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Expression and cellular distribution of baculovirus-expressed bovine herpesvirus 1 (BHV-1) glycoprotein D (gD) sequences

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Summary. Glycoprotein D (gD) of bovine herpesvirus 1 (BHV-1), a homolog of herpes simplex virus gD, represents a major component of the viral envelope and is a dominant immunogen. To study the antigenic properties of the different regions of gD, we have expressed the full-length gD encoding gene and overlapping fragments spanning various regions of the gD open reading frame in a baculovirus (*Autographa californica* nuclear polyhedrosis virus) – insect cell (*Spodoptera frugiperda*, SF-9) system. Maximum levels of expression for all proteins were obtained 48 to 72 h post infection of SF-9 cells by recombinant viruses. Full-length and truncated recombinant gD proteins reacted specifically with anti-gD monospecific serum as determined by immunoprecipitation and immunoblotting, indicating that the proteins retained their antigenicity. However, based on the reactivity with a panel of gD-specific monoclonal antibodies (Mabs), the full-length recombinant gD lacked proper expression for two highly neutralizing linear epitopes identified by Mabs R54 and 9D6. The rest of the epitopes appeared to be preserved and antigenically unaltered. Immunofluorescence studies of recombinant baculovirus infected SF-9 cells using gD monospecific serum, revealed no direct correlation between cellular localization of the expressed proteins and their amino acid sequences.

Introduction

Bovine herpesvirus 1 (BHV-1), a member of the *Alphaherpesvirinae* subfamily [16], is an economically important pathogen of cattle. The virus has been associated with a variety of clinical disease manifestations including rhinotracheitis, vulvovaginitis, abortions, conjunctivitis, and generalized systemic infections [4].

As in other alphaherpesviruses, BHV-1 glycoproteins are the major structural components of the viral envelope and virus-infected cell membranes. The glycoproteins play important roles in virus-cell interactions, including recognition, attachment, and penetration of the virion into susceptible cells [6, 9, 10], viral neutralization [5, 13] and immune destruction of infected cells. Glycoprotein D (gD), of BHV-1 is one of four major glycoproteins, namely gB, gC, gD, and gH that have been identified on the virus envelope and the plasma membranes of BHV-1-infected cells [14, 24]. Glycoprotein D, which is 417 amino acids long and approximately 77 Kd in molecular size, stimulates a potent neutralizing antibody response in animals and induces significant protection against BHV-1 induced disease. Immunization with affinity-purified gB, gC, and gD individually or in combination induced protective immune responses against BHV-1 infection [3, 25]. However, of the several glycoproteins, only anti-gD immune response significantly reduce viral replication and shedding [22].

To study the functional role of gD and evaluate its candidacy as a subunit vaccine, several expression systems and strategies have been used to express the protein [21]. Bacterial expression of gD in *Escherichia coli* produced high protein yields, but poor immunogenicity, mainly due to lack of proper folding, disulfide bond formation, and glycosylation [22]. Epitope mapping studies on *E. coli* recombinant gD using monoclonal antibodies (Mabs) showed that only linear epitopes are preserved [2]. Expression of gD in bovine kidney cells [19] and by vaccinia virus [20] yielded authentic protein indistinguishable from gD produced in BHV-1 infected MDBK cells. Antigenic analysis of different segments of gD using vaccinia virus has demonstrated that both linear and conformational epitopes are preserved [20]. A drawback of most mammalian expression systems is that they produce very low protein yield. Cloning and expression of gD in baculovirus under the polyhedrin promotor has yielded high level of protein expression [23]. Immunization of cattle with the crude recombinant protein resulted in lower neutralizing antibody responses when compared to the authentic gD [24]. However, the purified recombinant protein elicited neutralizing antibody responses similar to the authentic gD [22]. The purpose of the study reported here was to examine in detail the antigenic properties of BHV-1 gD amino acid sequences expressed by baculovirus and determine whether all epitopes are properly preserved and expressed in the insect cell environment. The full-length and portions of the BHV-1 gD ORF were recombined into baculoviruses under the control of the polyhedrin gene promoter and expressed as recombinant proteins in SF-9 cells. The recombinant proteins were tested for antigenicity and cellular distribution by western blotting, immunoprecipitation, and immunofluorescence using gD monospecific serum and a panel of Mabs.

Materials and methods

Viruses, cell lines, and antibodies

Recombinant baculoviruses were propagated in SF-9 cells maintained in serum free SF900 II medium (Gibco BRL). The Cooper (Colorado-1) strain of BHV-1 was obtained from the

American Type Culture Collection (Rockville, MD). The virus was propagated and titrated in Madin-Darby Bovine Kidney (MDBK) cells grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Mabs R54 and 111B specific for gD were developed and characterized previously [1, 2]. Anti-gD monospecific serum and the other Mabs were developed previously [7].

