Pseudorabies virus glycoprotein gI: \textit{in vitro} and \textit{in vivo} analysis of immunorelevant epitopes

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Overlapping fragments of the gene encoding glycoprotein gI of pseudorabies virus (PRV; herpesvirus suis 1) were expressed in bacteria. Using the fusion proteins and a panel of monoclonal antibodies (MAbs) against gI as well as swine sera we found that the N-terminal part of gI (residues 33 to approximately 100) contains a highly antigenic and immunogenic domain. Transfer of antibodies binding to this region as well as vaccination with fusion proteins containing the N terminus of gI are able to confer protection to mice against a lethal challenge of virus. The results show that gI, which is non-essential for virus replication in tissue culture, can induce neutralizing and protective antibodies. The potential suitability of fusion proteins encompassing N-terminal parts of gI as diagnostic tools is demonstrated.

Introduction

Pseudorabies virus (PRV), a member of the alphaherpes-virinae (herpesvirus suis 1), represents an important pathogen in swine, causing Aujeszky's disease. Current control of the disease includes vaccination with inactivated and live vaccines or test-and-removal procedures (Gustafson, 1975). During recent years much effort has been made to investigate the glycoproteins of PRV regarded as major targets for the host's immune response. To date seven glycoprotein genes have been mapped on the viral genome and sequenced (Hampl et al., 1984; Lukács et al., 1985; Mettenleiter et al., 1985a, 1986; Petrovskis et al., 1986a, b, 1988; Rea et al., 1985; Robbins et al., 1986a, 1987; Wathen & Wathen, 1984, 1986). Four of these glycoproteins, gI, gp63, gIII and gX, are not required for virus growth in cell culture (Ben-Porat et al., 1986; Mettenleiter et al., 1985a; Petrovskis et al., 1986c; Rea et al., 1985; Robbins et al., 1986b; Wathen & Wathen, 1986). Since no gI-negative field strain has been reported up to now (van Oirschot, 1989; van Oirschot et al., 1986; T. C. Mettenleiter & H.-J. Rziha, unpublished data), gI probably plays a role in the replication of PRV in its natural host. The importance of gI-specific antibodies in virus neutralization is still unclear. Results of Ben-Porat et al. (1986) indicate an inferior role of gI in the induction of virus-neutralizing antibodies, whereas Wardley & Post (1989) consider gI an important target for a protective immune response. Several attenuated PRV strains used as live vaccines have been found to carry deletions in the gI gene (Gielkens et al., 1982, 1985; Herrman et al., 1984; Lomniczi et al., 1984, 1987; Petrovskis et al., 1986c), and consequently fail to express gI (Mettenleiter et al., 1985b). Recently, attenuated strains have been constructed which do not express gI (Quint et al., 1987; Visser & Lüticken, 1989), gIII (Kit et al., 1987) or gX (Marchioli et al., 1987) due to the introduction of deletions in these genes. The vaccine strains allow the serological discrimination between vaccinated and field virus-infected swine by testing for the presence of antibodies against the product of the deleted gene (Eloit et al., 1989; Kit, 1989; McGinley & Platt, 1988; Platt et al., 1986; van Oirschot & Waal, 1987; van Oirschot et al., 1986, 1988). Recent results obtained with ELISA based on the use of gI-specific monoclonal antibodies (MAbs) suggest a long-lasting humoral immune response to gI in swine (van Oirschot, 1988, 1989; van Oirschot et al., 1988), and indicate the suitability of such assays to identify infected pigs in herds vaccinated with gI-negative vaccines. However, the binding sites on gI of either these MAbs or swine antibodies have not been characterized in detail. Furthermore, purified virions or PRV-infected cell lysate is used as an antigen in the existing ELISA. Considering previous results that gI can be expressed variably, both quantitatively and qualitati-
vely (Mettenleiter et al., 1987), a standardized gI preparation is needed to improve these tests.

The present study was designed to determine immunogenic domains on gI and to evaluate whether gI plays a role in inducing protective immunity. By the use of bacterial gI fusion proteins and a panel of anti-gI MAbs as well as swine sera, we demonstrate that the N-terminal part of gI represents a highly immunogenic region containing multiple epitopes. Preliminary experiments indicate the suitability of those fusion proteins in diagnostic tests (e.g. ELISA). In addition, we show that transfer of anti-gI MAbs and vaccination with gI-containing fusion proteins are able to protect mice from a lethal PRV challenge.

Methods

Cells and virus. PRV strain Phylaxia was propagated in Madin-Darby bovine kidney (MDBK) cells (Lukacs et al., 1985). Single plaque isolates of PRV strain Ka (Ka2 and Ka3) have been described (Mettenleiter et al., 1987).

