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Glycoprotein gp50-Negative Pseudorabies Virus: a Novel Approach toward a Nonspreading Live Herpesvirus Vaccine

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Essential herpesvirus glycoproteins are involved in membrane fusion processes during infection, e.g., viral penetration and direct cell-to-cell transmission. We previously showed that the gD-homologous glycoprotein gp50 of pseudorabies virus (PrV) is essential for virus entry into target cells but proved to be dispensable for direct viral cell-to-cell spread in cell culture (I. Rauh and T. C. Mettenleiter, J. Virol. 65:5348–5456, 1991). For gp50-negative (gp50$^-$) viruses, after phenotypic complementation necessary for primary infection, the only means of viral spread is by way of direct cell-to-cell transmission. In contrast, virus mutants lacking the essential gB-homologous glycoprotein gII after phenotypic complementation are only able to infect primary target cells and are blocked in further viral spread. To analyze how these in vitro phenotypes translate into virus replication in the animal, mice were infected intranasally with gp50$^-$ or gII$^-$ PrV mutants after prior phenotypic complementation by propagation on cell lines providing the essential glycoprotein in *trans*. Our results show that whereas the gII$^-$ mutants did not cause disease or any symptoms, gp50$^-$ mutants derived from two different PrV strains were fully virulent, with animals exhibiting severe symptoms ultimately leading to death. However, free infectious virus could not be recovered from either gp50$^-$ or gII$^-$ PrV-infected animals. We conclude that direct cell-to-cell transmission as the only means of viral spread of the gp50$^-$ mutants is sufficient for a full virulent phenotype in mice. After infection of pigs with phenotypically complemented gp50$^-$ PrV, only mild symptoms were observed, whereas the gII$^-$ mutant was totally avirulent. In both cases, shedding of infectious virus did not occur, in contrast to results with animals infected by gX$^-$ PrV that showed severe signs of disease and extensive virus shedding. After challenge infection with the highly virulent NIA-3 strain, the previously gII$^-$ PrV-infected animals exhibited severe symptoms, whereas the gp50$^-$ PrV-infected pigs showed a significant level of protection. In conclusion, vaccination with a PrV mutant lacking glycoprotein gp50, which is unable to spread between animals because of a lack of formation of free infectious virions, can confer on pigs protection against challenge infection. These results provide the basis for the development of new, nonspreading live herpesvirus vaccines based on gp50$^-$ PrV mutants.

Aujeszky's disease (AD), an illness that mainly affects the pig industry, leading to severe financial losses, is caused by pseudorabies virus (PrV), also named suid herpesvirus 1 (38). PrV belongs to the alphaherpesvirus subfamily of the *Herpesviridae* together with other important animal pathogens, e.g., bovine herpesvirus 1, or human pathogens such as herpes simplex virus (HSV) or varicella-zoster virus. Although great efforts are being made to eradicate AD by using a strategy involving differentiation of vaccinated and infected animals (34, 37), problems which are due in part to recombination between different avirulent live vaccine strains leading to virulent progeny (6, 7) or uncontrolled dissemination of vaccine strains among animals (1) are arising.

The proteins involved in both immunogenicity of herpesviruses and their ability to infect target cells are glycoproteins which are structural components of the viral envelope. In PrV up to now, six structural glycoproteins and one nonstructural glycoprotein that all constitute homologs of glycoproteins found in HSV have been identified (reviewed in reference 21). Four of them, gI (gE), gp63 (gI), gIII (gC), and gX (gG), are nonessential for viral replication in cell culture, whereas gII (gB), gp50 (gD), and gH are indispensable for viral infectivity. Functional analyses showed that gIII, like its homolog gC(HSV), is an important viral attachment protein and mediates adsorption of PrV to a heparin-like cellular surface receptor (25). Glycoproteins gI and gp63, which are present in the form of a noncovalently linked complex (41), determine efficiency of virus release in a cell-type-specific manner (24, 39) and also contribute to direct viral cell-to-cell transmission (40). However, so far, no function for the nonstructural glycoprotein gX has been found. gX-negative (gX$^-$) PrV mutants do not differ from wild-type PrV strains in their behavior either in cell culture or in the animal (11, 22, 32). Therefore, the gX gene has been used as an integration site for expression of marker genes such as β-galactosidase (23) or luciferase (13) without apparent alteration of viral growth characteristics or virulence.

The glycoproteins gII and gH are essential for virus entry into target cells and virus spread by way of direct cell-to-cell transmission (26–28). Analysis of PrV mutants deficient in expression of glycoprotein gp50 showed that whereas gp50 is also indispensable for virus penetration into target cells, as is its homolog gD (HSV), it is not necessary for direct viral cell-to-cell spread (27, 28). Whereas gp50-negative (gp50$^-$) Pr virions are noninfectious, as are the gII- and gH-negative (gII$^-$ and gH$^-$, respectively) virions (12, 26–28) after phenotypic complementation by propagation on cell lines providing gp50 in *trans*, the virus can enter primary target cells and effectively spread by direct cell-to-cell transmission. In contrast, the gD homologs in HSV and bovine herpesvirus 1

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are necessary for both penetration and viral spread (4, 15). This result not only showed that penetration and direct cell-to-cell spread are distinct processes that can be separated in the PrV system but also opened the way to analyze the relative contribution of direct cell-to-cell transmission to viral virulence in the animal. Because virions released from noncomplementing cells after infection with phenotypically complemented gp50$^-$ PrV are noninfectious, reinfection by cell-free released virus is not possible. This also implies that shedding of infectious virus should not occur and that, in addition, reisolation of free infectious virus from organs should be unsuccessful.

Glycoprotein gp50 has been shown to represent a major immunogenic protein of PrV, and several potent neutralizing anti-gp50 antibodies have been described (3, 8, 36). In addition, vaccination with recombinant gp50 has been shown to protect animals from AD (18, 30). However, experiments regarding the relative importance of gp50 for a protective immune response in the context of the viral envelope have never been performed. It was therefore of interest to determine the immunogenicity of a gp50$^-$ PrV mutant.

