Herpes Simplex Virus Type 1 Capsid Protein VP26 Interacts with Dynein Light Chains RP3 and Tctex1 and Plays a Role in Retrograde Cellular Transport

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Cytoplasmic dynein is the major molecular motor involved in minus-end-directed cellular transport along microtubules. There is increasing evidence that the retrograde transport of herpes simplex virus type 1 along sensory axons is mediated by cytoplasmic dynein, but the viral and cellular proteins involved are not known. Here we report that the herpes simplex virus outer capsid protein VP26 interacts with dynein light chains RP3 and Tctex1 and is sufficient to mediate retrograde transport of viral capsids in a cellular model. A library of herpes simplex virus capsid and tegument structural genes was constructed and tested for interactions with dynein subunits in a yeast two-hybrid system. A strong interaction was detected between VP26 and the homologous 14-kDa dynein light chains RP3 and Tctex1. In vitro pull-down assays confirmed binding of VP26 to RP3, Tctex1, and intact cytoplasmic dynein complexes. Recombinant herpes simplex virus capsids were constructed either with or without VP26. In pull-down assays VP26+ capsids bound to RP3; VP26− capsids did not. To investigate intracellular transport, the recombinant viral capsids were microinjected into living cells and incubated at 37 °C. After 1 h VP26+ capsids were observed to co-localize with RP3, Tctex1, and microtubules. After 2 or 4 h VP26+ capsids had moved closer to the cell nucleus, whereas VP26− capsids remained in a random distribution. We propose that VP26 mediates binding of incoming herpes simplex virus capsids to cytoplasmic dynein during cellular infection, through interactions with dynein light chains.

Herpes simplex virus type 1 (HSV-1) infects 40–80% of people worldwide and can cause potentially fatal meningencephalitis in adults or disseminated infection in neonates, in addition to common mucocutaneous disease. After inoculation of the skin or mucous membrane, HSV-1 is transported along sensory axons in a retrograde direction to the neuronal cell body, where it establishes life-long latent infection. Periodic reactivation results in HSV-1 being transported in an anterograde direction to nerve terminals, where it causes recurrent clinical disease or asymptomatic viral shedding (1). The double-stranded DNA virus HSV-1 has a 1250-Å icosahedral protein capsid, surrounded by a less structured protein tegument layer, in turn surrounded by a lipid envelope containing several glycoproteins. The major capsid proteins VP5, VP19C, VP23, and VP26 are self-assembling when expressed in vitro using recombinant baculoviruses (2, 3).

During infection, HSV-1 binds to cell surface receptors (via glycoproteins), enters the cell by membrane fusion, then most but not all tegument proteins dissociate from the nucleocapsid after phosphorylation (4, 5). The nucleocapsid-tegument complex is transported to the outer nuclear membrane where it docks and releases viral DNA into the nucleus, but the capsid itself does not enter the nucleus (6). There is evidence that the rapid "retrograde" transport of this complex to the cell nucleus involves microtubules and is mediated by the minus-end-directed molecular motor cytoplasmic dynein (7–9). Retrograde transport of the closely related alphaherpesvirus pseudorabies virus (PRV) has been observed in live cells to be saltatory, with brief, rapid transport events (10). The herpes viral proteins that mediate retrograde transport are unknown but are likely to involve outer capsid or inner tegument proteins. In support of this, HSV-1 capsids, stripped of envelope and much of the tegument protein by detergent lysis, move in a retrograde direction after injection into giant squid axons (11). The site of attachment for the dynein complex on the capsid has not been confirmed.

Cytoplasmic dynein is a large (1.2 MDa) complex, with heavy chains providing motive force, whereas intermediate and light chains contribute to cargo binding (12). Regulation of dynein function is not well understood but is thought to involve the multisubunit complex dynactin, which is also involved in binding membranous cargo (13). Light chain LC8 has been reported to interact with proteins from rabies virus (14, 15), African swine fever virus (16), human adenovirus, vaccinia virus, and human papillomavirus (17), whereas Tctex1 interacts with the poliovirus receptor CD155 (18). Despite a recently reported interaction between dynein intermediate chain (DIC) and HSV-1 protein UL34 (19), its role in retrograde transport has yet to be confirmed. The protein product of UL34 is absent from mature virions in HSV-1 (20) and PRV (21). Furthermore, deletion of UL34 from HSV-1 does not prevent infection of cells (22).
Previous work in our laboratory has concentrated on anterograde axonal transport of HSV-1. We have shown that newly formed HSV-1 capsids, having acquired much of their tegument in the neuron cell body (23), are transported in an anterograde direction along axons, separate from glycoproteins (24, 25). Similar observations have been made for PRV (26). Fast anterograde, microtubule-dependent transport is mediated by the kinesin family of molecular motors (27). We have shown previously (28) that the HSV-1 tegument protein US11 interacts with the ubiquitously heavy chain of the kinesin motor KIF5B and is likely to play an important role in anterograde axonal transport.

