Deletion of glycoprotein gM of pseudorabies virus results in attenuation for the natural host

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Glycoprotein M (gM) is one of the very few non-essential glycoproteins conserved throughout the herpesvirus family. Despite this conservation little is known about its function in virus replication. To test for the importance of gM in vivo in a natural virus–host system, 6-week-old piglets were intranasally infected with a gM mutant of the alphaherpesvirus pseudorabies virus (PrV). Following infection virus excretion from the nasal mucosa was decreased ca. 100-fold compared to wild-type or revertant virus. Clinical signs were limited to transiently elevated temperature. In contrast, animals infected by wild-type or revertant virus exhibited high fever, severe respiratory symptoms and affliction of the central nervous system. Prior infection with gM PrV conferred protection against challenge infection and animals mounted an antibody response against gM after wild-type virus infection. Thus, gM is important for efficient virus replication in vivo and deletion of gM may contribute to development of live attenuated, genetically marked vaccines.

The alphaherpesvirus pseudorabies virus (PrV) causes Aujeszky’s disease (AD) in pigs, characterized by respiratory symptoms and central nervous disorders. Manifestation of disease depends on the virulence and infectious dose of the virus strain, age of the animal and route of infection. Naturally, infection occurs oronasally resulting in primary replication in the respiratory tract leading to respiratory distress. In addition, the virus enters nerve endings in trigeminal and olfactory pathways and ascends into the central nervous system. Whereas younger animals may exhibit a high mortality due to severe central nervous disorders, older animals usually survive with only moderate respiratory symptoms (Pensaert & Kluge, 1989). To prevent economic losses, vaccination with live attenuated and inactivated vaccines is performed. Although PrV is able to infect most mammals except higher primates, including humans, pigs are the only animal species capable of surviving a productive PrV infection. They are, therefore, considered the natural host of PrV.

The observation that immunogenic glycoproteins are absent in several attenuated live vaccine strains prompted intensive research into the structure and function of PrV glycoproteins. Until now, eleven PrV glycoproteins, gB, gC, gD, gE, gG, gH, gI, gK, gL, gM and gN, have been identified (reviewed in Mettenleiter, 1996). Whereas homologues to most of them are present in other alphaherpesviruses, only gB, gH, gL, gM and gN are conserved throughout the herpesvirus family. gB, gH and gL are necessary for productive replication in all viruses analysed in this respect. In contrast, gM is dispensable for replication of herpes simplex virus 1 (HSV-1; Baines & Roizman, 1991, 1993, MacLean et al., 1991, 1993), equine herpesvirus 1 (Osterrieder et al., 1996) and PrV (Dijkstra et al., 1996). In these viruses, lack of gM resulted in a decrease of replicative ability in cell culture represented by 10- to 50-fold lower virus titres, a delay in entry into target cells, and slightly reduced plaque size. For gM of the betaherpesvirus human cytomegalovirus a role in attachment was inferred from its heparin-binding properties (Kari et al., 1994). These limited phenotypic alterations in the absence of gM contrast the high conservation of gM structure. All deduced gM homologues share a charged carboxy terminus, eight putative transmembrane domains and an N-glycosylation motif between the first and second hydrophobic domain (Dijkstra et al., 1996).

So far, only one investigation has dealt with growth properties of a herpesvirus gM− mutant in animals (MacLean et al., 1993). gM− HSV-1, after footpad inoculation into mice, was impaired in its ability to grow at the periphery, and spread to the nervous system. After intracranial infection, the LD<sub>50</sub> of the gM− mutant proved to be 35-fold higher than that of the isogenic revertant. Mice, however, are not natural hosts for HSV-1.

To analyse a gM− mutant in a natural virus–host system, we constructed a gM deletion/β-galactosidase expression cassette (Mettenleiter & Rauh, 1990;
Fig. 1. Animal infections. (A) Virus excretion from intranasally infected pigs. Six 6-week-old pigs per group were infected intranasally with $2 \times 10^8$ p.f.u. of PrV-Ka (C), PrV-AgMβ (○) or PrV-AgMβR (▲). Nasal swabs were taken daily and virus excretion was determined by titration on MDBK cells. Values represent average titres derived from all animals in one group. Standard deviations are indicated. (B) Body temperature in intranasally challenged pigs. All eighteen pigs from the virulence test, which had been infected with PrV-Ka (●), PrV-AgMβ (△) and PrV-AgMβR (▲), plus three naive age-matched controls (X) were infected intranasally with $10^8$ p.f.u. of PrV strain NIA-3. Virus excretion from the nasal mucosa was determined at the indicated times after challenge infection. Values represent average titres from all animals in one group. Standard deviations are also indicated. Control animals died on days 3 and 4 p.c. (one and two animals, respectively).

