Characterization of Pseudorabies Virus Glycoprotein B Expressed by Canine Herpesvirus

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ABSTRACT: A recombinant canine herpesvirus (CHV) which expressed glycoprotein B (gB) of pseudorabies virus (PrV) was constructed. The antigenicity of the PrV gB expressed by the recombinant CHV is similar to that of the native PrV. The expressed PrV gB was shown to be transported to the surface of infected cells as judged by an indirect immunofluorescence test. Antibodies raised in mice immunized with the recombinant CHV neutralized the infectivity of PrV in vitro. It is known that the authentic PrV gB exists as a glycoprotein complex, which consists of gBa, gBb and gBc. In MDCK cells, PrV gB expressed by the recombinant CHV was processed like authentic PrV gB, suggesting that the cleavage mechanism of PrV gB depends on a functional cleavage domain from PrV gB gene and protease from infected cells.—KEY WORDS: CHV, gB, PrV.

Pseudorabies virus (PrV), an alphaherpesvirus, is the etiologic agent of Aujeszky’s disease. PrV is an important pathogen of pigs, dogs, cattle and other animals. PrV has at least seven different glycoproteins. Glycoprotein B (gB) is a major envelope protein, and plays essential roles in membrane fusion events during not only virus infection but also cell-to-cell spread of viruses [14, 16]. PrV gB is initially synthesized as a monomer in the endoplasmic reticulum (ER), rapidly converted to an oligomer, and then transported as a dimer to the Golgi, in which it receives complex sugar modifications before being proteolytically processed. The complex, consisting of three polypeptide, gBa, gBb and gBc, is maintained by disulfide linkages and possibly hydrophobic interactions [5, 7, 20, 23, 25]. gBb and gBc are the cleavage products of gBa.

Those homologues to gB of herpes simplex virus type 1 (HSV-1) exhibit the highest conservation of primary structure [21, 22]. The amino acid sequence identity between the gB homologues of PrV [18] and bovine herpesvirus 1 (BHV-1) [10, 24] is 63%, whereas that between gB of HSV-1 and PrV is 50%. BHV-1 gB is able to complement functionally a gB- PrV mutant but PrV gB is unable to complement a gB- BHV-1 mutant. However both gB- mutants are complemented when the carboxy-terminal half of the chimeric gB was derived from BHV-1 gB and the amino-terminal half from PrV gB [9]. PrV gB can functionally substitute for HSV-1 gB whereas HSV-1 gB is unable to complement a gB- PrV mutant [8].

Canine herpesvirus (CHV), classified as a member of the subfamily alphaherpesvirinae [19], causes a fatal haemorrhagic disease in neonatal puppies as well as upper respiratory infection in adult dogs [1]. The unique characteristic of CHV is the low G+C content of its genome. The G+C contents of 33% have been reported for the entire CHV genome [15] and 26–29% have been observed in the gB, gC, gD, gE and gI sequences [6, 13]. The G+C contents of PrV, BHV-1 or HSV are about 70% in the entire genomes or in individual genes. In spite of the wide difference between the DNA sequences, the amino acid sequence identity between the gB homologues of CHV and PrV is 61% [6]. In this study, we constructed a recombinant CHV carrying the PrV gB gene. It was found that the PrV gB promoter functioned in the CHV system, PrVgB polypeptide was synthesized, processed authentically and antigenically authentic PrV gB was expressed.

MATERIALS AND METHODS

Cell and viruses: The DFD-6 strain of CHV [11] and its recombinants were propagated in Madin-Darby canine kidney (MDCK) cells in Eagle’s minimum essential medium (EMEM, Nissui, Tokyo) supplemented with 5% heat-inactivated fetal calf serum (FCS). The Bucharest strain of PrV was grown in monolayers of RK13 cells, which were cultured in EMEM supplemented with 7.5% FCS.

Construction of recombinant CHV transfer vectors: The plasmid pBB1, in which the BamHI1 fragment of the genome of PrV Bucharest strain was cloned at the BamHI site of pBR322, was partially digested with XbaI fragment containing the thymidine kinase (TK) gene of PrV. The Spnl fragment containing the complete gB expression unit of PrV was cloned at the Spnl site of multi-cloning sites (MCS) of pUC19 (pUCgB). The recombinant transfer vector of CHV co-expressing PrV gB gene together with lacZ gene was constructed as described earlier [27]. Briefly, the 6.5 kbp XbaI fragment containing the thymidine kinase (TK) gene of CHV YP11mu strain was cloned into the PvuII site of pUC19 (pCTK) (Fig. 1B). This procedure eliminated the MCS of pCTK. The plasmid pCTK was digested with EcoRI and HindIII at the unique sites within the TK open reading frame (ORF) sequence, and the EcoRI - HindIII

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fragment (485 bp) was replaced with EcoRI- HindIII fragment (485 bp) in the TK ORF replaced with EcoRI-HindIII fragment containing multi-cloning sites (MCS) of pUC19 resulted in pCTKdlEH/MCS; (D) 4.2 kbp fragment containing the lacZ gene controlled by SV40 early promoter inserted into XbaI site in MCS of pCTKdlEH/MCS, and the fragment containing PrV gB inserted into BamHI site in MCS of pCTKdlEH/MCS resulted in pCTK/Z/gB. Abbreviations for the restriction endonuclease site: X, XbaI; E, EcoRI; H, HindIII; B, BamHI.