By using a similar approach for retrograde transport, we report an interaction between the HSV-1 capsid protein VP26 and 14-kDa dynein light chains RP3 and Tctex1. These light chains are 55% homologous at the amino acid level and are mutually exclusive in cytoplasmic dynein complexes (29–31). We propose that VP26 mediates binding of the HSV-1 nucleocapsid to cytoplasmic dynein, via interactions with RP3 and probably Tctex1, during retrograde axonal transport of virus in neurons as well as during infection of non-neuronal cells.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—Genes were amplified by PCR using Geneamp® XL PCR Kit (Applied Biosystems). The entire open reading frame for each gene was cloned into recombinant expression vectors in all cases except for HSV-1 UL36, where the N-terminal two-thirds of the gene (amino acids 24–1874) were cloned. Oligonucleotide primers, incorporating EcoRI and/or XhoI restriction endonuclease sites for most constructs, were designed using Primer3 software (32) and BioManager by ANGIS (www.angis.org.au). For UL36 a 3’ BglII site was used for insertion into BamHI sites, and for UL32 NcoI sites were used. Human Dic1 was amplified from a pBluescript plasmid, kindly provided by Dr. Lap-Ching Tse, Faul University of Toronto (34). Genes for dynein light chains Tctex1, RP3, and LCS were amplified from human brain cDNA library (Display Biosystems Biotech). HSV-1 genes were amplified from overlapping DNA cosmids encoding strain 17 (35, 36), except for US11 which was amplified from plasmid pRB4766 (strain 17), kindly provided by Bernard Roizman, University of Chicago (36). UL19 was excised from plasmid pE19 (33) with BglII and inserted into a BamHI site. Digested PCR products were inserted into expression plasmids using a Clonable® kit (Novagen). Genes were cloned into pGEX-SX-1 (Amer sham Biosciences) for glutathione S-transferase (GST) tag fusion protein expression or pET-28a (Novagen) for hexahistidine (His6) tag fusion protein expression. For the LexA yeast two-hybrid system genes were amplified into displayBait and/or displayTarget vectors (Display Systems Biotech). All constructs were sequenced to confirm gene sequence.

**Preparation of Recombinant HSV-1 Capsids**—Recombinant HSV-1 capsids were prepared as described previously (3). Briefly, recombinant baculoviruses were used to express either five or six HSV-1 capsid genes (UL18, UL19, UL26, UL26.5, and UL38, with or without UL35) in insect Sf9 cells. The resulting capsids, purified by sucrose gradient centrifugation, have the same morphology as HSV B capsids (3, 39). All recombinant capsids contain major capsid proteins VP5, VP16C, and VP23 and scaffolding proteins VP21, VP22a, and VP24. Capsids formed in the presence of UL35 also contain capsid protein VP26. For microinjection, capsids were diluted 1:10 in 20 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM NaCl and 1 mM EDTA, subjected to sonication in a cup-horn water bath (three times for 40 s, 50% duty cycle), and centrifuged at 15,000 × g for 30 min. The approximate final protein concentration, measured by Bio-Rad Protein Assay, was 0.1 mg/ml.

