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The Pseudorabies Virus UL51 Gene Product Is a 30-Kilodalton Virion Component

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Positional homologs to the UL51 open reading frame of herpes simplex virus type 1 have been identified throughout the herpesvirus family. However, no respective protein has so far been described for any of the herpesviruses. With rabbit antisera directed against oligopeptides predicted to comprise antigenic regions of the deduced pseudorabies virus (PrV) UL51 protein, a polypeptide with a size of 30 kDa was identified in PrV-infected cell lysates and in purified virions. This molecular mass correlates reasonably well with the predicted mass of 25 kDa of the 236-amino-acid deduced UL51 protein. Antisera raised against peptides derived from different predicted antigenic regions all detected the 30-kDa protein in Western blot (immunoblot) analyses. Specificity was ascertained by peptide competition. Subcellular fractionation showed the presence of the UL51 protein mainly in the nucleus of infected cells. After separation of purified virion preparations into envelope and capsid, the PrV UL51 protein was detected in the capsid fraction. In summary, we identified the first herpesvirus UL51 protein and demonstrate that it represents a structural component of PrV virions.

Herpesviruses are complex animal viruses with a linear double-stranded genomic DNA with a size of ca. 120 to 250 kbp. The family *Herpesviridae* is divided into the three subfamilies *Alpha*-, *Beta*-, and *Gammaherpesvirinae* (23). Based on the increasing availability of complete genomic sequence information for several herpesviruses (1, 2, 5, 7, 8, 17, 19, 21, 24, 26), it has become clear that a set of genes is conserved among all three subfamilies of herpesviruses, whereas others appear to be unique for each subfamily. The genome of the prototypic alphaherpesvirus, herpes simplex virus type 1 (HSV-1), contains at least 84 different genes (17, 22). The majority of these genes are conserved in other members of the alphaherpesviruses, such as varicella-zoster virus (7), equine herpesvirus 1 (26), bovine herpesvirus 1 (24), or pseudorabies virus (PrV [18]). For most of the HSV-1 open reading frames, the corresponding gene products have been identified, and an increasing number of proteins are functionally more or less well characterized. However, several gene products still remain unknown, including the product of the UL51 open reading frame.

Recently, we sequenced a 7.4-kbp region at the left end of the unique long region of the PrV genome (4). Within this area, genes homologous to the HSV-1 UL51 to -54 open reading frames were localized in addition to an open reading frame conserved between PrV and equine herpesvirus 1 but absent from the other alphaherpesviruses. The PrV UL51 gene of 711 bp was predicted to encode a 236-amino-acid protein (4).

Open reading frames homologous to the HSV-1 UL51 gene are at least positionally conserved throughout the herpesvirus family (1, 2, 5, 7, 8, 17, 19, 21, 24, 26). However, to our knowledge, no UL51 gene product has been identified so far. Since the apparent conservation might be indicative of a prominent functional role for UL51 in the herpesvirus replicative cycle, we set out to identify the PrV UL51 protein by using rabbit antisera directed against oligopeptides comprising four antigenic regions of the UL51 protein predicted from the amino acid sequence. Shown in Fig. 1 is the prediction of secondary structure and antigenicity of the PrV UL51 protein (6, 10, 12). Peptides corresponding to amino acids 10 to 30, 50 to 70, 193 to 209, and 219 to 234 were synthesized, coupled to keyhole limpet hemocyanin, and used to immunize three rabbits each according to standard procedures (15). For coupling of peptide 3, the proline at position 193 had been altered to cysteine. Sera obtained after the third immunization were used for the experiments.

