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Glycoprotein D of Bovine herpesvirus 5 confers an extended host range to BoHV-1 but does not contribute to invasion of the brain

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Running title: gD5 extends the host range of BoHV-1

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Abstract
Bovine herpesviruses 1 and 5 (BoHV-1 and BoHV-5) are closely related pathogens of cattle but only BoHV-5 is considered a

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neuropathogen. We engineered intertypic gD-exchange mutants with BoHV-1- and BoHV-5-backbones in order to address their \textit{in vitro} and \textit{in vivo} host range, with particular interest on invasion of the brain. The new viruses replicated in cell culture with similar dynamics and to comparable titers as their wild type parents. However, gD of BoHV-5 (gD5) was able to interact with a surprisingly broad range of nectins. \textit{In vivo}, gD5 provided a virulent phenotype to BoHV-1 in AR129 mice, featuring high incidence of neurological symptoms and early onset of disease. However, only virus with BoHV-5-backbone, independent of the gD-type, was detected in the brain by immunohistology. Thus, gD of BoHV-5 confers an extended cellular host range to BoHV-1 and may be considered as a virulence factor but does not contribute to the invasion of the brain.

\textbf{Keywords}

Bovine herpesviruses; host-range; virulence

\textbf{Introduction}

Bovine herpesviruses 1 and 5 (BoHV-1 and BoHV-5) belong to the subfamily \textit{Alphaherpesvirinae} and are closely related pathogens of cattle (22). The protein repertoire of the two viruses averages 82\% amino acid identity (21). Both viruses are neurotropic but only BoHV-5 can significantly replicate in the central nervous system (CNS) to cause encephalitis of either naturally infected cattle or experimentally inoculated laboratory animals (2, 5, 6, 12, 40, 41, 44). Glycoprotein D (gD) is accepted as the critical and essential receptor-binding protein of many alpha herpesviruses (reviewed in (8, 48). The main gD
receptors identified to date include members of the tumor necrosis factor (TNF) receptor family (HveA) and the poliovirus receptor family (HveB or nectin 2 and HveC or nectin 1) (28, 42, 51). Furthermore, a modified form of heparan sulfate, 3-O-sulfated heparan sulfate, can mediate herpesvirus entry (46). J1.1-2 cells (J cells) represent a subpopulation of thymidine kinase negative baby hamster kidney (BHK) cells, selected for their property to be resistant against infection with herpes simplex virus type 1 (HSV-1), HSV-2 and BoHV-1. Expression of nectin 1 in those cells, rendered them susceptible to BoHV-1 infection and replication, which suggests that nectin 1 can serve as a receptor for BoHV-1 gD (gD1) (16, 18, 28). Interestingly, we observed that BoHV-5 was able to productively replicate in J cells without the nectin 1 receptor.

According to the published sequence comparison of BoHV-1 and BoHV-5 (21), the highest divergence between the two viruses map with the latency-related region and the immediate early proteins (less than 75% aa identity) BICP0, BICP4, and BICP22. Glycoprotein E (gE) was also listed in this category, with 74% aa identity between gE of BoHV-1 (gE1) and gE5. This fact gave also ample reason to try mapping the neurovirulent phenotype of BoHV-5 to the gE5 molecule (3, 4, 13). In contrast, the highest sequence similarities between the two viruses have been described in proteins involved in viral DNA replication and processing as well as certain virion proteins. Among others, the predicted aa sequences of gD1 and gD5 were listed as being identical to 98% (21). However, our own analysis, using the European Molecular biology Software suite (43), revealed only 79.9% aa identity. Obviously, the most extensive difference between gD1
and gD5 maps to a glycine-rich stretch located in the molecule’s ectodomain, between aa 280 and 330 of gD5, in the close vicinity to the transmembrane region. Based on these considerations, we hypothesized that BoHV-5 was able to make use of a cellular receptor that is unavailable to BoHV-1. To test this hypothesis, the gD genes were removed from bacterial artificial chromosomes (BACs) harboring the genomes of either BoHV-5 or BoHV-1 (27). In a second step, gD-exchange viruses were created by cotransfecting the gD-less BACs with appropriate plasmids carrying either gD1 or gD5 genes and appropriate flanking sequences. The newly generated viruses included an intertypic BoHV-5 mutant carrying gD1 in the place of gD5 and a corresponding BoHV-1 carrying gD5. These mutants, together with appropriate revertant mutants, were then used to explore in vitro their ability to infect J cells and in vivo, in a previously established mouse model, their ability to cause neurological disease and invade the brain (2). Our results indicate that gD5 confers an extended host range to BoHV-1 but is non-essential for invasion of the brain.

**Materials and Methods**

**Viruses and cells.**

Madin Darby bovine kidney (MDBK) cells, SV40 transformed African green monkey kidney (COS-1) cells and J1.1-2 cells (J cells) were grown at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with a mixture of penicillin and streptomycin and 5% to 10% fetal calf serum (FCS). J cells are derived from
BHKtk− cells and have been selected for resistance against HSV infection (16). Vero 2.2 cells were grown in DMEM supplemented with G418 (500 µg/ml), 10% FCS at 37°C and 5% CO2 (47). Wt BoHV-5 (strain N569), wtBoHV-1 (strain Jura); and recombinant (r) BoHV-5, rBoHV-5gD1HA, rBoHV-1, rBoHV-1gD5V5, recombinant-revertant (rr) BoHV-5gD5V5 and rrBoHV-1gD1YFP were propagated in MDBK cells as described previously (25, 40).

**Mice.**

All animals were cared for and used in accordance with Swiss laws for animal experimentation. The mouse strain AR129 used throughout this study is derived from 129Sv/Ev (H-2b) mice. AR129 mice are with genetically deleted type 1 interferon receptors combined with RAG-2 knockout (30).