**Microinjection of Recombinant HSV-1 Capsids**—HeP2 cells were grown at 37 °C (5% CO₂) on CEL Locate glass coverslips (Eppendorf) in Dulbecco’s modified Eagle’s medium (Invitrogen) with 9% fetal calf serum (JRH Biosciences). Microinjection was performed at room temperature, using Dulbecco’s modified Eagle’s medium with 2% fetal calf serum, buffered with 25 mM HEPES-NaOH, pH 7.4. For co-localization experiments PTK2 cells were grown in minimum Eagle’s medium (Invitrogen) supplemented with nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), and 10% fetal calf serum (JRH Biosciences). An Eppendorf FemtotJet® microinjector and InjectMan® N12 microinjection microscopy were used to inject recombinant HSV-1 capsids through a Femtotip® glass micropipette into HeP2 or PTK2 cells. The injection parameters used are as follows: compensation pressure, 50 kPa; injection pressure, 280–300 kPa (range 200–400 kPa); injection time, 0.2–0.4 s. Between 200 and 400 cells were injected for each experimental group or time point.

**Immunofluorescence and Confocal Microscopy**—Microinjected HeP2 cells were fixed and permeabilized as described previously (25), either immediately or after 2–4 h of incubation at 37 °C. Sensitive and specific labeling of intra-cytoplasmic capsids was obtained with rabbit polyclonal anti-VP5 antibody (NC1, kindly provided by Dr. G. Cohen and Dr. R. Eisenberg, University of Pennsylvania, Philadelphia (40)). Some microinjected capsids did not involve the nuclear membrane or cytoplasm. Mouse anti-bovine α-tubulin antibody (monoclonal antibody 236-10501, Molecular Probes) was used to label microtubules. Immunolabeling was as described previously (25), except antibodies were diluted in Tris-buffered saline with goat serum (1.5% v/v), bovine serum albumin (0.1% w/v), Tween 20 (0.1% v/v), and NaN₃ (0.02%). Secondary antibodies were Cy3® or Cy5® (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were mounted with Vectashield (Vector Laboratories). Images were captured with a cooled charge-coupled device camera (Photometrics) and processed with Metamorph software (Molecular Devices). Images were analyzed with ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD). Statistical analysis was performed using Welch’s t test. The level of significance was determined by ANOVA, with post hoc Tukey’s HSD test. Data were considered significant at the level of p < 0.05.

**Co-localization Experiments**—Microinjected PTK2 cells were incubated for 1 h at 37 °C fixed, and permeabilized (25). Dynein light chains were detected with rabbit polyclonal antibodies (a kind gift of Dr. Michael Fishkind, University of Connecticut Health Center, Farmington, CT). Primary antibody R5270 against RP3 (30) or R5205 against Tctex1 (41) was used as above, followed by Cy3®-conjugated anti-rabbit IgG (Amersham Biosciences). Microtubules were labeled as above. Capsids were detected using anti-VP5 antibodies, either rabbit polyclonal...
HSV-1 proteins, inserted in Bait vector, were tested for interaction with dynein subunits and inserted in Target vector in a LexA yeast two-hybrid system. *Positive interactions,* defined as growth on leucine-deficient media and blue color change of yeast colonies within 48 h, were confirmed using a quantitative β-galactosidase assay (shown in arbitrary units). The symbols used are as follows: +, 10–50; ++, 20–300; ++++, 300–1000; +++++, >1000; −−−−, no color change at 48 h; −−−−, no color change but protein was poorly expressed; AA, strong auto-activation at 48 h; ND, not determined due to autoactivation; −−−−ve, display Target with no protein insert.

**Table I**

| Bait   | DIC          | LC8          | RP3          | Tctex1       | UL6          | UL38         |
|--------|--------------|--------------|--------------|--------------|--------------|--------------|
| DIC    | −−−−         | +++++        | +            | +++++        | −            | −            |
| LC8    | −            | +            | +            | −            | −            | −            |
| US11   | −            | −            | −            | −            | −            | −            |
| UL6    | −            | −            | −            | −            | −            | −            |
| UL13   | (−)          | (−)          | (−)          | (−)          | (−)          | (−)          |
| UL17   | AA           | ND           | ND           | ND           | ND           | ND           |
| UL18 (VP23) | −            | +            | +            | ++++         | +            | −            |
| UL19 (VP5) | (−)          | (−)          | (−)          | (−)          | (−)          | (−)          |
| UL25   | AA           | ND           | ND           | ND           | ND           | ND           |
| UL32   | AA           | ND           | ND           | ND           | ND           | ND           |
| UL33 (VP26) | −            | +            | +            | ++++         | +            | −            |
| UL36 (VP12) | −            | −            | −            | −            | −            | −            |
| UL37   | −            | −            | −            | −            | −            | −            |
| UL38 (VP19C) | AA           | ND           | ND           | ND           | ND           | ND           |
| UL41 (VHS) | −            | −            | −            | −            | −            | −            |
| UL46 (VP11/12) | −            | +            | +            | ++++         | +            | −            |
| UL47 (VP13/14) | −            | −            | −            | −            | −            | −            |
| UL48 (VP16) | AA           | ND           | ND           | ND           | ND           | ND           |

*UL38 amino acids 1–1874.

