) of equine herpesvirus type 1 as a novel glycoprotein with extensive $O$-linked carbohydrate

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The high $M_r$ glycoprotein (gp300) of equine herpesvirus type 1 was found to have an $M_r$, estimated by SDS–PAGE, of over 400,000 and was confirmed as being a surface glycoprotein by $^{125}$I-labelling. In contrast to $[^3H]$glucosamine, gp300 showed very low levels of $[^3H]$mannose incorporation. The $M_r$ of gp300 showed no detectable change upon treatment of purified virus with $N$-glycanase, and showed only a small change in virus-infected cells treated with tunicamycin. In addition, gp300 failed to bind the lectin concanavalin A. Taken together, these results indicate a lack of $N$-linked carbohydrate on gp300. The major carbohydrate species were found to be composed primarily of $O$-linked chains, as indicated by the sensitivity of the protein to monensin, to exoglycanase enzymes specific for sugars present in $O$-linked chains and to mild alkaline borohydride treatment, which revealed three species of carbohydrate of $M_r$ of $>10,000$, 2400 and 1100, respectively. Neuraminidase treatment and binding of Helix pomatia lectin indicated the presence of $\alpha$-N-acetylglucosamine and sialic acid as terminal sugars. Immunological cross-reactivity of gp300 with a high $M_r$ protein of equine herpesvirus type 4 was shown and it also exhibited a marked $M_r$ variation in the vaccine strain Rhinomune.

Introduction

Structural polypeptides of equine herpesvirus type 1 (EHV-1) have been identified by various groups (Aboodeely et al., 1971; Kemp et al., 1974; Perdue et al., 1974; Turtinen & Allen, 1982; Meredith et al., 1989) and up to 13 of these have been considered to be glycoproteins. Although these groups used different virus strains and differing electrophoresis conditions, there is a reasonable consensus of opinion regarding the presence of a glycoprotein in the virion with an $M_r$ in excess of 200,000, termed either gp1, gp2, gp1/2 or gp300. The high $M_r$ of this glycoprotein has led to the suggestion that it might represent a mucopolysaccharide (Killington et al., 1977).

The closely related EHV-4 also has a high $M_r$ glycoprotein of over 200K, termed either gp1, gp2 or gp260 (Allen & Bryans, 1986; Meredith et al., 1989). The only other herpesviruses with characterized glycoproteins of $M_r$ greater than 200,000 are Epstein–Barr virus (EBV), with the glycoprotein gp350/220 (Edson & Thorley-Lawson, 1983), and infectious laryngotracheitis virus, with a protein of $M_r$ 205000 (York et al., 1987).

The gene for gp2 has been mapped to approximately 0·3 map units in the U1 region of the EHV-1 genome (Allen & Yeargen, 1987), a coordinate which does not correspond with that of any other identified glycoprotein in the alphaherpesvirinae. Monoclonal antibodies reactive with the gp300 region have been described (Sinclair et al., 1989; Edington et al., 1987; Allen & Yeargen, 1987). None of these antibodies neutralize virus infectivity but some have been shown to restrict plaque size, thus implicating gp300 in cell to cell spread (Edington et al., 1987).

The aims of the experiments reported here were to characterize gp300.

Methods

Cell culture, virus strains and virus purification. EHV-1 (strains Ab-1, Rac-H and Rhinomune) and EHV-4 (strain MD) were cultivated in either RK-13 cells or NBL-6 cells and purified from the extracellular growth medium using standard techniques (Meredith et al., 1989). The EHV-1 strain Rhinomune was obtained from Smith Kline Animal Health. All experiments were performed with strain Ab-1 unless otherwise indicated.

