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Molecular Characterization and Baculovirus Expression of the Glycoprotein B of a Seal Herpesvirus (Phocid Herpesvirus-1)

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A glycoprotein B (gB) gene homologue was identified in a 5.4-kb *Bam*HI genomic fragment of the phocid herpesvirus type-1 (PhHV-1) which represents a widespread and important pathogen of pinnipeds. Sequence analysis revealed a gB-specific open-reading frame comprising 881 amino acids. Phylogenetic analysis gave evidence for a close evolutionary relationship between PhHV-1 and members of the *Varicellovirus* genus of the *α-Herpesvirinae* and canid herpesvirus in particular. In PhHV-1-infected Crandell feline kidney cells gB is expressed as a 113-kDa glycosylated molecule which is proteolytically cleaved into at least two fragments of 67 and 53–59 kDa apparently forming disulfide-linked heterodimers of 140 kDa. Cell surface expression of PhHV-1 gB was confirmed by FACS analysis. Thus, synthesis and processing of the gB protein of PhHV-1 follows a pattern also observed in other *Varicelloviruses.* Since the gB protein of herpesviruses, expressed in the baculovirus system, has been shown to be a suitable target for vaccine design, we used this system for expression of PhHV-1 gB. Recombinant (rec) baculovirus-expressed gB was identified as a 105-kDa glycosylated molecule. Proteolytic cleavage into fragments of 62 and 52 kDa was markedly delayed compared to wild-type (wt) gB. Wt and rec gB harbored endoglycosidase H (precursor)- as well as N-glycosidase F-sensitive N-glycans (proteolytic fragments). Baculovirus-expressed gB appeared to be antigenically authentic, since it was recognized in radioimmunoprecipitation and immune peroxidase monolayer assays by PhHV-1-neutralizing seal sera and by gB-specific neutralizing murine monoclonal antibodies. Furthermore, PhHV-1-neutralizing antibodies were induced in mice following immunization with baculovirus-expressed gB, indicating its suitability for incorporation in a candidate vaccine for seals.

INTRODUCTION

Two species of authentic herpesviruses of pinnipeds, designated phocid herpesvirus type-1 and type-2 (PhHV-1, -2) have been described (Osterhaus *et al.,* 1985; Lebich *et al.,* 1994; Harder *et al.,* 1996). On the basis of partial sequence analysis, PhHV-1 was tentatively associated with the *Varicellovirus* genus of the *α-Herpesvirinae,* whereas PhHV-2 clustered with *γ-Herpesvirinae* (Harder *et al.,* 1996).

While PhHV-2 infections have not been associated with disease in seals, PhHV-1 represents an important pathogen: Following primary infection, self-limiting upper respiratory disease is most frequently observed. Neonate seals and immunocompromised animals, however, are prone to fatal generalization of the infection (Osterhaus *et al.,* 1985, Frey *et al.,* 1989). Serosurveys indicated high prevalences of PhHV-1 or other closely related herpesviruses among different seal species worldwide (Vedder *et al.,* 1987; Harder *et al.,* 1991; Stenvers *et al.,* 1992; Stuen *et al.,* 1994). In seal rehabilitation centers recurrent annual outbreaks of PhHV-1 infections causing losses of pups pose a severe problem and raise demands for a vaccine conferring protection against disease (Osterhaus *et al.,* 1985; Borst *et al.,* 1986; Harder *et al.,* submitted).

Herpesvirus membrane glycoproteins (gp's) mediate essential functions during the replication cycle and are important determinants of viral pathogenesis (Spear, 1993). Among several gp's which proved to be indispensable for viral replication of α-herpesviruses *in vitro,* the glycoprotein B (gB), in concert with other gp's, plays an essential role during the penetration process of virus into the host cell cytoplasm by a fusion event between the virion envelope and the host cell membrane as well as for virus spread by cell-to-cell fusion (Cai *et al.,* 1988; Pereira, 1994). Representatives of the gB family are expressed by members of all herpesvirus subfamilies and show considerable sequence and structural homologies (Gong *et al.,* 1987; Griffin, 1991; Niikura *et al.,* 1992; Maeda *et al.,* 1992; Ellinger *et al.,* 1993; Goltz *et al.,* 1994; Limbach *et al.,* 1994). Therefore, gB has also become an interesting tool for studies on herpesvirus evolution (Karlin *et al.,* 1994; McGeoch and Cook, 1994). Furthermore, gB represents a prominent target of both humoral and cellular immune responses particularly against α-herpesvirus infections, and in the mouse model of herpes simplex virus-1 (HSV-1) infection immunity solely to gB has been shown to confer protection against lethal chal-

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lenge (Ling Chan *et al.,* 1985; Cantin *et al.,* 1987; Blacklaws and Nash, 1990; Ghiasi *et al.,* 1992; Eis-Hubinger *et al.,* 1993). Thus, gB is considered a major constituent in the development of herpesviral subunit vaccines (Inglis, 1995).

Here we present the nucleotide and deduced amino acid sequences of the gene encoding the gB equivalent of PhHV-1 and show that it can be expressed in a baculovirus system as an antigenetically and immunogenically intact protein.

MATERIALS AND METHODS

Virus and cells

Crandell feline kidney cells (CrFK), maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, were used for culturing PhHV-1 (isolate PB84). A modified *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV–BacPAK 6) expressing β-glucuronidase (Kitts and Possee, 1993; obtained from Clontech) and other recombinant baculoviruses were grown and assayed in *Spodoptera frugiperda* clone 9 (Sf 9) monolayer cultures according to standard procedures (O'Reilly *et al.,* 1992).

