The cytoplasmic tail of herpes simplex virus envelope glycoprotein D binds to the tegument protein VP22 and to capsids

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INTRODUCTION

Herpes simplex virus (HSV) is a complex DNA virus. The viral genome is packaged within an icosahedral capsid surrounded by an amorphous layer of proteins termed the tegument. This in turn is contained within a lipid envelope bearing numerous virally encoded glycoproteins (Roizman & Pellett, 2001).

It is generally agreed that HSV capsids acquire their final tegument and envelope in the cytoplasm. However, the molecular mechanism of HSV-1 envelopment in the cytoplasm is poorly characterized. During infection, viral membrane glycoproteins are expressed and traffic via cytoplasmic organelles to the cell surface. Several lines of evidence suggest that the cytosolic domains of these glycoproteins may provide a platform for tegument assembly and recruitment of capsids followed by envelopment (Mettenleiter, 2002). In a recent study, a double mutant of pseudorabies virus (PrV) lacking viral glycoproteins gM and gE (or gM and the cytoplasmic tail of gE) showed a failure in envelopment, instead accumulating cytoplasmic nucleocapsids immersed in a dense layer of tegument (Brack et al., 1999). Following single deletion of either glycoprotein, the production of infectious virions was not affected, suggesting that the cytoplasmic tails of gE and gM act in a redundant fashion during assembly, most likely interacting with the tegument proteins and, indirectly, with the surface of nucleocapsids. In HSV-1, simultaneous deletion of the same two glycoproteins did not dramatically affect production of infectious virions (Browne et al., 2004), although simultaneous deletion of gD and gE resulted in a striking defect in particle assembly (Farnsworth et al., 2003). This apparent redundancy makes analysis of specific glycoprotein–tegument interactions difficult to perform in vivo. To overcome this obstacle, we developed a glutathione S-transferase (GST) fusion protein-binding assay to investigate the interaction between envelope glycoprotein tails and tegument partners in vitro. This approach has previously been used to demonstrate the interaction between the cytoplasmic tail of glycoprotein H (gH) and the tegument component VP16 (Gross et al., 2003). In the current study, we focused on analysing the tail of the glycoprotein D (gD) because it is a highly abundant component of the envelope (Spear & Roizman, 1972) with a short cytoplasmic tail of 30 amino acid residues that can easily be manipulated in mutagenesis studies. Moreover, it has been shown to play a key, albeit redundant, role in assembly (Farnsworth et al., 2003). Additionally, Pomeranz & Blaho (1999) reported that gD co-localizes with at least one tegument protein, VP22, during infection.

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We prepared GST fusion proteins that contained the cytoplasmic tail of gD or mutants thereof at their carboxy terminus. Following binding of the GST fusion proteins to glutathione–Sepharose beads and incubation with HSV-infected cell extracts, these fusion proteins were tested for their ability to interact with tegument proteins. Here we report that the cytoplasmic tail of gD bound to the tegument protein VP22 in a manner that was independent of other viral polypeptides. Additionally, our results indicated that VP22 may be one of the tegument components that mediate the interaction between the cytoplasmic tail of gD and the HSV capsid.