To answer these questions, mice and pigs were infected intranasally with phenotypically complemented gp50$^-$ or gII$^-$ PrV. Our results show that whereas infection by gII$^-$ PrV did not lead to any symptoms in either mice or pigs, infection of mice with two different gp50$^-$ PrV mutants led to severe symptoms similar to those found after gX$^-$ PrV infection and ultimately resulted in the death of the animals. This result proves that direct cell-to-cell transmission is the pathogenetically important mode of virus spread in mice. In contrast, pigs infected with the gp50$^-$ PrV showed only mild signs of disease compared with gX$^-$ PrV-infected animals. Reisolation of virus from organ suspensions or nasal swabs of gp50$^-$ or gII$^-$ PrV-infected animals invariably proved negative, whereas infectious virus could readily be isolated from the gX$^-$ PrV-infected animals. After challenge infection with wild-type PrV, the formerly gp50$^-$ PrV-infected pigs exhibited a significant level of protection.

MATERIALS AND METHODS

Viruses and cells. The moderately virulent PrV strain Ka (9) and the highly virulent strain NIA-3 (19) were used as parental wild-type strains. Isogenic gX$^-$, gp50$^-$, and gII$^-$ mutants carrying a β-galactosidase expression cassette have been described (10, 23, 28, 29). Briefly, the gX$^-$ and gII$^-$ mutants of both strains were obtained by insertion of a gX–β-galactosidase expression cassette into either the gX or gII gene (23, 29). In the gp50$^-$ NIA-3 mutant, the expression cassette was inserted into the gp50 gene, thereby interrupting its expression (10). The gp50$^-$ mutant of strain Ka, because of an unanticipated recombinational event, lacks both gX and gp50 (28).

Virus mutants were propagated on normal bovine kidney (MDBK) cells or MDBK derivatives that constitutively express gp50 (MT50-3 [28]) or gII (MT-3 [29]). Virus stocks were only passaged twice to reduce the amount of virions rescued by the resident glycoprotein gene in the cell line. For each batch of virus, the proportion of rescued virions was determined by either plaque assay on noncomplementing cells for the gII$^-$ mutants or two passages on noncomplementing cells for the gp50$^-$ mutants. Rescue frequency was at or less than 1:10^5 PFU.

Mouse experiments. For analysis of the virulence of the different mutants, 6-week-old female BALB/c mice were infected intranasally with 10 μl of virus suspension containing 10^4 PFU of either gX$^-$ or phenotypically complemented gp50$^-$ or gII$^-$ PrV. This infectious dose had previously been established as leading to 100% mortality in gX$^-$ PrV-infected animals (see Fig. 1A) while it was low enough to avoid inclusion of significant amounts of rescued virus in the inoculum. Mice were observed at least every 8 h for symptoms of PrV infection. Moribund animals were sacrificed, and brain homogenates were analyzed for the presence of infectious virus by titration on gII-expressing MT-3 cells in which wild-type and gX$^-$ PrV, phenotypically complemented gp50$^-$ and gII$^-$ PrV, and all putative rescued virions should be able to form plaques. To this end, brains were removed, snap frozen in liquid nitrogen, homogenized in minimum essential medium supplemented with 100 μg of gentamicin (Boehringer, Mannheim, Germany) per ml, and plated onto MT-3 monolayers. After 1 h of adsorption, the monolayer was extensively washed with phosphate-buffered saline (PBS) and overlaid with methylcellulose medium supplemented by gentamicin. Two to 3 days postinfection (p.i.), plaques were counted. Organs for histological examination were taken from moribund mice infected intranasally with 10^5 PFU in a 50-μl volume to increase the number of infected cells for microscopical analysis.

Histological examination. After being cut into smaller pieces, organs from mice that were sacrificed in a moribund state were fixed for 1 h in 2% formaldehyde–0.2% glutaraldehyde–0.02% Nonidet P-40–0.01% sodium deoxycholate in PBS and afterwards reacted with a substrate solution for β-galactosidase containing 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) per ml, 16 mM potassium ferricyanide, 16 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.02% Nonidet P-40, and 0.01% sodium deoxycholate in PBS (16). After overnight incubation at 37°C, organ pieces were embedded in paraffin and cut on a microtome into 4- to 8-μm sections. They were mounted on slides and analyzed under the microscope for the appearance of blue-staining cells.

Pig experiments. Six-week-old littermates (three animals per group) were infected intranasally with 10^6 PFU of gX-βgal (gX$^-$), gp50-βgal (gp50$^-$), or gII-βgal (gII$^-$) PrV mutants derived from strain NIA-3. They were observed for signs of disease such as respiratory or neurological symptoms and fever. Nasal swabs were taken daily and titrated on MT-3 cells to detect shedding of any infectious virus. Survivors of this experiment (one gX$^-$ PrV-infected animal died) were bled 27 days p.i., and neutralizing antibody titers as well as the presence of anti-gp50 and anti-gII antibodies were determined. Complement-dependent and -independent neutralization titers were determined with a plaque reduction assay as described (29). The titers indicated show serum dilutions yielding 50% plaque reduction. Eight weeks p.i., the animals were challenge-infected intranasally with 3×10^8 PFU of wild-type strain NIA-3. Again, they were observed for signs of disease, body temperature was recorded, and nasal swabs were analyzed for virus shedding.

Radioimmunoprecipitation. Either gp50-expressing MT50-5 (28) or gII-expressing MT-3 (29) cells were labelled for 24 h in medium supplemented with 50 μCi of [^{35}S]methionine per ml. Thereafter, cell lysates were prepared for immunoprecipitation as previously described (17). As controls, anti-gII(PrV) monoclonal antibody (MAb) 5/14 (kindly provided by H.-J. Rziha, Tübingen, Germany [17]) and anti-gp50 MAb MCA50-1 (kindly provided by M. W. Wathen, Kalamazoo, Mich. [36]) were used. Precipitates were separated in nonreducing sodium dodecyl sulfate–10% polyacrylamide gels