Western blots with one representative serum specific for peptides 1 to 3 are shown in Fig. 2A to C. Purified virions were used as antigen. To this end, supernatant from Vero cells infected with wild-type PrV Ka (14) was harvested after the cells had exhibited a complete cytopathic effect. Virions were purified by centrifugation through a discontinuous sucrose gradient, a procedure which results in high-quality virion preparations (15). Virion lysates were separated in sodium dodecyl sulfate (SDS)–12% polyacrylamide gels under reducing conditions. Compared to matching preimmune sera (lanes 1), all sera at a dilution of 1:500 detected a 30-kDa protein in virion lysates (Fig. 2, lanes 3). The apparent molecular mass of 30 kDa is only slightly larger than the predicted mass of 25 kDa as calculated from the sequence of the UL51 gene. To ascertain the specificity of the reaction, competition experiments were performed with 50 μg of peptide per assay (15). As shown in Fig. 2, lanes 4, the homologous peptide competed efficiently in every instance, whereas heterologous peptides (Fig. 2, lanes 5) failed to inhibit the reactivity of the respective antiserum. One representative noncompeting peptide is shown for each antiserum. The reactivities of preimmune sera after competition with homologous peptide are shown in Fig. 2, lanes 2. The reactivity of antisera against peptide 4 was very weak and was impossible to reproduce photographically. We conclude that antisera specific for three of the four predicted antigenic domains specifically recognized a 30-kDa PrV virion protein. For

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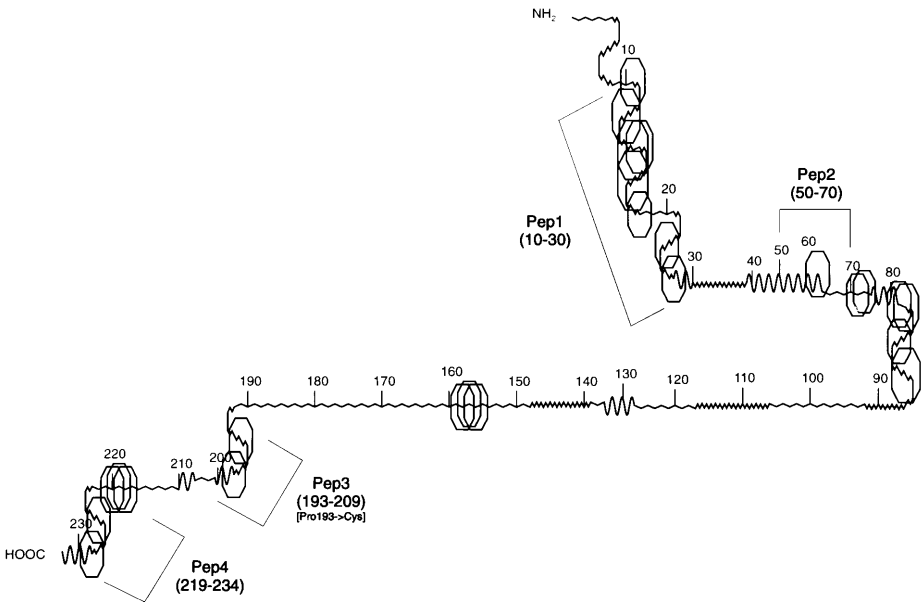

FIG. 1. Predictions of secondary structure and antigenicity of the PrV UL51 protein. Secondary structure and antigenicity were predicted from the deduced amino acid sequence of the UL51 open reading frame (4) by the methods of Chou and Fasman (6) and Jameson and Wolf (12), respectively. Octagons indicate local antigenic indices higher than 1.2. Pep1, Pep2, Pep3, and Pep4 denote regions contained in the corresponding oligopeptides used for immunization of rabbits. The positions of amino acids present in the oligopeptides are indicated. For coupling to keyhole limpet hemocyanin in Pep3, the proline at position 193 had been altered to cysteine.

further experiments, serum directed against peptide 2, which yielded the best signal, was used.