**RESULTS**

**VP26 and VP11/12 Bind to RP3 and Tctex1 in the Yeast Two-hybrid System.—**A yeast two-hybrid matrix approach was used to screen for interactions between HSV-1 capsid or tegument proteins and cytoplasmic dynein. Genes for dynein subunits DIC, LC8, RP3, and Tctex1, as well as for HSV-1 capsid and tegument proteins, were cloned into both Bait vector (fusion construct with LexA DNA binding domain), and Target vector (fusion construct with B42 DNA activation domain). The proteins were tested pairwise for interactions in all available combinations. A strong interaction was indicated by both growth on media lacking leucine and a blue color change of yeast colonies within 48 h. Interactions were further confirmed with a quantitative β-galactosidase assay. Known interactions between DIC and dynein light chains (31, 44, 45), as well as LC8 dimerization, were confirmed in our system (Table I).

With HSV-1 proteins in Bait vector a strong interaction was detected between VP26 (UL35) and VP11/12 (UL46) with homologous 14-kDa dynein light chains RP3 and Tctex1 (Table I). β-Galactosidase activity for each protein interaction was measured using a quantitative assay and statistically examined using analysis of variance (Fig. 1). β-Galactosidase activity was significantly greater for interactions with RP3 or Tctex1 than for DIC (p < 0.05), LC8 (p < 0.004), or the negative control (p < 0.003). The apparent small increase for interactions with DIC and LC8 was not statistically significant. The known interaction between HSV-1 capsid proteins VP23 and VP19C (46) was confirmed in our system.

With dynein subunits in Bait vector, RP3 and Tctex1 strongly auto-activated, so they could not be tested further. No interactions were detected between DIC or LC8 in Bait vector and any of the HSV-1 proteins tested in Target vector (data not shown). The previously reported interaction between DIC and UL34 could not be confirmed because UL34 was poorly expressed when inserted in Target vector.

His<sup>gro</sup>-VP26 Binds to GST-RP3 and GST-Tctex1 in Vitro—His<sup>gro</sup> fusions constructs of HSV-1 proteins VP26 and VP11/12 were expressed in *E. coli*. His<sup>gro</sup>-VP26 formed insoluble inclusion bodies, which were solubilized by denaturing with 8 M urea and then slowly refolded by dilution to 1 M urea. VP26 expressed in bacteria, denatured, and then refolded has been shown previously to bind to HSV-1 capsids, in either CHAPS buffer or 1 M urea (47, 48). His<sup>gro</sup>-VP11/12 was only expressed at low concentrations. In vitro pull-down assays were performed by incubating His<sup>gro</sup>-tagged HSV-1 proteins with GST fusion constructs of dynein subunits DIC, LC8, RP3, and Tctex1, bound to glutathione-Sepharose beads. Expression and subsequent elution from glutathione-Sepharose beads were confirmed for each GST-dynein construct, with GST-DIC present at lower concentrations than the other constructs (Fig. 2A). Refolded His<sup>gro</sup>- VP26 bound to GST-Tctex1 and GST-RP3, but not to GST-DIC, GST-LC8, or GST alone (Fig. 2, B and C). Binding was not mediated via the His<sup>gro</sup> tag, because a βHis<sup>gro</sup>-tagged kinesin heavy chain fragment (His<sup>gro</sup>-KIF5B residues 771–963) did not bind to RP3 or Tctex1 (Fig. 2B). There was no evidence of dynein...
Hep2 cells were cultured on glass coverslips and microinjected with a suspension of recombinant HSV-1 capsids. HSV-1 Capsid Protein VP26 Interacts with Dynein

**Fig. 1.** HSV-1 proteins VP26 and VP11/12 interact with dynein light chains RP3 and Tctex1 in a yeast two-hybrid system. Positive interactions in a yeast two-hybrid screen (Table I) were confirmed using a quantitative β-galactosidase assay. This figure summarizes the mean β-galactosidase activity for interactions with VP26 (A) and VP11/12 (B), including standard errors, in replicate experiments. Analysis of variance and Tukey’s correction for multiple comparisons confirmed that β-galactosidase activity was significantly greater for interactions with RP3 or Tctex1 than for DIC (p < 0.05), LC8 (p < 0.004), or the negative control (p < 0.003). The apparent small increase for interactions with DIC and LC8 was not statistically significant.

