Identification and characterization of glycoprotein gp1 of bovine herpesvirus type 4

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Three major bovine herpesvirus type 4 (BHV-4) glycoproteins have been described previously. By using monoclonal antibodies produced against BHV-4 envelope proteins from which the three major antigens had been removed by immunoaffinity, a fourth glycoprotein was identified. This protein (gp1) has a high Mₐ (>300K), is detected about 8 h post-inoculation of infected cells and is strictly expressed as a gamma protein. Moreover, gp1 was identified by a polyclonal antiserum from an infected animal, indicating that this glycoprotein is an antigen recognized by the immune system of infected animals.

Bovine herpesvirus type 4 (BHV-4) (Bartha et al., 1987) has been classified as a cytomegalovirus primarily because certain features of its replication cycle are characteristic of cytomegalovirus replication (Storz et al., 1984). However, more recent molecular data on the genome suggest that BHV-4 may be more correctly classified as a gammaherpesvirus (Staczek, 1990; Thiry et al., 1990).

BHV-4 is distributed world-wide and has been isolated from animals with a variety of clinical diseases and even from healthy cattle (reviewed in Thiry et al., 1989). BHV-4 is not strictly species-specific, having also been isolated from sheep, African buffalo (*Syncerus caffer*), American bison (*Bison bison*), cats and owl monkeys (*Aotus trivirgatus*) (reviewed by Thiry et al., 1990; Bublot et al., 1991). Most workers have failed to reproduce experimental disease, but there is evidence for an association between BHV-4 infection and genital disease (Thiry et al., 1990).

The BHV-4 structural proteins have been described recently (Dubuisson et al., 1989b), and three major glycoproteins have been identified by using monoclonal antibodies (MAbs) (Dubuisson et al., 1989b, 1992). The first is a complex of three glycopolypeptides (gp6, gp10 and gp17), in which gp10 and gp17 are linked by disulphide bonds and gp6 is non-covalently linked to the others. The second is a single glycopolypeptide (gp11) linked to a 16-5K non-glycosylated polypeptide (VP24), and the third is a single glycopolypeptide (gp8). A precursor has been identified for some components of the first glycoprotein [p(gp10/gp17)] (Dubuisson et al., 1989b) and another has been identified for gp11 (Dubuisson et al., 1991). The temporal expression of these three glycoproteins shows that the gp6/gp10/gp17 complex appears as early as 6 h post-inoculation (p.i.), and gp11 and gp8 are detected about 8 h p.i. (Dubuisson et al., 1991). Moreover, the precursor of gp10 and gp17 [p(gp10/gp17)] is expressed as a beta-gamma protein, whereas the gp11/VP24 and gp8 glycoproteins are strictly expressed as gamma proteins.

Various roles have been attributed to the glycoproteins of herpes simplex virus: they mediate entry into the host cell as well as virus egress, induce the production of neutralizing antibodies, and determine interactions between the host immune system and virus-infected cells (reviewed by Spear, 1984). Ten glycosylated polypeptides have been identified in purified BHV-4 preparations by SDS–PAGE (Dubuisson et al., 1989b). Of these proteins, only five (gp6, gp8, gp10, gp11 and gp17) were detected by MAbs. The production of MAbs against the other glycoproteins should be possible by immunizing BALB/c mice with purified envelope proteins from which the major antigens have been removed by immunoaffinity. In this paper, we report the identification and characterization of a new BHV-4 glycoprotein by using this technique.

The BHV-4 strains used in this work were the V.Test strain isolated from a case of orchitis (Thiry et al., 1981), the LVR140 strain isolated from a case of post-partum metritis (Wellemans et al., 1984), the European reference strain Movar 33/63 (ATCC VR-842) isolated from a cow with keratoconjunctivitis (Bartha et al., 1966), the American reference strain DN 599 (ATCC VR-631)
isolated from a calf with respiratory disease (Mohanty et al., 1971) and the K10/82 strain isolated from foetal lung cell culture (Metzler & Wyler, 1986), kindly provided by A. Metzler (University of Zürich, Switzerland). The HVA-2 isolate OMKI 68-69 was isolated from a kidney cell culture obtained from an apparently healthy owl monkey (Barahona et al., 1973) and was kindly provided by M. D. Daniel (Regional Primate Research Center, Harvard Medical School, Southborough, Mass., U.S.A.). Madin Darby bovine kidney (MDBK) cells were cultured in MEM as described previously (Dubuisson et al., 1987) for BHV-4 multiplication. Each virus except K10/82 was plaque-purified three times.

