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Short Communication

Deletion or green fluorescent protein tagging of the pUL35 capsid component of pseudorabies virus impairs virus replication in cell culture and neuroinvasion in mice

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To facilitate tracing of virion movement, the non-essential capsid proteins pUL35 of herpes simplex virus type 1 and pseudorabies virus (PrV) have been tagged with green fluorescent protein (GFP). However, the biological relevance of PrV pUL35 and the functionality of the fusion proteins have not yet been investigated in detail. We generated PrV mutants either lacking the 12 kDa UL35 gene product, or expressing GFP fused to the N terminus of pUL35. Remarkably, both mutants exhibited significant replication defects in rabbit kidney cells, which could be corrected in pUL35-expressing cells. After intranasal infection of mice both mutants showed delayed neuroinvasion, and survival times of the animals were extended to 3 days, compared with 2 days after wild-type infection. Thus, fusion of pUL35 with GFP resulted in a non-functional protein, which has to be considered for the use of corresponding mutants in tracing studies.

Pseudorabies virus (PrV, *Suid herpesvirus 1*) is a member of the genus *Varicellovirus* within the subfamily *Alpha-herpesvirinae* of the family *Herpesviridae* (Davison *et al.*, 2005). It is the causative agent of Aujeszky's disease in pigs but also leads to fatal neurological disorders in many other mammalian species (Mettenleiter, 2000). Since the PrV genome is similar to other alphaherpesvirus genomes regarding gene content and arrangement, most gene designations originally introduced for herpes simplex virus type 1 (HSV-1) have been adopted (Klupp *et al.*, 2004; McGeoch *et al.*, 1988).

Roughly half of the approximately 70 gene products of alphaherpesviruses are components of virus particles, which consist of an icosahedral capsid surrounded by a tegument protein layer and a lipid envelope of cellular origin containing viral membrane proteins (Mettenleiter, 2002). While several of the envelope and tegument proteins are only present in the alphaherpesviruses, the capsid components are highly conserved between all subfamilies of mammalian and avian herpesviruses. Mature, DNA-containing C-capsids essentially consist of four viral proteins, which, for HSV-1, were named VP5 (pUL19), VP26 (pUL35), VP19C (pUL38) and VP23 (pUL18) (Roizman & Knipe, 2001).

The smallest capsid protein of HSV-1 (VP26) is encoded by the open reading frame (ORF) UL35 (Davison *et al.*, 1992; McNabb & Courtney, 1992). The protein does not contain a nuclear import signal, but it does require association with VP5 to enter the nucleus, where it accumulates in areas of capsid assembly (Rixon *et al.*, 1996; Ward *et al.*, 1996). VP26 is present in 900 copies per virus particle, since hexameric rings of VP26 are bound to the tips of all 150 hexons, but absent from pentons (Wingfield *et al.*, 1997; Zhou *et al.*, 1995). However, VP26 is required neither for *in vitro* capsid assembly, nor for virion formation *in vivo* (Desai *et al.*, 1998; Tatman *et al.*, 1994; Trus *et al.*, 1995), and its biological function is largely unknown.

Yeast two-hybrid studies indicated interactions of HSV-1 VP26 with several subunits of the dynein complex, which is involved in intracytoplasmic transport of herpesvirus capsids along microtubules (Douglas *et al.*, 2004; Sodeik *et al.*, 1997). Active capsid transport to the nucleus is considered to be essential for host cell infection, and for transneuronal spread of alphaherpesviruses. However, UL35-negative deletion mutants of HSV-1 and PrV are replication competent in cell culture and actively transported (Antinone *et al.*, 2006; Desai *et al.*, 1998; Döhner *et al.*, 2006), suggesting that VP26 cannot be the only viral protein interacting with cellular motor proteins. On the other hand, deletion of UL35 leads to reduced virus titres in cell culture, and productive replication of UL35-negative HSV-1 in the central nervous system of mice is severely affected, indicating a possible role of VP26 during reactivation from latency (Desai *et al.*, 1998).

Supplementary material is available with the online version of this paper.

