Proteins of bovine herpesvirus type 4 released into the culture medium of productively infected cells: identification of a 135K glycoprotein involved in viral attachment

Jean Dubuisson,† Igor Koromyslov,‡ Paul-Pierre Pastoret and Etienne Thiry*

Department of Virology-Immunology, Faculty of Veterinary Medicine, University of Liège, Institut de Chimie (B6), 4000 Liège, Belgium

Three bovine herpesvirus type 4 (BHV-4) proteins released into the culture medium of infected cells were identified, with Mr values of 135K, 16K and 14.5K. Among these three proteins, two were precipitated by the monoclonal antibodies characterized in this work. One is a glycoprotein of 135K (gp8) which does not seem to be involved in BHV-4 neutralization. Moreover, this 135K glycoprotein adsorbed onto uninfected susceptible cells. The attachment of gp8 to cells was totally inhibited by the prior adsorption of unlabelled viral proteins. Moreover, anti-gp8 monoclonal antibodies were effective in inhibiting the adsorption of gp8. These results indicate that gp8 could be involved in BHV-4 attachment.

Bovine herpesvirus type 4 (BHV-4) (Bartha et al., 1987) is a member of the herpesviridae family and has been classified tentatively as a cytomegalovirus, primarily because certain features of its replication cycle are characteristic of cytomegalovirus replication (Storz et al., 1984). Nevertheless, recent data suggest that it may be more appropriately classified as a gammaherpesvirus (Honess, 1984; Thiry et al., 1990; Staczek, 1990). BHV-4 is found worldwide and it includes a large number of antigenically related isolates recovered from diseased or healthy cattle (reviewed by Ludwig, 1983; Thiry et al., 1989; Staczek, 1990). In contrast to BHV-1 and BHV-2, BHV-4 has not yet been established as the aetiologic agent of a distinct disease; however a role in the aetiology of some diseases of the genital tract has been suggested (reviewed by Thiry et al., 1989, 1990).

Twenty-nine BHV-4 structural proteins have been identified previously by SDS-PAGE of purified labelled virus, 10 being glycosylated. Two major glycoproteins were identified by the use of anti-BHV-4 monoclonal antibodies (MAbs) (Dubuisson et al., 1989b). The first is a complex of three glycoproteins (gp6/gp10/gp17) in which gp10 and gp17 are linked by disulphide bonds and gp6 is non-covalently linked to this complex. The second is made up of one glycopolypeptide (gp11) non-covalently linked to a non-glycosylated polypeptide (VP24). Moreover, a precursor has been identified for gp10 and gp17 in the first glycoprotein [p(gp10/gp17)] (Dubuisson et al., 1989b) and another has been identified for gp11 (Dubuisson et al., 1991a, b).

Cells infected with some other herpesviruses release a subset of virus-specified polypeptides in a soluble form into the tissue culture medium (Ben-Porat & Kaplan, 1970; Kaplan, 1975; Norrild & Vestergaard, 1979; Randall et al., 1980; Randall & Honess, 1980; van Zaane et al., 1982; Okuno et al., 1983; Bennett et al., 1986). Some of these polypeptides are glycosylated. In the case of herpes simplex virus type 1 (HSV-1), these secreted polypeptides are capable of absorbing out all virus-neutralizing antibody raised against glycoproteins of the virus particle and of the infected cell (Randall et al., 1980). The humoral immune response of cattle to BHV-4 infection is characterized by low neutralizing antibody levels or an absence of such antibodies (reviewed by Thiry et al., 1989). In order to test whether such secreted glycoproteins could explain the weak neutralizing activity of anti-BHV-4 antisera, BHV-4 proteins released into the culture medium of productively infected cells were identified and characterized.

The BHV-4 strain V. Test isolated from a case of orchitis (Thiry et al., 1981) was used in this work. Madin Darby bovine kidney (MDBK) cells cultured in MEM containing 5% newborn calf serum were used for virus multiplication. In some experiments, Georgia bovine kidney (GBK) cells were also used. Each virus was

† Present address: Department of Molecular Microbiology, Washington University School of Medicine, 660 South Euclid Avenue (Box 8230), St Louis, Missouri 63110-1093, U.S.A.
‡ Present address: Zvenigorodskoye Road 5, Institute for the Control of Veterinary Products, 123022 Moscow, U.S.S.R.
plaque-purified three times and used at passage level 14 to 16.

For the preparation of purified labelled virus, MDBK cells grown to confluence were infected at a multiplicity of 10 p.f.u. per cell and incubated for 2 h at 37 °C in MEM with 2% newborn calf serum. The virus was labelled and purified as previously described (Dubuisson et al., 1989b). The virion envelope was removed as described by Hampl et al. (1984).