Purification and restriction endonuclease digestion of PhHV-1 DNA

PhHV-1 viral DNA was extracted from mature extracellular virions enriched by ultracentrifugation through a sucrose cushion as described previously (Lebich *et al.,* 1994). Recovery of DNA followed the protocol of Engels *et al.* (1983). Aliquots of approximately 1 μg of viral DNA were endonuclease-digested, separation of fragments was by electrophoresis in 0.7% agarose slab gels at 1.0 V/cm overnight, and fragments were visualized after ethidium bromide staining.

Southern blot hybridization

Restriction fragments of PhHV-1 DNA were separated in 0.7% agarose gels and blotted using standard procedures (Sambrook *et al.,* 1989). A 1.6-kb PCR amplificate derived from the PhHV-1 (isolate PB84) gB equivalent gene served as a probe in nonradioactive hybridizations (ECL; Amersham). Selection of degenerate primers (upstream, 5′-ACA ACT gTA Tgg TCT gg; downstream, 5′-ggT AgA AAT TCA CgA TC[C/T] TC) from conserved parts of the canid and felid herpesvirus (CHV and FHV) gB genes as well as PCR conditions were exactly as described previously (Harder *et al.,* 1996).

Cloning and DNA sequencing

A 5.4-kb *Bam*HI restriction fragment of the PhHV-1 PB84 genome hybridized to the gB-specific probe and was cloned into pBluescript (SK^+) phagemid (Stratagene). Nested sets of deletions were generated from the 5′-end of the insert using an exonuclease III system (Erase-a-Base; Promega). Deletion mutants were cycle-sequenced using dye-dideoxy terminator reactions (PRISM; Applied Biosystems) and analyzed on an automatic sequencer (373A; Applied Biosystems). Specific primers (17-mers) were then selected to sequence the complement strand. Sequences were finally assembled and further analyzed using the GCG software package, Release 8.0 (Devereux *et al.,* 1984).

Construction of transfer vectors and recombinant baculoviruses

The entire ORF encoding the PhHV-1 gB gene was amplified by PCR from full-length viral DNA. Primers were selected according to the newly established sequences (upstream, 5′-CgC *ggA TCC* AgT TAT gTA TTT AAT TAC; downstream, 5′-Cgg *CTC gAg* TTA gTT ATT AAA CCT CAT C). Italics indicate *Bam*HI and *Xho*I restriction sites enabling directional cloning of the amplificate into baculovirus transfer plasmid pBacPak8 (Clontech) downstream of the polyhedrin promoter (plasmid pBacPBgB). PCR was performed employing a modified polymerase (Expand High Fidelity; Boehringer Mannheim) to improve the fidelity of the reaction (Barnes, 1994). Sequence integrity of the *Bam*HI–*Xho*I gB insert was confirmed using specific 17-mer primers. Liposome-mediated cotransfection of Sf 9 insect cells with linearized baculovirus BacPAK 6 DNA (lacking a *Bsu*36I fragment) and pBacPBgB according to the manufacturer's recommendations (Clontech) yielded recombinants which were plaque-purified. Recombinants were screened for the 2.7-kb gB ORF by PCR using primers Bac 1 (5′-ACC ATC TCg CAA ATA AAT AAg) and Bac 2 (5′-ACA ACg CAC AgA ATC TAg Cg) (Clontech) located in the polyhedrin promoter and terminator regions, respectively. Selected recombinants were further plaque-purified and expanded for expression analysis.

Northern blot hybridizations

The full length 2.7-kb PCR amplificate of the PhHV-1 gB was used as a probe in a nonradioactive Northern blot assay (ECL; Amersham). Total RNA was extracted from mock-infected and PhHV-1 (PB84)-infected CrFK cells (24 hr postinoculation, p.i.). Hybridization at 42° was allowed for 16 hr. Subsequent washing steps were carried out under high stringency conditions.

Immune peroxidase monolayer assay (IPMA)

Semiconfluent Sf 9 cell cultures were infected with recombinant BacPBgB or BacPAK 6 or were mock-infected. CrFK cell cultures, infected with wt PhHV-1 PB84 or mock-infected, were prepared in parallel. After 3 days of incubation confluent monolayers were heat-fixed at 80° for 2 hr. Serial twofold dilutions in PBS containing 0.05% (v/v) Tween 20 of PhHV-1-specific convalescent seal sera

or monoclonal antibodies (mabs) raised against PhHV-1 (Lebich *et al.,* 1994) were applied to the wells and incubated at 20° for 2 hr. Wells to which seal sera had been added were processed with protein A–peroxidase conjugates while goat anti-murine IgG peroxidase conjugates were used to detect bound mabs. 3-Amino-9-ethylcarbazole served as a chromogene. Between all incubation steps plates were washed extensively with double-distilled water. Results were read by light microscopy.

Radioimmunoprecipitation assay (RIPA)

CrFK cells either mock-infected or infected with wt PhHV-1 (m.o.i. = 1) and Sf 9 cells infected with BacPBgB or BacPAK 6 (m.o.i. = 4) were metabolically labeled with L-[^{35}S]methionine/cysteine (Promix; Amersham: 400 μCi per 10^7 cells) for 4 hr at 24 and 48 hr p.i., respectively. A parallel set of cultures was maintained in the presence of tunicamycin (10 μg/ml), added when cultures were starved for 1 hr in methionine/cysteine-deficient medium. Cell lysis and immunoprecipitations were exactly as described (Barrett *et al.,* 1989). For precipitations mabs 1.1H3, 2.2F2, and 3.4B3 raised against PhHV-1 by Lebich *et al.* (1994) were used since these mabs were found to react to BacPBgB-infected Sf 9 cells in IPMA. Mabs 1.1H3 and 2.2F2 were also known to neutralize PhHV-1 isolates. Additionally, precipitations were performed with various pinniped sera obtained from seals of different species with or without virus-neutralizing (VN) antibodies to PhHV-1. For pulse–chase studies CrFK cells infected for 24 hr with wt PhHV-1 and Sf 9 cells infected for 48 hr with BacPBgB were pulsed with L-[^{35}S]methionine/cysteine (400 μCi per 10^6 cells) for 15 min and chased for various times with cold methionine/cysteine. All immunoprecipitates were analyzed in 12.5% SDS–PAGE gels.