**Plasmids.**

More details of all constructions are provided in supplementary data. All primers are listed in Table 1. p118 contains a gene expression cassette providing kanamycin resistance flanked by two FRT sites (see also supplementary data). p302ΔgD1HA contains a gene expression cassette providing kanamycin resistance flanked by two FRT sites, and homology arms to target the glycoprotein D locus of fBoHV-5 BAC (gD5). The primers P1, P2, P3, and P4 were used for amplification and cloning of the homology arms, i.e. 477 bp upstream and 421 bp downstream of gD5, respectively.
p302ΔkanR contains the same homology arms as p302ΔgD1HA. In this construct, they flank the gD1 ORF, which is fused to an HA-epitope coding sequence at its 3' end. Primers P5 and P6 were used for amplification and cloning of the gD1HA ORF.

p309 contains homology arms to target the gD5 locus, which flank the gD5 ORF fused to a V5 epitope sequence at its 3' end. Primers P7 and P8 were used for amplification and cloning of the gD5V5 ORF.

p307 contains a gD5V5 ORF, flanked with extended homology arms to target the gD1 locus. The upstream 1134 bp homology arm was amplified with primers P9 and P10. The downstream 1136 bp homology arm was amplified with primers P11 and P12.

pCS156 has a total size of 7107 bp and contains a 4182 bp fragment consisting of gD1 fused to the yellow florescent protein (YFP) coding sequences, flanked by homology arms to target the gD1 locus. The upstream homology arm, together with the gD1 coding sequence without stop codon, was amplified with primers P13 and P14. The downstream homology arm was amplified with primers P15 and P16. The YFP sequence was amplified with primers P17 and P18.

Red recombinations for gD deleted BoHV BACs.

fBoHV-5ΔgDkanR BAC: The vector p302ΔgD1HA was used for construction of fBoHV-5ΔgDkanR BAC by Red recombination (19). The 2832 bp transfer fragment containing 477 bp upstream of gD5 start codon, kanamycin cassette flanked by FRT sites and 421 bp downstream of gD5 stop codon was excised with HpaI from p302ΔgD1HA, gel purified and electroporated into arabinose-induced
E. coli strain DH10B harboring fBoHV5 BAC and pKD46 (19).

Electroporated cells were selected on LB agar plates containing 25 µg/ml kanamycin. The resulting recombinant BoHV-5 BAC was designated fBoHV-5ΔgDkanR.

fBoHV-1ΔgDkanR BAC: The 2010 bp DNA transfer fragment was amplified by PCR using p118 as a template. It contains a kanamycin cassette for selection in E. coli, flanked by 50 bp homology upstream of the start codon of gD1 ORF and 50 bp homology downstream of the stop codon of the gD1 ORF. The P19 and P20 primers used for amplification are listed in Table 1. The resulting PCR product was DpnI digested in order to remove residual template DNA. Next, the transfer fragment was electroporated into arabinose-induced E. coli strain DH10B harboring fBoHV-1 BAC and pKD46 (19). Electroporated cells were selected on LB agar plates containing 25 µg/ml kanamycin. The resulting recombinant BoHV1 BAC was designated fBoHV1ΔgDkanR.

DNA preparation from virions.

Herpesviral DNAs were extracted as described previously (23).

Generation of recombinant viruses.

rBoHV-5gD1HA: To generate the gD-exchanged recombinant, BoHV-5 expressing BoHV-1 gD, p302ΔkanR was digested with Hpall, gel purified and cotransfected with fBoHV-5ΔgDkanR BAC DNA into Vero 2.2 cells by Lipofectamin (Invitrogen) as described before (45). fBoHV-5ΔgDkanR BAC DNA transfected alone did not lead to viral progeny. However, after cotransfection with the purified fragment
from p302ΔkanR, rBoHV-5gD1HA emerged and was passaged three times in MDBK cells.

**rrBoHV-5gD5V5:** To generate the BoHV-5 gD revertant, *Hpa*I digested and gel purified fragment derived from p309 was cotransfected with fBoHV-5ΔgDkanR BAC DNA into Vero 2.2 cells as described before. The resulting recombinant virus progeny-rrBoHV-5gDV5 was passaged three times in MDBK cells.

**rBoHV-1gD5V5:** To generate the gD-exchanged BoHV-1 recombinant expressing BoHV-5 gD (rBoHV-1gD5V5), a *Hpa*I digested and gel purified fragment derived from p307 vector and fBoHV-1ΔgDkanR BAC DNA were cotransfected into COS-1 cells as described before. The resulting recombinant virus progeny, rBoHV-1gD5V5 was passaged three times in MDBK cells.

**rrBoHV-1gD1YFP:** To generate the BoHV-1 gD revertant mutant, the vector pCS156 was *XbaI/EcoRI* digested, gel purified and cotransfected with fBoHV-1ΔgDkanR DNA into MDBK cells as described before. The resulting recombinant virus progeny-rrBoHV-1gD1YFP was passaged three times in MDBK cells.

**Cre mediated excision of F-plasmid in BAC derived BoHV recombinants.**

MBDK cells were cotransfected with DNA of selected viral mutants and *cre* expressing vector p116.006 (20). Three days post transfection five viral plaques were randomly collected and plaque purified three times. Finally, viral DNA from non-GFP fluorescent progeny was extracted and characterized by restriction enzyme analysis in order to verify the deletion of the BAC cassette.
For the *in vitro* experiments, the BAC cassette from rrBoHV-
1gD1YFP was deleted by *cre* mediated recombination and selection
of GFP-negative, YFP-positive progeny virus. However, the BAC
cassette in the other recombinant viruses used in this study was
retained in order to have permanent *in vitro* tracers. For the *in vivo*
experiments performed in this study the BAC cassette in rBoHV-
1gD5V5 and rBoHV-5gD1HA viruses was deleted in order to obtain
virulence close to respective wild type viruses (data not shown).