Preparation of radiolabelled infected cell extracts and purified virus. Virus preparations labelled with either $[^3H]$mannose (2 Ci/ml) or $[^3H]$glucosamine (2 Cu/ml) were prepared by labelling cell monolayers infected with 0·1 p.f.u./cell from 8 to 26 h post-infection (p.i.), in medium containing one-tenth the normal level of glucose. Infected cell extracts for immune precipitation were prepared by infection with 5 p.f.u./cell and subsequent labelling from 8 to 21 h p.i. with either 10 Cu/ml $[^35]$S) methionine (>600
et al., prepared using purified EHV-1 as an immunogen by the method of
containing one-tenth the normal level of glucose. Infected cell extracts
were incubated for 2 h at 0 °C and terminated by the addition of 100 μL 0.2 M-
glycine. All isolates were obtained from Amersham.

Monoclonal antibodies. Monoclonal antibodies P19 and βA4A1 were prepared using purified EHV-1 as an immunogen by the method of Killington et al. (1981) and isolated as described previously (Meredith et al., 1989).

SDS–PAGE and Western blotting. Protein samples were analysed on gels cross-linked with N,N'-diallyltartardiamide with a final concentration of 9% acrylamide (Heine et al., 1974). x2-Macroglubulin (340K non-reduced, 170K reduced), phosphorylase b (97K), glutamate dehydrogenase (55K), lactate dehydrogenase (36K) and soy bean trypsin inhibitor (20K) (Boehringer Mannheim) were used as Mr markers. Protein was transferred to nitrocellulose by standard techniques (Towbin et al., 1979) and antibody binding revealed by peroxidase-conjugated sheep anti-mouse antibody reacted with 4-
galactoside galactohydrolase, EC 3.2.1.22; Boehringer Mannheim) and incubated at 37 °C for 16 h. Fluorography was by the method of Laskey & Mills (1975).

Immune precipitation. Soluble infected cell extract was prepared by treatment of infected cells with RIPA buffer (0.1% SDS, 0.1% sodium deoxycholate, 0.5% Nonidet P40, 0.1 M–NaCl, 50 mM-Tris–HCl pH 7.4). Protein A-Sepharose suspension (10 μL, Pharmacia) was incubated with 10 μL rabbit anti-mouse immunoglobulin (Dako) for 30 min at room temperature. This was washed three times with phosphate-buffered saline (PBS) and incubated with 10 μL of monoclonal antibody ascitic fluid for 1 h at room temperature, followed by three washes with PBS. Antigen was added and incubated at 4 °C for 16 h with shaking, followed by five washes with RIPA buffer. Bound material was then eluted with SDS–PAGE sample buffer at 100 °C for 5 min prior to electrophoresis.

Treatment with glycosylation inhibitors. Confluent monolayers of RK
were infected with 10 p.f.u./cell EHV-1, or were mock-infected. Cells were treated with 2 μg/ml tunicamycin (Sigma) or 0.2 μM
monensin (Sigma) from the end of the adsorption period (1 h) until 21 h p.i., or remained untreated.

Treatment with glycosidases
(i) N-glycanase. Purified EHV-1 (10 μL) was treated with 10 μL 1%
(w/v) Triton X-100 at 0 °C for 10 min and centrifuged at 10 000 g for 10
min. The supernatant was treated with 15 μL 0.2 M–sodium phosphate
buffer pH 8-6, 3 μL 100 mm-1,10-phenanthroline and 10 units (U)/ml N-
glycanase [peptide-N-acetyl-β-glucosaminidase] (Genzyme) and incubated at 37 °C for 16 h.
(ii) Neuraminidase. Purified EHV-1 was treated with N-glycanase as above and adjusted to pH 5 with 0.1 M–citric acid. Neuraminidase (acylneuraminyl hydrolase, EC 2.2.1.18) Type V; Sigma (0.2 U) was added and incubated at 37 °C for 4 h.
(iii) Exoglycanases. Purified EHV-1 was digested with N-glycanase followed by neuraminidase as above. β-N-Acetylhexosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucopyranosidase, EC 3.2.1.20; Sigma) (0.01 U), 0.01 U α-D-fucosidase (α-D-fucosidase fucohydrolase, EC 3.2.1.51; Sigma) and 0.01 U β-D-galactosidase (β-D-galactosidase galactohydrolase, EC 3.2.1.22; Boehringer Mannheim) were added and incubated at 25 °C for 4 h.