Construction of transfer plasmids

The 1.3 Kb MaeI DNA fragment containing the full-length BHV-1 gD encoding gene was blunt ended by Klenow fragment DNA polymerase and cloned into the StuI site of pFastBacHTa plasmid (Gibco BRL) to yield plasmid pgD1–417. Cloning in this plasmid added 6 histidine codons in frame and upstream of the first gD methionine codon. The histidine codons were added to provide a binding site for Ni^{2+}-nitrilotriacetic acid (NTA) agarose resins for possible use in purification protocols. Subsequently, portions of the gD ORF were generated either directly or indirectly from plasmid pgD1–417. The numbers in the plasmid designations indicate the gD amino acid residues expressed. All cloning and DNA manipulation techniques were performed following standard procedures [2, 17]. Proper in frame insertion and orientation were verified by DNA sequencing and restriction site analysis.

Construction of pgD107–417. Plasmid pgD1–417 was digested with NdeI and BamHI. The larger fragment containing the 3′ 933 bp of the gD ORF was purified, blunt ended, and religated.

Construction of pgD1–216. Plasmid pgD1–417 was digested with SmaI. The larger fragment containing the plasmid backbone and the 5′ 649 bp of the gD ORF was purified and religated.

Construction of pgD31–242. Plasmid pgD1–417 was digested with SalI. The 639-bp fragment was purified and inserted in frame into SalI-opened pFastBacHTc plasmid.

Construction of pgD218–416. Plasmid pgD1–417 was digested with SmaI. The 599-bp fragment was purified and inserted in frame into StuI-opened pFastBacHTc plasmid.

Construction of pgD244–417. Plasmid pgD1–417 was digested with SalI and NsiI. The 545-bp fragment was purified and inserted in frame into SalI/PstI-digested pFastBacHTc plasmid.

Construction of pgD107–216. Plasmid pgD107–417 was digested with SmaI. The larger fragment containing the plasmid backbone and the NdeI-SmaI 333 bp of the gD ORF was purified and religated.

Construction of pgD1–105. Plasmid pgD1–417 was digested with NotI and NdeI. The larger fragment containing the plasmid backbone and the 5′ 316 of the gD ORF was purified, blunt ended by Klenow, and religated.

Construction of pgD321–417. Plasmid pgD1–417 was digested with NarI. The larger fragment containing the plasmid backbone and the 3′ 292 of gD ORF was purified and religated.

Development of recombinant baculoviruses

Recombinant baculoviruses were generated using the Bac-to-Bac system developed by Gibco BRL [12] following the manufacturer's recommendations. Briefly, DNA from pFastBac plasmids were used to transform DH10Bac competent *E. coli* cells already containing the baculovirus composite (shuttle) plasmid vector bMON14 272 called bacmid. By transposition, the gD gene and ORF subfragments were inserted into the bacmid plasmids to yield recombinant bacmids. DNA from recombinant bacmids was isolated and used for transfection of SF-9 insect cells. SF-9 cells were transfected with 1 μg of bacmid DNA using cationic liposomes (Lipofectin; Gibco BRL). The transfection mixture was added to 1×10^6 SF-9

cells in a well of a 6-well tissue culture plate supplemented with SF900 II medium without serum and incubated for 5 h at 27 °C. Baculovirus harvested from transfected cells was plaque titrated, analyzed for gene recombination by hybridization, and assayed for protein expression by immunoprecipitation and immunoblotting following standard techniques as described previously [1, 2].

Protein expression

Recombinant proteins were prepared by infecting a suspension of SF-9 cells with each recombinant baculovirus at a multiplicity of infection (moi) of 5. Infected cells were incubated in SF900 II medium with shaking at 27 °C. Cells were harvested 48 to 72 h post infection when viability reached approximately 50% as determined by Trypan blue dye exclusion. The harvest time which gives the highest yield for each protein was predetermined by a time course of protein expression.

Radiolabeling and immunoprecipitation

Immunoprecipitation of recombinant proteins was performed as described previously [18]. Briefly, six-well tissue culture plates containing SF-9 cell monolayers in SF900 II medium were infected with each recombinant baculovirus at an moi of 10 and incubated at 27 °C. At 24 h post infection, the medium was removed and replaced with methionine-free medium for 1 h. ^{35}S-methionine (DuPont NEN) was added to a final concentration of 50 μCi/well and incubation was continued until 48 to 60 h post infection. Radiolabeled cells were harvested and immunoprecipitated with monospecific serum or Mabs as previously described [1, 2]. BHV-1 infected MDBK cells were also labeled with ^{35}S-methionine [1] and used as a positive control.