Bacterial strains and vectors. Escherichia coli strain DH5α [Gibco/Bethesda Research Laboratories (BRL)] was used for transformation with plasmids derived from vectors pTZ19R and pSPT19 (Pharmacia/LKB). E. coli strain BMH71-18 (Rüther & Müller-Hill, 1983; kindly provided by H. Wolf, Max-von-Pettenkofer Institute, Munich, F.R.G.) was used to propagate pSS20 plasmids (kindly provided by S. Scholtissek, Max Planck Institute, Göttingen, F.R.G.). Plasmid pSS20 allows the production of fusion proteins with β-galactosidase at the N terminus followed by a recognition sequence (CRS) for site-specific protease collagenase and the foreign protein at the C terminus (Scholtissek & Grosse, 1988). The pEx plasmids were used for the production of heterologous proteins fused to the N-terminal part (99 amino acids) of the MS2 polymerase and controlled by the inducible λ P1 promoter (Remaut et al., 1981, 1983; Strebel et al., 1986). Hybrid plasmids were constructed, transformed into E. coli C600/W6 and DNA was isolated for characterization. Fusion proteins were expressed in E. coli K12 strain 537 (provided by R. Braun, University of Heidelberg, F.R.G.) carrying the gene for the temperature-sensitive λ repressor cI857 on a kanamycin resistance plasmid (Strebel et al., 1986).

Enzymes. Restriction enzymes were purchased from Gibco/BRL, Boehringer Mannheim, or Amersham-Buchler and used as specified by the manufacturers.

Cloning procedures. Viral DNA fragments were cloned by standard procedures using T4 DNA ligase (Gibco/BRL). Vector DNA was treated with calf intestinal phosphatase (Boehringer Mannheim) after restriction enzyme cleavage. Transformation of competent bacteria was done according to Hanahan (1983). Plasmid DNA was isolated by procedures using T4 DNA ligase (Gibco/BRL). Vector DNA was isolated for characterization. Fusion proteins were expressed in E. coli K12 strain 537 (provided by R. Braun, University of Heidelberg, F.R.G.) carrying the gene for the temperature-sensitive λ repressor cI857 on a kanamycin resistance plasmid (Strebel et al., 1986).

Plasmid construction. The plasmids expressing parts of gI are shown in Fig. 1. The plasmid pTM-7A (Fig. 1 b) containing the SaI–BamHI fragment 7A (Mettenleiter et al., 1985b) was cleaved with DruI and BamHI, and the resulting 2.9 kbp fragment was isolated from an agarose gel using DEAE-paper (Dretzen et al., 1981). The eluted fragment was inserted into pTZ19R cleaved with BamHI and SmaI resulting in plasmid pRZ-7DB (Fig. 1 b) which contains the complete gI gene (Petrovskis et al., 1986b). Starting with pRZ-7DB derivatives were constructed after isolation of the required restriction fragments. Vector pSS20b was cleaved with BglII and ligated in-frame with the compatible ends of either the 885 bp Sau3A fragment or the 448 bp Sau3A fragment of pRZ-7DB to obtain plasmids pWF-18 and pWF-19, respectively (Fig. 1c). Both Sau3A fragments were also ligated into the BamHI site of pEx31c resulting in plasmids pWF-18P and pWF-19P (not shown). After double digestion of pWF-18P with Smal and HindIII (located in pEx31c), followed by filling the recessed HindIII ends with Klenow polymerase and religation, the plasmid pWF-24 was obtained, containing the viral 394 bp Sau3A–Smal fragment in pEx31c (Fig. 1c). To construct plasmid pWF-25, pWF-19P was digested with SpH1 and HindIII, and the ends were filled and religated to yield plasmid pWF-27 which contains the 268 bp Smal–Bse11 fragment of the gI DNA (Fig. 1c). Recombinant plasmid DNA was tested for correct insertion by restriction enzyme cleavage and Southern blot hybridization.

Expression and purification of fusion proteins. Recombinant clones derived from pSS20b were grown in broth (LB) containing 100 μg/ml ampicillin and 0.5% glucose at 37 °C and expression of fusion protein was induced with 1 mM-isopropyl-β-D-thiogalactopyranoside (Scholtissek & Grosse, 1988). E. coli strain 537 was transformed with hybrid pEx plasmid DNA and grown under selection overnight at 28 °C. After dilution with prewarmed LB the culture was incubated for 3 h at 42 °C with good aeration. Bacteria were pelleted and used as lysates for SDS-PAGE. Fusion protein was released by Triton X-100 lysis of lysozyme-treated bacteria followed by differential urea extraction as described (Strebel et al., 1986) and further purified by excision of the fusion...
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protein band from preparative SDS–PAGE gels and electroelution in 0.05 M Tris–glycine, 0.1% SDS, 10 M NH₄HCO₃, pH 8.6. The eluate was lyophilized and resuspended in phosphate-buffered saline (PBS).