**Indirect Immunofluorescence.**

MDBK infected cells or J1.1-2 cells were infected and incubated until
discernible plaques had formed. Then, they were fixed in 3%
paraformaldehyde and permeabilized with 0.2% Triton X-100 (Fluka
Chemie). After blocking with 2% albumin from bovine serum (BSA)
(Sigma-Aldrich), they were incubated with mouse monoclonal IgG$_{2a}$
(1:500 in PBS) against either the HA-epitope (Santa Cruz
Biotechnology) or the V5-epitope (Invitrogen). For visualization Cy3$_{TM}$
conjugated AffiniPure goat anti-mouse IgG was used (*Jackson
ImmunoResearch* Laboratories).

**Southern Blot Hybridization.**

Digested virus and BAC DNAs were separated on 0.7% agarose gels
for 24 to 48 h at 30 V and then transferred overnight to nylon
membrane (Roche). Transferred DNA was then hybridized with gD5-
DIG or gD1-DIG labeled probes overnight at 68 °C. For detection by
chemiluminescence, CDP-Star substrate (Roche) was used. The
gD5-DIG and the gD1-DIG labeled probes were amplified by PCR
(PCR DIG Probe Synthesis Kit, Roche). The primers used for amplification (gD5upper; gD5lower and gD1upper; gD1lower) are listed in Table 1.

**Virus growth analysis.**

MDBK cells were infected with respective viruses either at moi 0.01 or moi 5. After 2 h at 4 °C, the temperature was shifted for 1 h to 37 °C to allow virus penetration. The inoculum was removed before the cells were washed twice with PBS and overlaid with fresh DMEM. At 0, 24, 48, 72, and 96 h (moi = 0.01) and 0, 6, 12, 18, and 24 h (moi = 5) following the temperature shift, the infected cells were scraped into the culture medium and clarified by centrifugation (311 x g for 10 min). The supernatant was then removed to be analyzed separately. The cell pellet was resuspended in fresh DMEM. Infectious virus was harvested following three cycles of freezing/thawing and low speed centrifugation to remove cell debris. Infectivity in the supernatants and the pellets was titrated separately in 96 well plates. Each titration was performed in three independent assays. Viral titers were determined as TCID$_{50}$/ml in MDBK cells.

**Soluble receptors.**

The following soluble receptors carrying either the entire ectodomain (VCC) or the single N-terminal V domain (V) of human nectins were used as previously described: human nectin 1 (N1(V)-Fc); human nectin 2 (N2(V)-Fc); human nectin 3 (N3(VCC)-Fc); human nectin 4 N4(VCC)-Fc (14, 15, 24, 38). Briefly, the PCR amplification products were cloned in the COS Fc Link vector (SmithKline Beecham) and
transfected in COS cells with FuGENE6, according to the manufacturer’s instructions. The SARS-S–Fc construct was a gift from dr. F. Neipel, Erlangen University. The ectodomain of SARS-S was cloned in pAB61 vector (7) and transfected in HEK 293T cells. Soluble proteins were affinity purified from cell media on Affigel-protein-A or HiTrap-protein-A, as detailed and referenced elsewhere (7, 14, 37).

Inhibition of infection.
Soluble receptor proteins were incubated with stocks of infectious virus in order to test for inhibition of infection. Triplicate aliquots of rBoHV-5 and rBoHV-1gD5V5, corresponding to a concentration of moi 5 for 20,000 MDBK cells, were preincubated individually with each of the soluble receptors at a 200 nanomolar (nM) concentration. 200 nM SARS-S spike glycoprotein, 200 nM BSA or D’MEM without added proteins were used as controls. After 1 hour incubation at 37°C, the aliquots were transferred to J-cell monolayers in 96 well plates. After 2 hours, these inocula were removed and the cells were supplemented with 100 µl fresh medium before incubating at 37°C for 48 hours. Emergence of plaques was recorded and GFP-positive cells were counted under the fluorescence microscope.

Design of the animal experiments.
43 AR129 mice (8 weeks of age, all female) were divided into groups of 7 to 10 and used for intraperitoneal (i.p.) inoculation with 10^7 50% tissue culture infective doses (TCID_{50}) of the viruses specified in
Table 2. Four siblings of the same mice were used as mock-infected controls.

Individual mice were euthanized as soon as they developed severe disease symptoms. Exceptions are listed in Table 2. At necropsy the brain, kidney, liver, spleen, intestine and lung were collected. Each organ was divided into two parts. One part was fixed with formalin solution in order to be used for hematoxylin and eosin (HE) staining or immunohistochemistry (IHC). The remaining part was shock-frozen in liquid nitrogen and conserved at -80 °C for further analysis by real time PCR.

**DNA detection and quantification.**

Primers and probes for quantitative real-time PCR (TaqMan) used to amplify sequences within the open reading frames of the glycoprotein B of BoHV-1 and BoHV-5, PCR conditions, and usage of plasmid positive controls were performed as previously described (2). For the internal control, the previously developed TaqMan assay reagent for the 18S rRNA kit was used (AB), according to the manufacturer. The data were analyzed on a 7900 HT Fast Real Time System detector (AB). The absolute quantitation of virus copies per cell was done according to AB User Bulletin 2 as previously described (2).