Because of their abundant expression and incorporation, pUL35 of HSV-1 and PrV were utilized for the tagging of capsids by fusion of the coding sequences of green or red fluorescent proteins to their 5′-ends. The labelled virus mutants were used for *in situ* studies of virion morphogenesis and live recordings of axonal spread in infected neurons (Antinone *et al.*, 2006; Desai & Person, 1998; Smith *et al.*, 2001, 2004; Snyder *et al.*, 2006; Wolfstein *et al.*, 2006). However, detailed studies on structure, localization and function of PrV pUL35 have not yet been carried out. Therefore, a monospecific antiserum, a pUL35-expressing cell line and a panel of PrV recombinants were generated and characterized.

The complete UL35 ORF of 104 codons was amplified from genomic DNA of PrV strain Kaplan (PrV-Ka; Kaplan & Vatter, 1959) and inserted into prokaryotic and eukaryotic expression vectors to yield pcDNA-UL35, pIRES-UL35 and pGex-UL35 (Fig. 1c). From pGex-UL35, a 37 kDa glutathione *S*-transferase–pUL35 fusion protein was expressed in *Escherichia coli*, and used for rabbit immunization (Fuchs *et al.*, 2002). The antiserum specifically detected the expected 12 kDa protein in Western blot analyses of rabbit kidney (RK13) cells infected with wild-type PrV-Ka, and in purified virions (Fig. 2a). The protein was also detectable in RK13-UL35 cells, which were obtained after transfection with pIRES-UL35 (Fig. 1c)

