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Envelope Glycoprotein gp50 of Pseudorabies Virus Is Essential for Virus Entry but Is Not Required for Viral Spread in Mice

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Phenotypically complemented pseudorabies virus gp50 null mutants are able to produce plaques on noncomplementing cell lines despite the fact that progeny virions are noninfectious. To determine whether gp50 null mutants and gp50+gp63 null mutants are also able to replicate and spread in animals, mice were infected subcutaneously or intraperitoneally. Surprisingly, both gp50 mutants and gp50+gp63 double mutants proved to be lethal for mice. In comparison with the wild-type virus, gp50 mutants were still highly virulent, whereas the virulence of gp50+gp63 mutants was significantly reduced. Severe signs of neurological disorders, notably pruritus, were apparent in animals infected with the wild-type virus or a gp50 mutant but were much less pronounced in animals infected with a gp50+gp63 or gp63 mutant. Immunohistochemical examination of infected animals showed that all viruses were able to reach, and replicate in, the brain. Examination of visceral organs of intraperitoneally infected animals showed that viral antigen was predominantly present in peripheral nerves, suggesting that the viruses reached the central nervous system by means of retrograde axonal transport. Infectious virus could not be recovered from the brains and organs of animals infected with gp50 or gp50+gp63 mutants, indicating that progeny virions produced in vivo are noninfectious. Virions that lacked gp50 in their envelopes, and a phenotypically complemented pseudorabies virus gII mutant (which is unable to produce plaques in tissue culture cells), proved to be nonvirulent for mice. Together, these results show that gp50 is required for the primary infection but not for subsequent replication and viral spread in vivo. These results furthermore indicate that transsynaptic transport of the virus is independent of gp50. Since progeny virions produced by gp50 mutants are noninfectious, they are unable to spread from one animal to another. Therefore, such mutants may be used for the development of a new generation of safer (carrier) vaccines.

Pseudorabies virus (PRV) is a highly neurotropic herpesvirus that causes Aujeszky's disease in domestic and wild animals (19, 31). Pigs are relatively resistant to PRV and therefore are considered the natural host of the virus. The natural portal of entry is the nasopharyngeal region. The virus is able to replicate in cells of the nasal and pharyngeal mucosa (34); after infection of peripheral nerves, it is transported to the central nervous system (5, 8, 12, 13, 26, 38), where it causes severe encephalitis that is often fatal in young pigs (1, 11). Older pigs usually survive the infection but may develop fever and pneumonia. Infection of sensory ganglia generally results in the establishment of latency (37).

Infection of cells by PRV is mediated by several glycoproteins that are located in the envelope of the virus. PRV synthesizes at least seven glycoproteins, designated gI, gII, gIII, gp50, gp63, gX, and gH, which, with the exception of gX, are located in the virus envelope (27). These glycoproteins are conserved to various degrees among PRV and other herpesviruses (41). It has been shown that three envelope glycoproteins of PRV, i.e., gII, gp50, and gH, are essential for virus entry (29, 30, 36). When phenotypically complemented mutants are used to infect noncomplementing cells, gp50 null mutants are still able to produce plaques, but gII or gH null mutants are not (29, 30, 36). This finding indicates that cell-to-cell spread of the virus is dependent not only on infectious progeny virions but also on the biological properties of the glycoproteins in the cell membrane of infected cells. In contrast to PRV, cell-to-cell spread of herpes simplex virus is dependent not only on gB (gII homolog) and gH but also on gD (gp50 homolog) (3, 15, 17, 20, 24, 28).

Although viral spread by means of cell-to-cell transmission seems to be a common characteristic of herpesviruses, its role and relative importance in natural infections and its pathological implications are unknown. It has been shown that the (neuro)pathogenicity of herpes simplex virus is modulated by gB (18, 40). Since gB and its PRV homolog gII play a critical role in penetration and cell fusion, this finding may indicate that interactions at the cell membrane are an important determinant of pathogenesis of herpesvirus infection.

We reasoned that if cell-to-cell transmission occurs not only in tissue culture cells but also in vivo, infection of animals with a high dose of a phenotypically complemented gp50 mutant may result in local replication at the primary inoculation site, perhaps followed by infection of peripheral nerves. Progeny virus released from the infected cells should be noninfectious since it lacks gp50, thus eliminating the possibility of secondary infections and shedding of infectious virus. Furthermore, if transsynaptic transport of the virus involves reinfection of postsynaptic neurons by infectious progeny virions (5, 25), transport of the mutant virus to the central nervous system might be blocked at synapses. This blockage would preclude the virus from entering the central nervous system, thus prohibiting its devastating effect in the brain.