Separation of proteins by SDS–PAGE in 10%, or 12% acrylamide gels (Laemmli, 1970), transfer to nitrocellulose filter and Western blot analysis were performed as described (Lukács et al., 1985) except for using 5% (w/v) non-fat milk in PBS for filter blocking and for dilution of antibodies. Bound primary antibody was detected with horseradish peroxidase-conjugated second antibody (Dianova) and visualized with 4-chloro-1-naphthol.

### Monoclonal antibodies and antisera

Monoclonal antibodies and antisera. Production and characterization of MAbs and the goat antiserum against PRV was as described by Lukács et al. (1985) and Thiel et al. (1981). Antibody isotypes were determined by immunodiffusion with subclass-specific antisera (Nordic Immunological Laboratories). The virus-neutralizing activity of MAbs and antisera was tested in a 50% plaque reduction assay (Metteneiter et al., 1987) performed on MDBK cells in the absence or presence of 5% rabbit serum as a source of complement. Neutralization titre was expressed as the dilution of antibody giving 50% plaque reduction.

Pig sera were collected from swine at different times post-infection (p.i.) with PRV.

A standard ELISA was performed as described (Lukács et al., 1985).

### Mouse protection experiments

For all experiments approximately 8-week-old C57BL/10 mice were used. Passive immunization was performed by intraperitoneal (i.p.) inoculation of 1:3 diluted ascites fluid (Table 1). By competition ELISAs (not shown) the five MAbs were able to neutralize PRV in the presence of 5% rabbit serum as a source of complement. Neutralization titre was expressed as the dilution of antibody giving 50% plaque reduction.

Mouse sera were collected from swine at different times post-infection (p.i.) with PRV.

A standard ELISA was performed as described (Lukács et al., 1985).

### Results

### Monoclonal antibodies

A panel of 13 MAbs directed against gI (Lukács et al., 1985) was analysed for in vitro and in vivo neutralizing activity. The results and the properties of the MAbs are given in Table 1. Plaque reduction assays revealed that five MAbs were able to neutralize PRV in the presence of complement, whereas none of them neutralized the virus without complement. The neutralizing MAbs belonged to either the 2a, 1 or 3 subclass of IgG (Table 1). By competition ELISAs (not shown) the five neutralizing MAbs could be assigned to at least three different topological groups designated A, B and C (Table 1).

### Reactivity of MAb with bacterial fusion proteins of gI

To localize the epitopes on gI recognized by the available MAbs, overlapping DNA fragments of the gI open reading frame (ORF) were expressed in bacteria and the fusion proteins were tested in Western blots for reactivity with the various MAbs. At first most of the gI ORF was expressed in only two segments by inserting either an 885 bp Sau3A fragment (pWF-18; Fig. 1c; Table 2) or the adjacent 448 bp Sau3A fragment (pWF-19; Fig. 1c; Table 2) in-frame with the 3’ end of the β-galactosidase–CRS of pSS20b. Both fragments were satisfactorily expressed leading to fusion proteins with sizes of approximately 155K and 140K, respectively (Table 2). Although both polypeptides tended to be degraded as judged by analysis in SDS–PAGE, they could be used in Western blot experiments. As indicated in Table 3, goat

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>Mₙ × 10⁻³</th>
</tr>
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<tbody>
<tr>
<td>pWF-18 pSS20b</td>
<td>156-54</td>
</tr>
<tr>
<td>pWF-19 pSS20b</td>
<td>139-65</td>
</tr>
<tr>
<td>pWF-24 pEx31c</td>
<td>31-26</td>
</tr>
<tr>
<td>pWF-25 pEx31c</td>
<td>19-95</td>
</tr>
<tr>
<td>pWF-26 pEx31a</td>
<td>40-36</td>
</tr>
<tr>
<td>pWF-27 pEx31a</td>
<td>21-25</td>
</tr>
</tbody>
</table>

* See Fig. 1; the total Mₙ of gI products fused to β-galactosidase (β-Gal) or to MS2 polymerase (MS2 pol) is given as calculated by computer (calculated) and observed in SDS–PAGE (apparent).