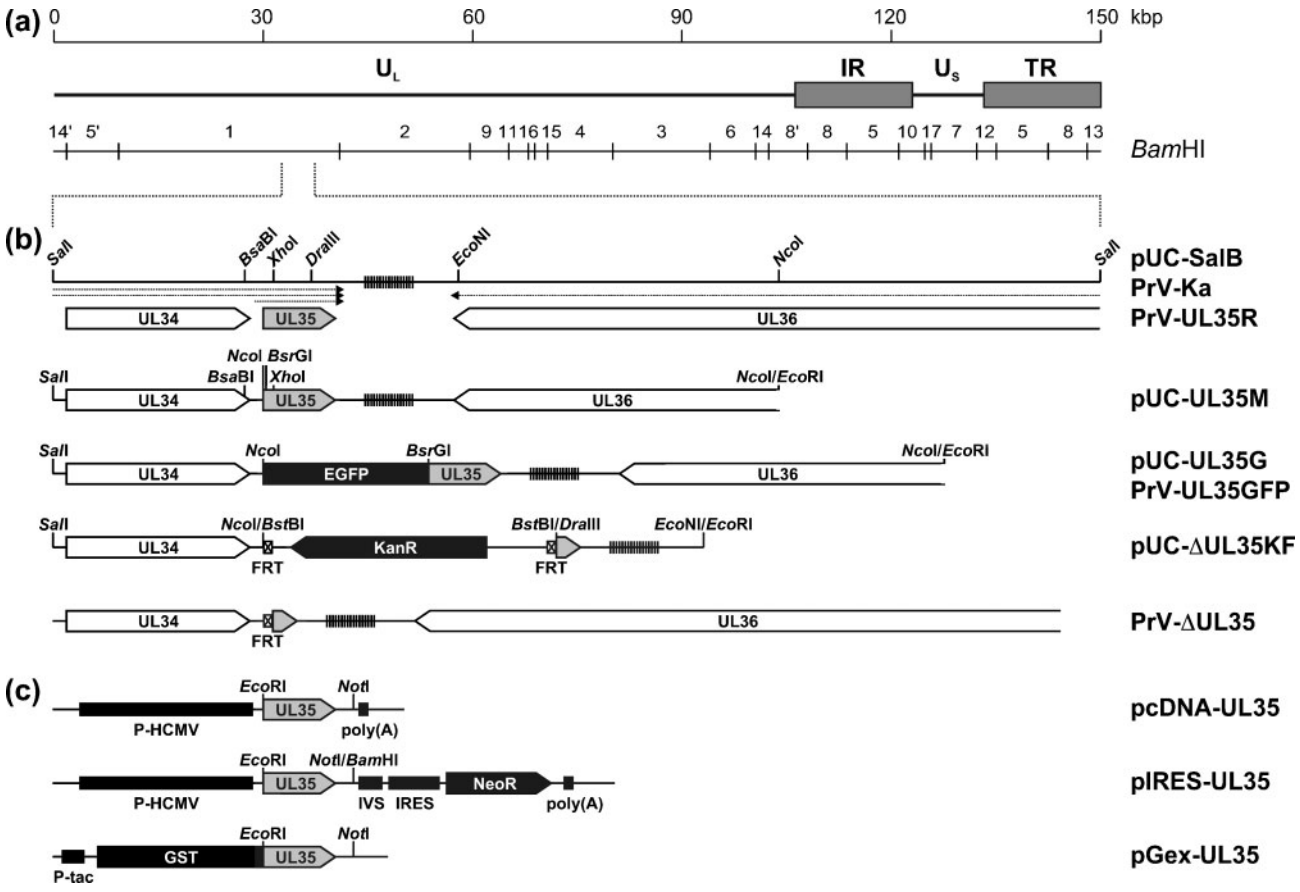

Fig. 1. (a) Map of the PrV genome. Long and short unique regions (U_L, U_S), inverted internal and terminal repeat sequences (IR and TR), and *Bam*HI restriction sites are shown. (b) Enlarged section with restriction sites used for construction of plasmids and virus recombinants. Tandem repeat sequences (vertical lines), viral transcripts (dotted arrows) and open reading frames (pointed rectangles) are indicated. Artificial *Nco*I and *Bsr*GI sites were introduced in pUC-UL35M for in-frame fusion of UL35 with the coding sequence of enhanced green fluorescent protein (EGFP) in PrV-UL35GFP, or for substitution of UL35 by a kanamycin resistance gene (KanR) for mutagenesis of the BAC-cloned PrV genome in *E. coli*. Flp recognition target (FRT) sites were used for subsequent removal of the resistance gene in PrV-ΔUL35. (c) For expression cloning, UL35 was provided with *Eco*RI and *Not*I sites by PCR-amplification. The vectors contained eukaryotic (P-HCMV) or prokaryotic promoters (P-tac), polyadenylation signals [poly(A)], as well as an intron (IVS) and an internal ribosomal entry site (IRES), which allowed expression of UL35 and a neomycin resistance gene (NeoR) from a bicistronic mRNA. For preparation of a monospecific antiserum, UL35 was expressed as a fusion protein with glutathione *S*-transferase (GST). For a more detailed description of plasmid and virus construction see Supplementary Material (available in JGV Online).

and selection with neomycin. However, RK13-UL35 cells proliferated very slowly, and were repeatedly overgrown by cells expressing either no detectable or mutated pUL35, indicating a detrimental effect of its high level constitutive expression on the cells (results not shown). The anti-pUL35 serum was also suitable for indirect immunofluorescence tests, and revealed a predominantly nuclear localization of its target protein in PrV-infected cells (Supplementary Fig. S1 available in JGV Online).

Three PrV recombinants derived from PrV-Ka were constructed and analysed. In PrV-UL35GFP the coding sequence of enhanced green fluorescent protein (EGFP) was fused in-frame to the 5′ end of UL35 (Fig. 1b), with concomitant loss of the UL35 initiation codon, and replacement of a threonine codon by serine at position two. The deduced 340 aa fusion protein of calculated 38 kDa was detected by the UL35-specific antiserum in cells infected with PrV-UL35GFP, as well as in purified virions (Fig. 2a). The same protein was also recognized by GFP-specific antisera (data not shown). Thus, as described previously for similar HSV-1 and PrV mutants (Desai & Person, 1998; Smith *et al.*, 2001), addition of a 26 kDa GFP-tag to the N terminus of the 12 kDa UL35 protein does not substantially affect expression and virion incorporation.