Mice were used as test animals to establish whether gp50 mutants and gp50+gp63 mutants are able to replicate and spread not only in tissue culture cells but also in vivo. In this report, we show that both gp50 mutants and gp50+gp63

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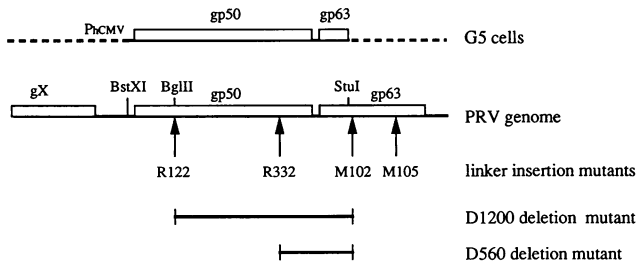

FIG. 1. Physical map of part of the PRV genome. The arrows indicate the positions of the premature stop codons, introduced by linker insertion mutagenesis, in the gp50 gene of gp50 null mutants R122 and R332 (30) and in the gp63 gene of gp63 null mutants M105 and M102 (previously called 233 and 149, respectively [9]). The horizontal bars show the positions and the extent of the deletions in the gp50+gp63 double mutants D560 and D1200. The upper line shows the *Bst*XI-*Stu*I fragment of PRV that is present in G5 cells that constitutively express gp50 (30).

mutants are lethal for mice after subcutaneous or intraperitoneal infection and are able to reach, and replicate in, the peripheral and central nervous systems. Furthermore, progeny virions produced in vivo by such mutants proved to be noninfectious. These observations indicate that cell-to-cell transmission is an important means of in vivo viral spread in herpesvirus infections and that transsynaptic transport of the virus is not dependent on gp50.

MATERIALS AND METHODS

Cells and viruses. The swine kidney cell line SK-6 (21) was routinely used for the propagation of virus. SK-6 cells were grown in Dulbecco's modification of Eagle's medium containing 5% fetal calf serum, glutamine (0.3 mg/ml), and the antibiotics penicillin (200 U/ml), streptomycin (0.2 mg/ml), and mycostatin (100 U/ml). SK-6 cells expressing gp50 or gII (cell line G5 or 10B2, respectively [30]) were grown in the same medium supplemented with 2.5 mM histidinol (Sigma). The wild-type PRV strain NIA-3 has been described elsewhere (1). The gp50 mutant viruses R122 and R332 (30) and the gp50+gp63 mutant viruses D1200 and D560 (see below) were grown on G5 cells to produce phenotypically complemented virus stocks containing gp50. The gII mutant virus B145 (30) was grown on complementing 10B2 cells. The gp63 linker insertion mutant M105 (previously called 233 [9]) was grown on SK-6 cells. The gp50+gp63 deletion mutants D560 and D1200 were constructed by means of overlap recombination (30, 39) after deletion of the fragments between the *Eco*RI sites of linker insertion mutants R332 and M102 (previously called 149 [9]) and R122 and M102, respectively (Fig. 1).

A batch of phenotypically complemented R122 virus that was virtually free from wild-type revertants was prepared by infecting SK-6 cells with a single plaque produced by R122 on G5 cells. Viral DNA was isolated from the infected SK-6 cells and used to transfect monolayers of G5 cells (which have a greatly reduced plating efficiency for PRV [30]). From the transfected cells, a virus batch that contained 2.1×10^7 PFU/ml, as determined by titration on SK-6 cells, was prepared. This batch was designated R122[+] to indicate that the virus was phenotypically complemented. A virus batch lacking gp50 was prepared by infecting SK-6 cells with 200 μl (4.2×10^6 PFU) of the undiluted R122[+] stock. The resulting virus batch was designated R122[−] to indicate that it was derived from noncomplementing cells. The physical titer of the R122[+] and R122[−] stocks was determined with the aid of electron microscopy by using latex beads (diameter of 91 nm; Serva) as an internal standard.

Animal experiments. Groups of five 6- to 8-week-old female BALB/c mice (Charles River, Suldzfeld, Germany) were infected by intraperitoneal injection or subcutaneous injection in the neck with 10^5 PFU of the respective virus strain in 100 μl (experiment 1) or 200 μl (experiments 2 and 3) of Hanks medium lacking serum and antibiotics. A sample of the inoculum was titrated on SK-6 cells or, in the case of the B145 mutant, on 10B2 cells to verify the amount of virus. The animals had free access to food and water throughout the experiments. Observations for clinical signs of Aujesky's disease were made every 2 h during daytime (starting from 6:00 a.m. till 6:00 p.m.) and at midnight. When it was obvious that animals would die between standard observation times (during daytime), the animals were visited more frequently. The brains and organs (liver, spleen, lung, kidney, and intestine) of animals were collected shortly after the animals had died (usually within 30 min), embedded in paraffin, and immediately frozen in liquid nitrogen. The presence of virus in the brain and organs was determined by immunohistochemistry (see below) and by titration of extracts that were prepared by grinding the tissues in a mortar in culture medium followed by low-speed centrifugation. Cryostat sections of the brains and organs of infected mice were fixed and processed for immunohistochemistry as described previously (34). Immunohistological detection of viral antigens was done essentially as described by Pol et al. (35). When monoclonal antibodies against gp50 were used as primary antibodies, peroxidase-conjugated goat anti-mouse immunoglobulin G antibodies were used in the second incubation (30).