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**Table 1. Reactivity of gI-specific MAbs**

<table>
<thead>
<tr>
<th>MAb</th>
<th>Isotype</th>
<th>Neutralization*</th>
<th>C'</th>
<th>C' Expt. 1</th>
<th>Expt. 2</th>
</tr>
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<tbody>
<tr>
<td>3/6 (A)†</td>
<td>G2a</td>
<td>-</td>
<td>4-8</td>
<td>9/10</td>
<td>9/10</td>
</tr>
<tr>
<td>4/17 (A)</td>
<td>G2a</td>
<td>-</td>
<td>3-8</td>
<td>5/10</td>
<td>8/10</td>
</tr>
<tr>
<td>1/14 (B)</td>
<td>G2a</td>
<td>-</td>
<td>4-6</td>
<td>9/10</td>
<td>8/10</td>
</tr>
<tr>
<td>1/8 (C)</td>
<td>G1</td>
<td>-</td>
<td>3-9</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>4/4 (C)</td>
<td>G3</td>
<td>-</td>
<td>1-7</td>
<td>7/10</td>
<td>8/10</td>
</tr>
<tr>
<td>N-11 (D)</td>
<td>G2a</td>
<td>-</td>
<td>8/10</td>
<td>9/10</td>
<td></td>
</tr>
<tr>
<td>S-4</td>
<td>G2b</td>
<td>-</td>
<td>6/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-1</td>
<td>G2b</td>
<td>-</td>
<td>3/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/5</td>
<td>G1</td>
<td>-</td>
<td>0/10</td>
<td>0/10</td>
<td></td>
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<tr>
<td>4/15</td>
<td>G1</td>
<td>-</td>
<td>2/10</td>
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</tr>
<tr>
<td>3/2</td>
<td>G1</td>
<td>-</td>
<td>2/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IN-2</td>
<td>G2b</td>
<td>-</td>
<td>0/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/9</td>
<td>G1</td>
<td>-</td>
<td>0/10</td>
<td>0/10</td>
<td></td>
</tr>
</tbody>
</table>

C' represents 5% rabbit serum as source of complement.

† If available, the results of two independent mouse protection experiments using different preparations of ascites fluid are indicated.

‡ MAbs assigned to different groups (see text).

* Neutralization titres are given as log₁₀ dilution resulting in 50% plaque reduction.

**Table 2. gI Expression constructs**

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>Vector</th>
<th>Insert (bp)</th>
<th>Fused to</th>
<th>Calculated</th>
<th>Apparent</th>
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<tbody>
<tr>
<td>pWF-18 pSS20b</td>
<td>pSS20b</td>
<td>885</td>
<td>β-Gal</td>
<td>156-54</td>
<td>155</td>
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<tr>
<td>pWF-19 pSS20b</td>
<td>pSS20b</td>
<td>448</td>
<td>β-Gal</td>
<td>139-65</td>
<td>140</td>
</tr>
<tr>
<td>pWF-24 pEx31c</td>
<td>pEx31c</td>
<td>394</td>
<td>MS2 pol</td>
<td>31-26</td>
<td>32</td>
</tr>
<tr>
<td>pWF-25 pEx31c</td>
<td>pEx31c</td>
<td>214</td>
<td>MS2 pol</td>
<td>19-95</td>
<td>17</td>
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<tr>
<td>pWF-26 pEx31a</td>
<td>pEx31a</td>
<td>619</td>
<td>MS2 pol</td>
<td>40-36</td>
<td>42</td>
</tr>
<tr>
<td>pWF-27 pEx31a</td>
<td>pEx31a</td>
<td>268</td>
<td>MS2 pol</td>
<td>21-25</td>
<td>27</td>
</tr>
</tbody>
</table>

* See Fig. 1; the total Mₙ of gI products fused to β-galactosidase (β-Gal) or to MS2 polymerase (MS2 pol) is given as calculated by computer (calculated) and observed in SDS–PAGE (apparent).
Table 3. Reactivity of expressed gI regions

<table>
<thead>
<tr>
<th>Amino acids expressed</th>
<th>Antibody</th>
<th>33-122</th>
<th>33-238</th>
<th>108-238</th>
<th>108-402</th>
<th>403-471</th>
<th>403-552</th>
</tr>
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<tbody>
<tr>
<td>pWF-27</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWF-26</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWF-24</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWF-18</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWF-25</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWF-19</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
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</table>

3/6 + * + - - - -
4/17 + + - - ND + - -
S-4 + + - - - - -
1/14 - - - - - - -
4/4 + + - - ND - -
1/8 + + - - - - -
N-11 + + - ND ND ND
S-1 + + - - - - -
2/5 + + - - - - -
4/15 + + - - - - -
3/2 + + - ND ND ND
IN-2 - - - - - +/-
2/9 - - - - - +/-
Ant-PRV +++ ++ ++ (+) +

* The reactivity of MAb in Western blots with the indicated constructs is shown: a plus symbol (+) indicates presence and a minus sign (-) absence of reactivity without estimation of signal strength, except for IN-2 and 2/9 which reacted very poorly with pWF-19.
† ND, Not determined.
‡ The goat hyperimmune serum (anti-PRV) reactions are estimated from strong (+ + +) to weak (+).