J. M. Dijkstra, T. C. Mettenleiter & B. G. Klupp, unpublished results). One-step growth kinetics confirmed that, compared to wild-type PrV-Ka and revertant PrV-AgMβR, titres of PrV-AgMβ consistently remained approximately 10- to 50-fold lower (data not shown). These results parallel those obtained with a different PrV gM mutant (Dijkstra et al., 1996).

To test for importance of gM in PrV replication in the natural host, three groups of six 6-week-old piglets were each infected intranasally with $2 \times 10^8$ p.f.u. of PrV-Ka (group 1), PrV-AgMβ (group 2) or PrV-AgMβR (group 3). Starting at day 1 post-infection (p.i.), virus excretion from the nasal cavity was determined daily by titration of nasal swabs on MDBK cells. In Fig. 1A mean virus titres of each group are depicted. Average virus excretion after infection with PrV-AgMβ was 100- to 1000-fold less compared to PrV-Ka- or PrV-AgMβR-infected animals. This demonstrates a significant impairment of replication of the gM- mutant in the nasal mucosa, and indicates that the defect is due to the absence of gM. After infection with PrV-Ka or PrV-AgMβR there was a sharp rise in mean body temperature with average values above 41 °C (Fig. 1B). After infection with PrV-AgMβ a transient slight elevation in body temperature was observed, and in only two of the animals did body temperature rise above 41 °C at 2 days p.i. No clinical symptoms were observed in the PrV-AgMβR-infected group. In contrast, all animals in groups 1 and 3 showed severe respiratory symptoms, and one animal in each group exhibited central nervous disorders such as ataxia and convulsions. However, all animals survived (Table 1).

Seven weeks after the experimental infection all 18 animals were intranasally challenged with $10^8$ p.f.u. of the highly virulent PrV strain NIA-3 (Baskerville et al., 1973). As controls, three naive age-matched animals were also infected. One control animal died on day 3 post-challenge (p.c.) and two on day 4 p.c. from acute disease with severe neurological disorders, whereas none of the animals in groups 1 and 3 exhibited any signs of AD. Group 2 animals showed light fever at days 2 and 3 p.c. (mean temperature 40.7 °C at 40.8 °C) and decreased appetite. Virus shedding after challenge (Fig. 1C) was quite variable, even among animals in the same group, except for the control animals, which shed high amounts of virus before death. Generally, titres of excreted virus were higher in group 2 compared to groups 1 and 3. However, none of the animals shed virus after day 7 p.c. Weight gain is a sensitive measurement of protection in pigs. At day 10 p.c., the average weight gain was 5.7 ± 3.2 kg for the animals in groups 1 and 3, and 4.8 ± 2.2 kg in group 2. Thus, survival of a primary wild-type infection yielded the best protection from challenge. However, infection with PrV-AgMβ, despite its diminished replication, also resulted in significant protection from challenge (Table 1).

Deletion of gM leads to a pronounced attenuation of PrV, indicating its usefulness as a live vaccine. Since the absence of gM in vaccine strains could also be used as a marker to differentiate vaccinated from wild-type virus-infected animals, sera from animals which survived infection by gM- viruses were tested for reactivity with baculovirus-expressed gM. For expression of gM in baculovirus the UL10 gene [Dijkstra et al., 1997; EMBL X97257, nt positions 3585 (start)–2406 (stop)] was excised with NolIII (nt positions 3586 and 2385) and inserted into vector pVL1393 resulting in pVL1393-UL10.
Virulence of gM-negative PrV

Table 1. Clinical scores in pigs after PrV infection

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Infection I: virulence test*</th>
<th>Infection II: challenge†</th>
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<tr>
<td></td>
<td>Fever</td>
<td>Respiratory</td>
</tr>
<tr>
<td>PrV-Ka</td>
<td>+++++</td>
<td>+++++</td>
</tr>
<tr>
<td>PrV-AgMβ</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>PrV-AgMβR</td>
<td>+++++</td>
<td>+++++</td>
</tr>
<tr>
<td>None</td>
<td>NA</td>
<td>NA</td>
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* In the first experiment three groups of six 6-week-old animals were intranasally infected with 2 × 10⁶ p.f.u. of PrV-Ka, PrV-AgMβ or PrV-AgMβR. Clinical signs such as fever, respiratory distress or neurological disorders were recorded daily, as was survival of the animals, and were graded as: +++, severe; ++, moderate; +, weak; −, none.

† Seven weeks after the first infection all animals including three age-matched controls were intranasally challenged with 10⁷ p.f.u. of PrV strain NIA-3. Again, clinical signs and survival of the animals were monitored.

NA, Not applicable.

After lipofection of BaculoGold DNA (PharMingen) with pVL1393-UL10 into High Five cells (Invitrogen), recombinant baculovirus was identified by immunofluorescence and Western blot with a gM-specific anti-peptide serum (Dijkstra et al., 1996). Clinical signs such as fever, respiratory distress or neurological disorders were recorded daily, as was survival of the animals, and were graded as: +++, severe; ++, moderate; +, weak; −, none.