Endoglycosidase treatment of immunoprecipitated proteins

Lysates of ^{35}S-radiolabeled CrFK cells infected with PhHV-1 PB84 or Sf 9 cells infected with BacPBgB were immunoprecipitated with mab 3.4B3. Extracted proteins were eluted from protein A–Sepharose beads by boiling in Endo H/F buffer (10 m*M* Tris, pH 8.0, 0.5% SDS, 0.1% 2-mercaptoethanol) for 3 min. Digestion with 2 mU endoglycosidase H (cleaves high mannose and hybrid N-linked oligosacharides) or 100 mU N-glycosidase F (removes all N-glycans except those with α-1,3-linked core fucose residues) proceeded at 37° overnight in the presence of protease inhibitors (Complete; Boehringer Mannheim). Digests were analyzed by SDS–PAGE in 12.5% gels.

Immunization of mice

Sf 9 cells infected with BacPBgB or wild-type BacPAK6 for 72 hr were washed twice in PBS and resuspended at 10^7 cells/ml PBS. Mice were injected subcutaneously with 0.1 ml (equivalent to 10^6 cells). Injections were repeated 14 and 28 days later. Sera were recovered 5 days after the final injection.

Herpesvirus neutralization assay

VN antibodies to PhHV-1, CHV, and FHV in seal and mice sera were measured by means of a complement-enhanced virus microneutralization assay as described (Harder *et al.,* 1991).

FACS analysis

Native CrFK and Sf 9 cells infected at high m.o.i. with PhHV-1 (20 hr) or BacPBgB (72 hr), respectively, were labeled with mab 3.4B3 for 1 hr on ice in PBS supplemented with 1% (w/v) BSA (B-PBS). Cell viability exceeded 90% on basis of trypan blue dye exclusion. FITC-labeled goat anti-murine IgG was used as a second antibody. Between all incubation steps cells were washed gently in B-PBS. Mock-infected controls were treated identically. Cells were analyzed in a FACScan (Becton–Dickinson).

GenBank accession

The nucleotide sequence of the PhHV-1 gB gene has been assigned Accession No. Z68147.

RESULTS

Identification and nucleotide sequence analysis of the PhHV-1 gB equivalent gene

Southern blot hybridizations using a 1.6-kb gB-specific PCR amplificate of the PhHV-1 isolate PB84 as a probe led to the identification of gB-specific sequences in a 5.4-kb *Bam*HI fragment of the PhHV-1 genome (not shown). Figure 1 presents the nucleotide and deduced amino acid (aa) sequences of open reading frames identified within a 3-kb part of the respective *Bam*HI fragment which comprises the entire gB gene homologue of PhHV-1 capable of encoding 881 aa. The sequence context according to Kozak's rule (Kozak, 1991) around the putative gB initiation codon (ATT *ATG* T) is well conserved at position -3, but not at $+4$, which is similar to the gB gene of CHV (Limbach *et al.,* 1994) and other members of the *Varicellovirus* genus. Analysis of sequences upstream of the putative gB ORF revealed homology to members of the UL 28 gene family of α-herpesviruses which encodes the ICP18.5 (Pellett *et al.,* 1986). An overlap of ICP18.5 and gB ORFs as described for several members of the *Varicellovirus* genus including FHV, bovid herpesvirus-1 (BHV-1), pseudorabies virus (PRV), and equine herpesvirus-1 and -4 (EHV-1 and -4) was not found. Thus, PhHV-1 gene arrangements in this region again resemble those of CHV (Limbach *et al.,* 1994). Downstream of the gB ORF several potential initiation codons were located. However, homologies to herpesvi-