Lectin binding analysis. This was essentially by the method of Jackson & Tijan (1988). Briefly, 10 μl purified EHV-1 was separated by SDS–PAGE followed by Western blotting. The nitrocellulose was blocked for 1 h in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 3
mg/ml non-fat dried milk. Biotin-labelled lectins (Sigma) were added at 10 μg/ml for 4 h, followed by streptavidin-peroxidase conjugate (Sigma) for 1 h and colour was developed with chloronaphthol/H2O2.

Alkaline borohydride treatment. Immune-precipitated material was eluted from the Sepharose beads with 1% SDS, 50 mM-Tris–HCl pH 7.4 at 100 °C for 5 min, followed by dialysis against 20 mM-HEPES, 0.1 M–NaCl pH 6-8, incubation with 0.05 mg/ml Bt12, M 1350 (Bio-Rad). Further analysis of low M1 components was carried out using a column of Bio-Gel P10 (Bio-Rad) equilibrated in 10 mM-HEPES, 0.1 M–NaCl pH 6-8, and 0.5 ml fractions were collected. Markers were blue dextran, M 200000, cytochrome c, M 12400, aprotinin, M 6500 and vitamin B12, M 1350 (Sigma).

Results

Radioactive labelling of gp300
(i) Carbohydrate labelling. EHV-1 was purified from infected cells labelled with [3H]glucosamine and [3H]mannose and analysed by SDS–PAGE followed by fluorography. Fig. 1 shows that, overall, incorporation of mannose was lower than that of glucosamine, but this was particularly significant for gp300. In the region of gp300, two weak bands can be seen which may indicate two forms of this protein which have a very low, but measurable, mannose content.

(ii) Protein labelling. Purified EHV-1 was labelled with 125I-Bolton and Hunter reagent to reveal surface components, then subjected to SDS–PAGE followed by autoradiography (Fig. 1b). The iodinated bands seen correspond to the major [125I]glucosamine-labelled glycoproteins described previously (Meredith et al., 1989). This confirms that gp300, along with the other major glycoproteins, is a surface component of the virion.

Immune precipitation of gp300

To identify polypeptides in EHV-1-infected cells that are immune-precipitated with monoclonal antibody P19, [35S]methionine- and [14C]glucosamine-labelled infected cells were subjected to immune precipitation (Fig. 2). A single heavily labelled band of M1 > 300000 was visible when labelled with glucosamine. The mobility of this species remained constant whether reducing or non-reducing conditions were used for sample preparation.
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When [35S]methionine was used for labelling, only a very weak band of $M_r > 300000$ was visible. This antibody did not precipitate any protein from extracts prepared from mock-infected cells, and non-immune ascitic fluid does not react with gP300 under any conditions (data not shown).

**Determination of the $M_r$ of gp300 using low percentage acrylamide gels**

To attempt to determine an accurate $M_r$ for gp300, EHV-1 structural proteins were separated by electrophoresis on a 6% acrylamide gel, transferred onto nitrocellulose and gp300 was located using monoclonal antibody P19 (Fig. 3). A single diffuse species that migrated slower than the marker of $M_r$ 340000 was revealed. A precise measurement of $M_r$ by SDS-PAGE was not possible by this technique as markers with $M_r$s above 340000 are not commercially available. Although the position of gp300 would suggest a true $M_r$ in excess of 400000, the nomenclature of gp300 used previously has been maintained (Meredith et al., 1989).

**Precursor forms of gp300 synthesized in infected cells treated with glycosylation inhibitors**

To study the type of glycoprotein present, and to attempt to identify precursor forms of gp300, cultures of EHV-1-infected cells were treated with either tunicamycin or monensin, or remained untreated. These extracts were solubilized, analysed by SDS-PAGE followed by Western blotting and the location of gp300 was revealed using monoclonal antibody P19 (Fig. 4). Treatment with tunicamycin produced an alteration in the mobility of immunoreactive material with a distinct band with an $M_r$ of approximately 250000, in contrast to an apparent $M_r$ of approximately 300000 for untreated gp300 in this gel system.
Treatment with monensin resulted in a marked reduction in the amount of gp300 seen upon Western blotting, indicating a significant loss of immunoreactivity.