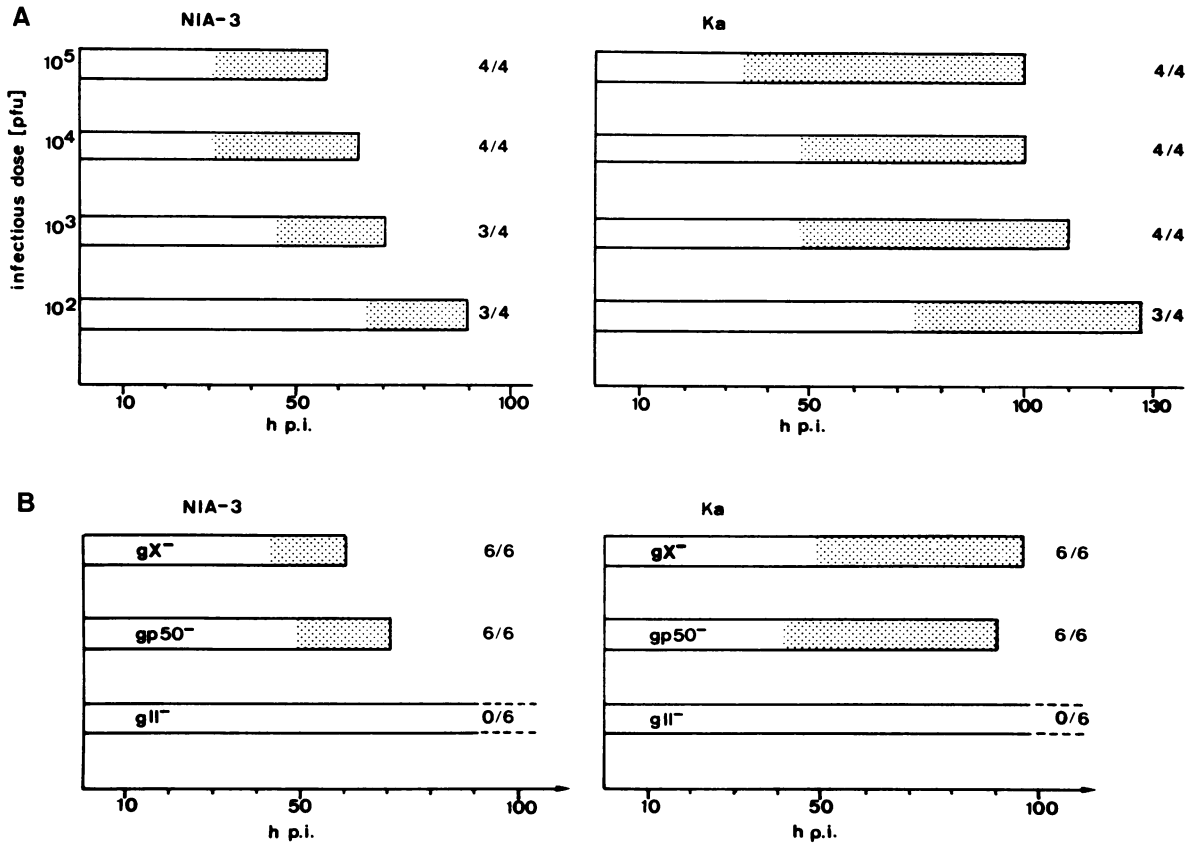

FIG. 1. Determination of minimal lethal infectious dose of PrV (A) and virulence of gp50⁻ and gII⁻ PrV mutants (B) for mice. (A) Mice were infected intranasally with different PFU of gX-βgal (gX⁻) mutants derived from either wild-type strain NIA-3 or Ka. The mean time to death for each group of four animals is indicated. Shaded areas indicate presence of symptoms. Numbers to the right of the vertical bars denote dead mice from a total of four infected animals per group. (B) Mice were intranasally infected with 10^4 PFU of either gX-βgal (gX⁻) or phenotypically complemented gp50-βgal (gp50⁻) or gII-βgal (gII⁻) mutants of PrV strains NIA-3 and Ka. Vertical bars indicate mean time to death of each group of six animals. Shaded areas denote presence of symptoms. Numbers to the right of the vertical bars indicate dead mice from a total of six infected mice per group. Animals infected by phenotypically complemented gII⁻ PrV all survived for more than 4 weeks (when the experiment was terminated) without showing any symptoms.

(14), and fluorographic images were obtained on Kodak XAR-5 film.

RESULTS

Determination of minimal lethal infectious dose for mice. When using viruses mutated in essential genes that have to be propagated on complementing cell lines, it is imperative to test for and to minimize the proportion of virions whose genome had been rescued by incorporation of the viral gene resident in the cell line. In our virus stocks, rescue frequency approximated 1:10^5 PFU. To establish the infectious dose necessary for a lethal intranasal infection, mice were infected with 10-fold dilutions of gX-βgal mutants of either strain NIA-3 or Ka and observed for symptoms. Results are shown in Fig. 1A. Whereas strain NIA-3, as expected, was more virulent than strain Ka (i.e., earlier onset of symptoms and shorter mean time to death), 10^4 PFU given intranasally was the lowest dose of virus leading to death of all infected animals. In the following experiment, animals were therefore inoculated with 10^4 PFU, giving a safety margin of 10-fold, not to inadvertently include rescued virus in the inoculum.

Lack of gp50 does not significantly alter the virulence of PrV for mice, whereas lack of gII does. In vitro, phenotypically complemented gp50⁻ PrV after primary infection is able to spread by direct cell-to-cell transmission. In contrast, phenotypically complemented gII⁻ PrV is restricted to infection of primary target cells (27, 28). To analyze how this in vitro phenotype translates into in vivo characteristics, mice were infected intranasally with 10^4 PFU of either gX⁻ PrV (which behaves like the normal wild type [32]) or phenotypically complemented gp50⁻ or gII⁻ mutants of both strains NIA-3 and Ka. As can be seen in Fig. 1B, in both cases after phenotypic complementation, the gp50⁻ mutants were able to induce symptoms and to cause death in all animals, similar to the isogenic gX⁻ mutants. In addition, the onset of symptoms and the mean time to death between wild-type and gp50⁻ mutant virus infection were not significantly different. In contrast, both gII⁻ mutants did not cause any symptoms over a period of more than 4 weeks, at which time the experiment was terminated. Noncomplemented gp50⁻ or gII⁻ virions, as expected, did not lead to any signs of disease, reflecting their inability to infect target cells (data not shown). These results indicate that after primary infection, gp50 is not necessary for virulence of PrV after intranasal infection of mice.

Insertion of a β-galactosidase expression cassette in all viruses used facilitated studies of viral spread in the animal.