RESULTS

gp50+gp63 null mutants are lethal for mice. Previously, we and others have shown that phenotypically complemented gp50 null mutants of PRV are able to produce plaques on noncomplementing cells despite the fact that progeny virions are noninfectious (30, 36). This phenotype is independent of the cell type, since similar phenotypes were found on porcine (SK-6), bovine (MDBK), monkey (Vero), and mouse (3T3) cell lines (unpublished results). To establish whether these mutants were also able to spread in animals, mice were chosen as test animals because they are highly susceptible to herpesviruses and have been used extensively as a model system with which to study virulence and neuronal spread (8, 10, 12, 13, 16, 23, 33). Figure 1 shows the mutants used in this study. The gp50 linker insertion mutants R122 and R332 have been described elsewhere (30). The gp50+gp63 deletion mutants D1200 and D560 contain deletions extending from the linker insertion sites of R122 and R332, respectively, to that of strain M102 (previously called 149 [9]) (Fig. 1). The gp63 mutant M105 (previously called 233) has been described by de Wind et al. (9).

In the first experiment, we used the gp50+gp63 deletion mutants D560 and D1200, instead of the linker insertion mutants R122 or R332, to exclude the presence of wild-type revertants in the inoculum. Such revertants may arise in stocks of linker insertion mutants by homologous recombination of viral sequences present in the complementing cell line and the virus genome (3, 29, 30). Since sequences at the 3′ side of the gp50+gp63 deletion of strains D560 and D1200 have no homologous counterpart in the complementing G5

TABLE 1. Mean time to death and detection of virus in brain and organs of mice infected with different PRV strains

Expt	Virus strain	Genotype	Route[a]	Mean time to death (h) ± SD	Presence of virus			
					Immunohistochemistry		Titration on SK-6 cells	
					Brain	Organs	Brain	Organs
1	NIA-3	Wild type	s.c.	70.8 ± 12.9	+	ND[b]	+	ND
	M105	gp63$^-$	s.c.	91.2 ± 18.2	+	ND	+	ND
	D560	gp50$^-$ gp63$^-$	s.c.	132.2 ± 28.6	+	ND	−	ND
	D1200	gp50$^-$ gp63$^-$	s.c.	141.4 ± 22.4	+	ND	−	ND
2	NIA-3	Wild type	s.c.	55.6 ± 6.8	+	−	ND	+ (3 of 5 mice)
	R122[+]	gp50$^-$	s.c.	67.9 ± 6.8	+	−	ND	−
	M105	gp63$^-$	s.c.	78.6 ± 18.7	+	−	ND	−
	D1200	gp50$^-$ gp63$^-$	s.c.	160.0 ± 20.4	+	−	ND	−
	NIA-3	Wild type	i.p.	67.3 ± 9.0	+	+	ND	+
	R122[+]	gp50$^-$	i.p.	77.7 ± 5.6	+	+	ND	−
	M105	gp63$^-$	i.p.	91.6 ± 15.6	+	+	ND	+
	D1200	gp50$^-$ gp63$^-$	i.p.	134.8 ± 27.0	+	+	ND	−
3	R122[+]	gp50$^-$	s.c.	60.0 ± 4.2	+	ND	ND	ND
	R122[−]	gp50$^-$	s.c.	4× ∞, 1× 84[c]	+	ND	ND	ND
	B145	gII$^-$	s.c.	∞	ND	ND	ND	ND
	R122[+]	gp50$^-$	i.p.	85.2 ± 5.0	+	ND	ND	ND
	R122[−]	gp50$^-$	i.p.	∞	ND	ND	ND	ND
	B145	gII$^-$	i.p.	∞	ND	ND	ND	ND

[a] s.c., subcutaneous; i.p., intraperitoneal.
[b] ND, not determined.
[c] One of five mice died.

cells (Fig. 1), the generation of wild-type revertants should be impossible during replication of these mutants in complementing G5 cells.

Mice were infected subcutaneously in the neck with 10^5 PFU of strains NIA-3, D560, and D1200 and the gp63 mutant M105. Mice inoculated with strain NIA-3 developed severe signs of Aujeszky's disease such as vigorous scratching with the hind legs, face washing, and paralysis and died at about 70 h after infection. Animals infected with strain M105 did not show extensive pruritus but were sitting apart with a crooked back and an increased respiration rate. The animals died at about 90 h after infection. Mice infected with strain D560 or D1200 showed symptoms similar to those of animals infected with strain M105. They became apathetic and showed signs of paralysis before entering a moribund state that sometimes lasted for up to 42 h until the animals died at about 130 to 140 h after infection (Table 1, experiment 1). These results show that gp50+gp63 null mutants are lethal for mice. They are, however, significantly less virulent than the wild-type virus or a gp63 mutant virus.

gp50+gp63 null mutants are able to reach, and replicate in, the central nervous system. Because symptoms of neurological disorders were much less apparent in mice infected with gp63 or gp50+gp63 mutants than in mice infected with strain NIA-3, the brains of infected animals were collected shortly after death and examined for the presence of virus by means of immunohistochemistry (see Materials and Methods). To our surprise, we observed that large numbers of infected neurons were present in brain sections of mice infected with mutants D1200 and D560, whereas only small numbers of infected neurons were present in brain sections of mice infected with strain NIA-3 or M105 (Fig. 2). Infected neurons, which could be distinguished from glial cells by their size and morphological appearance, were predominantly present in the brainstem, although viral antigens were also found in other parts of the brain. The virus that was present in the brains of animals infected with D1200 and D560 did not express gp50, as determined by differential immunostaining (data not shown). These results indicate that gp50+gp63 null mutants, which produce noninfectious progeny in tissue culture, are still able to reach, and replicate in, the central nervous system of mice.