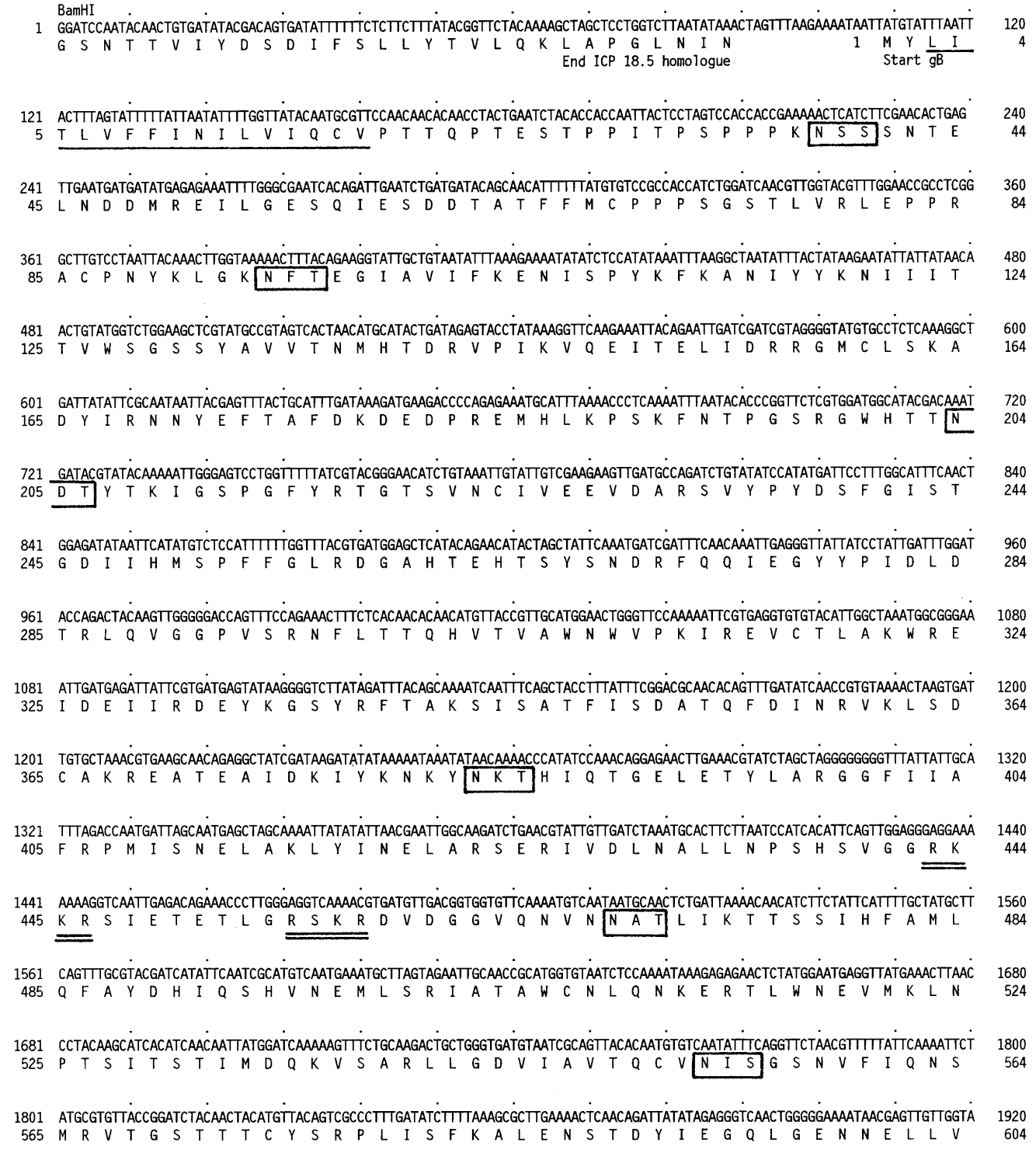

FIG. 1. Nucleotide sequence of a part of a 5.4-kb *Bam*HI fragment of PhHV-1 (isolate PB84) and deduced amino acid sequences of open reading frames. A polyadenylation signal is indicated by dashed lines (2760–2765). Putative N-linked glycosylation sites are boxed. Regions of primarily hydrophobic amino acids capable of spanning membranes are underlined. Subtilisin-like serine endoprotease motifs (-R-X-K/R-R-) are double underlined.

ral proteins were not found for products translated from any of the putative start codons.

Northern blot analysis gave evidence for at least two gB-specific mRNA species of 3.6 and 2.8 kb in infected CrFK cells at 24 hr postinfection (not shown). Similar gB transcript patterns have been detected in FHV-infected cells (Maeda *et al.,* 1992). A polyadenylation signal (McLauchlan *et al.,* 1985) is present just downstream of