**METHODS**

**Construction of a GST–gD-expressing vector.** The predicted cytoplasmic tail of HSV-1 gD, encoded by the 3′ end of the US6 gene, was amplified by PCR from genomic DNA using the oligonucleotides 5′-GGCGAATTCTACACCGCCGCACTCGGAAAAAGGCGATACGGCTTTGGGGCTTTCC-3′ (upstream primer) and 5′-GGGAAGGTTAATTAGTCATCACTAAGTTCA-3′ (downstream primer). The PCR product encoded the entire carboxyl-terminal tail of gD with two stop codons, flanked by EcoRI and HindIII restriction sites. Digestion with these two restriction enzymes, followed by ligation to EcoRI/HindIII-digested pGEX-KG plasmid (Guan & Dixon, 1991), resulted in an in-frame fusion of GST with the gD cytoplasmic tail ORF. This construct will be referred to as gDc. A truncation mutant expressing only the first 17 amino acids of the cytoplasmic tail of gD, termed T17, was constructed using complementary oligonucleotides 5′-AAATTCTACACCGCCGCACTCGGAAAAAGGCGATACGGCTTTGGGGCTTTCC-3′ and 5′-AGGCTTCTACGAGGTATGAGGTAACTGTTGGGCTTTCCGAGTTGGGCGGTATTGAATTT-3′. To anneal these oligonucleotides, 5 μg of top and bottom strands was mixed in TE (10 μM Tris, 1 mM EDTA) and placed in a 93 °C water bath. This was then allowed to cool slowly to room temperature to produce a dsDNA fragment with EcoRI- and HindIII-terminal ends. The dsDNA fragment was ligated in frame into pGEX-KG. Generation of alanine mutants of T17 was conducted in the same manner using annealed complementary oligonucleotides, except that, where appropriate, codons were mutated to encode alanine. The oligonucleotides were annealed in the same manner as described above and ligated in frame to the GST gene in pGEX-KG. gDκR, gDκR60, gDR and gDK mutants were generated using the upstream primers 5′-GGCGAATTCTACACCGCCGCACTCGGAAAAAGGCGATACGGCTTTGGGGCTTTCC-3′, 5′-GGGAAGGTTAATTAGTCATCACTAAGTTCA3′ and 5′-GGGAAGGTTAATTAGTCATCACTAAGTTCA3′, respectively, with a common downstream primer, 5′-GGACCCGACTCTAGTAAAAACA-3′. The Gdc plasmid expressing the full-length gD cytoplasmic tail was used as the DNA template.

**Expression of GST fusion proteins.** Appropriate expression plasmids were transformed into Escherichia coli strain BL21 (Stratagene). Overnight stationary-phase cultures were used to inoculate fresh medium the following day to an OD₆₀₀ of 0.2 and cultures were grown on a shaker to an OD₆₀₀ of 0.9. IPTG was added to the cells to a concentration of 1 mM to induce expression of the fusion protein. After 3 h, cells were collected by centrifugation at 8000 r.p.m. for 10 min at 4 °C in a JA20 rotor, the medium was discarded and the pellet was frozen at −20 °C overnight. The following day, the pellet was thawed and resuspended in lysis buffer (0.05% Tween 20, 50 mM EDTA, 1 mM PMSF, 5 μg aprotinin ml⁻¹, 5 μg leupeptin ml⁻¹ and 1 mg lysozyme ml⁻¹ in PBS) and sonicated using a probe sonicator six times for 10 s each, with 30 s intervals on ice. Debris and unbroken cells were pelleted at 10 000 r.p.m. for 10 min at 4 °C in a JA20 rotor. Supernatants were subjected to SDS-PAGE and stained with Coomassie blue to visualize the fusion proteins.

**Preparation of HSV-1-infected cell extracts.** Confluent monolayers of COS cells were infected with HSV-1 strain SC16 at an m.o.i. of 10 in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS). After 1 h, fresh medium was added and the incubation proceeded for an additional 18 h at 37 °C. Cells were collected and a post-nuclear supernatant (PNS) was prepared as previously described (Harley et al., 2001). Briefly, cells were washed twice with cold homogenization buffer (0.25 M sucrose, 2 mM MgCl₂, 10 mM Tris/HC1 pH 7.6), scraped, pelleted at 1000 g for 5 min at 4 °C and resuspended in 1 ml homogenization buffer containing 5 μg leupeptin ml⁻¹, 5 μg aprotinin ml⁻¹ and 1 mM PMSF. Cells were broken by passing through a 25 gauge 5/8 inch needle eight times and then spun at 2000 g for 10 min at 4 °C to remove unbroken cells and nuclei. The PNS was adjusted to 150 mM NaCl, 0.5% NP-40, 50 mM Tris/HC1 pH 7-6, incubated for 1 h on ice to solubilize tegument proteins and centrifuged at 53 000 r.p.m. in a Beckman TLA 100.3 rotor for 1 h. The supernatant (S100) was aliquotted and frozen at −70 °C.