Treatment of EHV-1 with endoglycanase and exoglycanase enzymes

Purified EHV-1 was treated with glycanase enzymes, analysed by SDS–PAGE followed by Western blotting, and the location of gp300 revealed using monoclonal antibody P19 (Fig. 5). Treatment with N-glycanase failed to produce any change in the $M_r$ of gp300. Treatment with neuraminidase gave a species of gp300 with lowered mobility on the gel. Unfortunately, an accurate $M_r$ could not be assigned, as markers of such high $M_r$ are not commercially available. Treatment with the exoglycanases $\beta$-$N$-acetylhexosaminidase, $\alpha$-fucosidase and $\beta$-galactosidase resulted in a complete lack of reactivity of gp300 in a high $M_r$ form and the appearance of a new species with an $M_r$ of 67000.

As a control, samples from each digest were also probed with a monoclonal antibody against gp88. The $N$-glycanase reaction produced a mobility shift indicative of the presence of significant levels of $N$-linked carbohydrate on this protein (data not shown). Samples of purified herpes simplex virus type 1 (HSV-1) were also treated with the glycanases listed above and probed with a monospecific antibody raised against VP14, a tegument protein of HSV-1. No degradation of protein was observed with any enzyme (data not shown).

Lectin binding specificities of EHV-1 glycoproteins

To study the types of sugar residue present on gp300, purified EHV-1 was analysed by SDS–PAGE followed
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(a) Western blot of a 5 to 15% acrylamide gel of purified EHV-1, probed with monoclonal antibody P19, treated with neuraminidase (lane 2), with N-glycanase (lane 3) or left untreated (lane 1). The position of immunoreactive species is indicated (<>). (b) Western blot of a 9% acrylamide gel of purified EHV-1, probed with monoclonal antibody P19, treated with the exoglycanases β-N-acetylhexosaminidase, α-fucosidase and β-galactosidase (lane 2), or left untreated (lane 1). The position of immunoreactive species (<>) and M_r s are indicated.

by Western blotting and binding of lectins. Lectin from Helix pomatia specifically bound to gp300 alone. Competition with this binding was seen in co-incubation with either N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc) (Fig. 6a). Neither wheatgerm agglutinin nor concanavalin A showed any binding to gp300, whereas other components of EHV-1 did bind these lectins (Fig. 6b). Neither Dolichos biflorus lectin or Glycine max lectin bound any structural component of EHV-1 (data not shown).

Size analysis of carbohydrate chains present on gp300

As monoclonal antibody P19 had been shown to immune precipitate only a single glycosylated species, corresponding to gp300 (Fig. 2), this technique was used to isolate the protein from [3H]glucosamine-labelled infected cells. Carbohydrate released from gp300 by treatment with mild alkaline borohydride, together with an untreated control, were analysed by gel filtration on a Bio-Gel TSK 250 HPLC column (Fig. 7a, b). Untreated material eluted with a single peak with an M_r of 360000, whereas alkaline borohydride-treated material eluted with a main peak in the region of M_r 1000 to 1500.

To examine further this low M_r carbohydrate, a duplicate sample of alkaline borohydride-treated gp300 was analysed on Bio-Gel P10 (Fig. 7c). Three major peaks of radioactivity were seen. Peak 1 corresponds to the void volume of the column (M_r >100000), peak 2 corresponds to an M_r of 2400 and peak 3 to an M_r of 1100.

Antigenic relationships between high M_r glycoproteins of EHV-1 and EHV-4

Purified EHV-1 and EHV-4 were separated by SDS-PAGE, followed by Western blotting with a variety of
monoclonal antibodies to gp300. Certain monoclonal antibodies raised against EHV-1 or EHV-4 showed immunological cross reactivity between gp300 of EHV-1 and gp260 of EHV-4. Monoclonal antibody βA4A1 typifies this reaction (Fig. 8). This antibody reacts with a single species of $M_r > 300000$ in EHV-1 and a single species with an $M_r$ of approximately 260000 in EHV-4. Monoclonal antibody P19 reacted only with EHV-1 on Western blotting (data not shown). To date no immunological cross-reactivity between gp300 and any structural protein of HSV-1 or pseudorabies virus has been detected by Western blotting with any monoclonal antibody available in this laboratory (data not shown).