Transfection and isolation of recombinant virus: MDCK cells were transfected with 40 µg of the transfer vector, pCTKdlEH/Z/gB by electroporation, and then infected with wild type CHV (DFD-6 strain). The transfection progeny was subjected to plaque assay and X-gal staining at a concentration of 200 µg/ml. The recombinant CHV was selected as the blue plaques and designated as dlTK/Z/gB. The expression of PrV gB gene was prepared from PrV gB gene in tandem was designated as pCTK/gB. The plasmid containing PrV gB inserted into TK locus.

Expression of PrV gB in recombinant CHV-infected MDCK cells: MDCK cells were infected at 1 PFU/cell with CHVdlTK/Z/gB or its parent strain, DFD-6. After incubating for 48 hr, cell extracts were prepared and analyzed by Western blotting method using anti-PrV gB rabbit serum under reducing and non-reducing conditions (Fig. 3). Under the non-reducing condition, anti-PrV gB serum reacted with two major bands in PrV-infected MDCK cell extracts and CHVdlTK/Z/gB-infected MDCK cell extracts, but not in CHV DFD-6- or mock-infected cell extracts, suggesting that these two bands corresponded to mature and precursor forms of PrV gB (Fig. 3A). Under the reducing condition, anti-PrV gB serum reacted with three major proteins, gBa, gBb and gBc in PrV-infected MDCK cells (Fig. 3B lane 2). In CHVdlTK/Z/gB-infected MDCK cells, gBa and gBb were detected and gBc were faint (Fig. 3B lane 3). The growth rate of CHVdlTK/Z/gB was slightly lower than that of parental strain, CHV DFD-6, suggesting that a little decrease of the growth rate may be due to the insertion of the foreign gene into TK locus.
cell extracts, indicating that the β-gal was expressed in CHVdlTK/Z/gB (Fig. 3C). CHV gB was expressed in CHVdlTK/Z/gB-infected MDCK cells, indicating that lacZ and PrV gB insertion had no effect on expression of CHV gB (data not shown).

To determine whether recombinant CHV expressed antigenically authentic gB, indirect immunofluorescence test using a panel of MAbs raised against PrV-derived gB was performed. As shown in Table 1, PrV gB expressed by CHVdlTK/Z/gB reacted with all five MAbs, suggesting that PrV gB expressed by CHVdlTK/Z/gB had the antigenic structure of authentic PrV gB. The reactivity of CHVdlTK/Z/gB was slightly lower than that of native PrV. This result may be due to difference of growth rate between CHV and PrV, since the growth rate of PrV is a little higher as compared with that of CHV in MDCK cells (data not shown).

Intracellular localization of PrV gB in recombinant CHV-infected MDCK cells: To determine whether CHV-expressed PrV gB was transported to the infected cell surface, recombinant CHV-infected MDCK cells were examined by

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**Fig. 2.** Growth curves of the wild type CHV DFD-6 strain (□) and CHVdlTK/Z/gB (●). Monolayers of MDCK cells were infected at a multiplicity of infection of 3. Samples were taken from the culture supernatant and cells at the time points indicated and titrated in duplicate on MDCK cells. The zero time point was taken from the virus suspension prior to infection of the monolayer.

**Fig. 3.** Western blot analysis of CHV recombinant expressing PrV gB. (A) Extracts of MDCK cells infected with DFD-6, CHVdlTK/Z/gB and PrV were analyzed using anti-PrV gB serum [12] under a non-reducing condition. Lane 1, DFD-6-infected; Lane 2, PrV-infected; Lane 3, CHVdlTK/Z/gB-infected; Lane 4, Mock-infected. mgB, mature gB; pgB, precursor gB. (B) Extracts of MDCK cells infected with DFD-6, CHVdlTK/Z/gB and PrV, were analyzed using anti-PrV gB serum under a reducing condition. Lane 1, DFD-6-infected; Lane 2, PrV-infected; Lane 3, CHVdlTK/Z/gB-infected; Lane 4, Mock-infected. (C) Extracts of MDCK cells infected with DFD-6, CHVdlTK/Z/gB and PrV were analyzed using anti-β-gal serum [28] under a reducing condition. Lane 1, DFD-6-infected; Lane 2, PrV-infected; Lane 3, CHVdlTK/Z/gB-infected; Lane 4, Mock-infected. Molecular masses of markers are given in kDa.
Table 1. Antigenic properties of recombinant PrV gB

<table>
<thead>
<tr>
<th>MAAb</th>
<th>Epitope</th>
<th>dTK/Z/gB</th>
<th>PrV</th>
<th>DFD-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.21</td>
<td>A (gB complex)</td>
<td>++b)</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>1F2</td>
<td>B (gBa, gBb)</td>
<td>+</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>6C1</td>
<td>B (gBa, gBb)</td>
<td>++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>1A6</td>
<td>B (gBa, gBb)</td>
<td>++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>1C3</td>
<td>C (gBa, gBc)</td>
<td>++</td>
<td>+++</td>
<td>–</td>
</tr>
</tbody>
</table>

a) Specific for PrV gB [13, 26].
b) Interpreted as the intensity of immunofluorescence on acetonefixed infected cells: no fluorescence (–), weak (+), medium (++), and intense (+++).

indirect immunofluorescence test with or without acetone fixation. Specific fluorescence for PrV gB was detected not only in fixed but also on unfixed cells (data not shown).