Western blotting

To identify the recombinant proteins expressed by baculoviruses and test their reactivity with monospecific serum or Mabs, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as previously described [2]. Briefly, proteins extracted from baculovirus-infected SF-9 cells and from BHV-1 infected MDBK cells were electrophoresed on a 12% gel, transferred to nitrocellulose (NC) membrane, and probed with monospecific serum or Mabs specific to gD. NC membranes were blocked with 3% non-fat milk, 0.05%Tween 20 in TBS (TBST). Subsequent steps were performed with a biotin-avidin peroxidase-substrate system using a kit from Vector Laboratories (Burlingame, CA).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to determine the antigenic characteristics of the recombinant proteins expressed by baculoviruses and confirm the presence of specific epitopes. To determine optimum volumes and reagent concentrations, preliminary checker-board titration experiments were conducted with gD- specific Mabs and recombinant baculovirus-infected SF-9 cells or BHV-1 infected MDBK cell proteins. The best results were obtained when approximately 300 ng of recombinant baculovirus-infected SF-9 cells were used to coat each well. Afterwards, the antigens were diluted in 0.05 M sodium carbonate pH 9.6, 100 μl was added to each well and incubated for 1 h at 37 °C and then overnight at 4 °C. Plates were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 2 h. Ten-fold serial dilutions of the Mabs were prepared in PBS containing 0.05%Tween 20 (PBST) and added to the plate. A prestandardized dilution of peroxidase-conjugated goat anti-mouse IgG was added to the wells and the reaction was developed using o-phenylenediamine dihydrochloride (OPD) sub-

strate in citrate buffer (Sigma) at 0.4 mg/ml. Optical densities at 405 nm wave length were determined by using a microplate reader (Dynatech Laboratories, Inc., Alexandria, VA).

Immunofluorescence

The localization and distribution of proteins expressed by recombinant baculoviruses in infected SF-9 cells were determined by indirect immunofluorescence. Briefly, 2×10^6 SF-9 cells in suspension culture were infected with the appropriate recombinant baculovirus at an moi of 5 and incubated with shaking at 27 °C. At 24 h post-infection, cells were counted and 3×10^4 cells were transferred to LabTech II slide chambers (Fisher) precoated with poly-D-lysine hydrobromide (Sigma), and the incubation was continued. Cells were fixed at 48 h post infection with a mixture of 70% acetone and 30% methanol for 10 min at −20 °C. Anti-gD monospecific serum diluted 1:100 in PBS was added and incubated in a humidified incubator for 40 min at 37 °C. Cells were washed 3× in PBS and fluorescein isothiocyanate-(FITC) conjugated goat anti-rabbit IgG (Sigma) was added and incubated for another 40 min at 37 °C. The cells were finally washed in PBS and mounted in 60% glycerol solution for examination with an epifluorescent microscope.

Results

Development and characterization of recombinant proteins

Baculovirus recombinants containing the full-length BHV-1 gD ORF or ORF subfragments were developed and confirmed to bear BHV-1 gD DNA sequences by DNA hybridization using a ^{32}P-labelled 1.3 Kb MaeI fragment as a probe (data not shown). All recombinant baculoviruses were designated with the gD amino acid sequences they express and are represented schematically in Fig. 1. To further identify the recombinant baculoviruses and confirm proper gD DNA sequence insertion, the protein products expressed by these viruses were analyzed by radioimmunoprecipitation (Fig. 2) and immunoblotting (Fig. 3).

Radioimmunoprecipitation of recombinant baculovirus BacgD1–417 (expressing the full-length gD ORF) infected SF-9 cells with gD monospecific serum revealed a protein band of approximately 75 Kd in molecular size (Fig. 2, lane 2) which is slightly less than the authentic gD protein (77 Kd) precipitated from BHV-1 infected MDBK cells (Fig. 2, lane 1). This indicates that the recombinant gD has properties similar to that of the authentic gD, but is slightly less glycosylated. All recombinant baculoviruses expressing portions of gD ORF were properly immunoprecipitated with gD monospecific serum, indicating that they carry their native antigenic properties (Fig. 2, lanes 3 through 10). All recombinant proteins were detected at, approximately, the expected molecular sizes. However, in some instances additional band(s) were observed, which may be degradation products or precursor forms of the expressed proteins.

Immunoblotting analysis of the recombinant proteins with gD monospecific serum gave similar results (Fig. 3) as those observed with immunoprecipitation (Fig. 2). All proteins were recognized consistently by the serum, except for one protein expressed by BacgD 244–417 which showed reduced reactivity (Fig. 3, lane 7). This could either be due to partial loss of antigenicity because of denaturation, degradation by proteolytic activity, or lower protein concentration in the sample.