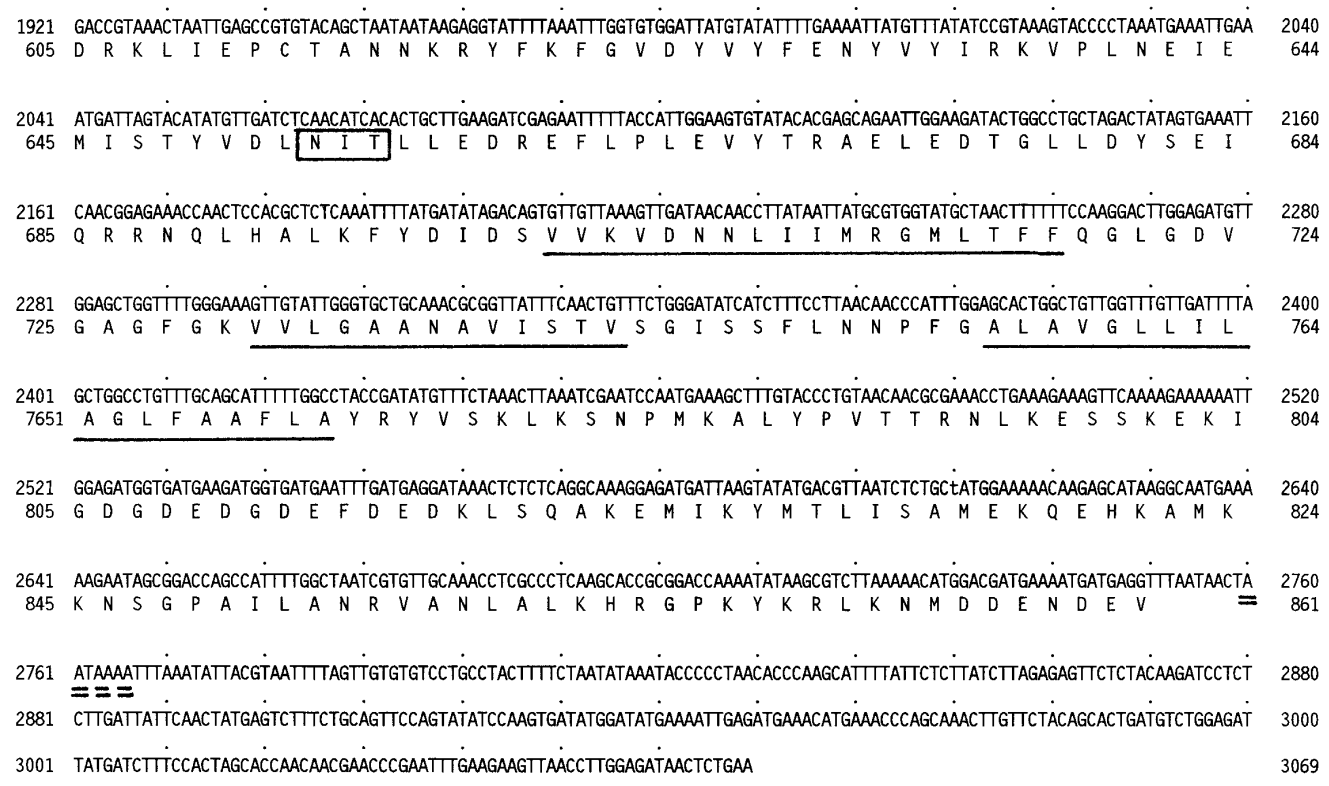

FIG. 1—*Continued*

the gB termination codon (Fig. 1). Possible gB promoter regions were not identified in this study.

Predicted PhHV-1 gB amino acid sequence and phylogenetic inferences

Secondary structure analysis of the deduced amino acid sequence of PhHV-1 gB revealed characteristics of a type-I transmembrane glycoprotein featuring three C-terminal clusters of hydrophobic amino acids (Fig. 1). The most N-terminal stretch of these, however, harbors an arginine in a central position and, therefore, is highly unlikely to function as a membrane anchor. Though an N-terminal hydrophobic translocation signal was identified, a possible signal cleavage site was not unambiguously predicted (Nakai and Kaneshisa, 1992) using either the Van Heijne matrix (Van Heijne, 1987) or the method of McGeoch (1985).

Within the putative extracytoplasmic domaine eight potential N-linked glycosylation sites are predicted. A motif of subtilisin-like serine endoproteases (-R-X-K/R-R-; Garten *et al.,* 1994) is found at aa positions 443–446 and 455–458. Cleavage at or near either of these sites would yield two fragments of approximately 51/49 or 52/48 kDa from a 100-kDa precursor (molecular masses calculated for full-length unglycosylated forms).

To study evolutionary relationships of PhHV-1, deduced aa sequences of gB equivalents of several herpesvirus species were aligned using "Pileup" (GCG). Stretches which could not be unambiguously aligned were removed and phenetic analysis finally was based on 645 aa residues. Inferences were performed by use of the neighbor-joining principle (NJ) based on distance matrices calculated by Kimura's method and by use of maximum parsimony (PARS) employing a branch and bound approach. The best trees computed by these methods (Phylip software package; Felsenstein, 1989) displayed similar topologies placing PhHV-1 among the members of the *Varicellovirus* genus (NJ, Fig. 2; PARS, not shown). HSV-1, an α-herpesvirus of the *Simplexvirus* genus was chosen as an outgroup. According to the distances calculated and confirmed by the high bootstrap values, CHV was identified as the closest relative of PhHV-1 (Fig. 2).

Synthesis and processing of authentic and baculovirus-expressed PhHV-1 gB

Baculoviruses carrying the complete gB ORF of PhHV-1 were constructed and the recombinant BacPBgB was selected for expression studies. Several mabs (including 1.1H3, 2.2F2, 3.4B3) and all five PhHV-1-neutralizing pinniped sera strongly reacted in IPMA with Sf 9 cells infected by BacPBgB, but not with control cells (BacPAK6 expressing β-glucuronidase, mock-infected Sf 9 cells) (data not shown).

Synthesis and processing of authentic and rec gB were analyzed by RIPA. Mab 3.4B3 specifically coprecipitated

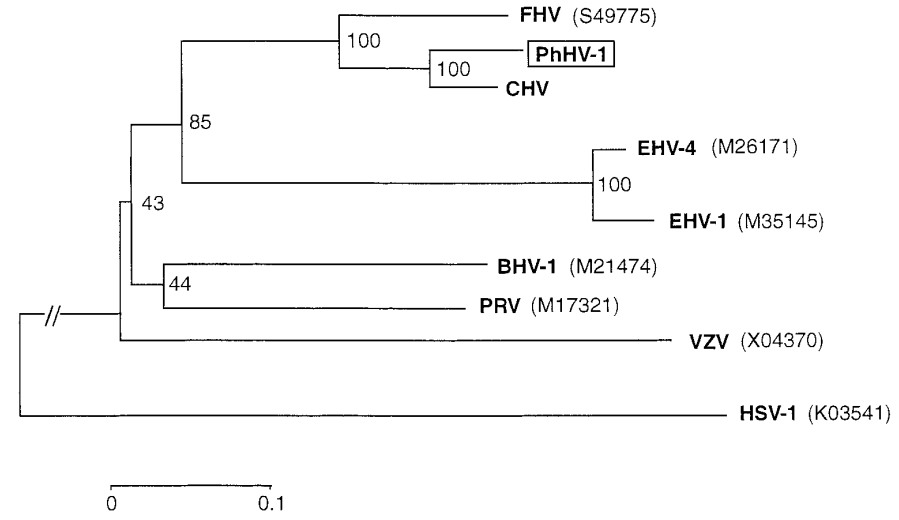

FIG. 2. Inferred evolutionary relationships of PhHV-1 based on gB aa sequences. Alignments were computed by "Pileup" included in the GCG software package (Version 8.0) setting gap creation weights to 3.0 and gap extension weights to 0.1. Further editing included the removal of stretches which could not be unambiguously aligned. Distances were calculated by the Kimura method on the basis of 645 residues and used to construct trees according to the neighbor-joining method. Branches are drawn in proportion to distances as indicated by the bar. Numbers at tree branchings represent bootstrap *P* values after 200 replications. Values ⩾95% indicate a statistically significant separation of the respective cluster from the rest of the tree. Calculations were carried out using the "Phylip" software package (Felsenstein, 1989). Sequences were extracted from the GenBank database (Accession No. indicated) or from recent publications (CHV, Limbach *et al.,* 1994). VZV, varicella zoster virus.