**Production of radiolabelled VP22.** A VP22/myc–His-encoding plasmid (Invitrogen) and Redivue [³H]methionine were added to the TnT Rabbit Reticulocyte Lysate in vitro transcription/translation system (Promega) following the manufacturer’s protocol. The resulting in vitro-translated VP22 was mixed with PBS containing 5 μg leupeptin ml⁻¹ and 5 μg antipain ml⁻¹ and incubated with GST fusion-protein-coated glutathione–Sepharose beads as described below. Material bound to the beads was subjected to SDS-PAGE and the gel treated with 30% methanol, 10% acetic acid for 30 min, En3Hance (NEN Life Sciences Products) for 1 h and 1% glycerol for 30 min. The gel was then dried using a Bio-Rad gel drier and exposed to film for 1–2 days.

**Preparation of [³H]thymidine-labelled capsids.** Prior to infection, confluent monolayers of COS cells were overlaid with DMEM containing 1% dialysed FBS and 1% PS for 2 h and then infected with HSV-1 strain SC16 at an m.o.i. of 10. After 1 h, fresh medium with [³H]thymidine (NEN) was added to a final concentration of 25 μCi ml⁻¹ and incubated for 18 h. Cells were harvested as above, and a PNS was prepared and adjusted to a final concentration of 2.5 mM magnesium acetate, 50 mM potassium acetate, 150 mM NaCl, 0.5% Triton X-100 and 5 mM DTT and incubated on ice for 1 h. The treatment removed the membranes and solubilized some of the tegument associated with the cytoplasmic virions. Following incubation, debris was removed by a clearing spin at 1500 g. The treated PNS was used to test for binding of capsids to fusion proteins bound to glutathione–Sepharose beads. The protein concentration of the PNS was determined using a BCA assay (Pierce) with BSA as a protein standard.

**GST in vitro-binding assay.** Glutathione–Sepharose beads were dispensed into a microcentrifuge tube and washed three times with cold PBS. BSA (100 μl of a 10 mg ml⁻¹ solution), an equivalent amount of GST fusion protein-containing bacterial extract and PBS to a total volume of 1 ml were added to the washed beads and incubated at 4 °C for 1 h on a rotator. The beads were then washed twice with wash buffer 1 (50 mM Tris/HC1 pH 7-6, 150 mM NaCl, 0.5% Triton X-100) and then with wash buffer 2 (50 mM Tris/HC1 pH 7-6, 1-3 M NaCl, 0.5% Triton X-100) and again with wash buffer 1. Infected-cell cytosol (500 μl), in vitro-translated VP22 or cell extracts containing [³H]thymidine-labelled capsids was then added to the GST fusion protein-bound beads, incubated at 37 °C for 1 h and then washed six times with wash buffer 1. For detection of bound tegument proteins, the beads were resuspended in 500 μl supernatant.
50 mM Tris/HCl pH 7.6. SDS-PAGE sample buffer was added and the mixture was heated to 95°C for 5 min, subjected to SDS-PAGE and Western blotted using appropriate antibodies.

**Measurement of packaged viral DNA.** A TCA precipitation assay to measure DNA packaging was carried out as previously described (Church et al., 1998). Briefly, after performing the GST in vitro-binding assay with $[^3]H$thymidine-labelled capsid-containing extracts, beads were incubated with 2 mM MgCl$_2$ and 280 U DNase I (type II; Sigma) ml$^{-1}$ for 90 min at 37°C. EDTA and SDS were added to final concentrations of 10 mM and 0.3%, respectively, and incubation continued for an additional 15 min. The treated material was then spotted on to individual GF/C Whatman filter papers and allowed to dry. Each filter was subjected to one 4°C wash and two consecutive 65°C washes in TP buffer (5% TCA, 20 mM sodium pyrophosphate) before rinsing in 70% ethanol and drying. The level of TCA-precipitable radioactivity was determined by liquid scintillation counting.

**Electron microscopy analysis of bead-associated capsids.** GST or GST fusion proteins were bound to glutathione–Sepharose beads and incubated with HSV capsid-containing extracts and the beads were washed, pelleted and fixed in 2:5% glutaraldehyde in SC (100 mM sodium cacodylate, pH 7.43) at room temperature for 45 min. The pellet was then rinsed in SC, post-fixed in 1% osmium tetroxide in SC followed by 1% uranyl acetate, dehydrated through a graded series of ethanols and embedded in LX112 resin (LADD Research Industries). Ultrathin sections were cut on a Reichert Ultracut E, stained with uranyl acetate followed by lead citrate and viewed on a JEOL 1200EX transmission electron microscope at 80 kV.