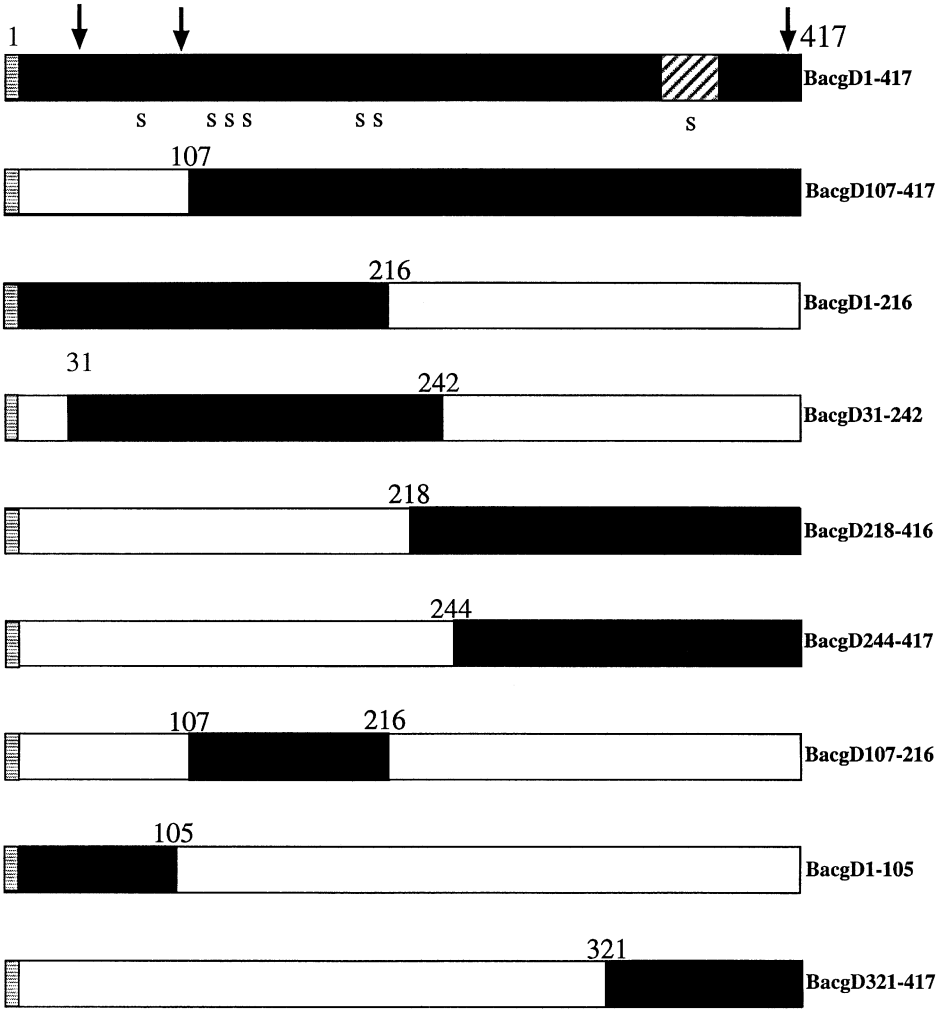


Fig. 1. Schematic representation of the BHV-1 gD glycoprotein and its segments that were expressed by baculovirus recombinants. The full-length gD is depicted on top showing the transmembrane domain (▨), cysteine residues(s), and N-linked glycosylation sites (↓). Glycoprotein D segments expressed are shown as solid bars identified by amino acid numbers on top and by the recombinant baculovirus designation on the right. Each protein is fused with a polyhistidine sequences at its aminoterminal end (▯) for purification purposes

Antigenic analysis of recombinant proteins by monoclonal antibodies

To identify antigenic determinants on the recombinant proteins, recombinant baculovirus infected SF-9 cells were analyzed by immunoblotting and ELISA against a panel of gD specific Mabs.

Mabs R54 , 9D6, and 3D9S, which react with linear epitopes [2, 20], were examined by western blotting (Fig. 4A–C). The rest of the Mabs namely, 111B, 136, 3E7, 2C8, 3C1, 4C1, and 10C2, were analyzed by ELISA. The results of Mab reactivities with recombinant proteins are summarized in Table 1. Mab R54

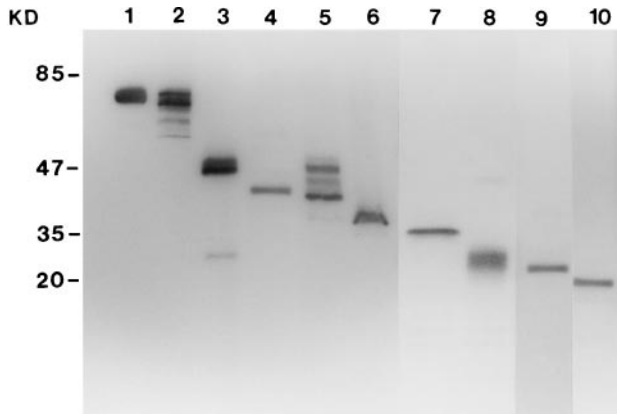


Fig. 2. Immunoprecipitation of baculovirus recombinant proteins expressing gD amino acid sequences. Lysates from ^{35}S-methionine labeled MDBK cells infected with BHV-1 (*1*) or from SF-9 cells infected with recombinant baculovirus expressing full-length gD (*2*), amino acid (aa) residues 107–417 (*3*), aa residues 1–216 (*4*), aa residues 31–242 (*5*), aa residues 218–416 (*6*), aa residues 244–417 (*7*), aa residues 107–216 (*8*), aa residues 1–105 (*9*), and aa residues 321–417 (*10*) were immunoprecipitated with gD monospecific serum. The precipitated proteins were analyzed by SDS-PAGE (12%) and autoradiography