The following constructs all used the pEx plasmid resulting in MS2 polymerase fusion proteins. Attempts to obtain more stable expression of the fragments inserted in pWF-18 and pWF-19 in the pEx31 vector were unsuccessful. Plasmid pWF-24 contained the 394 bp Sau3A–SmaI fragment (amino acids 108 to 238); plasmid pWF-25 expresses amino acids 403 to 471 encoded by the 214 bp Sau3A–SphI fragment (Fig. 1c; Table 2). Both fusion proteins were synthesized with a size of 32K and 17K, respectively (Fig. 2), which corresponds well with the predicted size (Table 2). After Western blotting the product of pWF-24 and pWF-25 was recognized by the goat hyperimmune serum, whereas no reaction occurred with goat preimmune serum (Fig. 2; Table 3). None of the MAb tested reacted with pWF-18, pWF-24 and pWF-25 (Table 3). Reaction of MAb IN-2 and 2/9, respectively, with the hybrid protein of pWF-19 generated a signal hardly detectable in Western blots (data not shown; Table 3). Although this faint signal was reproducible and not observed with the controls, its specificity has to be further proven unequivocally.

The remaining N-terminal part of gI, apart from the putative signal sequence, was expressed from plasmids pWF-26 and pWF-27 (Fig. 1c; Table 2). Plasmid pWF-26 contains a 619 bp SmaI fragment representing amino acids 33 to 238, plasmid pWF-27 expresses a 268 bp SmaI–BstEII fragment (amino acids 33 to 122). After heat induction of E. coli cells transformed with these plasmids, fusion proteins with a size of approximately 42K and 27K were produced in large amounts. The discrepancy between the calculated and the observed size of the protein expressed from pWF-27 (Table 2) is explained by the usage of a stop codon in the vector plasmid sequence further downstream of the inserted gI sequence. Western blot analysis revealed excellent reactivity of pWF-26 and pWF-27 not only with the goat hyperimmune serum but also with most of the MAb. Fig. 3 represents some of the results obtained by Western blotting. As controls, the goat preimmune serum (not shown), an unrelated MAb (not shown) and a gII-specific MAb (Fig. 3g) were included. These results are
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summarized in Table 3. As shown, pWF-26 and pWF-27 were able to bind the MAbs, except for IN-2 and 2/9 (Fig. 3e and f) which, however, reacted strongly in Western blots with purified PRV virions or infected cell lysates (data not shown). The MAb 1/14 reacted with pWF-26, but not with pWF-27 (Fig. 3d), or with pWF-24 (not shown). Taken together, these results indicate that the N-terminal part of gI, particularly between amino acids 33 and 108, contains several epitopes binding 11 out of 13 gI-specific MAbs (Table 3).

Although not all MAbs against gI have been tested thoroughly (e.g. in competitive ELISA) to achieve a complete topological grouping, at least four distinguishable binding sites appear to exist in this N-terminal region: binding sites A, B and C for the neutralizing MAbs (Table 1) and at least one additional site D for the MAb N-11 (not shown). The results are also compatible with computer prediction which indicates a region of high antigenicity, hydrophilicity and surface probability between amino acids 50 and 70, and a second highly antigenic sequence around amino acid 100 (Fig. 4). The poor antigenicity of the peptides synthesized by pWF-25 and pWF-19 is not surprising because both contain the putative transmembrane region (amino acids 429 to 453) and anchor sequence (amino acids 454 to 577) of gI (Fig. 4).

Reactivity of swine antibodies with pWF-26 and pWF-27

To investigate whether humoral antibodies against the N-terminal part of gI are induced in infected pigs, swine sera were tested on Western blots with pWF-26 and pWF-27. To minimize background, both fusion proteins had been partially purified by differential urea extraction. Fig. 5 shows the reaction obtained with sera of pigs collected at different times after infection with strain Phylaxia (Fig. 5, lanes 3 to 10) and with two sera of field-infected animals (Fig. 5, lanes 2 and 12). Sera collected at 103 and 148 days p.i. were obtained from immunosuppressed pigs. Sera derived from animals vaccinated with a gI-negative live vaccine (strain NIA-4; Fig. 5, lane 11) and sera from non-infected animals did not react with pWF-26 or pWF-27 (Fig. 5, lane 13). In total, 21 sera of swine intranasally infected with different PRV strains (Phylaxia, Ka, NIA-3) and collected between 77 days and 148 days after infection were found to react specifically with pWF-26 and pWF-27 in Western blotting.