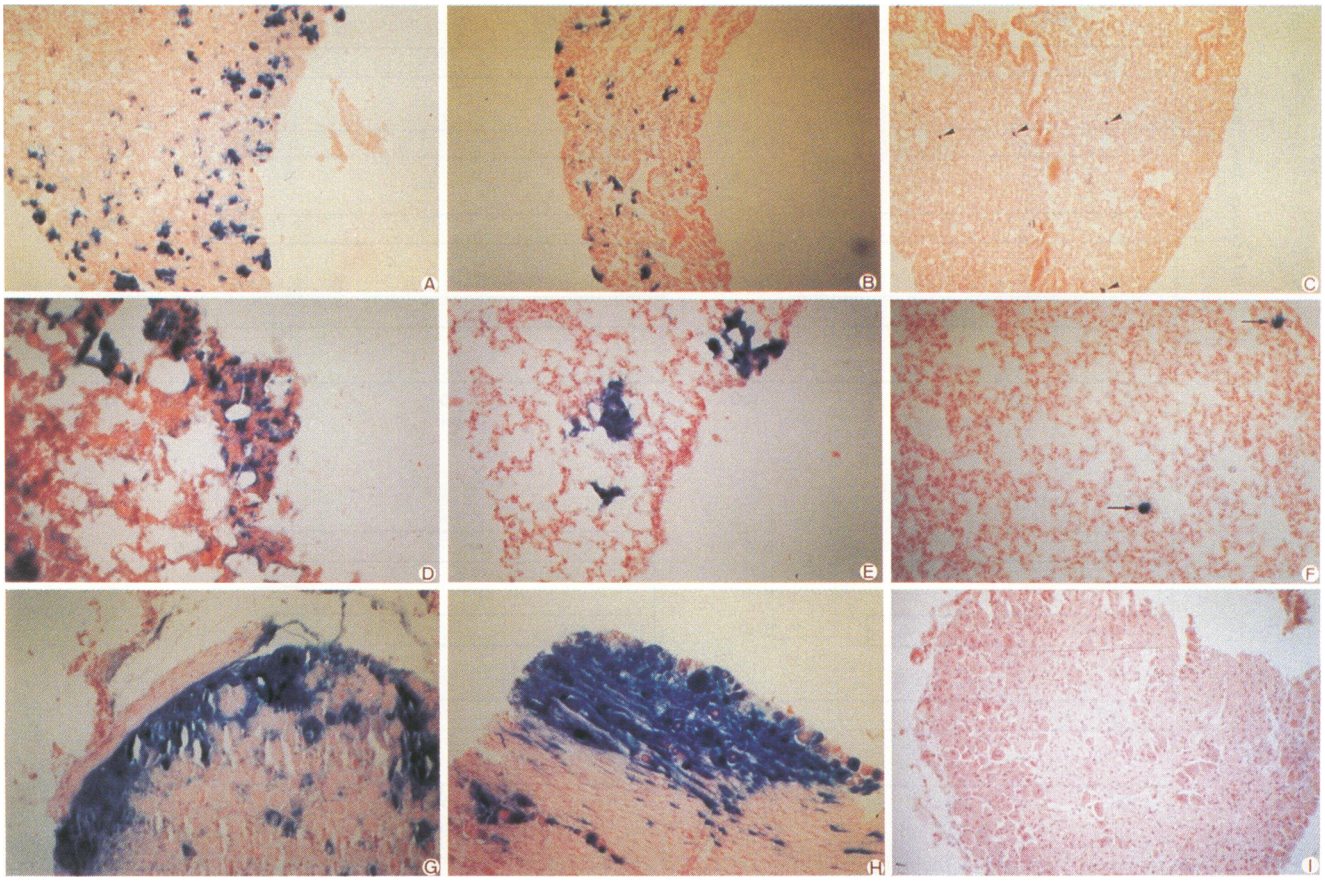

FIG. 2. Histological demonstration of PrV-infected cells in mice. Histological sections of mice infected by either gX-βgal (gX$^-$; A, D, and G) or phenotypically complemented gp50-βgal (gp50$^-$; B, E, and H) or gII-βgal (gII$^-$; C, F, and I) PrV were analyzed microscopically after being stained with X-gal. Examples of lung tissue (A through C, ×100; D through F, ×400) and trigeminal ganglion (G through I, ×200) are shown. Arrows denote single blue-staining cells observed after infection with phenotypically complemented gII$^-$ PrV.

Animals were infected as described in Materials and Methods, organs were removed immediately after euthanization of moribund animals, and histological sections were examined under the microscope after staining with X-gal. As shown in Fig. 2A through F, in the lungs of both gX$^-$ and gp50$^-$ PrV-infected animals, widespread virus infection was seen. In contrast, only single blue-staining cells were observed after infection with gII$^-$ PrV. The phenotype seen in the animal therefore mimics that observed in cell culture (27, 28). Invasion of the nervous system (e.g., the trigeminal ganglion; Fig. 2G through I) was invariably seen in both gX$^-$ and gp50$^-$ PrV-infected animals but was only rarely observed in animals infected by gII$^-$ PrV.

Free infectious virus is not detectable after infection of mice with phenotypically complemented gp50$^-$ or gII$^-$ PrV. Both gII and gp50 are required for penetration of virions into target cells in vitro. Therefore, virions released after primary infection of noncomplementing cells by both phenotypically complemented virus mutants are noninfectious (27, 28). To analyze whether similar results could be observed in vivo, brain homogenates of moribund gX$^-$ or gp50$^-$ PrV-infected mice or gII$^-$ PrV-infected mice which were killed at the same time without exhibiting any signs of disease were used for reisolation of virus on gII-expressing MT-3 cells (Table 1). On these cells, all putative infectious virions, either gX$^-$ or phenotypically complemented or rescued gp50$^-$ or gII$^-$ PrV, should be able to form plaques. Whereas reisolation of virus from all gX$^-$ PrV-infected animals proved to be successful, no free infectious virus was detected after infection by phenotypically complemented gp50$^-$ PrV despite the fact that the animals were moribund, showing profound

TABLE 1. Virus isolation from gX$^-$, gp50$^-$, or gII$^-$ PrV-infected mice

Group	Days p.i.	Severity of symptoms[a]	Virus reisolation[b]
Ka			
gX$^-$	4	++	2.6×10^4
gp50$^-$	4	++	0
gII$^-$	4	−[c]	0
NIA-3			
gX$^-$	3	++	1.0×10^4
gp50$^-$	3	++	0
gII$^-$	3	−[c]	0

[a] Symptoms of AD prior to sacrifice. ++, severe; −, none.