**Co-immunoprecipitation of endogenous gD and VP22.** COS cells were infected with HSV-1 strain PAAr5 (Jones et al., 1995) at an m.o.i. of 10. At 15 h post-infection, cells were collected and resuspended in lysis buffer [50 mM Tris/HCl pH 7.6, 150 mM NaCl, 0.5% NP-40, 0.5 mM EDTA, 0.5 mM PMSF, 2 mM DTT, 200 μg protease inhibitor cocktail tablets (Roche Diagnostics) ml$^{-1}$] and incubated on ice for 30 min. The resulting lysate was cleared by centrifugation for 20 min at 15,000 g and pre-cleaned by incubation with Protein G–agarose beads (Sigma) for 20 min at 37°C. After pelleting the beads, the supernatants were collected and mixed with fresh Protein G beads with either anti-gD (Virusys) or anti-α-HA (Roche Diagnostics) monoclonal antibody (mAb). After a 15 min incubation at 37°C, beads were washed four times with a wash buffer and once in PBS. Bound material was then subjected to SDS-PAGE and Western blotted using anti-VP22 antibody.

**RESULTS**

**Generation of a GST–gD tail fusion protein to test for interaction with tegument proteins**

A PCR-amplified DNA fragment encoding the gD cytoplasmic tail was cloned in frame to the coding sequence of GST using the vector pGEX-KG (Guan & Dixon, 1991) (Fig. 1a) to form the plasmid gDc. E. coli (strain BL21) was transformed with pGEX-KG or gDc, treated with IPTG to induce expression and cell extracts were subjected to SDS-PAGE. In Fig. 1(b), Coomassie blue staining shows the fusion protein of the predicted size of approximately 30 kDa as the most abundant protein in the gel.

**Fig. 1.** Construction and expression of the GST–gD fusion protein. (a) Schematic diagram of the fusion protein-expressing region of pGEX-KG. Ptac is an IPTG-inducible promoter with the arrow indicating the direction of transcription. The GST ORF is indicated by a filled box, followed by a polyglycine flexible hinge (GGGGG). The gD cytoplasmic tail was cloned between the EcoRI and HindIII sites. The protein sequence of the gD tail is shown followed by two stop codons. (b) After transforming E. coli BL21 cells, expression of the fusion proteins was induced with IPTG and cell extracts were subjected to 10% SDS-PAGE. Coomassie blue staining readily showed GST and the fusion protein as the most abundant proteins in the cell extract (arrows). The positions and sizes (kDa) of standard molecular mass markers are indicated on the left. (c) Equivalent amounts of GST-, gDc- or gHc-expressing bacterial extracts were added to the in vitro GST-binding assay and incubated with the S100 fraction from HSV-infected cells. Bound (B) and unbound (U) polypeptides were resolved by SDS-PAGE, transferred to a PVDF membrane and probed with various antibodies as indicated on the left (α-Tub. , α-tubulin). Input indicates the total amount of S100 fraction added to each binding reaction.