Fig. 3. Reactivity of gI-containing fusion proteins obtained after induction of recombinant clones pWF-26 (lanes 2) and pWF-27 (lanes 3) with gI-specific MAbs 2/5 (a), 4/15 (b), S1 (c), 1/14 (d), IN-2 (e) and 2/9 (f). As controls, lysates of non-induced pWF-26 bacteria (lanes 1) as well as a gII-specific MAb (g) were used. Equal amounts of protein (corresponding to approximate absorbence at 260 nm of 0.3) were separated by 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose. The filter was cut into strips (a to g) which were reacted separately with the indicated MAbs.
Furthermore, both fusion proteins were used as antigens in ELISA. After urea extraction and electro-elution from SDS-PAGE gels each fusion protein was coated onto microtitre plates (0.1 µg per well) and were found to react specifically with different gI MAbs and sera of infected swine (data not shown). In addition, preliminary tests demonstrated that the products of pWF-26 and pWF-27 can be used in blocking ELISA and sandwich ELISA with a specificity and sensitivity comparable to existing ELISAs (not shown).

**Protective activity of gI-specific MAbs**

To test for protective activity of anti-gI MAbs, mice were passively immunized i.p. with 0.25 ml ascites fluid containing the individual MAbs and challenge-infected with a lethal dose of PRV strain Phylaxia as described in Methods. As shown in Table 1, several MAbs were able to protect mice to a different extent with protection rates ranging from 20 to 100%. Notably, the five MAbs exhibiting complement-dependent neutralizing activity conferred the most effective protection without correlation to the respective neutralization titre. Only one of the non-neutralizing MAbs, N-11, also protected 80% of the immunized mice (Table 1). Different preparations of ascites fluids used to repeat the protection experiments showed similar results (Table 1).

An additional experiment was performed using two different combinations of gI-specific MAbs for passive immunization. For this purpose, ascites fluids (0.1 ml of each MAb) were combined and 0.25 ml of the mixture was injected i.p. Both combinations of MAb conferred significant protection (80% and 70%) against lethal virus infection, although each of the MAbs alone (0.25 ml of ascites fluid) did not protect at all or only to a limited extent (Table 4).

Finally, groups of passively immunized mice were challenge-infected with PRV isolates which previously had been shown to express variable amounts of gI, and which are resistant against in vitro neutralization with gI-specific MAbs (Mettenleiter *et al.*, 1987). Ka2 and Ka3 represent single plaque isolates of strain Ka expressing significantly lower (Ka2) to undetectable (Ka3) amounts of gI compared to wild-type virus (Mettenleiter *et al.*, 1987).
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Table 4. Mouse protection by combinations of anti-gI MAb

<table>
<thead>
<tr>
<th>MAb</th>
<th>Single</th>
<th>Combined</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>4/15</td>
<td>2/10*</td>
</tr>
<tr>
<td></td>
<td>2/5</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>2/9</td>
<td>0/10</td>
</tr>
<tr>
<td>B</td>
<td>3/2</td>
<td>2/10</td>
</tr>
<tr>
<td></td>
<td>S-1</td>
<td>3/10</td>
</tr>
</tbody>
</table>

* Groups of mice (protected/total number of animals) were passively immunized with 0.25 ml of diluted ascites fluid of each MAb (a mixture of 100 μl of diluted ascites fluid of each MAb) and challenged i.m. with approximately 22 LD₅₀ of PRV strain Phylaxia. For comparison the protection obtained with each MAb (0.25 ml diluted ascites fluid) alone is given (single).

Table 5. Mouse protection against challenge infection with different PRV isolates

<table>
<thead>
<tr>
<th>Challenge virus</th>
<th>MAb*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ka2</td>
<td>3/6 (gI)</td>
</tr>
<tr>
<td>Ka3</td>
<td>2/10</td>
</tr>
<tr>
<td>Phylaxia</td>
<td>9/10</td>
</tr>
</tbody>
</table>

* Mice were passively immunized with 0.25 ml of diluted ascites fluid of either gI-specific MAb (3/6 or 1/14) or a gII-specific MAb (N-12), and 24 h later challenge-infected with the indicated virus strains. † Mice protected/total.

Table 6. Protection of mice after active immunization with gI-containing fusion proteins

<table>
<thead>
<tr>
<th>Fusion protein (μg)*</th>
<th>Mice protected/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWF-26 (200-0)</td>
<td>9/10</td>
</tr>
<tr>
<td>pWF-26 (50-0)</td>
<td>4/10</td>
</tr>
<tr>
<td>pWF-26 (12-5)</td>
<td>1/10</td>
</tr>
<tr>
<td>pWF-27 (150-4)</td>
<td>10/10</td>
</tr>
<tr>
<td>pWF-27 (37-6)</td>
<td>4/10</td>
</tr>
<tr>
<td>pWF-27 (9-4)</td>
<td>1/10</td>
</tr>
<tr>
<td>No protein</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* Mice were inoculated (i.m.) with the indicated amounts of purified fusion protein synthesized from pWF-26 and pWF-27, respectively, and 4 weeks later challenge-infected (i.m.) with approximately 130 LD₅₀ of PRV strain Phylaxia.