Virus samples were prepared from brains of infected animals and titrated on SK-6 cells. Infectious virus was present in brain samples of NIA-3- or M105-infected animals, as evidenced by plaque formation on SK-6 cells, but was not present in brain samples of D560- or D1200-infected animals (Table 1, experiment 1). These results indicate that progeny virions produced by gp50+gp63 null mutants are noninfectious, not only after replication in tissue culture cells but also after replication in animals.

gp50 null mutants are highly virulent. Although gp63 is dispensable for viral growth (32), plaques produced on complementing G5 cells and noncomplementing SK-6 cells by gp50+gp63 mutants were significantly smaller than plaques produced by gp50 mutants (data not shown). Furthermore, gp63 has been shown to be involved in virulence in pigs (22). Since these findings suggested that gp50 mutants are more virulent than gp50+gp63 mutants, we also wanted to examine the virulence of gp50 mutants in mice. However, to use a gp50 mutant for the infection of mice, we had to be absolutely sure that the inoculum did not contain wild-type revertants (see above). Therefore, a stock of phenotypically complemented gp50 mutant R122 (designated R122[+]) was prepared by using a limited number of passages in complementing G5 cells (see Materials and Methods). To establish whether the virus batch contained wild-type revertants, 200 μl (4.2×10^6 PFU) was used to infect SK-6 cells and a virus batch was again prepared. This batch, designated R122[−], contained 150 PFU/ml. However, when we performed an immunoperoxidase staining by using monoclonal antibodies against gp50 (30), these plaques proved to be gp50 negative (data not shown). This finding indicated that the virus stock did not contain wild-type virus but still contained some infectious virus particles. It is possible that these particles are derived from the inoculum (R122[+]) that was used to