**Extraction of viral DNA from mouse brains.**

The tissue from one half-brain, sagitally divided was homogenised by TissueRuptor (QIAGEN) and DNA from 50 mg of brain was extracted by using the QIAamp DNA Mini kit (QIAGEN).
**Immunohistology and antigen detection.**

Immunohistology was performed with paraffin-embedded brain, kidney, liver, spleen, intestine and lung tissue sections fixed in formalin solution (4%). 3 to 4 micrometer thick organ sections were mounted on positively charged slides and then deparaffinized in xylene and rehydrated by being dipped in a graded ethanol series (100, 96, and 70%). After a counterstaining in hemalaun for 4 min and washing in water at ambient temperature, the sections were digested with a Proteinase K solution (DAKO REAL™ Proteinase K diluent, S2032 and 40x concentrate, S2019) for 10 min at room temperature (RT). Afterwards endogenous peroxidase activity was blocked by incubation in 3% H$_2$O$_2$ for 15 min and nonspecific binding of antibodies was eliminated by treatment with blocking solution (protein block serum-free, DAKOCytomation, X0909) for 10 min at RT. The sections were then incubated over night with the monoclonal antibody 141 (specific for gC of both BoHV-1 and BoHV-5 (35) at a 1:20 dilution in phosphate buffered saline (PBS) (pH 8) and for 30 min with a anti-mouse secondary antibody conjugate containing a dextran polymer labeled with horseradish peroxidase (Dako, EnVision K4001). Incubations were done at room temperature. Between every step slides were rinsed with PBS (Phosphate Buffer Saline, pH 8). The development of the color reaction by the aminoethyl carbazole substrate (AEC (Red) substrate kit, Invitrogen 00-2007), added after washings, was under the microscope and stopped by rinsing with PBS. As positive control, wtBoHV-1-infected cells with were used.
Results

**Generation and genotypic characterization recombinant BoHV-5 and BoHV-1 with intertypic gD.**

A two-step strategy was used to exchange the gD encoding sequences between BoHV-5 and BoHV-1. In a first step, previously described BACs (27), harboring the genomes of either BoHV-1 (fBoHV-1) or BoHV-5 (fBoHV-5), were used to replace the gD-coding sequences by a kanamycin resistance cassette (see supplementary information S1).

In a second series of experiments, infectious viruses were rescued by homologous recombination. Notably, gD-less BACs did not provide infectious progeny upon transfection. Therefore, gD-less BAC DNAs were cotransfected with plasmids containing gD sequences supplemented with marker tags and appropriate flanking sequences. As a result, the following four new viruses were generated and characterized by restriction enzyme analysis and Southern blot as well as by identification of their markers (see supplementary information S1). (1) rBoHV-1gD5V5, a BoHV-1, which encoded gD5 with a V5 tag at its C-terminus; (2) rrBoHV-1gD1YFP, a rescue virus, which encoded gD1 with a yellow fluorescent protein fused to its C-terminus; (3) rBoHV-5gD1HA, a BoHV-5 mutant expressing gD1 with an HA-tag fused to its C-terminus; (4) rrBoHV-5gD5V5, a rescue mutant differing from its ancestor for a gD5 fused to a C-terminal V5-tag.
Marker-tag analysis of the newly generated viruses.

Three of the new viruses carried a gD fused to a C-terminal epitope, which was expected to be recognized by appropriate monoclonal antibodies. The fourth virus carried a yellow fluorescent protein marker. To test for the presence and functionality of those markers, MDBK cells were infected with parent, mutant, and rescue viruses and analyzed for plaque development by fluorescent microscopy (Fig. 1, panels A through F). Emerging green fluorescence was due to replication of the viruses and based on the expression of a GFP cassette embedded in the BAC sequences. Replication and plaque formation of those viruses provided evidence that they all contained a functional gD. To avoid interference between yellow and green fluorescence in rrBoHV-1gD1YFP, the GFP cassette was removed from this virus by Cre recombination and this rescue virus was shown to provide yellow fluorescence in the absence of green fluorescence (panel F). After successful plaque development, the monolayers were fixed for immunolabeling with appropriate monoclonal antibodies. As shown in panels A’ through E’, each virus with a predicted tag did react properly in this assay. Thus, each of the new viruses could be addressed by fluorescence and/or immunohistology. (See also supplementary information Fig. S2 and S3)

Viral growth analysis.

Both cell-free and cell-associated virus production was determined by growth curve experiments. Each analysis was done in triplicate. The results, shown in Fig. 2, indicated that all mutant and revertant viruses grew with similar kinetics and to similar titers as their wild type
ancestors. Thus, the growth in MDBK cells of the different viruses was not significantly affected, neither by the exchange of gD nor from retaining the BAC cassettes within the viral genome. (See also supplementary information Fig. S4)

**Type-specific gD determines the host range of BoHV-1 and -5 in vitro.**

J1.1-2 cells (J cells) are known to be resistant against infection with HSV-1, HSV-2 and BoHV-1 (16). However, we had previously observed that they were susceptible to BoHV-5. To test, whether this property could be mapped to gD5, we produced viral stocks in MDBK cells and inoculated J cells with the different viruses, using equal infectious doses (moi 5) as determined by titration in MDBK cells. The results at 72 hpi are shown in Fig. 3. Mock infection or infection with BoHV-1 did not lead at all to plaque formation (panels A, B, C). In contrast, fluorescent plaques developed continuously over time upon inoculation of J cells with either BoHV-5 or BoHV-1gD5 (panels E, G). Plaques due to infection with the latter virus could also be stained immunologically by using a monoclonal antibody against the V5-tag (panel H). Wild type gD5 (panel F) did not react with monoclonal antibodies against the HA-tag. However, inoculation with BoHV-5gD1 did occasionally lead to the detection of single green fluorescent J cells (panel I). As expected, such cells could also be stained with a monoclonal antibody against the HA-tag (panel J). However, neither spread of the virus infection to neighboring cells nor plaque formation was observed.
In summary, these data strongly indicate that BoHV-5 can use host entry receptors, which are generally not accessible for BoHV-1 and that gD5 contributes a major part to the apparent host-range divergence of the two viruses in vitro.