To analyze intracellular UL51 protein and to assess the time course of UL51 synthesis, Vero cells were infected at a multiplicity of infection (MOI) of 10 with wild-type PrV and harvested at the time of infection (Fig. 3, lanes −1), after a 1-h attachment period at 4°C (Fig. 3, lane 0), as well as 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 h after a temperature shift to 37°C (lanes 1 to 8, 12, and 24). The UL51 protein was first detected 3 h postinfection (Fig. 3A), correlating with the first detection of putative UL51 mRNA 2 hpi (4). However, whereas mRNA levels appeared to decrease at later time points (4), the amount of UL51 protein appeared to remain constant. Two proteins with sizes of 42 and 55 kDa, present in noninfected as well as in infected cells, also reacted with the antiserum. These signals are probably due to nonspecific reactivity by the polyclonal rabbit antiserum. As controls, sera specific for PrV dUTPase (Fig. 3B, UL50 [13]) and virion membrane glycoprotein gC (Fig. 3C [16]) were used. In infected cells, dUTPase became detectable at approximately the same time as the UL51 protein. In contrast, the precursor polypeptide for glycoprotein gC, which is expressed with late kinetics, first appeared 12 h after the temperature shift in this assay, and more highly glycosylated forms, including mature approximately 95-kDa gC, were observed at 24 hpi.

Unfortunately, rabbit antisera tend to create high background levels in immunofluorescence studies. This was also true for our sera (data not shown). Therefore, subcellular localization of the UL51 protein by immunofluorescence was impossible. To analyze the subcellular distribution of the PrV UL51 protein by Western blotting, Vero cells were infected with an MOI of 10 and separated into nuclear, soluble cytoplasmic, and cytoplasmic membraneous fractions (20) 12 h after infection, when UL51 protein has already accumulated to a significant extent (Fig. 3A). Briefly, cells were trypsinized, washed with phosphate-buffered saline, and resuspended in 10 mM Tris-HCl (pH 7.4)–10 mM NaCl–1.5 mM $MgCl_2$–1 mM phenylmethylsulfonyl fluoride (PMSF). After incubation on ice for 15 min, cells and nuclei were pelleted at 2,000 × *g* for 10 min, resuspended in buffer B (0.32 M sucrose, 1 mM $MgCl_2$, 1 mM potassium phosphate [pH 6.8], 1 mM PMSF), and homogenized in a Dounce homogenizer. Release of nuclei was verified by phase-contrast microscopy. After centrifugation, nuclei were washed twice in buffer B supplemented with 0.3% Triton X-100. The pellet was then resuspended in 20 mM Tris-HCl (pH 9.0)–300 mM NaCl–10% glycerol–1 mM $CaCl_2$–0.5 mM $MgCl_2$–2 mM EDTA–0.5% Nonidet P-40–1 mM PMSF. Supernatants of the first two centrifugation steps were collected and spun at 45,000 rpm in a TLA-45 rotor (Beckman Instru-

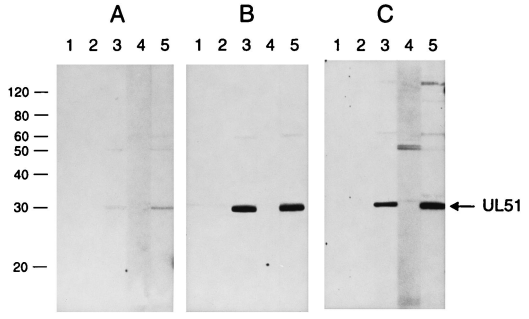

FIG. 2. Western blotting and peptide competition analysis of UL51 antipeptide sera. Rabbit sera directed against peptide 1 (A), peptide 2 (B), and peptide 3 (C) were tested at a 1:500 dilution in a Western blot on lysates of purified virions separated by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide) under reducing conditions. The reactivity of preimmune serum without or with competition by 50 μg of homologous peptide is shown in lanes 1 and 2, respectively. The reactivity of the respective antiserum obtained after the third immunization is shown without competing peptide (lanes 3) and after addition of 50 μg of competing homologous peptide (lanes 4). Lanes 5 show reactivity after addition of the same amount of heterologous peptide. The straight arrow indicates the position of the 30-kDa UL51 protein. Molecular mass markers are indicated on the left in kilodaltons.