Production and characterization of MAbs to BHV-4 glycoproteins gp6/gp10/gp17 and gp11/VP24 have been described previously (Dubuisson et al., 1989a,b, 1990). Other MAbs were produced by another immunization procedure. Briefly, inbred BALB/c mice were immunized with purified BHV-4 envelope proteins from which the two previously identified major glycoproteins gp6/gp10/gp17 and gp11/VP24 were extracted by immunoaffinity using MAbs as described in Harlow & Lane (1988). At intervals 2 weeks apart, they received three injections of antigen emulsified in an equal volume of complete Freund’s adjuvant for the first injection and in incomplete Freund’s adjuvant for the last two. After a further 15 days, they were boosted by the intraperitoneal route and the fusion was performed as previously described (Dubuisson et al., 1989a).

The BHV-4 glycoprotein gp8 was purified by immunoaffinity as described by Harlow & Lane (1988) using supernatant from BHV-4-infected cells (centrifuged at 100,000 g for 90 min) and injected subcutaneously into a rabbit according to the method described by Israel et al. (1988). Previously produced (Dubuisson et al., 1988) hyperimmune polyvalent antiserum to BHV-4 structural proteins was used in this work.

Antigen used for immunoprecipitation was prepared as described by van Drunen Littel-van den Hurk et al. (1984) and the immunoprecipitation procedure was as previously described (Dubuisson et al., 1989b). Labelled proteins were separated by SDS-PAGE (Laemmli, 1970). After electrophoresis, gels were treated with sodium salicylate (Chamberlain, 1979), dried and exposed to Kodak XAR-5 films. The Mr values of radioactive bands were determined by running, in parallel, 14C-labelled Mr standards (Amersham). Western blotting was performed as previously described (Dubuisson et al., 1989b).

For the production and radioactive labelling of polypeptides released from infected cells, confluent MDBK cells cultured in 75 cm2 flasks were infected at an m.o.i. of 10 p.f.u. per cell and incubated for 2 h at 37 °C in MEM containing 2% newborn calf serum. The culture medium was then replaced by methionine-free medium with 2% newborn calf serum and 8 h after infection, 10 μCi/ml of [35S]methionine (Amersham) was added. When the c.p.e. involved about 20% of the monolayer (about 72 h post-inoculation), the supernatant was harvested and centrifuged at 100,000 g for 90 min. The resulting supernatant was harvested and stored at −80 °C. Control supernatant was harvested from uninfected cell culture.

For the neutralization tests, twofold dilutions (50 μl) of ascites fluid or non-decomplemented sera were mixed with 50 p.f.u. of BHV-4 from either infected cell culture supernatant or pelleted virus from the same infected cell culture (100,000 g for 90 min) in the same volume of MEM. In some neutralization tests using MAbs, baby rabbit complement (Serotec; 5% final concentration) was added. Mixtures were incubated in duplicate in 96-well plates for 2 h at 37 °C, then 1·3 × 104 MDBK cells were added per well, and 16 h later 0·6% carboxymethylcellulose in MEM was added. After 8 days, the cells were fixed and stained with an aqueous alcoholic solution of crystal violet. The neutralizing titres were expressed as the reciprocal of the highest dilution of sera giving 50% reduction in plaque number.

Adsorption studies were carried out by a modification of the procedure developed by Sabara et al. (1985) as described by Okazaki et al. (1987). Briefly, MDBK cell monolayers in 96-well plates were fixed with 4% formalin in PBS (pH 7·4) for 15 min at 4 °C. The cells were then washed and incubated with MEM containing 5% newborn calf serum. Radiolabelled BHV-4 envelope proteins were incubated for 1 h at 37 °C with MAbs or a polyclonal antiserum to gp8. Then the reaction mixtures were added to the cells in 96-well plates. Competitive binding to the cells between radiolabelled BHV-4 envelope proteins and unlabelled purified virus (lysed by 1% Triton X-100 in PBS) was also examined by 1 h incubation of serially diluted unlabelled lysed virus. After adsorption at 4 °C for 2 h, cell monolayers were washed with cold PBS. In order to identify adsorbed proteins, 30 μl of Laemmli sample buffer was added per well, and the proteins were analysed by SDS-PAGE.