_Inhibition of infection by pre-incubation with soluble nectins._

J cells lack nectin 1, nectin 2, and HevA but express the nectin 3 ortholog of human nectin 3 (15, 16). Since gD5 was able to mediate infection of J cells with BoHV-5 and rBoHV-1gD5, the question arose, whether or not saturating gD5 with the ectodomain of human nectin 3 could block infectivity. Therefore, replicate aliquots of infectious virus stocks were preincubated before inoculation of J cells with each one of the following soluble receptors: human nectin 1, human nectin 2, human nectin 3, and human nectin 4, respectively (for details see materials and methods) (14, 15, 24, 38). Preincubations with equal concentrations of BSA or SARS-S glycoprotein or DMEM without added protein were used as controls. 48 hrs after inoculation, emergence of plaques was recorded and GFP-positive cells were counted under the fluorescence microscope. The results are summarized in Figure 4 (see also Figures S5-S7). Human nectin 3 reduced the number of infectious events with rBoHV-5 and rBoHV-1gD5V5 in J cells almost completely. When compared to human nectin 3, human nectin 1 exerted a somewhat lower effect, whereas human nectins 2 and 4 provided an intermediate effect (80 to 90% reduction). As expected, BSA (in comparison to incubation without protein) affected infectivity only marginally. As a further negative control, we used a totally unrelated protein, the surface protein S of
Severe Acute Respiratory Syndrome Coronavirus (SARS-S).

According to our experience, addition of such negative controls may inhibit virus infection by about 25% (14). Addition of SARS-S-Fc reduced virus infectivity between 25% and 40% as compared to BSA. Using the one-way analysis of variance and either Dunnet’s or also Newman-Keuls multiple comparison test, we found that the difference between BSA and SARS-S was not statistically significant. Inhibition of BoHV-5gD with nectin 1 was significant against BSA (p<0.05) but not against SARS-S. All other competitors provided significant inhibition compared to either BSA or SARS-S. There was no significant difference in between of the effects of various nectins. The results indicate that gD5 was able to interact with a variety of soluble human nectins, even though their native counterparts were not expressed in J cells. The differences between nectin 3 on one side and nectin 1, 2, 4 on the other side may reflect different affinity of gD5 to the various nectins. Altogether, these experiments suggested that gD5 carries binding sites for a number of receptors and their human orthologs. Thus, the gD5 receptor-binding domain(s) seem to act in a rather promiscuous manner, which is surprising when compared to wt HSV-1 and unrestricted HSV-1 mutants (15).

**Infection of AR129 mice**

Having established that rBoHV-1gD5 had an extended host range *in vitro*, it was of interest to know its properties *in vivo*. For this purpose, groups of 7 to 10 AR129 mice were inoculated intraperitoneally with $10^7$ TCID$_{50}$ of either BoHV-5, BoHV-1, rBoHV-1gD5, rBoHV-5gD1, or rrBoHV-1. For animal welfare reasons, all animals were checked twice daily for clinical symptoms and were euthanized upon...
development of severe disease. The healthy animals at a given time point post inoculation were considered “survivors”. A chart showing the survival times of the animals in each group is presented in Fig. 5. Both clinically and in terms of survival, the mice could be divided into two statistically significant different groups. Mice inoculated with wtBoHV-5, BoHV-5gD1, and BoHV-1gD5 experienced an early onset of disease and had to be euthanized significantly earlier (mean 12 to 15 dpi) than the mice inoculated with either wtBoHV-1 or rrBoHV-1 (mean 22 to 25 dpi). The calculated p-values for significant differences varied between 0.001 and 0.005 (Gehan-Breslow-Wilcoxon Test).

Erected fur was the single clinical symptom that was observed in all mice that succumbed to disease. Surprisingly, all diseased mice showed central nervous symptoms, although two different types could be distinguished and grouped with the virus used for inoculation. Diseased mice of three groups (BoHV-1, rrBoHV-1, and rBoHV-1gD5) showed ataxia, weakness in the rear limbs, and hunched back. The mice inoculated with either BoHV-5 or rBoHV-5gD1 showed even more severe neurological symptoms, i.e. stumbling, body tremor, ptosis, distorted body shape. Only mice inoculated with BoHV-5 showed also body-turn-over and apparent pain during walking.

Taken together, two surprises came with these experiments. First, the fact that BoHV-1-infected mice developed a disease with neurological symptoms, although with a significant delay. Second, the mice inoculated with BoHV-1gD5 grouped with BoHV-5 in terms of survival time and with BoHV-1 in terms of clinical disease signs.
We conclude from this experiment that BoHV-1gD5 was significantly more virulent in AR129 mice than either its parent BoHV-1 or its rescue mutant rrBoHV-1gD1. Indeed, it seemed that the BoHV-1 mutant carrying gD5 was almost equally virulent as the neurotropic virus BoHV-5. However, BoHV-5gD1 was indistinguishable from BoHV-5 both in terms of survival time and type of clinical disease.

**Invasion of the brain.**

To initially test whether or not the disease signs in the mice could be associated to the ability of the respective viruses to invade the brain, DNA was extracted from brain samples and subjected to real-time PCR analysis. Based on the results (Fig. 6), it was possible to divide the mice into two significantly different groups (unpaired t test; p<0.01), those with high yields of viral DNA in the brain (BoHV-5 and rBoHV-5gD1) and those with low or no yields of viral DNA in the brain (BoHV-1, rBoHV-1gD5, and rrBoHV-1gD1). As expected for this type of mice, no histological signs of encephalitis could be detected in any of the brain samples upon HE staining (Fig. 7).

However, upon immunohistological staining, using a monoclonal antibody recognizing gC of both BoHV-1 and BoHV-5, viral antigen could be detected in neurons in 7 out of 10 brain stems of mice inoculated with BoHV-5 and all seven brains of mice inoculated with BoHV-5gD1 (Fig. 7). In contrast, we did not detect any viral antigen in the brain sections from all the other mice.