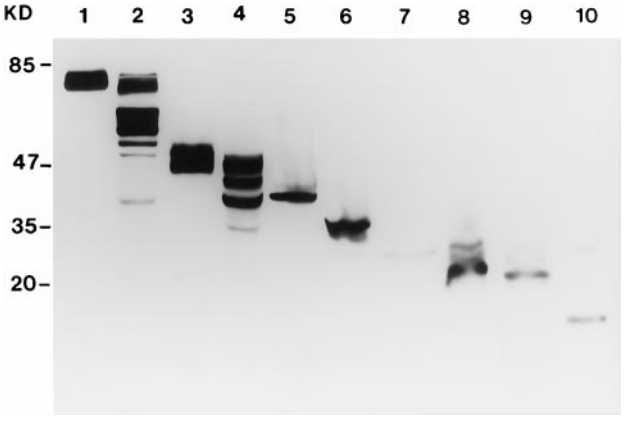
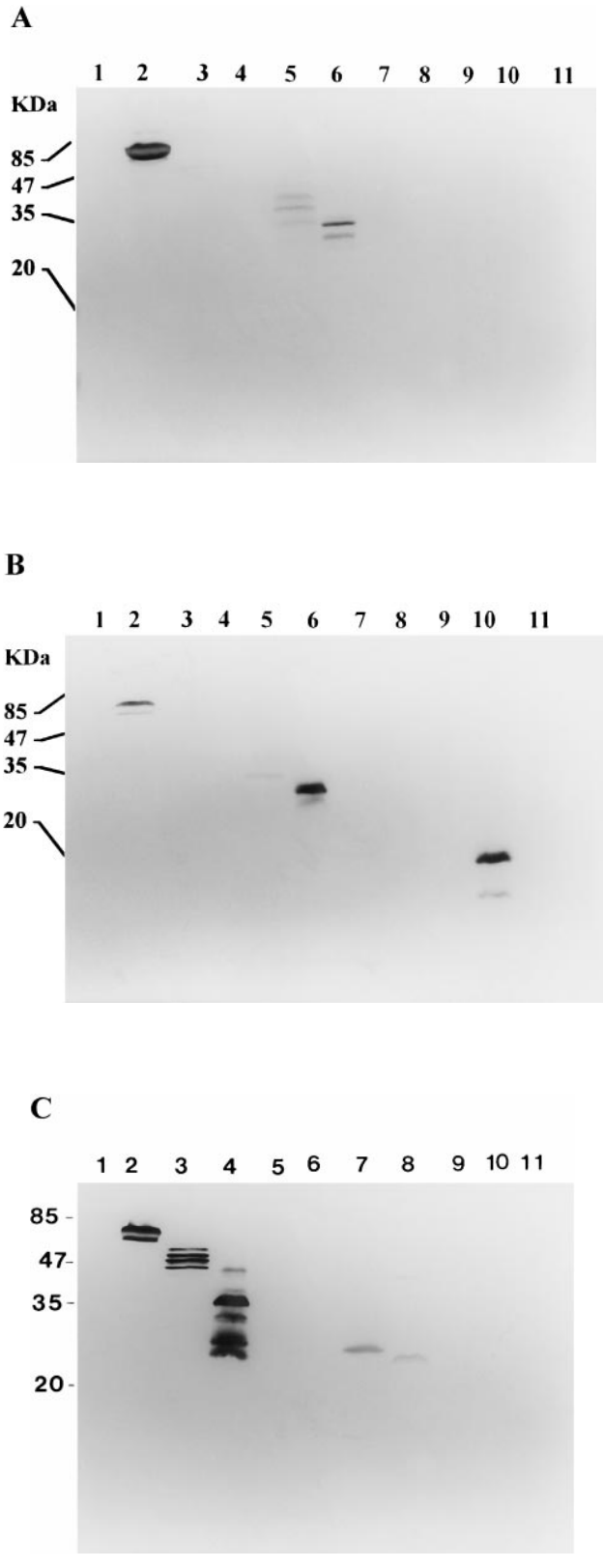


Fig. 3. Immunoblotting analysis of baculovirus-expressed BHV-1 gD amino acid sequences. Control BHV-1 infected MDBK cells (*1*), or recombinant baculovirus infected SF-9 cells expressing BHV-1 gD aa residues 1–417 (*2*), aa residues 107–417 (*3*), aa residues 1–216 (*4*), aa residues 31–242 (*5*), aa residues 218–416 (*6*), aa residues 244–417 (*7*), aa residues 107–216 (*8*), aa residues 1-105 (*9*), and aa residues 321–417 (*10*) were electrophoresed on 12% denaturing SDS-PAGE, transferred to nitrocellulose membranes, and probed with gD monospecific serum. Biotinylated secondary anti-rabbit antibodies were added and the reactive protein bands were detected with peroxidase/4-Chloro-1-Naphthol enzyme-substrate using a kit from Vector laboratories


A
1 2 3 4 5 6 7 8 9 10 11
KDa
85
47
35
20
B
1 2 3 4 5 6 7 8 9 10 11
KDa
85
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35
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C
1 2 3 4 5 6 7 8 9 10 11
85
47
35
20