proteins of 53–59, 67, and 113 kDa from CrFK cells infected with the parent PhHV-1 isolate (PB84) and proteins of 52, 60–62, and 105 kDa from Sf 9 cells infected with BacPBgB when precipitates were analyzed under reducing conditions (Fig. 3A). Under nonreducing conditions, proteins of approximately 113 and 140 kDa (wt gB) and 105 kDa (rec gB) were depicted (Fig. 3B). Mabs 1.1H3 and 2.2F2, known to neutralize PhHV-1 isolates (Lebich *et al.,* 1994), showed identical reactivities (data not shown). Sera collected from free-ranging pinnipeds reacted to a 105-kDa protein expressed in BacPBgB-infected Sf 9 cells only when containing neutralizing antibodies (titers exceeding 1/20 ND_{50}) against PhHV-1 (Fig. 3A). All seal sera tested also reacted weakly to a 135-kDa protein in Sf 9 cells infected with BacPAK 6.

Possible relationships of gB-specific proteins coprecipitated by single mabs from PhHV-1-infected CrFK cells were investigated in pulse–chase experiments which gave evidence that the 53- to 59- and 67-kDa protein species are generated by proteolytic cleavage from a 113-kDa precursor (Fig. 4A). Cleavage products were demonstrated already after a 15-min chase. However,

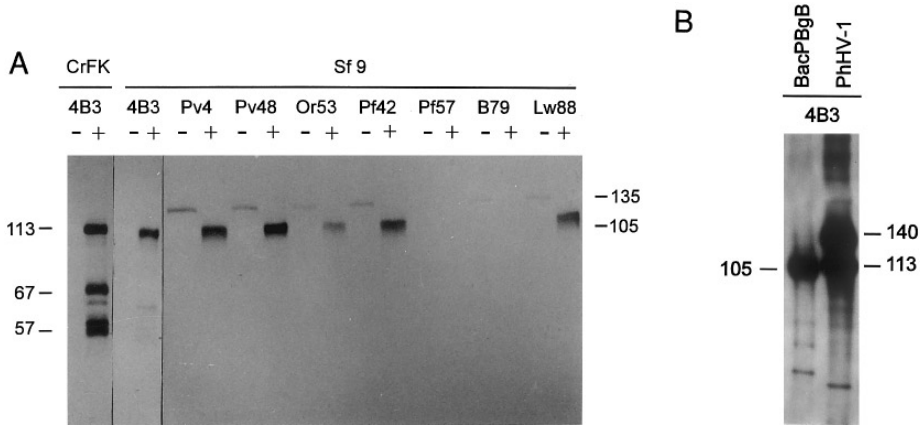

FIG. 3. Detection of wt and rec PhHV-1 gB by RIPA. [^{35}S]Methionine/cysteine-labeled lysates of CrFK cells infected with PhHV-1 PB84 (+) or mock-infected (−) and Sf 9 cells infected with BacPBgB (+) or BacPAK6 (−) were precipitated using the PhHV-1-specific mab 3.4B3 (4B3) or various pinniped sera obtained from different species worldwide. Selection of sera was based on presence of neutralizing antibodies to PhHV-1 (titer exceeded 1/20 ND_{50}): Pv4 and Pv48 (common harbor seal, *Phoca vitulina,* U.S. Atlantic coast and Dutch Wadden Sea), Or53 (walrus, *Odobenus rosmarus,* Bering Sea), Pf42 (ribbon seal, *Phoca fasciata,* Bering Sea), Lw88 (Weddell seal, *Leptonychaetes weddellii,* Antarctica). Sera from Pf57 (ribbon seal, *Phoca fasciata,* Bering Sea) and B79 (bearded seal, *Erignathus barbatus,* Norton Sound) had no detectable VN antibodies to PhHV-1. Separation was by SDS–PAGE (12.5% polyacrylamide) under reducing (A) or nonreducing (B) conditions. The apparent M_r of precipitates is indicated.

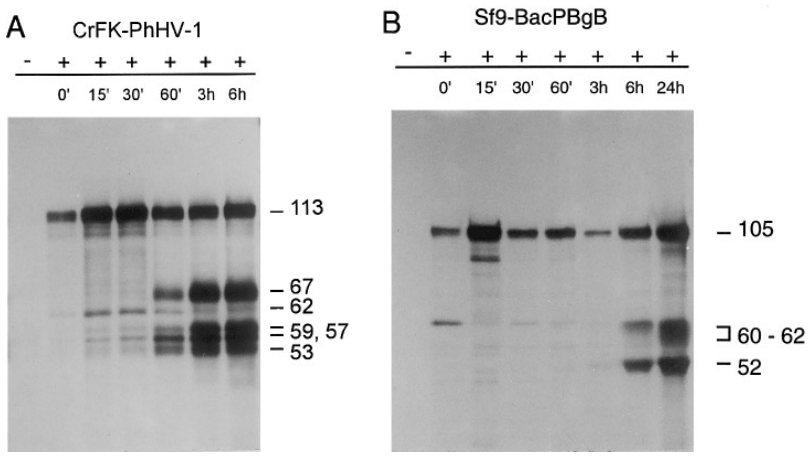

FIG. 4. Synthesis and processing of PhHV-1 gB. CrFK cells infected with wt PhHV-1 (A) or Sf 9 cells infected with BacPBgB (B) were pulse-labeled at 24 and 48 hr p.i., respectively, with [^{35}S]methionine/cysteine at 400 μCi per 10^6 cells for 15 min and then chased for the indicated time periods. Cell lysates were precipitated using mab PhHV-1 3.4B3. Mock-infected CrFK cells or BacPAK6-infected Sf 9 cells served as controls (−). Separation was by SDS–PAGE (12.5% polyacrylamide) under reducing conditions. The apparent M_r of precipitates are indicated.

processing was incomplete since after a 6-hr chase at least 50% of precursor molecules still remained uncleaved. In addition, a 62-kDa protein was weakly coprecipitated from PhHV-1-infected cell lysates by gB-specific mabs; the intensity of this band decreased during the chase (Fig. 4A).