We conclude from these experiments that invasion of the brain is a property of BoHV-5 but does not reside within gD5. Indeed, gD5 is apparently non-essential for invasion of the brain.
Discussion

The present study focused on the contributions of the type-specific entry-mediator-binding glycoproteins gD, specified by either BoHV-1 or BoHV-5, to infectivity of cell cultures and virulence \textit{in vivo}, which led to the following new insights:

(1) gD was interchangeable between BoHV-1 and BoHV-5 without significantly affecting the replication properties of the respective viruses in conventional cell cultures.

(2) However, type-specific gD was able to determine the host range of both BoHV-1 and -5. Interestingly, gD1 was associated with a narrow host range, whereas gD5 provided the ability to infect cells that were not accessible through gD1.

(3) To explore this further, viruses carrying gD5 were tested for their ability to be blocked by a range of soluble receptor molecules, i.e. nectins 1, 2, 3, 4 or control proteins, such as SARS glycoprotein S or BSA. Surprisingly, all four nectins were able to abolish the infectivity of viruses carrying gD5. In contrast, the control protein BSA had almost no effect. Interestingly, the SARS-S glycoprotein was able to interfere with infectivity, at least to a limited extent. Since SARS-S glycoprotein targets the angiotensin-converting enzyme 2 (ACE-2), which is expressed on the surface of kidney cells, we suggest that this interference may be attributed to steric hindrance (34). Thus, gD5 was blockable by an extraordinary broad range of nectins.

(4) \textit{In vivo}, we found that a BoHV-1 mutant carrying gD5 was almost equally virulent as the neurotropic BoHV-5. However, replacement of gD5 by gD1 in BoHV-5 did not reduce the ability of this virus to
invade the brain, while substitution of gD1 with gD5 in BoHV-1 did not
enhance the ability to infect secondary neurons.

Among all of the different herpesvirus entry mediators known to date,
nectin 1 has been shown to serve as a receptor for BoHV-1 (9, 10,
16-18, 26, 28, 49). In contrast, the potential receptors for BoHV-5
have not yet been determined. This study can only partially help to
shed light on this issue. However, the successful inhibition of
infectivity with soluble nectins together with our host-range studies in
vitro, suggests that gD5 may bind to and make use of an extremely
broad range of receptors when compared to its counterparts in other
alpha herpesviruses, such as wild type HSV-1 and unrestricted HSV-
1 mutants (15).

The crystal structures of BoHV gDs have not been determined.
However, on the basis of overall similarity and conservation of
cysteine residues throughout the molecules, which greatly determine
the three dimensional structure through disulfide bonds, one may
assume that the general formation of the BoHV gDs should be similar
to HSV gDs (10). Binding of HSV gDs to their entry mediators has
been mapped predominantly to the N-terminal portion of the
molecule, although more C-terminally located fragments also
contribute to entry functions (10, 17, 26, 49). Therefore, it is
interesting to note that only few differences between gD1 and gD5,
I.e. 6 aa substitutions, map within the first 100 aa from the N-terminus
of the mature BoHV gD molecules (1 and our data). However, gD5
differs from gD1 in an N-linked glycosylation site (aa 23 to 25, NYT).
Threonine at position 25 in gD1 is replaced by isoleucine in gD5,
which abrogates the glycosylation site (1, 50). We suggest that, due
to lesser glycosylation, gD5 might have a more flexible three-dimensional structure, which may facilitate binding to a broader range of entry mediators. However, glycosylation may not be the one and single determinant of the broader host-range attributed to gD5. The highest divergence between gD1 and gD5 maps to a fragment between aa 280 and 330 of the molecules, which still belongs to the ectodomain but is located close to the transmembrane region (1). In HSV gD, this fragment (aa 260 to 310) has been referred to as the profusion domain (PFD), which is required for interaction with other viral glycoproteins, i.e. gB, gH, gL, to proceed with the entry process (26, 29). Interestingly, aa 60 to 210 of HSV gD can be replaced, while the 210 to 310 segment is essential (39). Having this in mind, it is interesting to note that, despite of the differences in their PFDs, gD1 and gD5 apparently interacted successfully with their heterologous interaction partners. However, the details of one gD type interacting with the other partners of the entry complex may influence the entry pathway as well as the entire dynamics of viral replication.

The *in vivo* studies revealed a number of interesting new findings. Based on our previously established model, we inoculated AR129 mice with our new viruses (2). Unexpectedly, almost all of them developed neurological symptoms and had to be euthanized before the planned end of our study. Thus, all of our BAC-derived viruses had retained a significant degree of neurovirulence. However, disease developed early or late, depending on the virus type used for inoculation. Wild type BoHV-1 and the corresponding rescue mutant showed a delayed onset of disease, whereas wild type BoHV-5 and BoHV-5gD1 as well as BoHV-1gD5 showed an early onset. Thus,
gD5 appeared to increase the virulence of BoHV-1, whereas gD1 did not abrogate the virulence of BoHV-5. Clinically and virologically (PCR and immunohistology), the mice could also be separated into two groups. However, in this case BoHV-1gD5 did not group with BoHV-5 and BoHV-5gD1 but rather with the viruses having BoHV-1 backbones. Only BoHV-5 viruses, regardless of their gD types, were able to invade and replicate in the brain. This observation is in agreement with previous reports, which attributed the phenotype of neuroinvasion to gE, rather than gD (3, 4, 11-13, 36). However, in the case of BoHVs, the contribution of gD to neuroinvasiveness had not been addressed previously and it was, therefore, important to look at it. Nevertheless, these results and considerations cannot explain the occurrence of neurological disease symptoms in the mice inoculated with BoHV-1-backbone. In this context, we accidentally observed that both BoHV-1- and BoHV-5-derived viruses were able to target the adrenal gland of the mice, causing severe necrosis (data not shown). The adrenal medulla consists of differentiated post-ganglionic cells, which may provide direct access to the CNS (33). Indeed, intraperitoneal inoculation of C3H/HeN mice with HSV-1 leads to severe adrenal necrosis due to viral replication (31, 32). The same authors reported that HSV-1 was able to advance from the medullar gland to the spinal cord and brain stem, thus, causing neurological symptoms. Presently, we can only speculate that this pathway may also be open to BoHV-1 and BoHV-5 but this issue presents an interesting topic for further research. Future work may also focus on the fine mapping of the receptor-binding fragment of either gD type
and on the identification of the receptor used by gD5, for example a bovine ortholog to human nectin 3.