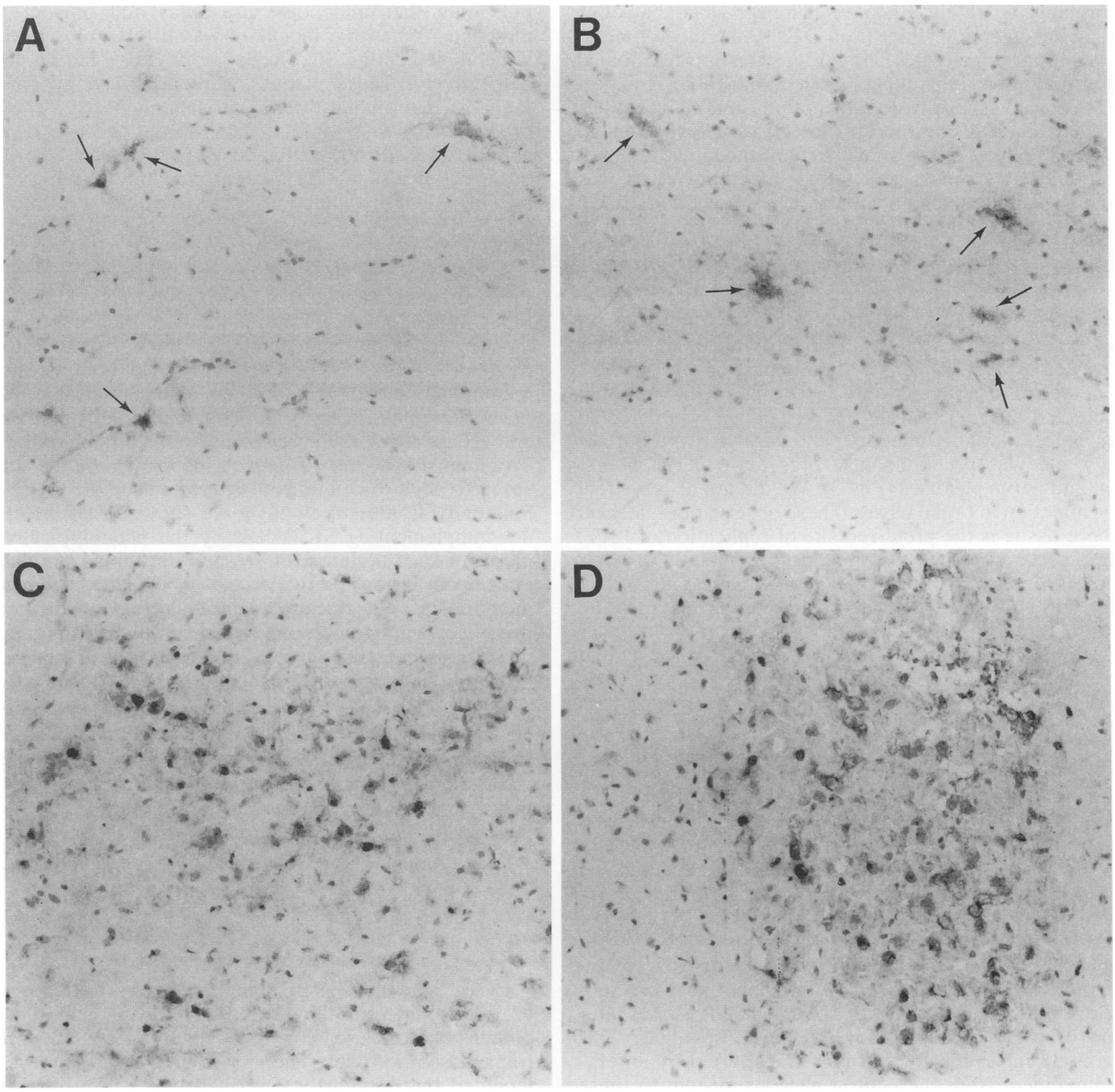

FIG. 2. Photomicrographs (magnification, ×200) of sections of the brainstems of mice infected subcutaneously with different PRV strains (Table 1, experiment 1). Viral antigens were stained immunohistochemically by using rabbit anti-PRV serum (35). (A) Few scattered neurons (arrows) containing viral antigens after infection with the virulent wild-type strain NIA-3. The animal died 66 h after inoculation. (B) Few scattered neurons (arrows) containing viral antigens after infection with strain M105. The animal died 92 h after inoculation. (C) Widespread neuronal degeneration with viral antigens after infection with strain D560. The animal died 136 h after inoculation. (D) Widespread neuronal degeneration with viral antigens after infection with strain D1200. The animal died 138 h after inoculation.

prepare the R122[−] batch. Alternatively, progeny virions may be able to reincorporate gp50 that was deposited into the plasma membrane of the SK-6 cells by the infecting R122[+] virions. Another possibility is that virions lacking gp50 are taken up by endocytosis (4) and occasionally escape degradation, resulting in a productive infection.

In addition to testing the virulence of R122[+], we tested whether virulence of the different viruses was dependent on the route of infection. Groups of five mice were inoculated with 10^5 PFU of strains NIA-3, M105, R122[+], and D1200 by subcutaneous or intraperitoneal injection. Mice that were infected with strain R122[+] developed signs of Aujeszky's disease that resembled those seen in animals infected with the wild-type strain NIA-3 (see above). In animals subcutaneously infected with NIA-3, the first symptoms became apparent at approximately 34 to 40 h after infection; in animals subcutaneously infected with R122[+], symptoms appeared at about 42 h after infection. The animals died at about 56 and 68 h after infection, respectively (Table 1, experiment 2). These results indicated that gp50 mutant R122[+] was still highly virulent for mice and was much more virulent than gp50+gp63 mutants D560 and D1200 or gp63 mutant M105. Intraperitoneal injection of strains NIA-3, R122[+], and M105 resulted in a mean time to death

that was approximately 10 to 13 h longer than that after subcutaneous injection. For strain D1200, the mean time to death was approximately 25 h shorter after intraperitoneal injection than after subcutaneous injection (Table 1, experiment 2). For all strains tested, symptoms and clinical signs were independent of the route of infection. Thus, although there are differences in the time course of the infection, both inoculation routes result in a lethal infection. Virus was detected by immunohistochemistry in brain sections of all animals used in experiment 2 (Table 1). Again, the number of infected neurons was much larger in brains of animals infected with D1200 than in brains of animals infected with NIA-3, R122[+], or M105 (data not shown).

Preferential replication of virus in peripheral nerves of infected organs. When sections of organs were examined for the presence of viral antigens by immunohistochemistry, we could detect viral antigens only in organs of animals that were infected intraperitoneally. Viral antigen was detected in liver, spleen, kidney, intestine, and adrenal gland but not in lungs and was predominantly present in nerve fibers, ganglia, and nerve plexuses (Fig. 3). We did not observe foci of virus infection in organ tissue. These observations suggest that nerve tissue is the preferred site of replication of PRV. Whether nerve tissue is also the primary target of PRV infection is unknown. Since the samples were taken postmortem, the observed antigen-positive neurons may be second- or third-order infected cells.

The presence of viral antigens in nerve fibers suggests that the virus is transported from the organs to the central nervous system by means of retrograde axonal transport, as has been shown previously (5, 8, 12, 13, 26, 38). The observation that a gp50 null mutant is efficiently transported to the central nervous system indicates that transsynaptic transport is not dependent on gp50.