![Graph showing β-Galactosidase Activity](image)

**Fig. 2.** His$_8$-VP26 binds GST-RP3 and GST-Tctex1 in vitro. Purified His$_8$-VP26 and His$_8$-KIF5B (amino acids 771–963, His$_8$ tag negative control) were incubated with GST-tagged dynein subunits, bound to glutathione-Sepharose beads, in a pull-down assay. His$_8$-VP26 bound to dynein light chains GST-RP3 and GST-Tctex1 but not to GST-DIC, GST-LC8, or GST control. His$_8$-VP26 was initially detected with Coomassie Blue stain (A), before confirmation on Western blots using anti-His$_8$ (B) and anti-VP26 (C) antibodies. Expression, binding, and elution of all GST-dynein fusion constructs were confirmed with Coomassie Blue stain (A). GST-DIC was present at lower concentrations than other fusion constructs.

![Western Blot Comparison](image)

binding to His$_8$-VP11/12, but this may simply reflect low expression levels (data not shown).

**His$_8$-VP26 binds to Intact Dynein Complexes in Vitro**—To confirm that VP26 binds to dynein light chains in a dynein complex, as well as in solution, an in vitro pull-down assay of cytoplasmic dynein complexes was performed. His$_8$ fusion constructs of HSV-1 proteins VP26 (refolded as above) and US11 (negative control) were bound to nickel beads and incubated with lysates from uninfected Hep2 cells, and the eluted complexes were analyzed by SDS-PAGE and Western blot, with dynein complexes detected by anti-DIC antibody. Dynein complexes, detected in cell lysates, bound to His$_8$-VP26 but not to His$_8$-US11 (A). An equivalent concentration of His$_8$-VP26 and His$_8$-US11 in eluted fractions was confirmed by Coomassie Blue stain (B).

![Western Blot Comparison](image)

**Recombinant HSV-1 Capsids bind to GST-RP3**—To investigate the behavior of capsid proteins in their usual tertiary and quaternary structure, recombinant HSV-1 capsids were constructed with or without VP26 (VP26+ or VP26−). These capsids were examined by electron microscopy and shown to be in single particle suspension (data not shown). They were used in pull-down assays with GST-tagged dynein subunits bound to beads, as above. The presence or absence of VP26 was confirmed by Coomassie Blue stain and Western blot (Fig. 4, A and B). VP26+ capsids bound to GST-RP3, but not to other dynein subunits, in replicate experiments (Fig. 5). VP26− capsids did not bind significantly above background in either experiment. The presence of capsids bound to any of the dynein chains was detected using polyclonal (NC1) antibody against the major capsid protein VP5.

**Recombinant HSV-1 Capsids Co-localize with RP3, Tctex1, and Microtubules in Living Cells**—To confirm an in vivo interaction between HSV-1 capsids and cytoplasmic dynein, PTK2 cells were cultured on glass coverslips and microinjected with a suspension of recombinant HSV-1 capsids (VP26+). Cells were fixed after 1 h at 37 °C and labeled for capsids (anti-VP5 antibody), as well as for either dynein light chains (anti-RP3 or anti-Tctex1 antibody) or microtubules (anti-α-tubulin antibody). Image overlays show the distribution of capsids within each cell and demonstrate co-localization of capsids with dynein light chains RP3 and Tctex1 (Fig. 6A), as well as with microtubules (Fig. 6C). Formal image analysis using the Boolean “AND” operator confirmed co-localization with dynein and microtubules for the majority of HSV-1 capsids (Fig. 6, B and D). RP3 and Tctex1 were also present in the cytoplasm of Hep2 cells (data not shown).