To produce MAbs, inbred BALB/c mice were immunized with envelope proteins of purified BHV-4 strain V.Test (Dubuisson et al., 1989b), from which the three major glycoproteins identified previously had been removed by immunoaffinity using MAbs as described by Harlow & Lane (1988). Briefly, MDBK cells grown to confluence were infected at a multiplicity of 10 plaque-forming units (p.f.u.) per cell. Three days later, virus (10¹⁰ p.f.u.) was harvested and purified from the supernatant fluid by differential centrifugation through a cushion of 30% sucrose in PBS (3 mM-KCl, 1·5 mM-KH₂PO₄, 0·14 M-NaCl, 6·5 mM-Na₂HPO₄, pH 7·2) as described by Misra et al. (1981). The virion envelope was removed as described by Hampl et al. (1984). The three major glycoproteins were then extracted by immunoaffinity and the resulting antigen was resuspended in 2 ml PBS with 1% Triton X-100. At 2-week intervals the mice received three injections of 250 μl of antigen emulsified in an equal volume of complete Freund’s adjuvant for the first injection and incomplete Freund’s adjuvant for the following two. After a further 2 months they were boosted with antigen by the intrasplenic route (Spitz, 1986) and fusion was performed as described previously (Dubuisson et al., 1989a).

Antigen used for immunoprecipitation was prepared as described previously (Dubuisson et al., 1989b). Briefly, two sources of antigen were used: [³⁵S]methionine-labelled purified virus prepared as above, and [³⁵S]methionine- or [³H]glucosamine-labelled BHV-4-infected cell culture (harvested 48 h p.i.) in radio-immunoprecipitation assay (RIPA) buffer (0·15 M-NaCl, 0·05 M-Tris-HCl pH 7·2, 1% Triton X-100, 0·1% SDS, 1% sodium deoxycholate, 0·1% NaN₃). The immunoprecipitation procedure was as described previously (Dubuisson et al., 1989b).

Ten MAbs were produced. One (MAb 123) precipitated four virus proteins (Mr > 300K, 110K, 49K and 37K) when [³⁵S]methionine-labelled infected cell lysate was used as antigen (Fig. 1). The 49K and 37K bands were faint and diffuse, suggesting that these bands could be degradation products. Under non-reducing conditions the immunoprecipitate showed the same profile, suggesting that no disulphide bonds are involved in the quaternary structure. When [³H]glucosamine-labelled infected cell lysate was used as antigen, two bands were detected (> 300K and 37K), indicating that these proteins are glycosylated (Fig. 1). When the envelope fraction of purified labelled virus was used as antigen, only the band of Mr > 300K was detected (Fig. 1). The results above were obtained when samples were boiled in the presence of PMSF (0·4 mM) and EDTA (1 mM) in the last washing step of the RIPA before adding Laemmli sample buffer, otherwise a large smear was detected. Under these conditions we were also able to identify a glycosylated band (> 300K) after immunoprecipitation using a polyclonal antiserum from a BHV-4-infected rabbit (data not shown).

In another experiment, immunoprecipitated proteins bound to Protein A-Sepharose (Pharmacia) in the appropriate buffer (50 mM-potassium phosphate buffer pH 7·4, 20 mM-EDTA, 0·5% Triton X-100, 0·4 mM-PMSF) were released by boiling and digested with 0·5 units of endoglycosidase F and N-glycosidase F (Boehringer-Mannheim) at 37 °C overnight. After incubation, an equal volume of 2 x Laemmli sample buffer was added to the reaction and samples were boiled for 2 min prior to SDS–PAGE (Laemmli, 1970). Control samples without enzyme were treated identically. After electrophoresis, gels were treated with sodium salicylate...
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(Chamberlain, 1979), dried and exposed to Kodak XAR-5 films. The $M_r$ of radioactive bands was determined by running $^{14}$C-labelled $M_r$ standards in parallel (Amersham). Endoglycosidase F and N-glycosidase F treatment of the immunoprecipitated proteins produced a decrease in $M_r$ only of the 37K band (14K instead of 37K), as measured by its migration in SDS–PAGE (Fig. 1), indicating that this protein contains N-linked glycans.