Titration on SK-6 cells of virus samples prepared from organs of animals that were infected intraperitoneally with strains NIA-3 and M105 yielded plaques. As expected, no plaques were obtained after titration of virus samples prepared from organs of animals infected with strain R122[+] or D1200 (Table 1, experiment 2). This observation again indicates that progeny produced by these viruses is noninfectious. Surprisingly, three of five animals that were subcutaneously infected with strain NIA-3 yielded plaques after titration of organ extracts (Table 1, experiment 2). This finding could mean that the virus is transported by anterograde movement from the central nervous system to these organs. The absence of infectious virus from organs of mice that were infected subcutaneously with strain M105 probably indicates that transport of this virus was delayed.

gp50 is required for primary infection in vivo. Previously we showed that gp50 is essential for virus entry in tissue culture cells (30). To determine whether gp50 is also essential for virus entry in animals, we prepared a batch of strain R122 that was grown on noncomplementing SK-6 cells and thus lacked gp50 (R122[−]; see above). We used 10^5 PFU of R122[+] and 3.3×10^6 physical particles of R122[−] (corresponding to 10^5PFU of R122[+]) for inoculation. As expected, all mice injected intraperitoneally or subcutaneously with R122[+] died (Table 1, experiment 3). However, all mice infected with R122[−] survived after intraperitoneal infection, whereas only one of five animals died after subcutaneous infection. These results indicated that the presence of gp50 in the envelope of the virus was required for successful infection of the animals.

Examination of the one animal that died after infection with R122[−] showed that virus was present in the brain, indicating that infectious virus was still present in the inoculum. When the inoculum was titrated in duplicate on noncomplementing SK-6 cells, we found 9 and 16 plaques, respectively. These plaques were produced by gp50 mutants, as determined by immunohistochemistry. The possible origin of these infectious virions has been discussed above. Since the 50% lethal dose of PRV strain NIA-3, after intraperitoneal infection, is approximately 70 PFU (2), it is possible that the infectious virus particles present in the R122[−] inoculum are responsible for the lethal infection of the one animal that died.

A pseudorabies virus gII mutant is nonvirulent for mice. Previously we showed that, similarly to gp50 null mutants, replication of gII or gH null mutants of PRV in noncomplementing cell lines resulted in the production of noninfectious progeny virions (29, 30). However, in contrast to gp50 mutants, gII and gH mutants were unable to produce plaques on noncomplementing cells. This finding indicated that gII and gH are required for cell-to-cell transmission of the virus. To establish whether virulence is dependent on the ability to spread by cell-to-cell transmission, we used the PRV gII null mutant B145 (30). Previously we showed that infection of noncomplementing SK-6 cells by the phenotypically complemented gII mutant B145 resulted in the accumulation of enveloped virions between the inner and outer nuclear membranes (30). Recent experiments have indicated that this effect is apparent only when the infected cells are grown under a methylcellulose overlay. When the infected cells are grown in liquid media, production of extracellular virus seems normal. The cause of this discrepancy is unknown; we are currently studying this phenomenon in more detail. When mice were injected intraperitoneally or subcutaneously with 10^5 PFU of the phenotypically complemented gII mutant B145, none of the animals developed any signs of Aujeszky's disease (Table 1, experiment 3). This result indicates that a PRV gII mutant is nonvirulent for mice and suggests that cell-to-cell transmission in vitro is a marker for virulence.

DISCUSSION

Despite the fact that gp50 null mutants of PRV are unable to produce infectious progeny, they are still able to produce plaques on noncomplementing cells by means of cell-to-cell transmission (30, 36). The gp50 mutants used in this study included the previously described linker insertion mutants R122 and R332 (30) and the newly constructed gp50+gp63 deletion mutants D560 and D1200 (Fig. 1). In a first characterization of the in vivo replication properties of gp50 and gp50+gp63 null mutants, we used mice as test animals because they are highly susceptible to PRV infection (8, 10, 12, 13, 16, 23, 33). Before we started these experiments, we did not known to what extent the viruses were able to replicate in peripheral tissue. It was also unclear to what extent cell-to-cell spread contributed to the spread of herpesviruses in infected animals. Furthermore, it was doubtful whether the viruses would be able to reach the central nervous system, since doing so would involve transsynaptic transport that might be dependent on the ability of the virus to reinfect postsynaptic neurons. We were surprised to find that gp50 and gp50+gp63 mutants were virulent for mice. The virulence (expressed as mean time to death of infected animals) of gp50 mutants was only moderately reduced compared with that of the wild-type strain NIA-3 (Table 1). The virulence of gp50+gp63 mutants, however, was much more reduced, indicating the involvement of gp63 in viru-