Three new MAbs (103, 113 and 114) were produced (Table 1). These three MAbs precipitated a 135K protein when [35S]methionine-labelled infected cell lysate was used as an antigen. None of these MAbs recognized a protein was still detected, showing that disulphide bonds are not involved in its quaternary structure. In some preparations of purified virus, a faint and diffuse band of approximately 56K was detected suggesting that this band could be a degraded product. When [3H]glucosamine-labelled infected cell lysate or envelope proteins were used as an antigen, a 135K band was still detected indicating that this protein is glycosylated. Nevertheless,
Table 1. Characterization of anti-BHV-4 MAbs

<table>
<thead>
<tr>
<th>MAb designation</th>
<th>Isotype</th>
<th>M&lt;sub&gt;r&lt;/sub&gt; of protein</th>
<th>Western blotting</th>
<th>Glycosylation*</th>
<th>Present in virion envelope</th>
<th>Present in nucleocapsid</th>
<th>Protein†</th>
</tr>
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<tbody>
<tr>
<td>21</td>
<td>IgG1</td>
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<td>+</td>
<td>VP25</td>
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<td>IgG2a</td>
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<td>+</td>
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<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>gp8</td>
</tr>
</tbody>
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* Determined by intrinsic labelling.
† From Dubuisson et al. (1989b).

Fig. 1. (a) [35S]Methionine- and (b) [3H]glucosamine-labelled polypeptides immunoprecipitated from purified BHV-4 by MAb 29 raised against gp11 (lane 1), MAb 35 raised against gp6/gp10/gp17 (lane 2) and MAb 113 raised against gp8 (lane 3). The immunoprecipitates were analysed by SDS-PAGE (12%). M<sub>r</sub> standards are shown in the centre. The symbol towards the foot of (b) lane 1 indicates a degraded product of gp11.

the intensity of this band was weak when compared to the other identified glycoproteins gp10 and gp11 (Fig. 1b) suggesting that this protein is less glycosylated. The intensity of the band was the same as those of gp6 and gp17.

Two other MAbs (MAbs 21 and 34) previously produced (Dubuisson et al., 1989a) were also characterized (Table 1). MAb 21 recognized a 16K non-glycosylated protein present in the nucleocapsid fraction of the virion which was detected by Western blotting. MAb 34 recognized a 140K non-glycosylated protein present in the nucleocapsid fraction of the virion but this protein was not detected by Western blotting.

Using a polyclonal antiserum to BHV-4 structural components three viral proteins (135K, 16K and 14.5K) were identified in the supernatant of infected cells (Fig. 2a and b). MAbs raised against proteins showing approximately the same M<sub>r</sub> value as these secreted proteins were used in immunoprecipitation tests using the supernatant of infected cells as antigen; these were MAbs to gp6/gp10/gp17 which coprecipitated a 132K precursor [p(gp10/gp17)] and MAbs to the 135K, the 140K and the 16K proteins identified in this work. From these MAbs, those raised against the newly identified 16K non-glycosylated protein and the 135K glycoprotein were positive, showing that these two proteins are secreted into the supernatant of infected cell cultures (Fig. 2). The secreted and cell-associated form of these newly identified proteins showed the same M<sub>r</sub> value as those obtained by separation by SDS–PAGE.

None of the anti-135K MAbs was neutralizing alone or in the presence of complement, either when supernatant from infected cell culture or pelleted virus was used as an antigen. The polyclonal antiserum raised against this 135K protein showed a very weak neutralizing activity with the two viral preparations (neutralizing titre, 4).

Various experiments to study the adsorption of BHV-4 envelope proteins to cell surfaces were performed. A 135K protein adsorbed onto the MDBK cell surface when radiolabelled BHV-4 envelope proteins were reacted with the cell monolayer. This band showed the same M<sub>r</sub> value as the newly identified glycoprotein (Fig. 3, lanes 1 and 2). Similar results were obtained on GBK cells (data not shown). In order to confirm that the 135K component binds to the cell surface, competitive binding to the cells between the radiolabelled proteins and unlabelled purified virus lysed by Triton X-100 was investigated. In our experimental conditions, the binding of the 135K viral component to the cells was totally blocked by the prior adsorption of BHV-4 viral proteins. MAbs and the polyclonal antiserum raised against the 135K glycoprotein markedly inhibited its attachment, showing that this protein is the newly identified glycoprotein (Fig. 3). Moreover, MAbs raised against the 132K glycoprotein precursor of gp10/gp17 did not inhibit the attachment of the 135K glycoprotein.

Three BHV-4 proteins present in infected cells are released into the culture medium of productively infected cells. One of them, a glycosylated protein, is most
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Fig. 2. [35S]Methionine-labelled polypeptides immunoprecipitated from BHV-4-infected (v) or uninfected (c) cells (panels a and c) or from the supernatant of BHV-4 infected (v) or uninfected (c) MDBK cells after centrifugation at 100000 g for 90 min (panels b and d) by a polyvalent antiserum to BHV-4 or by MAbs 113 (anti-gp8), 21 (anti-VP25) 31 [anti-gp6/p(gp10/gp17)/gp10/gp17] and 34 (anti-VP7). The immunoprecipitates were analysed by SDS-PAGE (15% for a and b; 8% for c and d). M, values of the secreted proteins are shown to the left of (a).