This indicates that PrV gB expressed by recombinant CHV was transported to the cell surface as the case of PrV gB produced in PrV-infected cells [25].

**Immunogenic property of PrV gB expressed by recombinant CHV:** The immunogenicity of the PrV gB produced by recombinant CHV was examined by inoculating DFD-6 or CHVdlTK/Z/gB in the culture medium into mice. As shown in Table 2, sera from CHVdlTK/Z/gB-vaccinated mice showed high titers in indirect immunofluorescence test. This suggests that PrV gB produced by CHVdlTK/Z/gB had immunogenic properties of native PrV. In addition, sera from CHVdlTK/Z/gB and CHV-vaccinated mice neutralized the antigenic structure of authentic PrV gB. In fact, the MAbs against PrV gB, which recognize conformational epitopes. Thus, PrV gB expressed by CHVdlTK/Z/gB had the antigenic structure of authentic PrV gB. In fact, the mice inoculated with CHVdlTK/Z/gB produced neutralizing antibodies to PrV.

**DISCUSSION**

In this study, we have constructed recombinant CHV, CHVdlTK/Z/gB, in which the PrV gB gene with its promoter and coding region was integrated at the TK locus. Although there is a wide gap in the G+C contents between the CHV and PrV genomes, the gB promoter of PrV is functional in CHV system because PrV gB was expressed by CHVdlTK/Z/gB and transported to the cell surface as judged by an indirect immunofluorescence test as the case of PrV gB [26]. The recombinant gB reacted with all five MAbs against PrV gB, which recognize conformational epitopes. Thus, PrV gB expressed by CHVdlTK/Z/gB had the antigenic structure of authentic PrV gB. In fact, the mice inoculated with CHVdlTK/Z/gB produced neutralizing antibodies to PrV. Similar results have been reported for HSV gB and PrV gB expressed by recombinant baculoviruses [3, 4, 26] and for PrV gB by a recombinant vaccinia virus [17]. Since CHV dlTK/Z/gB was neutralized by anti-PrV gB serum (data not shown), PrV gB is probably present on the virion surface as the case of authentic PrV [16]. The growth rate of CHVdlTK/Z/gB was slightly lower as compared with that of parental strain. It was reported that the growth rate of recombinant CHV which lacZ gene or gene of rabies virus G protein was inserted into its TK locus was slightly lower than that of parental strain, CHV.

**Table 2. Neutralization titer of sera from mice immunized with recombinant CHV**

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>PrV in</th>
<th>DFD-6</th>
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<tbody>
<tr>
<td>CHV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFD-6</td>
<td>&lt;5</td>
<td>&gt;12800</td>
</tr>
<tr>
<td>dlTK/Z/gB</td>
<td>320</td>
<td>&gt;25600</td>
</tr>
</tbody>
</table>

a) Virus neutralization test was performed in the complement.

DFD-6 [27]. In addition, dog inoculated with recombinant CHV which expresses rabies virus G protein produced hight titers of virus neutralizing antibodies against rabies virus [28].

These results suggest that CHVdlTK/Z/gB may be used as a vaccine to control Aujeszky’s disease in dogs.

It is known that the authentic PrV gB exists as a glycoprotein complex, consisting of gBa, gBb and gBc [5, 7, 23, 25]. After glycosylation, PrV gB precursor is cleaved by cellular protease located in the Golgi apparatus [23] into two smaller glycoproteins, gBb and gBc. An 11-amino-acid segment of PrV gB as a functional cleavage domain has been defined. When a synthetic oligomer encoding these residues is inserted in an unrelated PrV glycoprotein gene, the resulting hybrid protein is cleaved [23]. In a recombinant baculovirus system [26] and a recombinant vaccinia virus system [17], the PrV gBa is cleaved to gBb and gBc. In this study, PrV gB was cleaved in MDCK cells, rabbit kidney (RK13) cells and Madin-Darby bovine kidney (MDBK) cells (data not shown), suggesting the influence of common protease from several cells. PrV gB expressed by CHVdlTK/Z/gB was processed like authentic PrV gB in MDBK cells. Since CHVdlTK/Z/gB contains PrV gene with its promoter and coding region, it is supposed that other viral factors from PrV does not participate in the cleavage of PrV gB. These results suggested that cleavage mechanism of PrV gB depended on a functional cleavage domain from PrV gB gene and protease from infected cells [23].

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**REFERENCES**