Acknowledgments

We thank Eva Loepfe and Kati Zlinszky for excellent technical support, Monika Engels and Sarah Albini for enlightening discussions. We thank Dr. F. Neipel, Erlangen University, for SARS-S-Fc construct. This work was supported by grant 3100A0-12498 from the Swiss National Science foundation to M. A.
Table 1. Synthetic oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
<th>Amplification product</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>5’gatcgagctcgttaacgacgc</td>
<td>5’ of gD5 homology arm</td>
</tr>
<tr>
<td>P2</td>
<td>5’gatcgagctcgttaactgcgctcgcaacgc</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>5’gatcgagctcgttaacgacgc</td>
<td>3’ of gD5 homology arm</td>
</tr>
<tr>
<td>P4</td>
<td>5’gatcgagctcgttaacgacgc</td>
<td>gD1 ORF fused to HA</td>
</tr>
<tr>
<td>P5</td>
<td>5’gatcgagctcgttaacgacgc</td>
<td>gD5 ORF fused to V5</td>
</tr>
<tr>
<td>P6</td>
<td>5’gatcgagctcgttaacgacgc</td>
<td>5’ of gD1 homology arm</td>
</tr>
<tr>
<td>P7</td>
<td>5’gatcgagctcgttaacgacgc</td>
<td>3’ of gD1 homology arm</td>
</tr>
<tr>
<td>P8</td>
<td>5’gatcgagctcgttaacgacgc</td>
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</tr>
<tr>
<td>P9</td>
<td>5’gatcgagctcgttaacgacgc</td>
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<td>P10</td>
<td>5’gatcgagctcgttaacgacgc</td>
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<td>P11</td>
<td>5’gatcgagctcgttaacgacgc</td>
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</tr>
<tr>
<td>P12</td>
<td>5’gatcgagctcgttaacgacgc</td>
<td></td>
</tr>
<tr>
<td>Primer</td>
<td>Sequence (5' to 3')</td>
<td>Function</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------</td>
<td>----------</td>
</tr>
<tr>
<td>P13</td>
<td>5' gatctctagaacccgcatacggggttgcttt3'</td>
<td>5' of gD1 flank and gD1 ORF w/o stop codon</td>
</tr>
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<td>P14</td>
<td>5' gatgagatccccggcagcgctgtgatt3'</td>
<td>3' of gD1 flank</td>
</tr>
<tr>
<td>P15</td>
<td>5' gctgagatccccggcagcgctgtgatt3'</td>
<td>3' of gD1 flank</td>
</tr>
<tr>
<td>P16</td>
<td>5' acggatctctgtagtgacaggaagggcaggc3'</td>
<td>YFP ORF</td>
</tr>
<tr>
<td>P17</td>
<td>5' gatctctctctgtagtgacaggaagggcaggc3'</td>
<td>YFP ORF</td>
</tr>
<tr>
<td>P18</td>
<td>5' gatgagatccccggcagcgctgtgatt3'</td>
<td>Kanamycin ORF flanked by 50 bp homology arms for gD1 locus</td>
</tr>
<tr>
<td>gDSupper</td>
<td>5' cggaggttgccgctgtgcttg3'</td>
<td>gDS-DIG labeled probe</td>
</tr>
<tr>
<td>gDSlower</td>
<td>5' acagctgtgcccccaacctgc3'</td>
<td>gDS-DIG labeled probe</td>
</tr>
<tr>
<td>gD1upper</td>
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<td>gD1-DIG labeled probe</td>
</tr>
<tr>
<td>gD1lower</td>
<td>5' cgggggtctgactctc3'</td>
<td>gD1-DIG labeled probe</td>
</tr>
</tbody>
</table>

* Underlining indicates restriction site used for cloning

** The letters in bold indicates HA or V5 tags respectively

*** Binding part of the primers is given in upper case
Table 2. Infection, incubation times, status at necropsy.

<table>
<thead>
<tr>
<th>Infection</th>
<th>Group</th>
<th>Diseased(^b) (dpi)(^c,d)</th>
<th>Healthy(^e) (dpi)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BoHV-1</td>
<td>8</td>
<td>5 (18, 20, 23)(^e)</td>
<td>3 (29)</td>
</tr>
<tr>
<td>rrBoHV-1</td>
<td>9</td>
<td>3 (21, 25, 29)</td>
<td>6 (15, 18)</td>
</tr>
<tr>
<td>rBoHV-1gD5</td>
<td>9</td>
<td>8 (14, 15, 18, 19, 23)</td>
<td>1 (26)</td>
</tr>
<tr>
<td>BoHV-5</td>
<td>10</td>
<td>10 (11, 12, 13, 14, 16, 19, 21, 28)</td>
<td>0</td>
</tr>
<tr>
<td>rBoHV-5gD1</td>
<td>7</td>
<td>7 (12, 17)</td>
<td>0</td>
</tr>
<tr>
<td>Mock</td>
<td>4</td>
<td>0</td>
<td>4 (17, 29)</td>
</tr>
</tbody>
</table>