The UL35-negative recombinant PrV-ΔUL35 (Fig. 1b) was generated by mutagenesis of an infectious clone of the PrV genome (Kopp *et al.*, 2003) in *E. coli*. Codons 2–71 of UL35 were replaced by an artificial sequence of 36 bp, and expression of the 3′-part was prevented by a stop codon within the insertion. Therefore, no UL35 gene products could be found in RK13 cells infected with PrV-ΔUL35, or in virions purified from RK13 cells (Fig. 2a). In contrast, expression and incorporation of wild-type pUL35 was restored in rescuant PrV-ΔUL35R (Fig. 2a).

PrV-ΔUL35 could be structurally complemented *in trans* by propagation in RK13-UL35 cells, and the obtained virions contained a comparable amount of authentic pUL35 as PrV-Ka (Fig. 2a). Virions isolated from RK13-UL35 cells infected with PrV-UL35GFP contained both the 12 kDa wild-type and the 38 kDa EGFP–pUL35 fusion protein (Fig. 2a).

Although deletion of the UL35 ORF of PrV-Ka resulted in a replication competent virus mutant, plaque diameters of PrV-ΔUL35 on RK13 cells were reduced by approximately 30 % compared with those of wild-type PrV-Ka and the rescuant PrV-ΔUL35R (Fig. 2b). Remarkably, plaque sizes of PrV-UL35GFP were reduced to a similar extent (Fig. 2b), indicating that the EGFP–pUL35 fusion protein, although expressed and incorporated into virions (Fig. 2a), might not be functional. One-step growth analyses also revealed similar defects of PrV-ΔUL35 and PrV-UL35GFP. Compared with PrV-Ka and PrV-ΔUL35R, final titres of both mutants were reduced nearly 10-fold on RK13 cells (Fig. 2c). Moderate, but significant reductions of virus yields have also been reported for a UL35 deletion mutant