Figure 7. (a and b) HPLC gel filtration of [3H]glucosamine-labelled gp300 treated with (a) mild alkaline borohydride or (b) left untreated. (c) Bio-Gel P10 gel filtration of mild alkaline borohydride-treated gp300 labelled with [3H]glucosamine. $M_r$s are indicated ($\times 10^3$).

Figure 8. Western blot of purified EHV-1 (lane 1) and purified EHV-4 (lane 2) probed with monoclonal antibody βA4A1. The position of immunoreactive species (<) and $M_r$s are indicated.

**$M_r$ variation of gp300 between EHV-1 strains**

A range of EHV-1 strains were examined for $M_r$ variability of gp300. One strain in particular showed a marked difference when purified virus was analysed by SDS-PAGE and Western blotting, followed by detection of gp300 using monoclonal antibody P19 (Fig. 9). This strain, Rhinomune, an attenuated vaccine strain highly passaged in tissue culture, is shown in Fig. 9 together with Ab-1 and Rac-H, its parental strain. It can be seen that, in addition to a small amount of gp300 in a slightly lowered $M_r$ form, a large proportion of the protein present in this strain occurs with an $M_r$ of 96000. When monoclonal antibody βA4A1 was used to detect gp300 in Rhinomune, only the high $M_r$ form was visible (data not shown).

**Discussion**

The experiments reported here were designed to characterize further the high $M_r$ glycoprotein (gp300) of EHV-1. Electrophoresis of purified virus in low percentage SDS-PAGE gels produced an $M_r$ estimate of 400000 for
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Fig. 9. Western blot of purified EHV-1 strain Ab-1 (lane 1), Rhinomune (lane 2) and Rac-H (lane 3) probed with monoclonal antibody P19. Positions of immunoreactive species (<~) and Mrs are indicated.

gp300, although this is probably not representative of the true size of the protein as the non-stoichiometric binding of SDS to heavily glycosylated proteins affects their mobility in the gel. Iodination of the protein using Bolton and Hunter reagent confirmed the location of the protein as, at least partly, external to the envelope. The technique failed to label either VP9, the major capsid protein, or VP10, the major tegument protein, which would indicate a lack of disrupted virus in the preparation.

The type of carbohydrate present was of particular interest as early experiments demonstrated that the protein labelled well with glucosamine but poorly with mannose, indicative of a protein that is either low in N-linked carbohydrate or one which undergoes extensive trimming of mannose units prior to maturation.

The processes involved in N-linked glycosylation are well characterized (Kornfield & Kornfield, 1980) and this characterization has been aided by the use of inhibitors such as tunicamycin, which inhibits the addition of core mannose units to protein (Elbein, 1987), and a range of endoglycanase enzymes (Maley et al., 1989). Tunicamycin treatment of EHV-1-infected cells resulted in the formation of small amounts of a form of gp300 with reduced Mr. This material appeared to have incorporated glucosamine (data not shown), which indicates the presence of O-linked sugars (Wenske & Courtney, 1983; Holmes et al., 1981). In conjunction with this result, the apparent resistance of gp300 to N-glycanase, which removes all N-linked carbohydrate, and the failure of gp300 to bind concanavalin A, which binds to internal mannose structures (Goldstein & Hayes, 1978), all point to this protein containing little N-linked carbohydrate in its mature form. The results obtained with tunicamycin-treated cells must be treated with some caution because O-linked carbohydrate is attached to protein in the Golgi region, whereas core N-linked glycosylation occurs in the endoplasmic reticulum. The immunoreactive material detected on tunicamycin treatment may therefore represent abberantly processed gp300 which has escaped a block in transport from the endoplasmic reticulum to the Golgi apparatus.