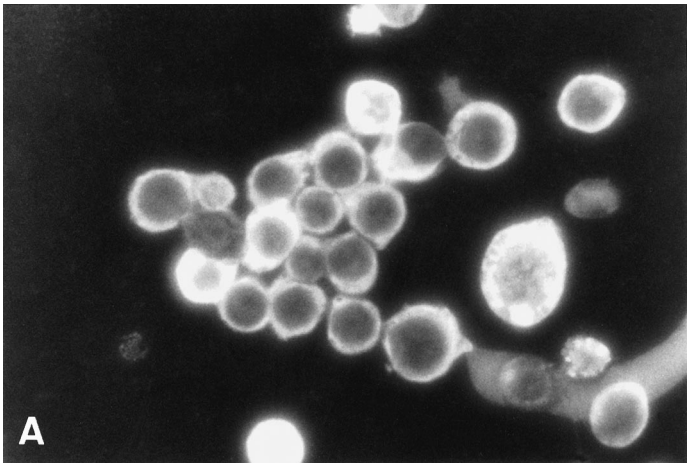
Table 1. Summary of the ELISA and immunoblotting reactivities of monoclonal antibodies with the recombinant proteins

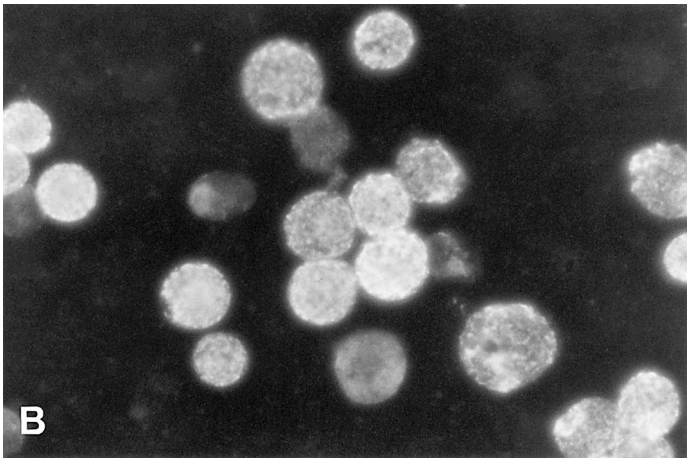
Baculovirus-expressed	Mab reactivity									
gD aa sequences	R54	9D6	3D9S	111B	136	3E7	2C8	3C1	4C1	10C2
1–417	+/−	−	+	+	+	+	+	+	+	+
107–417	−	−	+	−	−	−	−	−	−	−
1–216	+	+	−	−	−	−	−	−	−	−
31–242	+	+	−	−	−	−	−	−	−	−
218–416	−	−	+	−	−	−	−	−	−	−
244–417	−	−	+	−	−	−	−	−	−	−
107–216	−	−	−	−	−	−	−	−	−	−
1–105	−	+	−	−	−	−	−	−	−	−
321–417	−	−	−	−	−	−	−	−	−	−

Monoclonal antibodies R54, 9D6, and 3D9S were tested by western blotting while the remainder of Mabs were tested by ELISA. The +/− sign indicates reduced reactivity and aa indicates amino acids. Monospecific serum to gD reacted with all recombinant proteins by immunoprecipitation, immunoblotting, and ELISA

recognized proteins expressing amino acid residues 1–216 and 31–242 indicating that the Mab R54 binding site lies between amino acid residues 31–216 (Fig. 4A). Mab 9D6 recognized similar proteins as does Mab R54 except that it recognized an additional protein covering amino acid residues 1–105 (Fig. 4B). The two Mabs R54 and 9D6 appear to react better with short protein segments containing their epitopes than the full-length protein (Fig. 4A and B, Table 1). Mab 3D9S reacted against proteins expressing amino acid residues 1–417, 107–417, 218–416, and 244–417, narrowing its epitope between amino acid residues 244–417 (Fig. 4C and Table 1). The reactivities of the other Mabs with the respective recombinant proteins were determined by ELISA. An antibody titer of >100 is considered positive, <100 is considered negative, and 100 indicates weak reactivity. The titer cut-off at 100 was based on Mabs reactivity against BHV-1 infected MDBK and uninfected SF-9 cell lysates as positive and negative controls, respectively. Mabs 111B, 136, 3E7, 2C8, 3C1, 4C1, and 10C2 reacted only with the full-length protein expressing amino acid residues 1–417. No reaction was observed against other proteins. These results suggest that the epitopes for these Mabs require the intact gD to be expressed and that the structural conformation

Fig. 4. Immunoreactivity of monoclonal antibodies against recombinant proteins by western blotting. Proteins from uninfected SF-9 cells (*1*), from BHV-1 infected MDBK cells (*2*), or from SF-9 cells infected with BacgD (1–417) (*3*), BacgD (107–417) (*4*), BacgD (1–216) (*5*), BacgD (31–242) (*6*), BacgD (218–416) (*7*), BacgD (244–417) (*8*), BacgD (107–216) (*9*), BacgD (1–105) (*10*), and BacgD (321–417) (*11*) were electrophoresed, transferred to nitrocellulose membranes, and probed with Mabs R54 (**A**), 9D6 (**B**), and 3D9S (**C**). Reactive proteins were detected as described for Fig. 3