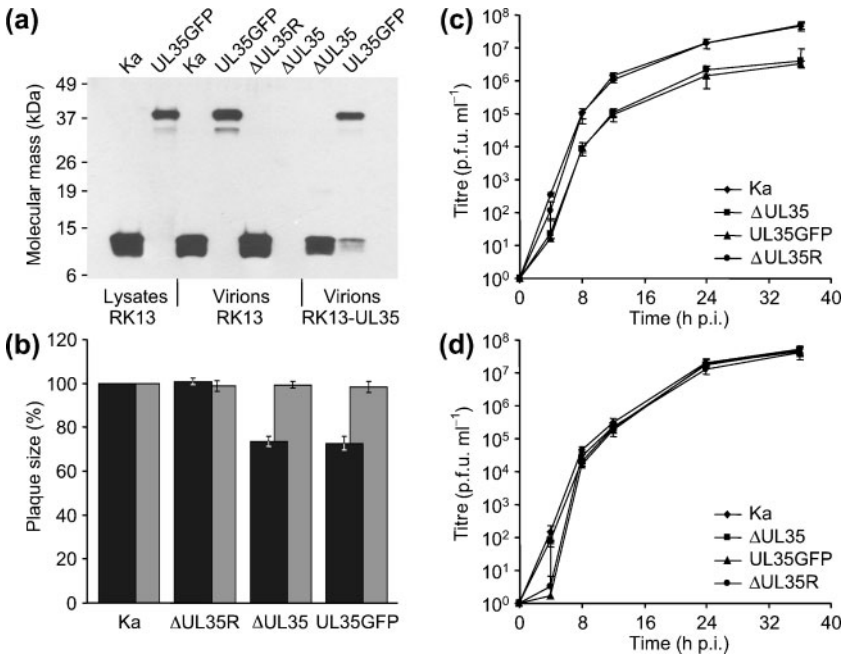

Fig. 2. Protein expression and *in vitro* replication of PrV-Ka, PrV-ΔUL35, PrV-ΔUL35R and PrV-UL35GFP. (a) RK13 or RK13-UL35 cells were harvested 24 h after infection at an m.o.i. of 5, and virions were purified from supernatants by sucrose gradient centrifugation (Karger *et al.*, 1998). Infected cell lysates (10^4 cells per lane) and virion proteins (3 μg per lane) were separated by 15 % SDS-PAGE. Western blots were probed with anti-pUL35 serum (dilution 1 : 50 000). Molecular mass markers are indicated on the left. (b) Plaque sizes on infected RK13 (black bars) and RK13-UL35 cells (grey bars), which were incubated for 48 h under semi-solid medium. Mean diameters and SD of 50 plaques per virus are shown as percentage values of the wild-type PrV-Ka sizes. For determination of one-step growth kinetics RK13 (c) or RK13-UL35 (d) cells were harvested together with the supernatant at indicated times after synchronized infection at an m.o.i. of 5. Mean progeny virus titres and SD of two experiments were calculated. All experiments were performed essentially as described previously (Fuchs *et al.*, 2002).

of PrV strain Becker (Antinone *et al.*, 2006), and for UL35-negative HSV-1 (Desai *et al.*, 1998). However, first infectious progeny virus of PrV-ΔUL35 and PrV-UL35GFP was detectable at 4 h post-infection (p.i.) as with PrV-Ka (Fig. 2c), and it could be confirmed that attachment and penetration were not affected (data not shown). Electron microscopy of infected RK13 also did not reveal inhibition of any specific step of capsid or virion morphogenesis, but showed decreased amounts of enveloped cytoplasmic and released virions of both mutants (data not shown). On *trans*-complementing RK13-UL35 cells, PrV-ΔUL35 and PrV-UL35GFP exhibited wild-type like growth properties (Fig. 2b, d). Taken together, these data demonstrate that pUL35 of PrV, although non-essential, fulfils an accessory function during virus replication, which cannot be provided by the EGFP–pUL35 fusion protein.

The relevance of pUL35 for neurovirulence of PrV was investigated in a mouse model (Klopfleisch *et al.*, 2004). Ten eight-week-old mice each were infected by bilateral intranasal instillation of 10^4 p.f.u. of PrV-Ka, PrV-ΔUL35, PrV-UL35GFP or PrV-ΔUL35R, and observed three times a day for clinical signs of disease. Animals infected with PrV-Ka and PrV-ΔUL35R developed symptoms like depression, anorexia and scratching of facial and nasal skin 2 days after infection, and died after a mean time of 50 and 52 h, respectively (Table 1). In contrast, mice infected with PrV-ΔUL35 or PrV-UL35GFP showed severe clinical signs at day 3 p.i. and died at 70 or 73 h p.i. (Table 1). The major capsid protein (pUL19) of PrV was detected by immunohistochemistry of skull sections of necropsied animals (Klopfleisch *et al.*, 2004) in epithelial cells of the nasal mucosa of mice infected with either virus at 24 h p.i. At this time, PrV-Ka and PrV-ΔUL35R were also found in first-order neurons of the trigeminal ganglia, whereas PrV-ΔUL35 and PrV-UL35GFP were detectable in neurons starting at 48 h p.i. In second-order neurons of the spinal trigeminal nucleus antigen of PrV-Ka, PrV-ΔUL35 and PrV-ΔUL35R could be first detected at day 2, and of PrV-UL35GFP at day 3 after infection. In mice infected with PrV-ΔUL35 also single third-order neurons in the cortex became positive at day 3 (Table 1). Since it has been shown that the velocity of retrograde axonal transport is not detectably impaired in the absence of pUL35 (Antinone *et al.*, 2006), the delayed neuronal spread of PrV-ΔUL35 may reflect impaired anterograde transport, virion formation and/or release at synaptic membranes.

Most significantly, PrV-UL35GFP exhibited almost identical replication defects as PrV-ΔUL35. Western blot analyses indicated that these defects were not caused by low expression levels or instability of the 38 kDa EGFP–pUL35 fusion protein. Virion incorporation of pUL35, which in HSV-1 requires interaction of the highly conserved C-terminal part (residues 79–100) of VP26 with the major capsid protein (Desai *et al.*, 2003; Rixon *et al.*, 1996), was also not significantly affected by tagging of the less conserved N terminus.