Pulse–chase studies performed with BacPBgB-infected Sf 9 cells likewise revealed proteolytic processing of the 105-kDa baculovirus-expressed PhHV-1 gB precursor (Fig. 4B). Significant amounts of fragments of 52 and 60–62 kDa, however, were present only after 6 hr of chase and even after 24 hr at least 50% remained unprocessed.

To examine glycosylation of wt and rec PhHV-1 gB, immunoprecipitates obtained with mab 3.4B3 from BacPBgB-infected Sf 9 cells and wt PhHV-1-infected CrFK cells were digested overnight with endoglycosidase H or N-glycosidase F or were left untreated. In parallel, precipitates from PhHV-1-infected CrFK cells labeled in the presence of tunicamycin to shut off N-linked glycosylation were analyzed. As shown in Fig. 5, proteins of 47/49, 59, and 98 kDa were precipitated from tunicamycin-treated PhHV-1-infected cell lysates. Both wt and rec gB were sensitive to Endo H and Endo F digestion. The shifts observed in molecular masses after digestion were similar in wt and recombinant gB and indicated that the gB precursor molecules were particularly sensitive to Endo H. The fragments, at least, of wt gB were fully sensitive to Endo F, but only partially to Endo H. The smallest wt gB fragment generally migrated as a broad band of 53–59 kDa. Following treatment with Endo H, however, a single protein of approximately 53 kDa was depicted (Fig. 5), indicating that different levels of Endo H-sensitive glycosylation may account for the varying molecular weights.

Cell surface expression of PhHV-1 gB was further examined by FACS analysis of CrFK and Sf 9 cells using mab 3.4B3. As shown in Fig. 6, expression of the protein was much more pronounced in the CrFK cells than in the Sf 9 cells when analyzed 24 and 48 hr after infection, respectively.

Immunogenicity of recombinant PhHV-1 gB in mice

Mice immunized with BacPBgB-infected Sf 9 cells developed neutralizing antibodies against PhHV-1 with titers ranging from 8 to 22 (reciprocal of ND_{50}). No reactivities in neutralization assays were detected with sera of

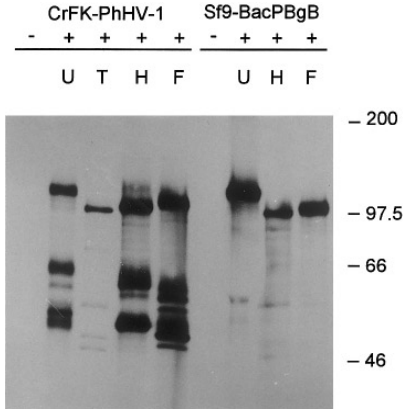

FIG. 5. Glycosylation of wt and baculovirus-expressed gB of PhHV-1. PhHV-1-infected CrFK cells or Sf 9 insect cells infected with BacPBgB were metabolically labeled with [^{35}S]methionine/cysteine at 24 and 48 hr p.i., respectively, for 4 hr. Lysates were reacted with mab 3.4B3. Precipitates were either left untreated (U) or digested with endoglycosidase H (H) or N-glycosidase F (F). In addition, precipitates were obtained from infected CrFK cells which were grown in the presence of tunicamycin (T). Mock-infected CrFK cells or BacPAK6-infected Sf 9 cells served as controls (−). Separation was by SDS–PAGE (12.5% polyacrylamide) under reducing conditions. The M_r of marker proteins are indicated.

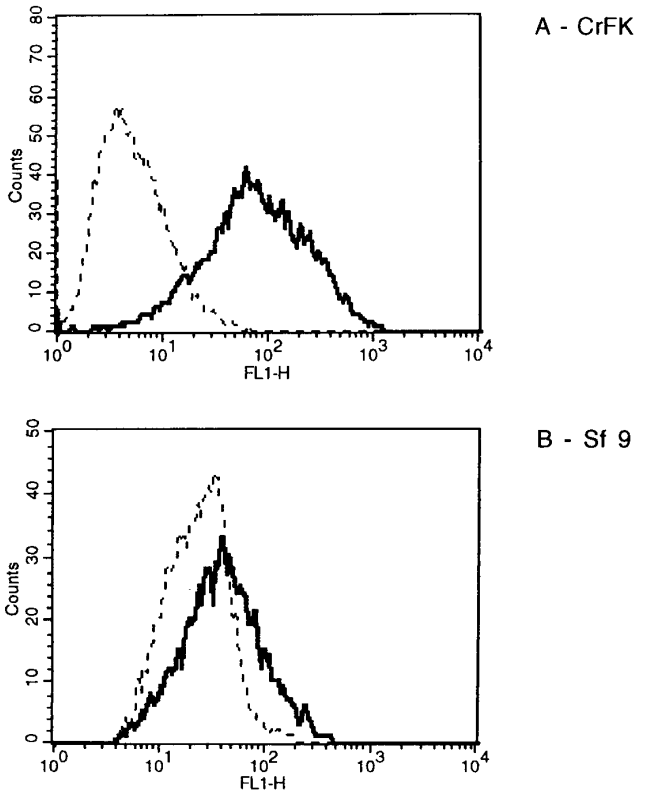

FIG. 6. Cell surface expression of PhHV-1 authentic and baculovirus-expressed gB. Viable PhHV-1-infected CrFK cells and BacPBgB-infected Sf 9 cells (bold lines) or mock-infected controls (dashed lines) were labeled with the gB-specific mab 3.4B3. Surface-bound mabs were detected using FITC-conjugated goat anti-mouse IgG and quantified by analysis in a FACScan.

control mice (immunized with BacPAK6-infected Sf 9 cells).