In experiments using a glycosylation inhibitor, tunicamycin (Sigma; 10 mg/ml) was added to BHV-4- or mock-infected cell cultures. Radioactive compound was added 1 h p.i., and the cells were labelled for 32 h prior to harvest. No virus protein was detected by RIPA using MAb 123 after tunicamycin treatment of infected cells.

To study the course of glycoprotein synthesis during the cycle of virus replication, MDBK cells infected with plaque-purified BHV-4 strain V.Test (m.o.i. > 10) were labelled with $^{35}$S]methionine (10 mCi/ml; Amersham) in methionine-free MEM from 0 to 4, 4 to 8, 8 to 12, 12 to 23, 23 to 32, 32 to 54 or 54 to 76 h p.i. At the end of each period, mock- and virus-infected cells were disrupted with RIPA buffer, cell lysates were cleared of cell debris by centrifuging at 100000 g for 1 h, and then immunoprecipitated and analysed by SDS–PAGE. The experiment showed that this glycoprotein was detected from 12 to 23 h until the end of the experiment (76 h), when there was approximately 30% c.p.e. (Fig. 2). To determine the time of appearance of the new glycoprotein, MDBK cells were infected with BHV-4 (m.o.i. > 10) and labelled with $^{35}$S]methionine (100 mCi/ml) at hourly intervals until 14 h p.i., as described previously (Dubuisson et al., 1991).

Briefly, the pulse–chase experiment was carried out as follows. Cells were incubated in methionine-free MEM for 30 min, labelled with $^{35}$S]methionine (100 mCi/ml; Amersham) for 1 h, and incubated with unlabelled methionine (5 mM) for 30 min. Cells were then harvested and analysed by RIPA. The pulse–chase experiment showed that this glycoprotein appears 8 h p.i. (Fig. 3). To characterize further the temporal control of this new BHV-4 glycoprotein, the DNA synthesis inhibitor phosphonoacetic acid (PAA) was used at a concentration of 300 mg/ml (Dubuisson et al., 1991). No band was detected after PAA treatment (data not shown).

Neutralization tests were performed as described by Dubuisson et al. (1990). No neutralizing activity was detected for MAb 123 (starting concentration 1 mg/ml) with or without complement.

Radioimmunoprecipitation of the BHV-4 proteins from reference strains (Movar 33/63, DN 599, LVR 140, K10/82 and HVA-2) using MAb 123 allowed the detection of the > 300K glycoprotein. No variation in $M_r$ was observed for this glycoprotein in these strains.

The protein identified by MAb 123 is a glycoprotein of $M_r$ > 300K which is present in the envelope fraction of purified virus. No glycoprotein of such a high $M_r$ has been identified previously (Dubuisson et al., 1989b). This could be due to the high sensitivity of the protein to proteases; proteins of high $M_r$ are more sensitive to proteolysis and the sensitivity of the > 300K glycoprotein was increased after denaturation. By using protease inhibitors at the end of the RIPA, this glycoprotein was detected after immunoprecipitation by either MAb 123.
or a polyclonal antiserum from a BHV-4-infected rabbit. These results indicate that the glycoprotein is an antigen recognized by the immune system of infected animals. This glycoprotein may correspond to VP1 (Mr > 300K), identified previously in purified virus (Dubuisson et al., 1989b), and was therefore called gp1.

Glycoprotein gp1 did not show any variation in Mr, after endoglycosidase F and N or glycosidase F treatment, but the coprecipitated 37K protein, which showed a large decrease in Mr (14K instead of 37K). This suggests that a degraded product of gp1 contains N-linked glycans. Moreover, no glycoprotein was detected after tunicamycin treatment, suggesting that the protein recognized by MAb 123 is affected by tunicamycin treatment and that N-linked oligosaccharides are involved in the conformation of the epitope of this protein. These results indicate that gp1 contains N-linked glycans.

This new glycoprotein appears 8 h.p.i., as has been observed for gp8 and gp11 (Dubuisson et al., 1991). Moreover, gp1 is not expressed in the absence of viral DNA replication, indicating that the gene encoding this protein is also expressed as a gamma gene.

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