Processes involved in O-linked glycosylation are poorly understood in comparison with those involved in N-linked glycosylation, and the methods used to study such carbohydrate addition are less specific. The ionophore monensin has been used extensively as an inhibitor of O-glycosylation (Johnson & Spear, 1982; Ledger & Tanzer, 1984) and treatment of EHV-1-infected cells with this agent almost completely abolished the presence of immunoreactive gp300. Under the conditions used, total virus protein synthesis was not significantly affected, but production of infectious virus was reduced by 99% (data not shown). Treatment of EHV-1 with a mixture of exoglycanases specific for sugars present in O-linked carbohydrate produced a shift in the Mr of immunoreactive material from >300,000 to 67,000. This dramatic shift in Mr, and a total loss of high Mr, immunoreactive material, suggests that the core protein of gp300 may have an Mr of 67,000 or less. However, this species cannot be rigorously defined as a precursor at the present time because it cannot be detected either in infected cell extracts or purified virus.

Digestion with O-glycanase, which removes O-linked sugars, has also been used to indicate the presence of O-glycosylated proteins (Lambert, 1988). However, this enzyme requires prior digestion with a range of exoglycanases. Treatment with exoglycanases gave a species with Mr of 67,000, with no subsequent shift in Mr detectable upon subsequent O-glycanase treatment (data not shown).

Another common method of determining the presence of O-linked sugar is treatment with mild alkali in the presence of sodium borohydride (Serafini-Cessi et al., 1985, 1989). When treated by this method gp300 released both short carbohydrate chains Mr 1100 and 2400 and longer chains (Mr >10,000). However, treatment of
proteins under such conditions has also been shown to release N-linked carbohydrate (Rasilo & Renkonen, 1981; Ogata & Lloyd, 1982), so the possibility that some of these chains represent N-linked sugar cannot be excluded.

Further evidence that the carbohydrate present on gp300 is predominantly O-linked is provided by the failure of a range of lectins, with the exception of H. pomatia lectin, to bind to this protein. This lectin has a relatively limited range of reactivity amongst herpesvirus glycoproteins, the only HSV proteins that bind the lectin are gC-1 (Olofsson et al., 1981) and gG-2 (Olofsson et al., 1986). It has a range of sugar specificities with α-GalNAc > α-GlcNAc > β-GalNAc > β-GlcNAc (Hammarstrom et al., 1977), probably as monosaccharides (Hammarstrom & Kabat, 1969). The failure of gp300 to bind to G. max lectin or D. biflorus lectin, both specific for α- and β-GalNAc (Hammarstrom et al., 1977), or to wheatgerm agglutinin, specific for β-GlcNAc (Goldstein & Hayes, 1978), would suggest that gp300 contains α-GlcNAc as a terminal sugar. This terminal α-GlcNAc raised possible problems in assessing the data obtained from the exoglycanase digestions because α-N-acetylgalactosaminidase would be necessary to cleave the terminal sugar. Such an enzyme has been characterized (von Figura, 1977) but is not commercially available. It is present, however, as a contaminant in the β-N-acetylgalactosaminidase used (manufacturer’s data) and so removal of this terminal carbohydrate residue could be occurring, permitting subsequent digestion of the carbohydrate chain.

The observation that neuraminidase treatment leads to an apparent increase in the M, of gp300 is due to an increase in the net positive charge of a protein, resulting in a decreased mobility on SDS-PAGE. This situation is analogous to that reported for gG-2 by Serafini-Cessi et al. (1985). The shift in mobility is indicative of sialic acid being present as terminal carbohydrate residues, along with α-GlcNAc.

Attempts have been made to identify precursor forms of gp300, by both Western blotting and immunoprecipitation, using infected cell extracts prepared at varying times after infection, but we have been unable to detect any precursor forms using the range of monoclonal antibodies available. It is likely that the identification of these forms will be assisted by in vitro translation of EHV-1 mRNA in conjunction with sequencing of the gp300 gene.