[b] Brain homogenates of animals that were sacrificed in a moribund state at the indicated time after infection were titrated on gII-expressing MT-3 cells. Values are mean titers for groups of four animals each.

[c] Animals infected by phenotypically complemented gII$^-$ PrV derived from both wild-type strains Ka and NIA-3 that did not show any signs of disease were killed at the same time as the corresponding gp50$^-$ PrV-infected animals.

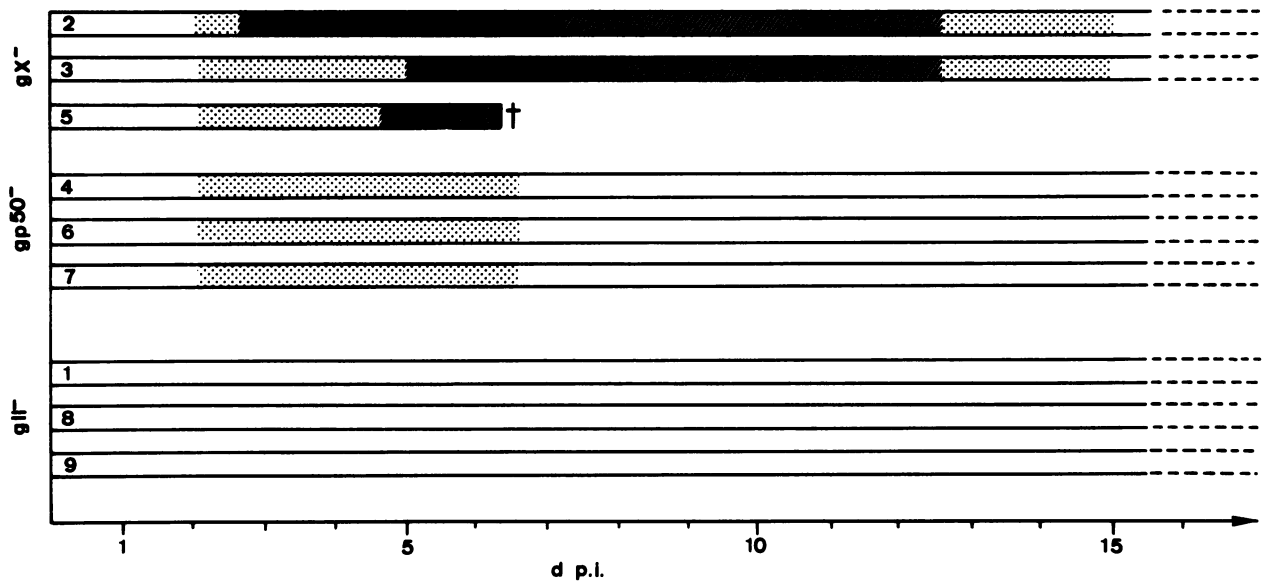

FIG. 3. Virulence of gp50⁻ and gII⁻ PrV mutants for pigs. Three pigs each were infected intranasally with 10^6 PFU of either gX⁻ or phenotypically complemented gp50⁻ or gII⁻ PrV mutants derived from wild-type strain NIA-3. The presence of mild general or respiratory symptoms (▩) or severe neurological signs (▨) is indicated. Numbers denote individual animals. Animal 5 was euthanized (†) 6.5 days (d) p.i.

symptoms. Infectious virus was also not recovered from the gII⁻ PrV-infected animals.

Deletion of gp50 decreases virulence of PrV for pigs. To establish the behavior of the virus mutants in PrV's natural host, three pigs, at 6 weeks of age, were intranasally infected with 10^6 PFU of gX⁻, gp50⁻, or gII⁻ mutants derived from the highly virulent NIA-3 strain. They were observed for signs of AD, and shedding of virus was monitored by analyzing nasal swabs. As can be seen in Fig. 3 and Table 2 (experiment A), pigs infected with the gX⁻ mutant exhibited severe respiratory and neurological symptoms. One moribund animal was sacrificed. All animals showed prolonged fever and virus excretion in nasal swabs. In contrast, after infection with phenotypically complemented gp50⁻ PrV, only mild, mainly respiratory symptoms occurred. The only other sign of disease was a slight weakness in the hind legs. Duration of fever was much reduced, and no infectious virus could be recovered from nasal swabs. The gII⁻ PrV-infected pigs did not show any symptoms or shedding of infectious virus. We conclude that deletion of gp50 from strain NIA-3 decreases its virulence for pigs, although the virus was obviously still able to cause respiratory distress and fever. Sera taken from the surviving animals at day 27 p.i. showed the presence of neutralizing antibodies in the gX⁻ PrV-infected group and in the gp50⁻ PrV-infected group (Table 2). The gII⁻ PrV-infected animals did not show seroconversion under our experimental conditions.

Immunoprecipitations with radiolabelled lysates of either gp50- (Fig. 4A) or gII-expressing cells (Fig. 4B) were performed to assay for the presence of anti-gp50 and anti-gII antibodies in sera from infected pigs. As shown in Fig. 4A, gp50 was specifically precipitated by sera from gX⁻ PrV-

TABLE 2. Signs of disease in infected and challenge-infected pigs

Group[a]	Neutralization titer[b]		Virus shedding[c]		Severity of symptoms[d]		Fever		Death (dead/total)
	Without complement	With complement	Days p.i.	Peak titer	Respiratory	Central nervous system	Days p.i.	Peak (°C)	
Experiment A									
gX⁻	1:20	1:1,000	2–10	1.5×10^4	++	++	2–9	41.4	1/3
gp50⁻	1:5	1:500	–[e]	–	+	(+)	2–5	40.5	0/3
gII⁻	–	–	–	–	–	–	–	–	0/3
Experiment B									
gX⁻	ND[f]	ND	–	–	(+)	–	1–3	41.2	0/2
gp50⁻	ND	ND	1 and 3	1×10^3	+	–	1–3	40.9	0/3
gII⁻	ND	ND	1–5	2.2×10^3	++	++	1–9	41.5	2/3

[a] For experiment A, animals were infected intranasally with 10^6 PFU of either gX⁻ or phenotypically complemented gp50⁻ or gII⁻ mutants of PrV strain NIA-3. For experiment B, survivors of experiment A were challenge-infected intranasally with 3×10^8 PFU of wild-type strain NIA-3.