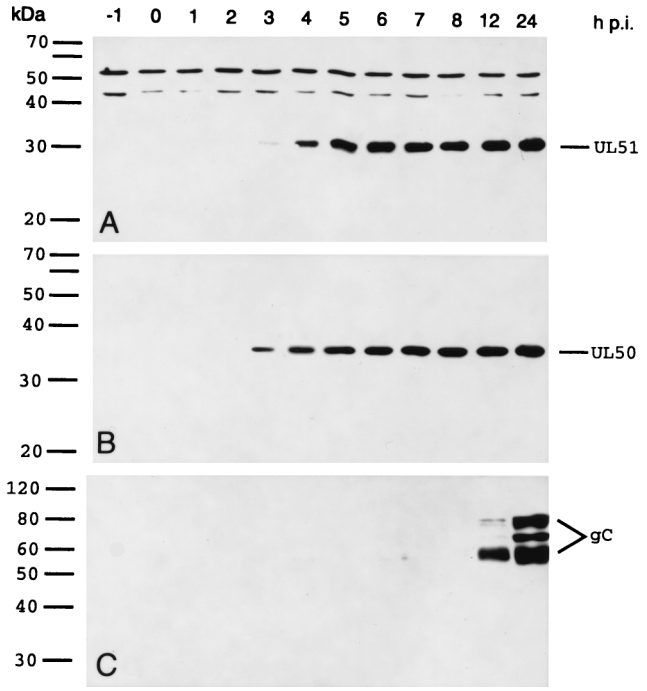

FIG. 3. Kinetics of expression of the UL51 protein. Vero cells were infected at an MOI of 10 for 1 h at 4°C. Thereafter, the inoculum was replaced by prewarmed medium to initiate penetration. Cell lysates obtained before infection (lanes −1), after 1 h of attachment at 4°C (lanes 0), as well as 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 h after the temperature shift were analyzed by Western blotting with the anti-UL51 Pep2 serum (A), a monospecific rabbit anti-UL50 serum (B [13]), and a monoclonal antibody specific for gC (C [16]).

ments) at 4°C for 1 h. The supernatant contained soluble cytosolic proteins. The membraneous pellet fraction was resuspended in buffer B. The protein concentration was determined (25), and equal amounts of protein were separated in SDS–12% polyacrylamide gels under reducing conditions. Whole-cell lysate (Fig. 4, lanes 1), the nuclear fraction (Fig. 4, lanes 2), the cytoplasmic membraneous fraction (Fig. 4, lanes 3), and cytoplasmic soluble protein (Fig. 4, lanes 4) were analyzed. Blots were probed with preimmune serum (Fig. 4A), anti-UL51 serum (Fig. 4B), anti-UL50 serum (Fig. 4C [13]), or anti-gC monoclonal antibody (Fig. 4D [16]).

At this stage of infection, most of the UL51 protein detected in whole-cell lysates (Fig. 4B, lane 1) was present in the nuclear fraction of cells (Fig. 4B, lane 2). In the cytoplasmic soluble

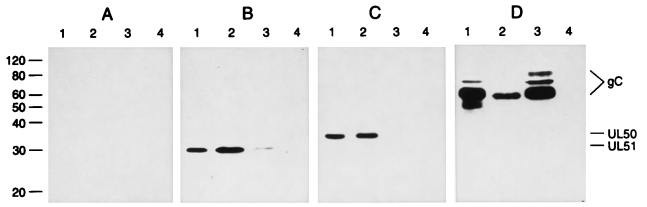

FIG. 4. Subcellular distribution of the PrV UL51 protein. Vero cells were infected at an MOI of 10 with PrV. At 12 hpi, cells were harvested and either lysed directly (lanes 1) or separated into nuclear (lanes 2), cytoplasmic membraneous (lanes 3), and cytoplasmic soluble (lanes 4) fractions. After separation by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide) under reducing conditions and Western blotting, nitrocellulose filters were incubated with preimmune serum (A), anti-UL51 serum (B), anti-UL50 serum (C), and anti-gC monoclonal antibody (D). Molecular mass markers are indicated on the left in kilodaltons.