A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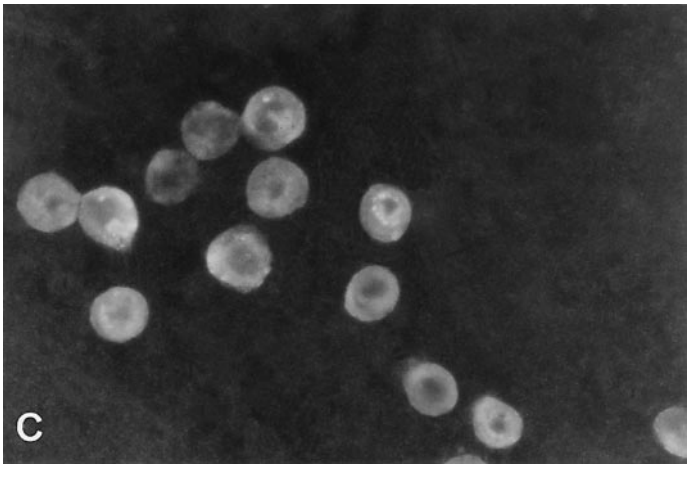
C

of the entire protein is critical for recognition by these Mabs. Similar results were obtained by immunoprecipitation (data not shown).

Cellular localization of recombinant proteins

To determine the subcellular localization and distribution of gD and ORF subfragments expressed by baculovirus, an indirect immunofluorescence test was performed on recombinant baculovirus infected SF-9 cells using anti-gD monospecific rabbit serum. Three patterns of fluorescence were observed in infected cells (Fig. 5). The intact gD expressed by BacgD (1–417) and other gD protein segments expressed by BacgD (107–417), BacgD (244–417), BacgD (107–216), BacgD (1–105), and BacgD (321–417) exhibited clear cell surface fluorescence (Fig. 5A), indicating that these recombinant proteins were efficiently transported to the insect cell surface. Recombinant proteins expressed by BacgD (1–216) and BacgD (218–416) showed only cytoplasmic fluorescence with no cell surface fluorescence (Fig. 5B), suggesting that these proteins were retained inside the cytoplasm as part of the virogenic stroma and were not transported to the extracellular surface. One protein expressed by BacgD (31–242) displayed a predominant perinuclear fluorescence (Fig. 5C), indicating that this protein was primarily localized in the cytoplasmic membranes of the perinuclear region. Correlations of the patterns of fluorescence with the gD amino acid sequences expressed by baculovirus showed no direct effect of the amino acid sequences on the distribution of these proteins in the infected insect cells.

Discussion

Glycoprotein D is an important envelope protein of BHV-1 that plays a central role in virus entry into animal cells and in the induction of protective humoral and cellular immune responses [21]. To study its functions, gD has been expressed previously in bacteria, insect, and mammalian cells [20, 22, 23]. Of these expression systems, the baculovirus-insect cell system is believed to produce recombinant proteins with properties similar to their authentic counterparts and still yield higher quantities [11]. Expression of gD in baculovirus and inoculation of the crude infected cell recombinant proteins into cattle elicited a lower neutralizing antibody response when compared to authentic gD [23]. However, when purified the recombinant gD gave similar antibody responses to the authentic gD [22]. The purpose of the present study was to examine in detail the antigenic characteristics of baculovirus-expressed gD, determine whether all antigenic determinants

Fig. 5. Immunofluorescence staining of SF-9 cells infected with recombinant baculoviruses. SF-9 cells infected with BacgD (1–417) (**A**), BacgD (1–216) (**B**), or BacgD (31–242) (**C**) were fixed with 70% acetone-30% methanol mixture, treated with gD monospecific serum, and then detected with fluorescein isothiocyanate-(FITC) conjugated goat anti-rabbit IgG. The staining of SF-9 cells infected with BacgD (107–417), BacgD (244–417), BacgD (107–216), BacgD (1–105), and BacgD (321–417) was similar to that shown in **A** and the staining of SF-9 cells infected with BacgD (218–416) was similar to that shown in **B**

are properly expressed, and also determine the localization of gD sequences expressed by recombinant baculoviruses in insect cells. To achieve this, we cloned the full-length and various segments of the BHV-1 gD ORF in baculovirus under the control of the polyhedrin gene promotor and expressed them in SF-9 cells (Fig. 1). The recombinant proteins were identified by immunoprecipitation and immunoblotting using gD monospecific serum. All proteins reacted specifically with the monospecific serum and were detected at approximately the expected molecular sizes, (Fig. 2 and Fig. 3) suggesting that they may carry properties similar to the authentic protein expressed by BHV-1. Some proteins showed more than one band which are believed to be degradation products or precursor forms of the expressed proteins. Reduced reactivity of the serum with recombinant protein expressed by BacgD(244–417) was observed on western blotting (Fig. 3 lane 7). However, the protein reacted well on immunoprecipitation (Fig. 2 lane 7).