[b] Neutralization titers were determined as described (29). Values are mean titers leading to 50% plaque reduction without or with addition of complement.

[c] Nasal swabs were taken daily and analyzed for presence of infectious virus by titration on gII-expressing MT-3 cells. Duration of virus shedding and peak titers per nasal swab are indicated.

[d] Severity of symptoms is indicated: –, none; (+), weak; +, intermediate; ++, severe.

[e] –, absence of neutralizing antibodies, virus shedding, symptoms, or fever.

[f] ND, not done.

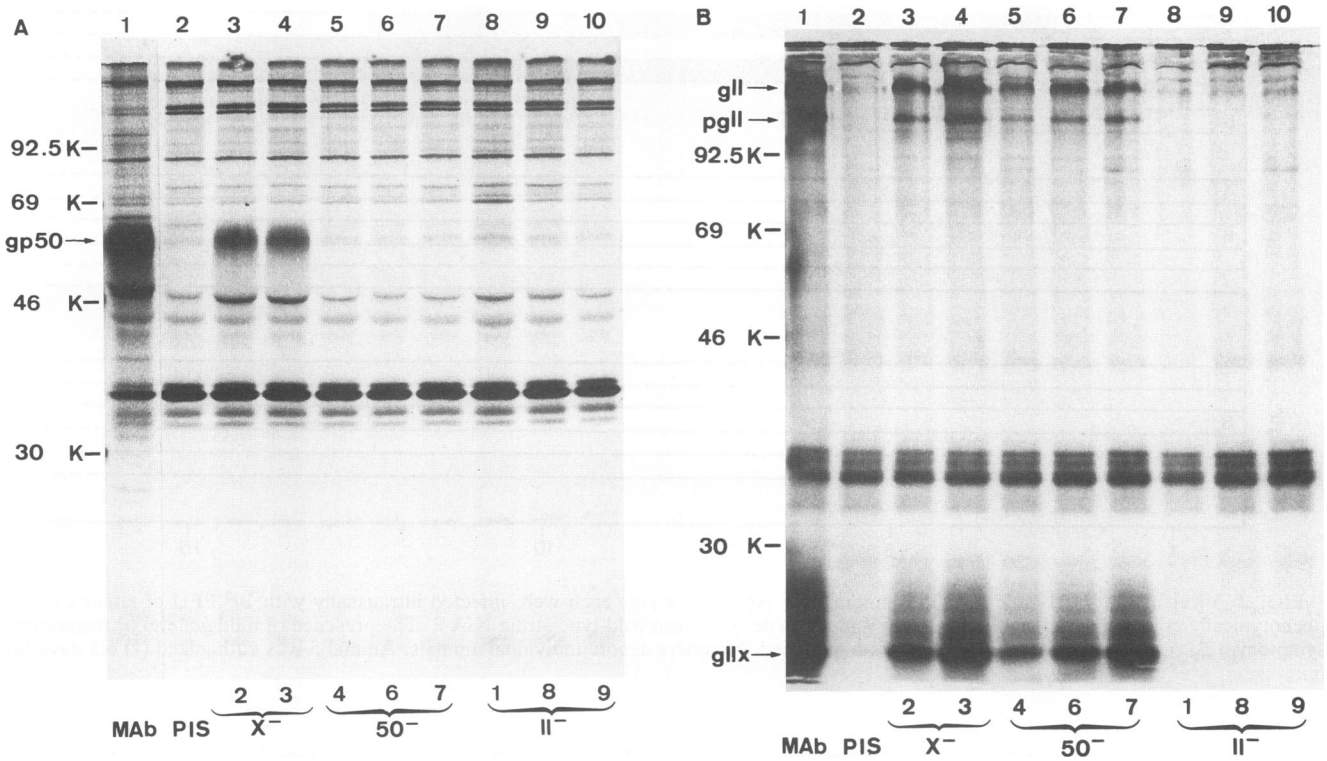

FIG. 4. Radioimmunoprecipitation analysis with sera from gX⁻, gp50⁻, and gII⁻ PrV-infected pigs. Radiolabelled extracts of cells expressing either gp50 (A) or gII (B) were precipitated with sera collected from gX⁻ PrV- (lanes 3 and 4), gp50⁻ PrV- (lanes 5 to 7), or gII⁻ PrV-infected pigs (lanes 8 to 10) at day 27 p.i. As controls, precipitations with an anti-gp50 MAb (A, lane 1) or an anti-gII MAb (B, lane 1) were performed. Lanes 2 of panels A and B show precipitations with a pool of preinfection sera (PIS) of these animals. Numbers below the lanes indicate individual pigs as designated in Fig. 3 and 5. Arrows mark the positions of mature gp50, mature gII, and the gII precursor pgII as well as the gII-related 27-kDa gIIx protein. The positions of the molecular weight markers are also indicated.

infected animals (Fig. 4A, lanes 3 and 4). These sera also precipitated the mature gII glycoprotein complex, the gII precursor protein pgII, as well as a gII-related 27-kDa protein, gIIx (Fig. 4B, lanes 3 and 4) that accumulates during extended labelling periods (20) and that is also recognized by the anti-gII MAb (Fig. 4B, lane 1). As expected, sera from the gp50⁻ PrV-infected animals recognized gII (Fig. 4B, lanes 5 through 7) but not gp50 (Fig. 4A, lanes 5 through 7), indicating the absence of anti-gp50 antibodies in these animals but the presence of antibodies to other virion components as exemplified by gII. Sera from the gII⁻ PrV-infected animals did not specifically recognize either gp50 or gII (Fig. 4, lanes 8 through 10) compared with preinfection serum (Fig. 4, lanes 2), which correlates with absence of seroconversion in these animals (Table 2). A protein comigrating with gII is nonspecifically precipitated by the preimmune serum (Fig. 4B, lane 2) as well as sera from the gII⁻ PrV-infected animals (Fig. 4B, lanes 8 through 10). As positive controls, precipitations with anti-gp50 MAb MCA50-1 (Fig. 4A, lane 1) or anti-gII MAb 5/14 (Fig. 4B, lane 1) were performed. A pool of preinfection sera (Fig. 4, lanes 2) served as a negative control. When tested individually, none of the preinfection serum samples from any pig exhibited specific reaction with either gp50 or gII (data not shown).