These results strongly indicate that EGFP-tagged pUL35 is non-functional. Non-functional fusions between GFP and pUL35 homologues have also been described for the betaherpesviruses human cytomegalovirus (HCMV) and murine cytomegalovirus, in which the small capsid protein is essential for virion formation (Borst *et al.*, 2001). In these studies it has been suggested that the small capsid protein is required for tegumentation, and that the GFP tag might prevent binding of tegument proteins to the capsid during virion formation. Whereas in betaherpesviruses tegument proteins are in close contact with the whole capsid surface, tegumentation of HSV-1 and, presumably, other alphaherpesviruses seems to initiate with binding of the large

Table 1. Neurovirulence of pUL35 mutants of PrV in mice

Mean time to death after intranasal infection with the indicated virus was calculated for 6–8 animals each. Clinical symptoms were scored as follows: +, slight depression, hunched position, ruffled hair coat; ++, apathy, anorexia, moderate dyspnoea, slight facial pruritus; +++, severe attacks of excitation, self mutilation, skin erosions, heavy dyspnoea; †, moribund or dead. Times of first pUL19 detection by immunohistochemistry are given [nasal cavity: respiratory mucosal epithelium; first-order neurons: trigeminal ganglion; second-order neurons: spinal trigeminal nucleus (Sp5); cortical neurons: ectorhinal cortex]. Most viruses were never detected in cortical neurons (−).

Virus	PrV-Ka	PrV-ΔUL35R	PrV-ΔUL35	PrV-UL35GFP
Mean time to death (SD)	50.3 h (1.09)	52.3 h (2.8)	70.2 h (1.53)	72.8 h (0.76)
Clinical symptoms				
1 day p.i.	−	−	−	−
2 days p.i.	+++	+++	++	++
3 days p.i.	†	†	+++	+++
4 days p.i.			†	†
Immunohistochemistry				
Nasal cavity	1 day p.i.	1 day p.i.	1 day p.i.	1 day p.i.
First-order neurons	1 day p.i.	1 day p.i.	2 days p.i.	2 days p.i.
Second-order neurons	2 days p.i.	2 days p.i.	2 days p.i.	3 days p.i.
Cortical neurons	−	−	3 days p.i.	−

tegument protein pUL36 to pentons, which do not carry pUL35 (Chen *et al.*, 1999; Trus *et al.*, 1999; Wingfield *et al.*, 1997; Zhou *et al.*, 1999). These observations are in line with the non-essential character of pUL35 in alphaherpesviruses, but do not exclude accessory interactions of pUL35 with other tegument proteins, which might be blocked by the GFP tag. Furthermore, binding of the considerably enlarged EGFP–pUL35 fusion proteins to the tips of hexons might sterically affect interactions of tegument proteins with pentonal sites.

Whereas the tagged small capsid proteins of cytomegaloviruses have been shown to induce dominant-negative effects, which could not be complemented by addition of the native gene products (Borst *et al.*, 2001), the *in vitro* and *in vivo* replication defects of PrV-UL35GFP, like those of PrV-ΔUL35, were completely corrected by propagation in cells expressing the native pUL35. PrV-UL35GFP virions prepared from *trans*-complementing cells contained the EGFP–pUL35 fusion protein as well as native pUL35, indicating similar affinities of both proteins to the capsid. The wild-type like replication properties of these particles indicate that functional, native pUL35 is not required in a correct copy number symmetrically attached to all hexons of the virion. This suggests a catalytic or regulatory function of pUL35, e.g. during particle transport (Douglas *et al.*, 2004), rather than a structural role in virion architecture.

In summary, we identified and functionally analysed PrV pUL35. The salient findings are as follows: (i) although PrV pUL35 is non-essential for viral replication, its absence impairs viral growth and plaque formation in cell culture, and delays neuroinvasion in mice; (ii) EGFP-tagging of PrV pUL35 results in similar defects, demonstrating that the fusion protein is non-functional; (iii) the defects associated with absence or expression of non-functional pUL35 were corrected on pUL35-expressing cells. These data are highly relevant, since GFP-tagged pUL35 is widely used to study virus entry and egress in cultured non-neuronal and neuronal cells.