**Recombinant HSV-1 Capsids Containing VP26 Migrate toward the Nucleus in Living Cells**—To confirm a functional biological significance for the interaction between VP26 and dynein, Hep2 cells were cultured on glass coverslips and microinjected with a suspension of recombinant HSV-1 capsids
Recombinant HSV-1 capsids, with (VP26+) or without (VP26−) the 12-kDa capsid protein VP26, were formed by expressing capsid proteins in Sf9 insect cells using baculovirus vectors and purified on a sucrose gradient. The presence of VP26 in only the VP26+ capsids was confirmed with Coomassie Blue stain (A) and Western blot (B). There was a similar content of VP5 in each capsid preparation, as shown by Western blot (B), using NC1 polyclonal antibody. Proteolytic fragments of VP5 were also present.

We initially demonstrated binding of VP26 to dynein light chains RP3 and Tctex1 in a yeast two-hybrid system, and we confirmed strong expression of the β-galactosidase reporter by quantitative assay. Subsequent work confirmed binding of recombinant, soluble VP26 to RP3 and Tctex1 in vitro, using bacterial expression and GST pull-down assays. We also confirmed that immobilized His6-VP26 is able to bind to intact cytoplasmic dynein complexes in cell lysates. Most important, we have confirmed binding of dynein (RP3) to VP26 in its biologically relevant form as part of a recombinant viral capsid bound to VP5.

Our results suggest that the binding of HSV-1 outer capsid protein VP26 to dynein light chains RP3, and probably Tctex1, is important in the retrograde transport of viral capsids toward the cell nucleus during infection.

Cytoplasmic dynein is the major minus-end-directed, microtubule-associated molecular motor, and each dynein complex contains two copies of the same 14-kDa light chain, either RP3 or Tctex1. The two chains, although 55% homologous at the amino acid level (30), are mutually exclusive in each complex, compete for binding sites on DIC, and have differing binding specificities (31).

We have demonstrated binding of VP26 to dynein light chains RP3 and Tctex1 in vitro. Furthermore, in microinjected cells we have shown colocalization of HSV-1 capsids containing VP26 with RP3, Tctex1, and microtubules, further supporting a functional role for VP26-dynein interactions.

Despite strong interaction between VP26 and Tctex1 in vitro and in vivo pull-down assays (using His6-VP26), and co-localization of HSV-1 capsids with Tctex1, we were unable to confirm binding of VP26+ capsids to Tctex1 in vitro. We believe that this result most likely reflects a lower sensitivity for this particular assay, in light of the other in vitro binding results. The ability to bind either RP3 or Tctex1 would be expected to offer HSV-1 an evolutionary advantage, by allowing retrograde transport of HSV-1 capsids on any cytoplasmic dynein complex in all cell types. We cannot exclude that recombinant Tctex1 may only bind to soluble VP26 in vitro and not to VP26 incorporated into recombinant HSV-1 capsids. However, the co-localization of VP26+ capsids with Tctex1 in live cells suggests that VP26 interacts with Tctex1, as well as
RP3, in this more biologically relevant model.

HSV-1 readily infects mucous membranes in both adults and children, before being transported in a retrograde direction along neuronal axons to establish latent infection. RP3 in particular is an attractive candidate to mediate this retrograde transport, as it is expressed at high levels in cells infected by HSV-1, including adult brain and many other tissue types, whereas Tctex1 is expressed predominantly in fetal tissue and testis (30). Furthermore, it has been proposed that RP3-containing dynein complexes play the predominant role during retrograde transport from post-synaptic nerve terminals (29), the site of HSV-1 entry.

VP26, at the hexon tips, is ideally located to interact with molecular motors (48), because most of the tegument is lost.

**Fig. 6. HSV-1 capsids co-localize with Tctex1, RP3, and microtubules.** PTK2 cells, grown on glass coverslips, were microinjected with recombinant HSV-1 capsids (VP26+). After 1 h at 37 °C the cells were fixed, immunolabeled, and examined by confocal microscopy. Capsids were labeled with anti-VP5 antibody (green), and cells with anti-Tctex1, anti-RP3, or anti-α-tubulin antibody (red). Over half the capsids were found to co-localize with dynein light chains Tctex1 (A and B), RP3 (A and B, insets), and microtubules (C and D). To look for co-localization, red and green images were initially overlaid (A and C), and co-localization was formally analyzed using the confocal software (B and D). The Boolean AND operator was used, and where a red and green pixel co-localized, a bright pixel was displayed; where there was no co-localization, a black pixel was displayed.
soon after cell entry (6). Tegument proteins VP11/12, VP13/14, VP16, and VP22 are known to dissociate from the virus at early stages of infection, whereas the major tegument protein VP1/2 and the minor capsid protein UL25 probably remain attached to incoming capsids (4, 5, 8, 50, 51). VP1/2 appears to attach to capsids at the vertical pentons (52), whereas VP26 is found exclusively on hexons (39, 48, 53) and does not interfere with capsid-tegument interaction (54). This specific binding is prob-