Infection by gp50⁻ PrV confers protection against virulent challenge. To analyze whether animals after prior infection by gX⁻ or phenotypically complemented gp50⁻ or gII⁻ PrV were protected from challenge with a virulent virus, all surviving pigs were intranasally challenged 8 weeks after the first infection with 3×10^8 PFU of the highly virulent NIA-3 strain (19). Results are shown in Fig. 5 and Table 2 (experiment B). In the previously gII⁻ PrV-infected animals, severe signs of disease were observed upon challenge. Two animals succumbed to the challenge virus infection. One animal was still in the recovery phase after 14 days when the experiment was terminated. The gX⁻ PrV survivors showed the least reaction upon challenge infection, with only limited fever and no shedding of challenge virus. The gp50⁻ PrV-infected pigs exhibited very mild symptoms of AD but only limited fever, although some virus shedding did occur. In summary, we demonstrate that infection of pigs with a phenotypically complemented gp50⁻ PrV mutant led to induction of a significant protective immune response.

DISCUSSION

In this study, the role of the essential glycoproteins gp50 and gII for PrV virulence in vivo and their importance for a protective immune response were examined. Both glycoproteins have previously been shown to be important immunogens (3, 5, 8, 18, 30) and have also been identified as necessary for the fusion of viral envelope and cellular cytoplasmic membrane during the process of infectious entry of the nucleocapsid into the target cell (27–29). Virions lacking gp50 or gII proved to be noninfectious. However, whereas gII was also required for direct viral cell-to-cell spread, which is thought to involve membrane fusion events,

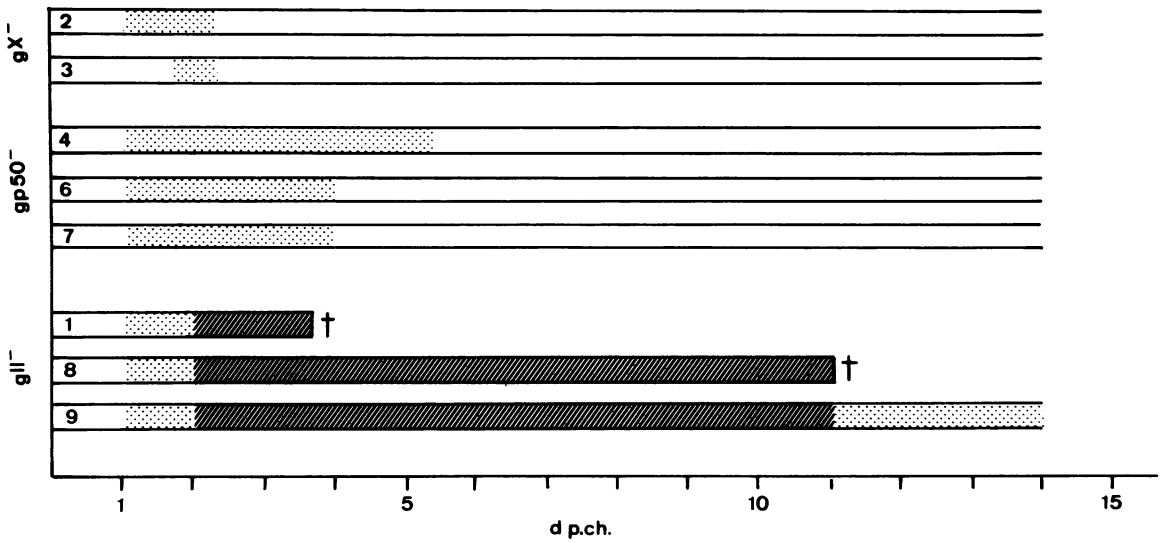

FIG. 5. Protective immunity induced by gp50⁻ and gII⁻ PrV mutants. Survivors from the experiment shown in Fig. 3 were challenge-infected intranasally with 3×10^8 PFU of wild-type PrV strain NIA-3. Mild general or respiratory symptoms (▨) or severe neurological signs (▨) exhibited at the indicated time after challenge are shown. Two gII⁻ PrV-infected animals had to be euthanized (†) during the course of the experiment, with one animal still in the recovery phase at the termination of the experiment (14 days postchallenge [d p.ch.]). Numbers identify individual animals as in Fig. 3.

gp50 could be dispensed with for this process (27, 28). In this respect, among herpesviruses the unique situation exists that after phenotypic complementation leading to infection of primary target cells, gp50⁻ PrV is able to spread exclusively by direct cell-to-cell transmission, whereas free infectious virions are not produced. This phenotype gives an ideal opportunity to analyze the importance of cell-to-cell transmission in the pathogenicity of the virus and should also have wide-ranging effects on the construction of a new kind of safe live vaccine unable to spread between animals.

For our studies, we used β-galactosidase insertion mutants to facilitate detection of virus-infected cells in the animal. Inactivation of the gene encoding the nonessential nonstructural glycoprotein gX has previously been shown not to interfere with the growth characteristics of PrV in vitro (22) or with virulence in vivo (11, 20, 32). We therefore used β-galactosidase insertion mutants in the gX gene as strains representative of wild-type PrV. The gII⁻ mutants of both strains NIA-3 and Ka and the gp50⁻ mutant of strain NIA-3 contained insertions of the β-gal expression cassette in the gII or gp50 gene, respectively, not affecting other genes (10, 23, 29). In contrast, the gp50⁻ PrV mutant based on strain Ka also lacks gX because of an unanticipated recombination event (28). However, because both gp50⁻ mutants exhibited similar behavior when tested in parallel in mice, it is unlikely that the absence of gX in the Ka mutant contributed significantly to the observed phenotype.