Fig. 2. Reactivity of porcine sera with baculovirus-expressed gM in Western blot. Lysates of insect cells infected with recombinant baculovirus were separated in SDS−10% PAGE, transferred to nitrocellulose filters and reacted with porcine sera A–D taken before the virulence test (lanes 0) or 2 weeks p.c., i.e. 9 weeks after start of the experiment (lanes 9). In lane R, reactivity of a gM-specific rabbit anti-peptide serum (Dijkstra, T. C. Mettenleiter & B. G. Klupp, unpublished results) is shown. Molecular masses of baculovirus-expressed gM products are indicated in kDa.

This serum reacts with the 33 kDa nonglycosylated gM precursor, a 35 kDa high-mannose form of gM, a 31 kDa breakdown product and probable dimers of ca. 60 kDa (J. M. Dijkstra, T. C. Mettenleiter & B. G. Klupp, unpublished results). Sera B–D showed reactivity primarily against the nonglycosylated 33 kDa gM precursor.

In PrV, several classes of proteins have been found to play a role in virulence in the natural host. These include enzymes involved in nucleotide metabolism, such as thymidine kinase (TK; Kit et al., 1985; McGregor et al., 1985), ribonucleotide reductase (de Wind et al., 1993) or dUTPase (Jöns et al., 1997), which are probably required for efficient replication of the virus in quiescent cells such as neurons. Deletion of TK or dUTPase completely attenuates the virus for young piglets. Despite this replication defect, prior infection with TK- or dUTPase-negative virus strains resulted in marked protection against a subsequent challenge infection (reviewed in Mettenleiter, 1994).

A second class of proteins involved in virulence is exemplified by a nonessential capsid component encoded by the UL21 gene (de Wind et al., 1992). Deletion of the UL21 gene leads to a striking attenuation of PrV without markedly impairing immunogenicity (Klupp et al., 1995).

A third group of proteins with a marked effect on virus virulence comprises nonessential viral glycoproteins. Of the eleven PrV glycoproteins six are nonessential for productive replication of the virus in cell culture (reviewed in Mettenleiter, 1996). Deletion of gE reduced PrV virulence, as did deletion of gI, which complexes with gE (Kimman et al., 1992). In contrast, no effect on virulence was observed after deletion of the gene encoding the nonstructural gG (Kimman et al., 1992). Of these proteins, none is conserved throughout the herpesvirus family. Here we show that the virion component gM (Dijkstra et al., 1996) is also required for the virulent phenotype of PrV. After infection of pigs with gM− PrV, clinical symptoms were limited to a slight transient elevation in body temperature and
neither respiratory distress nor central nervous symptoms were observed.

In addition to attenuation, the development of a live vaccine requires protection against challenge infection with virulent strains. Our protocol consists of intranasal challenge of pigs 7 weeks after the first infection with a high dose of the very virulent PrV strain NIA-3. This challenge invariably results in the death of naïve control animals and is more severe than the experimental protocols used by others (de Wind et al., 1993). Use of this challenge system allows us to better detect differences in the protective ability of different vaccine strains. Infection with PrV-AgMΔ resulted in protection of all animals from death by AD. However, as assessed by clinical parameters as well as excretion of challenge virus, the PrV-AgMΔ-infected animals were not as well protected as those which were infected with PrV-Ka or PrV-AgMΔR. This is not surprising, since it is generally accepted that the best protection is induced by infection with a wild-type virus, as long as the animal survives this infection. However, when compared to an isogenic PrV mutant lacking dUTPase (Jøns et al., 1997), the gMΔ mutant also appears to be slightly less efficient in conferring protection against clinical signs of AD after challenge infection. Nevertheless, gM represents the first nonessential glycoprotein conserved throughout the herpesvirus family whose inactivation has been demonstrated to markedly attenuate the virus for its natural host species. Therefore, inactivation of gM in other herpesviruses might be predicted to also lead to a significant attenuation.

Nonessential glycoproteins have also been used in the development of marked vaccines. The absence of a nonessential glycoprotein from the vaccine strain results in the absence of antibodies against this protein in the vaccinee. Since wild-type strains express all glycoproteins and thus induce the respective antibodies, presence or absence of antibodies against the marker protein discriminate wild-type virus-infected animals from vaccinated animals. So far, glycoproteins G, E and C have been used for this purpose, none of them being conserved within the herpesvirus family. We show here that after infection of pigs, serum antibodies against gM can be detected. But, somewhat disappointingly, only about half of the sera exhibited a gM-specific reactivity. However, our Western blot might not constitute the most sensitive assay system for anti-gM antibodies in porcine sera. Therefore, more sensitive tests have to be developed.

In summary, this study presents the first results of a gMΔ mutant virus infection in a natural virus–host system and demonstrates that deletion of gM leads to a marked attenuation of PrV for pigs. Results appear promising enough to pursue the development of gMΔ herpesviruses as live vaccines and to assess the ability of gM to serve as a universal herpesvirus vaccine marker protein.

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References


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