**Fig. 7.** VP26+ but not VP26− capsids move toward the nucleus in Hep2 cells. Recombinant HSV-1 capsids (VP26− or VP26+) were microinjected into Hep2 cells grown on glass coverslips. The cells were incubated for 0 or 2 h at 37 °C and then fixed. Capsids were labeled using NC1 polyclonal anti-VP5 antibody (green) and microtubules with monoclonal anti-α-tubulin antibody (red). Fluorescent particles (arrows) were clearly visible in the cytoplasm of injected cells (A–D), which were not present in un.injected cells (E). After 2 h, a redistribution of VP26+ (D) but not VP26− (B) capsids toward the cell nucleus was observed in these typical images. Nonspecific labeling of cell nuclei was observed with NC1 antibody (E), so intra-nuclear fluorescence was not included in our analysis. To quantify movement of HSV-1 capsids over time, the distance of individual fluorescent particles was measured from both the cell periphery b and nuclear membrane a, with the aid of image analysis software (F). Relative nuclear migration for each particle was expressed as a Nuclear Migration Index, calculated as $b/(a + b)$ (43). Nuc, nucleus. Thus particles near the cell periphery received values close to 0, and particles near the nucleus received values close to 1.
After 2 or 4 h, which was absent for VP26/H11002, but this may simply reflect diffusion to a truly protease such as UL25, may also contribute to dynein binding. We cannot exclude that VP1/2, or one of the minor capsid proteins to HSV-1 transport is unclear and requires further study.

Advantageous for the virus, and there are precedents for functional redundancy in other HSV-1 proteins (57). In our yeast two-hybrid system there was no evidence of interaction between dynein and a large fragment of VP1/2 (N-terminal two-thirds), but it was not possible to clone the rest of the protein. Neither was it possible to test UL25 in our system for possible interaction with RP3 or Tctex1, due to autoactivation.

The role of VP26 during viral transport in vivo remains to be determined. There has been one previous study, in a mouse eye model, dissecting the role of VP26 in retrograde transport of HSV-1 to, and replication in, trigeminal ganglia (56). Deletion of VP26 decreased the amount of infectious virus in trigeminal ganglia by 100-fold, whereas titer in cell culture (where retrograde transport is less critical) were decreased only 2-fold. Simultaneous deletion of thymidine kinase (and thus replication) ablated the effects of VP26 deletion at 72 h after ocular infection, suggesting the virus could still be transported in the absence of VP26. However, earlier time points may be needed to detect a decrease in transport velocity or efficiency, because wild-type virus can arrive as early as 19 h postinfection (58).

In our current study we set out to determine the viral and cellular proteins that mediate retrograde axonal transport of HSV-1. Our use of protein-protein interaction assays and minimal recombinant capsids should be a more sensitive way to detect HSV-dynein interactions than traditional approaches, relying on single gene null mutants, which can miss important interactions where multifunctional or redundant proteins exist (10).

We have demonstrated by two different assays that VP26 binds to dynein light chains RP3 and Tctex1 in vitro, and we confirmed binding of VP26 to intact dynein complexes. We have demonstrated co-localization in cells of micro-injected HSV-1 capsids with RP3, Tctex1, and microtubules. We have shown further that VP26 in its biologically relevant form is able to mediate binding of recombinant HSV-1 capsids to RP3 in vitro, in the absence of minor capsid or tegument proteins. Finally, we have shown that VP26, incorporated into recombinant capsids, is both necessary and sufficient to mediate retrograde intracellular transport of capsids. We therefore propose that VP26, although not essential for viral replication in vitro, is likely to be one of two or more HSV proteins mediating retrograde axonal transport in vivo, perhaps another example of redundancy for key viral functions.

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