The use of mutants lacking essential proteins that are dependent for productive replication on complementing cell lines inherently bears the risk of the presence in any virus stock of wild-type revertants rescued by the resident viral gene in the cell line. We therefore carefully avoided passaging of virus stocks more than two times and in every instance assayed for rescued virus in the inocula used for the experiments. The rescue frequency in all our virus stocks approximated $1{:}10^5$. Because, for the virulence tests, mice were infected with 10^4 PFU of either virus, a safety margin of 10 was present to avoid inadvertently including any wild-type rescuant. Inocula for pigs that were infected at 10^6 PFU on average could contain 10 PFU of rescued virus. However, it has previously been established that the infectious dose necessary for starting AD after intranasal infection of pigs this age by PrV was several orders of magnitude above this level (38). In addition, the lack of reisolation of infectious virus after gp50⁻ and gII⁻ PrV infection and the absence of anti-gp50 antibodies after infection with the phenotypically complemented gp50⁻ PrV stock, as demonstrated by immunoprecipitation, argue against a significant contribution of the few theoretically included wild-type rescuants to the outcome of our experiments.

Our results show that both the gp50⁻ and the gII⁻ PrV mutants after phenotypic complementation exhibit in vivo a phenotype similar to that exhibited in vitro. After intranasal infection of mice, the phenotypically complemented gp50⁻ PrV was as virulent as the gp50⁺ parental strain and was found to enter and replicate in the nervous system. Because the only means of intercellular transmission of the gp50⁻ mutant is by direct cell-to-cell spread, we conclude that it is indeed this mode of transmission that is pathogenetically most important in mice. A similar conclusion has been reached in studies with PrV mutants deficient in the nonessential glycoproteins gI and gIII (40) which exhibit an impairment in either attachment of extracellular infectious virions (gIII⁻) or direct cell-to-cell spread (gI⁻). However, these mutants were still capable of infecting cells by both ways—infection from outside and direct cell-to-cell spread—which is not the case in the gp50⁻ mutants. gp50⁻ PrV mutants are therefore excellent tools for studying the mechanism of direct viral cell-to-cell spread in vitro and in vivo.

The gII-deleted virus mutants after phenotypic complementation were able to infect primary target cells in the nasal mucosa (not shown) and the lung (Fig. 2) after intranasal infection of mice. In both organs, single infected cells were seen, reminiscent of the phenotype observed in cell culture (28). In two instances after infection by phenotypically complemented gII⁻ PrV, we observed one single blue-

staining trigeminal neuron after dissecting the whole trigeminal ganglion (data not shown). This indicates that trigeminal neurons are primary target cells for PrV because these are the only cells that can be infected by this viral mutant. Experiments to analyze the neural spread of the described virus mutants in more detail, including unequivocal identification of primary and secondary target cells, are in progress (2).

Although the gp50$^-$ PrV-infected mice showed severe symptoms ultimately leading to death, free infectious virus could not be recovered from brain homogenates (Table 1) or lungs (data not shown). This is in stark contrast to the situation after gX$^-$ PrV infection in which reisolation of virus proved to be successful in every instance. These data indicate that free infectious virus is not produced after infection of cells either in culture or in the animal by phenotypically complemented gp50$^-$ PrV. We conclude that in mice, once primary infection had occurred, the gp50$^-$ virus spread like a normal wild-type strain but free infectious virus did not arise.

Studies with pigs, the natural host of PrV, showed that lack of gp50 led to a decrease in viral virulence. Although respiratory distress and fever were observed upon intranasal infection by phenotypically complemented gp50$^-$ PrV, only weak neurological signs of disease (slight weakness in the hind legs) appeared, quite in contrast to the situation after infection by gX$^-$ PrV. Inactivation of the gp50 gene therefore led to an attenuation of the virus for pigs. This result allowed us to analyze the protection afforded by infection of animals with gp50$^-$ PrV. Both the gp50$^-$ PrV-infected animals and the gX$^-$ PrV survivors showed the presence of neutralizing antibodies, although the level was somewhat higher in the gX$^-$ PrV-infected group than in the gp50$^-$ PrV-infected animals. This is not surprising, because gp50 has already been described as a potent immunogen (8, 18). Challenge experiments using a very stringent protocol with intranasal infection by 3×10^8 PFU of the highly virulent NIA-3 strain proved that after prior infection with gp50$^-$ PrV, the animals showed a significant degree of protection against challenge infection, whereas the gII$^-$ PrV-infected animals showed severe signs of AD upon superinfection and two animals of three had to be euthanized.

Although the gp50$^-$ PrV-infected animals were less protected than the survivors of gX$^-$ PrV infection, a dramatic reduction in clinical symptoms was observed. In this context, it is generally acknowledged that the optimum protection against any virus infection is conferred after infection by wild-type virus. The performance of gp50$^-$ PrV in comparison with that of routinely used attenuated live vaccine strains remains to be established. Our results, however, indicate that gp50$^-$ PrV, despite the lack of a major immunogen, might indeed be used as a live vaccine that is able to stimulate in the animal efficient protective immune responses without the risk of shedding and uncontrolled spread of infectious vaccine virus among animals.

PrV is increasingly used as a live vector for expression of heterologous genes (31, 33, 35). However, uncontrolled spread of these genetically engineered recombinants is one of the major concerns about their practical use. On the basis of the described gp50$^-$ PrV mutants, safe PrV-based vectors that are unable to spread between animals could be devised. Presently, it is not known whether gp50$^-$ PrV is able to establish itself in a latent state in the animal and whether reactivation can be induced. On the basis of our results, however, it is likely that even after reactivation, only noninfectious virus that lacks gp50 could be shed from animals vaccinated with phenotypically complemented gp50$^-$ PrV.

In summary, our studies demonstrate that after primary infection gp50 is not required for the virulence of PrV in mice, showing that direct cell-to-cell transmission is the pathogenetically important mode of PrV spread in these animals. In pigs, lack of gp50 led to a decrease in PrV virulence. We also show that in no instance could infectious virus be recovered from animals infected by phenotypically complemented gp50$^-$ PrV despite full-blown disease in mice and respiratory symptoms in pigs. After prior infection by phenotypically complemented gp50$^-$ PrV, pigs showed a significant degree of protection against a heavy challenge infection. Lack of formation of free infectious virus concomitant with good protection despite the lack of gp50 are major prerequisites for the construction of a new generation of safe PrV live vaccines.

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