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References

Antinone, S. E., Shubeita, G. T., Coller, K. E., Lee, J. I., Haverlock-Moyns, S., Gross, S. P. & Smith, G. A. (2006). The herpesvirus capsid surface protein, VP26, and the majority of the tegument proteins are dispensable for capsid transport toward the nucleus. *J Virol* **80**, 5494–5498.

Borst, E. M., Mathys, S., Wagner, M., Muranyi, W. & Messerle, M. (2001). The genetic evidence of an essential role for cytomegalovirus small capsid protein in viral growth. *J Virol* **75**, 1450–1458.

Chen, D. H., Jiang, H., Lee, M., Liu, F. & Zhou, Z. H. (1999). Three-dimensional visualization of tegument/capsid interactions in the intact human cytomegalovirus. *Virology* **260**, 10–16.

Davison, M. D., Rixon, F. J. & Davison, A. J. (1992). Identification of genes encoding two capsid proteins (VP24 and VP26) of herpes simplex virus type 1. *J Gen Virol* **73**, 2709–2713.

Davison, A. J., Eberle, R., Hayward, G. S., McGeoch, D. J., Minson, A. C., Pellet, P. E., Roizman, B., Studdert, M. J. & Thiry, E. (2005). Family *Herpesviridae*. In *Virus taxonomy*, pp. 193–212. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball. San Diego: Academic press.

Desai, P. & Person, S. (1998). Incorporation of the green fluorescent protein into the herpes simplex virus type 1 capsid. *J Virol* **72**, 7563–7568.

Desai, P., DeLuca, N. A. & Person, S. (1998). Herpes simplex virus type 1 VP26 is not essential for replication in cell culture but influences production of infectious virus in the nervous system of infected mice. *Virology* **247**, 115–124.

Desai, P., Akpa, J. C. & Person, S. (2003). Residues of VP26 of herpes simplex virus type 1 that are required for its interaction with capsids. *J Virol* **77**, 391–404.

Döhner, K., Radtke, K., Schmidt, S. & Sodeik, B. (2006). Eclipse phase of herpes simplex virus type 1 infection: efficient dynein-mediated capsid transport without the small capsid protein VP26. *J Virol* **80**, 8211–8224.

Douglas, M. W., Diefenbach, R. J., Homa, F. L., Miranda-Saksena, M., Rixon, F. J., Vittone, V., Byth, K. & Cunningham, A. L. (2004). Herpes simplex virus type 1 capsid protein VP26 interacts with dynein light chains RP3 and Tctex1 and plays a role in retrograde cellular transport. *J Biol Chem* **279**, 28522–28530.

Fuchs, W., Klupp, B. G., Granzow, H., Osterrieder, N. & Mettenleiter, T. C. (2002). The interacting UL31 and UL34 gene products of pseudorabies virus are involved in egress from the host-cell nucleus and represent components of primary enveloped but not mature virions. *J Virol* **76**, 364–378.

Kaplan, A. S. & Vatter, A. E. (1959). A comparison of the herpes simplex and pseudorabies viruses. *Virology* **7**, 394–407.

Karger, A., Bettin, B., Granzow, H. & Mettenleiter, T. C. (1998). Simple and rapid purification of alphaherpesviruses by chromatography on a cation exchange membrane. *J Virol Methods* **70**, 219–224.

Klopfleisch, R., Teifke, J. P., Fuchs, W., Kopp, M., Klupp, B. G. & Mettenleiter, T. C. (2004). Influence of tegument proteins of pseudorabies virus on neuroinvasion and transneuronal spread in the nervous system of adult mice after intranasal inoculation. *J Virol* **78**, 2956–2966.

Klupp, B. G., Hengartner, C. J., Mettenleiter, T. C. & Enquist, L. W. (2004). Complete annotated sequence of the pseudorabies virus genome. *J Virol* **78**, 424–440.

Kopp, M., Granzow, H., Fuchs, W., Klupp, B. G., Mundt, E., Karger, A. & Mettenleiter, T. C. (2003). The pseudorabies virus UL11 protein is a virion component involved in secondary envelopment in the cytoplasm. *J Virol* **77**, 5339–5351.

McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., McNab, D., Perry, L. J., Scott, J. E. & Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J Gen Virol* **69**, 1531–1574.

McNabb, D. S. & Courtney, R. J. (1992). Identification and characterization of the herpes simplex virus type 1 virion protein encoded by the UL35 open reading frame. *J Virol* **66**, 2653–2663.

Mettenleiter, T. C. (2000). Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis – state of the art, June 1999. *Vet Res* **31**, 99–115.

Mettenleiter, T. C. (2002). Herpesvirus assembly and egress. *J Virol* **76**, 1537–1547.

Rixon, F. J., Addison, C., McGregor, A., Macnab, S. J., Nicholson, P., Preston, V. G. & Tatman, J. D. (1996). Multiple interactions control the intracellular localization of the herpes simplex virus type 1 capsid proteins. *J Gen Virol* **77**, 2251–2260.

Roizman, B. & Knipe, D. M. (2001). Herpes simplex viruses and their replication. In *Fields Virology*, pp. 2399–2459. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Smith, G. A., Gross, S. P. & Enquist, L. W. (2001). Herpesviruses use bidirectional fast-axonal transport to spread in sensory neurons. *Proc Natl Acad Sci U S A* **98**, 3466–3470.

Smith, G. A., Pomeranz, L., Gross, S. P. & Enquist, L. W. (2004). Local modulation of plus-end transport targets herpesvirus entry and egress in sensory axons. *Proc Natl Acad Sci U S A* **101**, 16034–16039.

Snyder, A., Wisner, T. W. & Johnson, D. C. (2006). Herpes simplex virus capsids are transported in neuronal axons without an envelope containing the viral glycoproteins. *J Virol* **80**, 11165–11177.

Sodeik, B., Ebersold, M. W. & Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J Cell Biol* **136**, 1007–1021.

Tatman, J. D., Preston, V. G., Nicholson, P., Elliot, R. M. & Rixon, F. J. (1994). Assembly of herpes simplex virus type 1 capsids using a panel of recombinant baculoviruses. *J Gen Virol* **75**, 1101–1113.

Trus, B. L., Homa, F. L., Booy, F. P., Newcomb, W. W., Thomsen, D. R., Cheng, N., Brown, J. C. & Steven, A. C. (1995). Herpes simplex virus capsids assembled in insect cells infected with recombinant baculoviruses: structural authenticity and localization of VP26. *J Virol* **69**, 7362–7366.

Trus, B. L., Gibson, W., Cheng, N. & Steven, A. C. (1999). Capsid structure of simian cytomegalovirus from cryoelectron microscopy: evidence for tegument attachment sites. *J Virol* **73**, 2181–2192.

Ward, P. L., Ogle, W. O. & Roizman, B. (1996). Assemblons: nuclear structures defined by aggregation of immature capsid and some tegument proteins of herpes simplex virus 1. *J Virol* **70**, 4623–4631.

Wingfield, P. T., Stahl, S. J., Thomsen, D. R., Homa, F. L., Booy, F. P., Trus, B. L. & Steven, A. C. (1997). Hexon-only binding of VP26 reflects differences between the hexon and penton conformations of VP5, the major capsid protein of herpes simplex virus. *J Virol* **71**, 8955–8961.

Wolfstein, A., Nagel, C. H., Radtke, K., Döhner, K., Allan, V. J. & Sodeik, B. (2006). The inner tegument promotes herpes simplex virus capsid motility along microtubules *in vitro*. *Traffic* **7**, 227–237.

Zhou, Z. H., He, J., Jakana, J., Tatman, J. D., Rixon, F. J. & Chiu, W. (1995). Assembly of VP26 in herpes simplex virus-1 inferred from structures of wild-type and recombinant capsids. *Nat Struct Biol* **2**, 1026–1030.

Zhou, Z. H., Chen, D. H., Jakana, J., Rixon, F. J. & Chiu, W. (1999). Visualization of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions. *J Virol* **73**, 3210–3218.