1987). From the results given in Table 5 it can be seen that the immunized animals were protected to a variable degree depending on the virus isolate used for challenge infection. Only two of 10 mice survived infection with Ka3 (expressing no detectable gI); however 60 to 70% of mice were protected against infection with Ka2 (which synthesizes reduced amounts of gI). After challenge with the Phylaxia strain a similar protection rate was found as in the preceding experiments described above. Although the challenge virus dose of Ka2 and Ka3 was not adjusted to obtain 100% lethality in control mice which were passively immunized with an unrelated MAb, complete lack of protection against challenge infection by Ka3 indicates a specific effect of the gI-specific MAb. Mice passively immunized with a PRV gI-specific MAb, N-12 (Lukács et al., 1985), were efficiently protected after challenge with either virus isolate (Table 5). Thus, gI-specific antibodies also display protective activity (Table 5; unpublished data).

Mouse protection after immunization with fusion proteins

Since we demonstrated passive protective activity of gI-specific antibodies in mice, we also used purified fusion proteins synthesized from pWF-26 and pWF-27 for active immunization of mice. The hind legs of groups of 10 mice were injected i.m. with different volumes of emulsified pWF-26 or pWF-27. After challenge infection i.m. with a lethal dose of strain Phylaxia 4 weeks after immunization, mice immunized by both fusion proteins were protected. As shown in Table 6, purified fusion proteins...
proteins pWF-26 and pWF-27 conferred protection to mice dependent on the amount of protein injected. Thus, the isolated immunodominant part of gI is able to induce protection in mice. The protective dose (PD_{50}, median protective dose against lethal challenge) was calculated protection in mice. The protective dose (PD_{50}) was calculated as 57 μg for pWF-26 and 33 μg for pWF-27.

Discussion

In the present study fragments covering the glycoprotein gI gene of PRV were expressed in bacteria and tested for reactivity with a panel of gI-specific MAbs to define linear epitopes. The presentation of those epitopes during virus infection in the natural host was analysed using sera from PRV-infected pigs. Furthermore, the role of gI in in vivo virus neutralization in an animal model system was investigated.

Reactivity of gI-specific MAbs with different gI fusion proteins demonstrated the presence of an immunodominant region in the N-terminal part of gI. In Western blotting experiments the majority of the MAbs (11 out of 13) reacted strongly with proteins synthesized from constructs pWF-26 and pWF-27 containing amino acids 33 to 238 of gI. Lack of reaction of any MAb with pWF-24 (amino acids 108 to 238) confines the antigenic region to amino acids 33 to 108, where at least three different epitopes binding complement-dependent neutralizing antibodies are located (Table 3). The binding site of MAb 1/14 probably resides between amino acids 108 and 122, since this antibody recognized pWF-26 (amino acids 33 to 238) but neither pWF-27 (amino acids 33 to 122) nor pWF-24 (amino acids 108 to 238), and therefore its binding site might be incompletely expressed in both constructs. At present, we cannot exclude the existence of additional epitopes close to the signal peptide which is predicted to extend to amino acid 21 (Fig. 4; Petrovskis et al., 1986a), since our most proximal fusion proteins starts with amino acid 33. These results correlate with Chou–Fasman predictions of gI delineating between amino acids 55 and 70 a region with a continuously high antigenic index (≥ 1.7). This region is contained in pWF-26 and pWF-27 and actually specifies several linear epitopes. A cluster of continuous epitopes has also been demonstrated in the N terminus of glycoproteins gD and gB of herpes simplex virus type 1 (HSV-1) (Kousoulas et al., 1988, 1989; Pereira et al., 1989; Weijer et al., 1988). Antibodies against gD with neutralizing activity against HSV-1 are also directed against N-terminal residues (Cohen et al., 1984; Strynadka et al., 1988). Additional antigenic sites are predicted, particularly in the C terminus of gI between amino acids 485 and 545 (Fig. 4). This region is expressed from pWF-19 which reacted very weakly in Western blotting with MAbs IN-2 and 2/9. However, the specificity of the reaction remains to be ascertained.