DISCUSSION

In the present paper we have molecularly characterized the gB of PhHV-1 and expressed it in a baculovirus system. Sequence analysis revealed greatest homology to α-herpesvirus species of the *Varicellovirus* genus which encode a proteolytically cleavable gB. Taxonomic clustering of PhHV-1 was confirmed by phylogenetic inference indicating that the genuine α-herpesviruses of carnivores (FHV, CHV, and PhHV-1), like the equine α-herpesviruses, form a separate cluster within this genus. In line with our previous data concerning cross-neutralization and B cell epitope patterns (Osterhaus *et al.,* 1985; Harder *et al.,* 1991; Lebich *et al.,* 1994), CHV was identified as the closest relative of PhHV-1. Considering recent evidence for a monophyletic origin of pinnipeds from a caniform carnivore ancestor (Lento *et al.,* 1995), the phylogenetic tree of α-herpesviruses from carnivores overlaps with the phylogeny of their natural hosts. These findings may contribute to the cospeciation hypothesis of α-herpesvirus evolution (McGeoch and Cook, 1994).

Biochemical characterization of PhHV-1 gB confirmed several features predicted from its sequence analysis: In infected CrFK cells the protein is synthesized as a 98-kDa transmembrane molecule which is cotranslationally glycosylated to yield a 113-kDa, Endo H-sensitive product. Further processing involves proteolytic cleavage into at least two, probably disulfide-linked fragments of 53–59 and 67 kDa which oligomerize to forms of approximately 140 kDa. PhHV-1 gB cleavage products were already present 15 min after chase in contrast to the gB of PRV which is cleaved after translocation into the Golgi apparatus, requiring at least 60 to 90 min (Whealy *et al.,* 1990). Similar, however, to PRV gB, the gB of PhHV-1 was incompletely processed and at least 50% of the precursor molecules still remained intact after 6 hr of chase. The proteolytic fragments of PhHV-1 gB predominantly contain complex, Endo H-resistant N-glycans. The identity of an additional 62-kDa molecule which was weakly coprecipitated from PhHV-1-infected cells remains to be elucidated. In PhHV-1-infected CrFK cells gB molecules follow the exocytic pathway and are expressed at the cell surface. Synthesis of PhHV-1 gB is, therefore, very similar to other members of the *Varicellovirus* genus, e.g., equine herpesvirus-1 (Sullivan *et al.,* 1989).

A signal cleavage site as well as the site of cleavage of the fragments of PhHV-1 gB was not unambiguously identified in this study. Most of the endoproteolytically cleavable gB molecules of the different herpesvirus species harbor a single motif (-R-X-K/R-R-) of subtilisin-like serine endoproteases located close to the middle of the primary sequence. The gB of genuine herpesviruses of carnivores, in contrast, contains this motif twice, separated by eight (FHV, PhHV-1) and six (CHV) aa, respectively (Maeda *et al.,* 1992; Spatz and Maes, 1993; Limbach *et al.,* 1994). Whether both sites actually are used and an endoproteolytic fragment is removed during maturation of these gB molecules, as has been suggested for equine herpesvirus-1 gB (Wellington *et al.,* 1996), remains to be investigated.

Synthesis and posttranslational processing of recombinant PhHV-1 gB expressed in baculovirus-infected Sf 9 insect cells resulted in products of slightly lower molecular weight (105, 62, 52 kDa) compared to wt gB, which may be due to differences in N-linkedglycosylation in insect cells (Davidson and Castellino, 1990; Kretzschmar *et al.,* 1994). The markedly delayed kinetics of endoproteolytic cleavage of rec gB into at least two fragments in comparison to wt gB cannot be explained by lack of endoproteases with -R-X-K/R-R- substrate specificity in Sf 9 cells (Du *et al.,* 1994), but probably is at the bottom of the absence of oligomerized forms of rec gB after a 4-hr labeling period. Trafficking of rec gB toward the cell surface seemed to be impaired as only small amounts were detected in the cell membrane of BacPBgB-infected Sf 9 cells in comparison to PhHV-1-infected CrFK cells. Since oligomerization appears to influence correct traf-

ficking of gB molecules via the exocytic pathway (Claesson-Welsh and Spear, 1986; Navarro *et al.,* 1993), the slow formation of the 52/62-kDa fragments of baculovirus-expressed PhHV-1 gB may be related to its low concentration at the Sf 9 cell surface.

Though certain biochemical differences were observed between baculovirus-expressed and authentic PhHV-1 gB, apparently they did not have a major impact on the antigenicity and immunogenicity of the recombinant protein. Possible protective effects of the PhHV-1 gB specific immunity induced in mice could not be evaluated since a challenge system has not been established. It should be noted, however, that the induced immune responses were comparable to those of mice vaccinated with baculovirus-expressed gB of HSV-1: These mice were shown to be fully protected from lethal intraperitoneal HSV-1 challenge and from induction of latency (Ghiasi *et al.,* 1992). The suitability of baculovirus-expressed gB as a compound of a future PhHV-1 subunit vaccine will be the subject of further studies.

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