The cross-reactivity between EHV-1 gp300 and gp260 of EHV-4 demonstrates that these two proteins are antigenically related, although the majority of monoclonal antibodies to gp300 available in this laboratory appear to be type-specific.

Variability in the M, of gp300 appears to be common amongst EHV-1 strains but was particularly apparent for the Rhinomune strain. The decreased amount of high M, material and the presence of the lower M, species of Rhinomune suggests that this gp300 may be either subject to proteolysis or blocked in carbohydrate processing. Further experiments are in progress to determine the cause of the gp300 alteration, which is of particular interest as Rhinomune has been used as a live vaccine and has reduced pathogenicity (Bass et al., 1973).

Many other herpesvirus glycoproteins have been shown to be partly composed of O-linked carbohydrate, such as HSV-1 gB, gC, gD and gE (Johnson & Spear, 1983; Olofsson et al., 1983; Wenske & Courtney, 1983; Serafini-Cessi et al., 1988), but this is of low abundance in comparison with N-linked carbohydrate. The EBV gp350 has approximately 50% of its carbohydrate present as O-linkages (Serafini-Cessi et al., 1989), but the only characterized alphaherpesvirus glycoprotein in which O-linked carbohydrate predominates is HSV-2 gG (Serafini-Cessi et al., 1985), which has approximately 25 times as much carbohydrate present in the O-linkage form than in the N-linkage form. The EBV gp350 has been shown to have homology to HSV-1 gC (Tanner et al., 1987) but, because a gC homologue in EHV-1 has already been identified (Allen & Coogle, 1988; Guo et al., 1989), a relationship here is unlikely. The HSV-2 gG shows most similarity to gp300 as, in addition to being heavily O-glycosylated, it binds H. pomatia lectin (Olofsson et al., 1986) and incorporates large amounts of glucosamine but relatively little mannose (Marsden et al., 1984). The gene coding for this protein, however, maps to a position of around 0.8 map units in the Us region of the HSV-2 genome (McGeoch et al., 1987), whereas gp300 maps to approximately 0.3 map units in the Ul region of the EHV-1 genome (Allen & Yeargen, 1987), so no obvious homology is present here.

Other extensively O-glycosylated proteins are present in viral and mammalian systems. Coronaviruses, such as mouse hepatitis virus, contain a glycoprotein (E1) which has O-linked carbohydrate only (Niemann & Klenk, 1981; Niemann et al., 1984) and respiratory syncytial virus G protein, which is responsible for attachment to cells, is heavily O-glycosylated (Wertz et al., 1985; Lambert, 1988). Vaccinia virus has a protein with approximately 25% O-linked carbohydrate which is responsible for the haemagglutinating activity of the protein (Shida & Dales, 1981) and the mouse egg ZP3 protein contains O-linked oligosaccharide which accounts for its sperm receptor activity (Florman & Wasserman, 1985). O-linked carbohydrate may, therefore, play essential roles in the biological activities of glycoproteins.

Amongst mammalian systems, proteins characterized with high levels of O-glycosylation are the mucins, which
contain numerous short carbohydrate chains present as 
O-linkages (Harp et al., 1979). The low Mₙ of much of the 
carbohydrate released from gp300, together with the high 
Mₙ of the protein, could indicate a mucin-like 
structure. Because of its large size, gp300 could also show 
features of proteoglycans. These consist of a polypeptide 
backbone substituted by N- and O-linked carbohydrate 
and also glycosaminoglycan disaccharide units, which 
usually contain sulphated sugars (Hook et al., 1984). No 
significant levels of sulphate have been shown to be 
present in gp300 (I. W. Halliburton, unpublished results), so 
a proteoglycan-like structure is unlikely. Experiments are currently under way to identify the role of 
gp300 in infection, to analyse the protein structure in 
more detail and to sequence the virus gene coding for this 
protein. We have recently isolated a monoclonal anti-
body to gp300 which neutralizes virus infectivity (L. 
Wheldon, unpublished results), which suggests that 
gp300 may have a role in initiating infection.

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