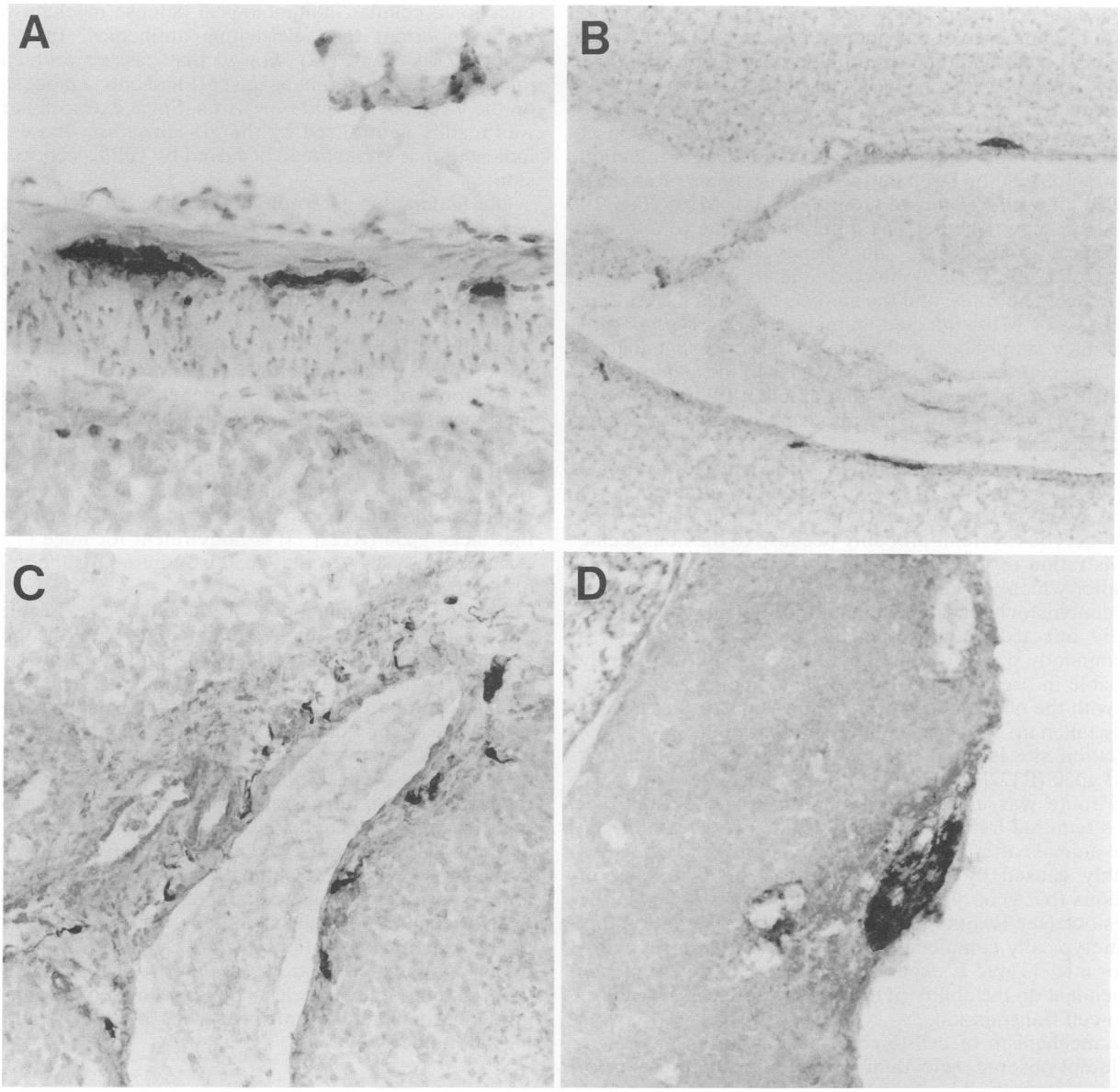

FIG. 3. Photomicrographs of enteric organs of mice infected intraperitoneally with different PRV strains (Table 1, experiment 2). Viral antigens were stained immunohistochemically by using rabbit anti-PRV serum (35). (A) Muscular layer of the small intestine showing viral antigens in the myenteric (Auerbach's) nerve plexuses (containing postganglionic parasympathetic ganglion cells) of a mouse that died 140 h after inoculation with strain D1200. (B) Hepatic blood vessel showing viral antigens in parasympathetic nerves along the vessel wall. The animal died 76 h after inoculation with strain R122[+]. (C) Hepatic blood vessel showing viral antigens in parasympathetic nerves along the vessel wall. The animal died 68 h after inoculation with strain NIA-3. (D) Adrenal parasympathetic nerve plexus containing viral antigens. The animal died 76 h after inoculation with strain R122[+].

lence for mice. The latter finding seems to be in agreement with the results of Kimman et al. (22), who showed that gp63 is involved in virulence in pigs. The finding that gp63 forms a complex with gI and the observation that the phenotypes of gI, gp63, and gI+gp63 mutants are similar (43) suggests that the complex of gp63 and gI is the functional unit. Evidence that gI (and thus gp63) is involved in cell-to-cell transmission of the virus was recently reported (42). Our observations concerning the reduction in virulence and the small plaque size of gp50+gp63 mutants are in agreement with the proposed function. The observation, however, that clinical signs of animals infected with gp63 or gp50+gp63 mutants differ from those of animals infected with either the wild-type virus or a gp50 mutant virus suggests that gp63, like gI (6), is involved in neurotropism.