Fig. 3. Identification of [35S]methionine-labelled BHV-4 envelope proteins which adsorb to MDBK cells after incubation with uninfected cell monolayers in 96-well-plates as described in the text. BHV-4 envelope proteins (representing about 10^7 p.f.u./well) were plated on the monolayers directly (lane 2 and 7) or after an incubation with anti-gp8 MAbs 103 (1 mg/ml; lane 3), 113 (125 μg/ml; lane 4) and 114 (500 μg/ml; lane 5) or with a polyvalent antiserum to gp8 (diluted 1:2; lane 6). In lane 8, the monolayer was pre-incubated with unlabelled BHV-4 proteins (representing 10^5 p.f.u./well). Lane 1 shows [35S]methionine-labelled polypeptides immunoprecipitated from BHV-4-infected MDBK cells by anti-gp8 MAb 113. Labelled proteins were analysed by SDS-PAGE (10%). M, standards are shown in the left margin.

probably gp8 which we have described as being present in purified virus (Dubuisson et al., 1989b). This protein was also present in the envelope fraction of the virion. It is therefore not a true secreted protein, but it could be released into the medium by proteolytic cleavage as is the case for gX of pseudorabies virus (Bennett et al., 1986). Nevertheless, no Mr variation was detected between the cell- or virion-associated forms and the secreted forms, which could be explained by the lack of cytoplasmic and transmembrane domains in the secreted form. The absence of any Mr difference could be due to abnormal behaviour of gp8 in SDS-PAGE as is the case for many glycoproteins, but it is also possible that gp8 is not an integral protein; indeed, it could be a peripheral protein which is anchored into the membrane by phosphatidylinositol (reviewed by Low et al., 1986). In this case, gp8 could be released by phosphatidylinositol-specific phospholipase C. Another mechanism that might explain these data is that gp8 is non-covalently bound to another molecule firmly anchored in the cell membrane and the 135K could be slowly released simply by mass action.

Sera from BHV-4 infected animals show a weak neutralizing activity (maximum titre: 64, reviewed by Thiry et al., 1989; Dubuisson et al., 1989c). The neutralizing activity of anti-gp8 MAbs or antiserum was tested to determine whether or not the soluble form of gp8 is capable of absorbing out most of the anti-BHV-4 neutralizing antibodies as was shown for HSV-1 (Randall et al., 1980). Only the antiserum raised against gp8 exhibited a weak neutralizing activity and no increase of the titre was observed with pelleted virus (from which the secreted form of gp8 was discarded). Therefore, gp8 does not play an important role in BHV-4 neutralization.

Glycoprotein gp8 was shown to adsorb to uninfected MDBK cells and the attachment of labelled gp8 was inhibited by unlabelled virus or by anti-gp8 MAbs,
suggesting that this glycoprotein is involved in BHV-4 attachment. Surprisingly, the three anti-gp8 MAbs had no measurable neutralizing activity. This could be explained if another glycoprotein plays a role in BHV-4 attachment. A similar result was observed by Kühn et al. (1990) for a MAb raised against gD of HSV-1. Moreover, the absence of a neutralizing activity for anti-gp8 MAbs suggests that gp8 could be non-essential in BHV-4 infectivity. Three glycoproteins (gB, gC and gD) have been shown to interact with the cell surface (Johnson et al., 1984; Fuller & Spear, 1985; Kühn et al., 1990). It is thus surprising that only one BHV-4 protein interacted with cell surface components. Other BHV-4 glycoproteins could be involved in BHV-4 attachment as previously suggested (Dubuisson et al., 1990) but the adsorption of these proteins could be inhibited by the fixation procedure or they may exhibit a weaker affinity than gp8. Okazaki et al., (1987) also identified only one glycoprotein (gIII) involved in BHV-1 attachment to susceptible cells by using the same procedure, but Liang et al., (1991) showed that BHV-1 attachment to permissive cells is mediated by its three major glycoproteins gI, gIII and gIV.

In conclusion, one BHV-4 glycoprotein (gp8) which was characterized in this work is released into the culture medium of productively infected cells. Moreover gp8 adsorbs to uninfected cells; this glycoprotein is probably involved in BHV-4 attachment and the secreted form of gp8 could interfere with BHV-4 infectivity. Further studies, however, will be needed to clarify this last point.

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