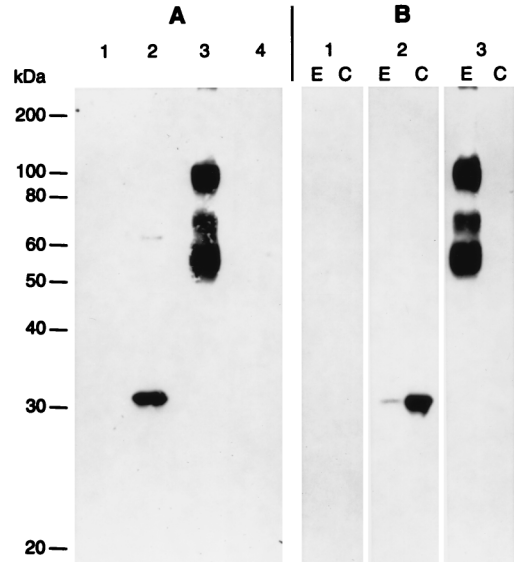

FIG. 5. Detection of UL51 protein in virions. Purified virion preparations were analyzed either directly (A) or after separation into envelope (B, lanes E) and capsid (B, lanes C) fractions by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide) under reducing conditions. After transfer, filters were incubated with preimmune serum (lanes 1), anti-UL51 serum (lanes 2), anti-gC monoclonal antibody (lanes 3), or anti-UL50 serum (lane 4). Molecular mass markers are indicated on the left.

fraction, no UL51 protein was detectable (Fig. 4B, lane 4), whereas small amounts of UL51 protein were found in the membraneous fraction (Fig. 4B, lane 3). This distribution is consistent with the hypothesis that as a virion component, the UL51 protein is primarily present intranuclearly during virus maturation, but is also detectable in membraneous structures in the cytoplasm in which virions are found (9). The UL50 protein, dUTPase, is present only in the nucleus (Fig. 4C, lane 2), which correlates with analyses of dUTPase activity in PrV-infected cells (13). Immature glycoprotein gC was present in the nuclear fraction (Fig. 4D, lane 2), and immature and mature forms were detected in cytoplasmic membranes (Fig. 4D, lane 3). As expected, no gC was detected in the soluble cytoplasmic fraction (Fig. 4D, lane 4). The relative distribution of the UL51 protein did not differ from these findings at any of the time points investigated (data not shown).

To identify the location of the UL51 protein in virions, purified virion preparations were analyzed either directly (Fig. 5A) or after separation into envelope (Fig. 5B, lanes E) and capsid (Fig. 5B, lanes C) fractions (11) by SDS-polyacrylamide gel electrophoresis under reducing conditions and Western blotting. Whereas the matching preimmune serum failed to react with a virion protein (Fig. 5, lanes 1), the anti-UL51 serum recognized UL51 protein in purified virions (Fig. 5A, lane 2), and the anti-gC monoclonal antibody detected gC (Fig. 5A, lane 3). As a control for a nonvirion protein, the anti-UL50 serum did not react with a virion component (Fig. 5A, lane 4). As shown in Fig. 5B, after separation into envelope and capsid fractions, gC is exclusively present in the envelope fraction (Fig. 5B, lane 3E) and absent from the capsid preparation (Fig. 5B, lane 3C). Mature 95-kDa gC and several breakdown products of gC were detected. In contrast, the UL51 protein was predominantly found in the capsid fraction (Fig. 5B, lane 2C), and only minimal amounts are present in the envelope preparation (Fig. 5B, lane 2E). From these data, it is evident that the UL51 protein is a virion component which is mainly asso-

ciated with the capsid. However, since the capsid fraction most likely contains nucleocapsids as well as adhered tegument material, it is unclear at present whether the UL51 protein represents a true capsid component of PrV virions or whether it is located in the tegument. The small amount of UL51 protein detected in the envelope preparation could thus indicate the location in the tegument and possible interactions with envelope constituents.

So far, no function has been assigned to any of the putative UL51 homologous proteins. However, it has been proposed that HSV-1 UL51 protein is dispensable for viral replication in cell culture (3). If this is also true for PrV, isolation of a UL51 mutant should be feasible on normal cells without the need for transcomplementation. Respective experiments are under way. Characterization of this mutant will likely help us understand the functional role of the conserved UL51 protein in the herpesvirus replication cycle.

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