Postmortem examination of infected animals showed that gp50 and gp50+gp63 null mutants were able to replicate in the brain. Surprisingly, virus replication in the brain was limited in animals that were infected with the highly virulent strains NIA-3, R122[+], and M105, whereas extensive virus replication was observed in animals that were infected with the less virulent strain D1200 or D560 (Fig. 3). Because the

animals died at different times after infection, it is difficult to judge the significance of this finding. It seems, however, that infection of neurons in the central nervous system is not the primary cause of death. It is possible that virulence is influenced by the severity of the inflammatory response evoked by the different viruses. We noted degeneration of neurons in the brainstems of animals that died after infection with strain D1200 or D560 but not in brainstems of animals that died after infection with strain NIA-3 or M105 (Fig. 2). We do not know whether this difference is due to different pathological properties of the various strains. Since the brains of D1200- or D560-infected animals contained many more infected neurons, and the animals died much later than did NIA-3- or M105-infected animals, the observed degeneration may simply be a reflection of the longer incubation time. Time course experiments may answer these questions.

Recently, it has been shown that different PRV strains are transported differentially in the rat visual system, suggesting that differential infection of ganglion cells is mediated by viral envelope glycoproteins (6, 7). In the present investigation, we have not been able to assign the differences in clinical symptoms to the infection of different parts of the central nervous system, probably because of the nonspecific administration of the inoculum and the extended period of time after which the animals were examined.

Immunohistochemical examination of organs of intraperitoneally infected animals showed that viral antigen was predominantly present in peripheral nerves and was hardly detectable in organ tissue (Fig. 3). This finding is in agreement with the observation of Field and Hill (13) that PRV is rapidly taken up by nerve endings and that replication at the inoculation site is only limited. When a gp50 mutant that lacked gp50 (R122[−]) was used, infection by the intraperitoneal route was unsuccessful. This finding indicates that gp50 is required for the primary infection. The death of one of five animals after subcutaneous infection with R122[−] is probably caused by the presence of a small number of infectious (but genotypically gp50 negative) particles in the virus stock (see Results). Furthermore, the observation that a phenotypically complemented PRV gII mutant is nonvirulent for mice (Table 1, experiment 3) suggests that virulence is dependent on the ability of the virus to spread by means of cell-to-cell transmission.

The mechanism of cell-to-cell transmission is unknown. We did not observe syncytium formation or the presence of multinucleate cells either in the brain or in peripheral organs. Therefore, cell-to-cell transmission is probably not the result of nonspecific cell-cell fusion. Cell-to-cell transmission is dependent, at least in tissue culture cells, on the presence of the virus-encoded envelope glycoproteins gII and gH (29, 30). These glycoproteins, which are involved in virus entry and membrane fusion, are present in the cell membrane of infected cells. It is conceivable that in the infected cell, membrane patches containing gII and gH (and probably others) are able to transiently fuse with the cell membrane of noninfected cells. Such cellular bridges might allow the passage of virus from infected to uninfected cells. However, since neuronal cells are polarized and specialized, it is questionable whether this mechanism can also account for transsynaptic transport.

Viral spread by means of cell-to-cell transmission or cell fusion seems to be a general characteristic of herpesviruses and other viruses such as members of the paramyxoviridae and coronaviridae (14). Possibly cell-to-cell transmission is a means of avoiding contact with circulating antibodies. This view would predict that for the clearance of herpesvirus infections, cellular immunological defense mechanisms are more important than circulating antibodies. In a recent report, Zsak et al. (42) showed that passive protection of mice by using antiserum against gII had only a modest effect on the virulence of wild-type virus. Since extracellular virus was readily neutralized by the gII antiserum, these results indicated that viral spread occurred by cell-to-cell transmission.

Our finding that gp50 mutants are able to reach the central nervous system shows that transsynaptic transport of the virus is possible without the involvement of gp50. This observation suggests that transsynaptic transport is not dependent on reinfection of postsynaptic neurons by extracellular virions that are released from presynaptic neurons. Formally, it is possible that PRV uses a different envelope glycoprotein for the infection of neuronal cells. In that case, gp50 would be needed only for penetration of nonneuronal (peripheral) tissue, whereas another glycoprotein is responsible for the penetration of nervous tissue. We cannot rule out this possibility at this moment. However, the finding of Field and Hill (13) that direct uptake of virus into nerve endings is possible and our observation that a gp50 mutant that lacks gp50 (R122[−]) is nonvirulent for mice (Table 1, experiment 3) suggest that gp50 is required for the infection of nerve cells.

The finding that infectious virus could not be isolated from any of the animals that were infected with the gp50 or gp50+gp63 mutants shows that progeny virions produced in animals by these mutants are noninfectious. This is a very desirable property for an Aujeszky's disease vaccine and even more so for a carrier vaccine. The application of a gp50 mutant as a recombinant carrier virus for the expression of heterologous genes would generate a safe vaccine that replicates only in the vaccinated animal and does not spread to other animals, including other species. Furthermore, if the heterologous gene is inserted at the location of the gp50 gene in the carrier virus, recombination with wild-type virus will always result in the generation of noninfectious recombinants. Because the available mutants are still too virulent for mice, additional mutations may have to be introduced in order to attenuate the strains so that they can be safely used in pigs and other species. Experiments to establish the virulence and immunogenic properties of the gp50 mutants in pigs are under way.

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