\(^a\) = number of animals per group; \(^b\) = number of animals with disease signs at the time of necropsy; \(^c\) = Day of necropsy in parentheses; \(^d\) = in this column, dpi equals incubation time; \(^e\) = number of animals without apparent disease signs before necropsy.
Figure captions

Fig. 1. Phenotyping of parent, mutant, and rescue viruses by fluorescence microscopy. MDBK cells were infected with various viruses to be analyzed at 72 hpi. The top two rows show plaques of BAC-derived rBoHV-5 and its mutants, whereas the bottom two rows show BAC-derived rBoHV-1 and its mutants. The top row shows green fluorescent plaques of A: rBoHV-5; B: rBoHV-5gD1HA; C: rrBoHV-5gD5V5. For the second row, the same dishes were stained with mcAbs and a red fluorescent Cy3 conjugate (A’ and B’: mcAb against HA-tag; C’ mcAb against V5-tag). In the third row, the following fluorescent plaques are shown: D: green fluorescent rBoHV-1 and E: rBoHV-1gD5V5; F: yellow fluorescent rrBoHV-1gD1EYFP. For the fourth row (D’, E’), the same viruses as in the third row were stained with mcAb against V5 and a red fluorescent Cy3 conjugate.

Fig. 2. Growth kinetics of recombinant BoHV-5 mutants and recombinant BoHV-1 mutants versus wt BoHV-5 and wt BoHV-1. MDBK cells were infected at moi of 0.01 with different viruses and harvested at various times post inoculation. The virus yields at each time point were determined by titration. Panels A and B: rBoHV-5 (full squares); rBoHV-5gD1 (open squares); rBoHV-1 (full circles); rBoHV-1gD5 (open circles). Panels C and D: wt BoHV-5 (full squares); rrBoHV-5gD5 (open squares); wt BoHV-1 (full circles); rrBoHV-1gD1 (open circles). Cell-free (supernatant, panels A and C) and cell-associated virus (pellets, panels B and D) were titrated separately. The x-axis represents the time scale post infection. Virus titers (
axis) are expressed as TCID$_{50}$/ml. Mean values with standard deviation (error bars) are shown.

**Fig. 3. Type-specific gD determines the host range of BoHV-1 and BoHV-5.** J cells were mock-infected (top row) or infected at moi 5 with BoHV recombinants, all of which express the green fluorescent marker upon successful infection and replication. At 72 hpi, cells were fixed and stained with monoclonal antibodies and a secondary antibody carrying Cy3. Mock infected cells did neither develop plaques (A) nor provide any green or red fluorescence (B). rBoHV-1 did neither give rise to green fluorescence (C) nor provide red fluorescence due to Cy3 conjugate binding to mcAb against V5 (D). rBoHV-5 caused development of green fluorescent plaques (E) but did not bind mcAb against HA (F). rBoHV-1gD5V5 caused green fluorescent plaque formation (G) and binding of mcAb against V5 (H). rBoHV-5gD1HA provided single green fluorescent cells (I), which also stained positive with mcAb against the HA-tag (J).

**Fig. 4. Inhibition of infection by pre-incubation with soluble nectins.** Relative infectivity rates are shown. Viruses were incubated with soluble proteins as described in materials and methods before being inoculated onto monolayers of J cells. Fluorescent cells were counted at 48 hpi. Fluorescent cell numbers emerging from virus incubated with 200 nM BSA were set as 100% (bars measured on y-axis). The identities of the proteins used for co-incubation with virus stocks are indicated beneath each pair of bars. Grey bars represent infection with rBoHV-5, black bars confer to rBoHV-1gD5V5. Each
experiment was done at least in triplicate (mean values are shown and the error bars represent the standard error of the mean).

**Fig. 5. Survival times of AR129 mice upon infection with different BoHV**s.** Groups of 7 to 10 mice were inoculated intraperitoneally \(10^7\) TCID\(_{50}\) of virus and checked daily twice for their health status. They were euthanized upon development of severe disease symptoms. The x-axis gives the survival times post inoculation. The y-axis gives the percentage of mice in each group at any given time point. The symbols used for each virus are given on the right of the figure.

**Fig. 6. DNA of BoHV-5 and BoHV-5gD1 in brain tissue.** DNA was extracted from the brains of each animal and subjected for real time PCR and quantification of the viral DNA load per cell as described in Materials and Methods. The viruses used for inoculation are listed on the x-axis. Box plots refer to the y-axis and show mean values, the 75th percentile (box) as well as maximal and minimal values (whiskers).

**Fig. 7. Histology (HE) and immunohistology of brain sections.** A) HE staining of a section of mouse brain from an animal inoculated with BoHV-1. This picture is representative for all sections analyzed, independent of the inoculated virus. B,C,D) Immunohistology using a monoclonal antibody, recognizing gC of both viruses BoHV-1 and BoHV-5. B) Negative brain section of a mouse inoculated with BoHV-1. This picture is representative for all brain sections from mice inoculated with either BoHV-1, BoHV-1gD5, and rBoHV-1. C) Representative picture of a brain section from a mouse inoculated with rBoHV-5gD1. This picture is also representative for animals
inoculated with BoHV-5. Neurons in the brain stem area show a positive intracytoplasmic staining (red cytoplasmic staining). D) Cutout of the section outlined in C. Bar: 100 µm.

Literature


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Fig. 1

Parent  Mutant  Rescue

A  B  C
A'  B'  C'
D  E  F

A'  B'  C'
D'  E'
Fig. 3
Infection of AR129 mice with different viruses

- BoHV-1
- BoHV-5
- BoHV-5gD1
- rrBoHV-1
- BoHV-1gD5

Percent survival vs Days post inoculation

Fig. 5
Fig. 6

BoHV-Neuroinvasion

Viral DNA copies per cell

BoHV-1  BoHV-5  BoHV-5gD1  rBoHV-1  BoHV-1gD5

Virus
Brain

Fig. 7