To test whether the N-terminal part of gI is also immunogenic in PRV infection of swine, pig sera were analysed for reactivity with pWF-26 and pWF-27. Sera collected from infected animals reacted in Western blot experiments with both fusion proteins, whereas no reaction was observed with the MS2 polymerase protein part. Specificity was also shown using sera of pigs vaccinated with gI-negative PRV (strain NIA-4), which did not react with any of the fusion proteins. Comparable results were obtained in ELISA using purified fusion protein expressed from pWF-26 or pWF-27 as antigens. We demonstrate that the N-terminal part of gI represents an important antigenic domain recognized by the immune system of swine, the natural host of PRV. This is consistent with recent data demonstrating the presence of gI-specific antibodies in pigs vaccinated with a PRV live vaccine (Omnimark; Kit, 1989) originating from the BUK strain which is deleted only in the C-terminal part of glycoprotein gI (Mettenleiter et al., 1988). Therefore, only vaccine strains that do not express the N terminus of gI should be used in programmes to control Aujeszky's disease based upon vaccination with gI-negative vaccines in combination with ELISA to detect gI-specific field virus antibodies in pigs. For the accompanying serological survey standardized antigens which can be produced inexpensively should be used. Considering the variable expression of gI in some PRV strains in vivo and in vitro (Mettenleiter et al., 1987) the infected cell lysates used as ELISA antigens must be tested for the quality and quantity of gI present. Fusion proteins synthesized by pWF-26 and pWF-27 offer a possibility to obtain standardized gI antigens. In addition, for the establishment of diagnostic assays those peptides will allow a systematic selection of suitable gI-specific MAbs.

Five MAbs against gI exhibit complement-dependent neutralizing activity (Table 1) which could be assigned by competition ELISA to three different groups (Table 1) binding to the N-terminal immunogenic domain. To investigate the role of gI-specific antibodies and gI in virus neutralization in vivo, mice were passively immunized with MAb and subsequently challenge-infected with a lethal dose of PRV. All virus-neutralizing MAbs protected the animals to a similar degree (80 to 100%). In addition, some of the non-neutralizing MAbs were able to confer protection (Table 1), again showing that in vitro neutralization and in vivo protection are not necessarily correlated, as found also for glycoprotein gp50 and gIII of PRV (Eloit et al., 1988; Marchioli et al., 1988; Wathen et al., 1985) and for HSV glycoproteins (Dix et al., 1981; Glorioso et al., 1984; Kümel et al., 1985; Rector et al., 1982, 1984). After simultaneous injection of two or three gI-specific MAbs which singly did not effectively
Epitope analyses of PRV glycoprotein gI

protect the mice, protection was observed pointing to a synergistic effect of antibodies directed against different epitopes (Lussenhop et al., 1988; van Drunen Littel-vanden Hurk et al., 1985; Roberts et al., 1985). The specificity of the protection mediated by the MAbs was monitored by application of an unrelated MAb (Tables 1 and 5) and by injecting higher dilutions of ascites fluid which resulted in decreasing protection rates (not shown). In addition, challenge infection of passively immunized mice with PRV isolates expressing different amounts of gl (Ka2 and Ka3; Table 4) showed a direct relationship between protection and the amount of gl present in the challenge virus. Plaque isolates Ka2 and Ka3 had been shown previously to contain no obvious alteration in the gl gene and appear to synthesize other viral glycoproteins comparable to wild-type PRV (Mettenleiter et al., 1987). Ka3 (with undetectable gl expression) proved to be resistant against in vitro neutralization with different gl-specific MAbs (Mettenleiter et al., 1987). Immunization with the purified fusion proteins pWF-26 and pWF-27 showed that the N terminus of gl is sufficient for the protection of mice against a lethal PRV infection. These experiments show that gl contributes to protective immunity and that antibodies directed against the N terminus of gl are involved in the protective immune response in vivo.

The role of individual herpesviral glycoproteins in the induction of neutralizing and protective antibodies in vivo is well known (Balachandran et al., 1982; Babik et al., 1987; Dix et al., 1981; Edson et al., 1985; Fuller et al., 1989; Guo et al., 1989; Pereira et al., 1980; Powell et al., 1974; Roberts et al., 1985; Spear, 1975; van Drunen Littel-vanden Hurk et al., 1989). Recently PRV glycoproteins gp50 (Eloit et al., 1988; Ishii et al., 1988; Kost et al., 1989; Marchioli et al., 1987, 1988, Petrovskis et al., 1986a), and gII (Eloit et al., 1988; Marchioli, 1988) were identified as important immunogens in mice and in swine. Results show that PRV gII-specific antibodies can also confer protection to mice (Table 4; unpublished data). However, it has been shown for PRV that the mouse model system does not correlate necessarily with the situation in the natural host (Marchioli et al., 1988). Data indicating that gl-specific antibodies play only an inferior role, if any, in virus neutralization in pigs have been reported (Ben-Porat et al., 1986). A virulent gl-negative PRV vaccine strains induce good protection when administered to swine but, similar to all currently available vaccines, do not prevent PRV infection. Therefore, the relative contribution of gl in the induction of protective immunity in swine with respect to the other PRV glycoproteins remains to be clarified.

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