To further study the antigenic characteristics of gD and subfragments expressed by baculovirus, we investigated the presence and locations of antigenic sites on these proteins using Mabs. The results were compared with previous epitope mapping studies using the same Mabs [2, 20]. We used western blotting to identify linear epitopes and ELISA to identify conformation-dependent epitopes. Two Mabs R54 and 9D6 recognized recombinant proteins expressing amino acid residues 1–216 and 31–242 (Fig. 4 A and B). Mab 9D6 recognized an additional recombinant protein expressing amino acid residues 1–105. These findings are consistent with previous mapping of the R54 epitope to amino acid residues 202–213 [2]. However, our results indicate that Mab 9D6 binding sites maps between amino acid residues 31–105, and not 164–216 as was determined previously [20]. Results of the reactivities of Mab 3D9S with the recombinant proteins (Fig. 4C) mapped its epitope between amino acid residues 244–416. This finding is consistent with previous mapping which has located this epitope between amino acids 320–355 [20]. Mab R54 showed reduced reactivity whereas Mab 9D6 did not react at all against recombinant protein expressing full-length gD. Nonetheless, the two Mabs reacted normally with short recombinant proteins expressing their epitopes (Fig. 4 A and B). Since R54 and 9D6 epitopes are believed to map on antigenic domain I [2, 20], this finding indicates that the linear epitopes on domain I, may be intact but may not be properly exposed on the full-length gD protein expressed by baculovirus. This is supported by the finding that sera from animals immunized with the crude baculovirus recombinant gD showed poor reactivity by ELISA against domain I epitopes [23]. The lack of proper expression of epitopes on domain I of the complete gD may be attributed to improper folding, inefficient glycosylation and/or disulfide bonding making these epitopes unaccessible to antibodies [20, 23]. It is also possible that the histidine tag at the amino terminus may have affected the proper folding of the protein.

Mabs 111B, 136, 3E7, 2C8, 3C1, 4C1, and 10C2, which do not react by western blotting, i.e. react with discontinuous epitopes, were analyzed by ELISA. All Mabs reacted with the recombinant protein expressing full-length gD, indicating that the conformation of baculovirus-expressed gD allows the expression of these discontinuous epitopes. However, no reaction of any of the Mabs was observed

against the remainder of the recombinant proteins. Although no conclusion could be drawn as to the locations of the epitopes, it is evident that the expression of the full-length recombinant gD is required in order for the Mabs to recognize their epitopes. Similar results were obtained previously using vaccinia virus recombinant protein [20]. The epitope mapping results obtained by ELISA were confirmed by immunoprecipitation (data not shown).

Immunofluorescence was used to determine the cellular distribution of the recombinant proteins in infected SF-9 cells and to examine if this distribution is influenced by the gD amino acid sequences expressed by the proteins. The results of this study demonstrated that the full-length gD protein is transported and expressed on the surface of infected SF-9 cells (Fig. 5A). The rest of the recombinant proteins were localized in different parts of the infected cells including cell surface, cytoplasm, and perinuclear membranes (Fig. 5). Correlations between the gD amino acid sequences expressed by the recombinant proteins and their cellular localization in infected cells revealed no direct relationship. For example, proteins expressed by BacgD (107–417), BacgD (244–417), BacgD (107–216), BacgD (1–105), and BacgD (321–417) all showed clearly visible cell surface expression as the one displayed by full-length recombinant gD (Fig. 5A). In another example, two proteins expressed by BacgD (1–216) and BacgD (218–416) were retained exclusively in the cytoplasm with no cell surface expression. These findings differ from previous results, using a vaccinia virus system, which showed that amino acid residues 245–320 of gD are required for gD transport to the cell surface [20]. The difference may be due to the variation between the baculovirus-insect cell and vaccinia virus systems in transport and processing of proteins. Although many baculovirus synthesized proteins are directed to the appropriate cellular locations [15], inappropriate cellular targeting has been reported [8, 15].

In summary, the immunoreactivities of gD monospecific serum and Mabs with the recombinant proteins expressing gD amino acid sequences, indicated that the full-length protein retained most of its antigenic properties. However, the full-length recombinant gD lacks two highly neutralizing linear epitopes for Mab R54 and 9D6 which make part of antigenic domain I. In contrast, most of the Mabs, specifically those recognizing conformation-dependent epitopes, did not recognize the other recombinant proteins, which indicates that these epitopes are not properly expressed. The immunofluorescence studies revealed no direct correlation between the cellular localization of the recombinant proteins and their amino acid sequences. It is likely that other factors are involved in the transport and cellular distribution of the recombinant proteins in baculovirus infected cells [8]. To further examine the potential of using the recombinant proteins as subunit vaccines we are presently purifying the ones that are highly antigenic to inoculate them into experimental animals and evaluate their immune responses.

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