**Binding of the tegument protein VP22 to the gD fusion protein**

To prepare a source of tegument polypeptides for the binding assay, we followed the methodology previously described for isolating HSV-1-containing organelles and putative assembly intermediates from the cytoplasm of HSV-1-infected cells (Harley et al., 2001). PNS from HSV-infected COS cells was first treated with NP-40 and NaCl to solubilize the viral structural proteins, as we have previously found many tegument polypeptides to be insoluble in the PNS, perhaps due to their incorporation into assembling virions. The detergent-solubilized extract was then incubated with glutathione–Sepharose beads to which had previously been bound equivalent amounts of GST, gDc or gHc, a fusion protein of the viral gH tail with GST that has been well characterized by our laboratory (Gross et al., 2003). Bound and unbound material was then resolved by SDS-PAGE, Western blotted and analysed by
probing with various antibodies. Fig. 1(c) shows binding of the tegument protein VP22 to the GST–gD fusion protein. VP22 remained mostly in the unbound fraction when incubated with GST alone, suggesting that the binding was tail specific. To test the specificity of binding further, we also examined the association of other abundant cellular and viral proteins with the gD tail. The cellular protein α-tubulin and the abundant capsid scaffold proteins Pra and ICP35 failed to bind to the gD and gH tails, indicating that the glycoprotein tails do not bind these cellular and viral proteins non-specifically: VP22 bound to the gD tail but not to the gH tail. We also observed that gD bound the tegument protein VP16 in a manner similar to gH. This was consistent with a previous study by Zhu & Coutney (1994), which demonstrated that VP16 could be cross-linked to both gD and gH in purified virions.

**Binding of VP22 to the gD tail is independent of other viral proteins**

The interaction between VP22 and gD may be direct or mediated by other viral proteins. To examine this question, we used a rabbit reticulocyte lysate in vitro transcription/translation system to generate 35S-labelled VP22 and added it to our in vitro GST-binding assay. Autoradiography showed that in vitro-translated VP22 (running at a molecular mass of 45 kDa) bound to the gD tail (Fig. 2). Since the rabbit reticulocyte lysate contained only the in vitro-translated VP22 and no other viral proteins, we could conclude that VP22 binding did not require any other viral polypeptides. Although we could not discount the possibility that a cellular polypeptide in the reticulocyte lysate mediated the gD–VP22 interaction, no host protein with the abundance of VP22 has ever been described in the HSV particle.

Smaller fragments of VP22 that might have been the result of either early termination of protein synthesis or partial degradation were also found in the bound lane of gDc. We assumed that the smallest fragment (23 kDa; Fig. 2) of the in vitro-translated VP22 contained the region required for binding to the gD tail.

**Alanine-scanning mutagenesis of the gD cytoplasmic tail**

To simplify the identification of amino acids within the gD cytoplasmic tail that play an important role in VP22 binding, we generated a truncation mutant of the tail that retained only the most amino-terminal 17 residues (named T17) (Fig. 3a). When the GST–T17 fusion protein was tested for its ability to bind VP22 present in cytosolic extracts prepared from HSV-infected cells, it was found to bind VP22 in a similar manner to the full-length gD tail (Fig. 3b). Next, to identify residues that might be critical for binding VP22, we carried out alanine-scanning mutagenesis. Clusters of amino acids were mutated simultaneously to alanine and named according to the amino acid residues that were mutated (Fig. 3a). These mutants, along with gDc and GST, were expressed and incubated with infected-cell extracts and the relative amounts of VP22 binding were detected by Western blotting. Fig. 3(c) shows that mutations in the arginine and lysine residues at positions 5/6 or 9/10 diminished the binding of VP22, indicating that these amino acid residues are important. Although the HRR mutant showed a slightly decreased binding of VP22, this level of binding was variable, and most commonly the HRR mutant bound VP22 at a level similar to the wild-type gD tail. Next, we examined the role of the key lysine and arginine residues in the context of the full-length gD tail (Fig. 3d). Similar to the truncated mutant, mutation of R5/K6 showed a dramatic decrease in binding of VP22. Individual mutation of these residues had a less dramatic effect, indicating that these amino acid residues both contribute to VP22 binding but may play a somewhat redundant role.

**Co-immunoprecipitation of endogenous gD and VP22**

So far, the gD–VP22 interaction was demonstrated using an in vitro GST-binding assay. In order to confirm this protein–protein interaction in vivo, a co-immunoprecipitation experiment was performed using infected-cell lysate prepared from COS cells infected with the wild-type HSV-1 strain PAAr5. Infected-cell lysates were incubated with protein G–agarose beads containing either an anti-gD or anti-HA mAb as described in Methods. The immunoprecipitated materials were then subjected to SDS-PAGE and Western blotted for VP22. The amount of VP22 in the total-cell lysate is shown in Fig. 4 (lane 1). VP22 was present when immunoprecipitation was carried out with the anti-gD mAb (Fig. 4, lane 2), but not when the anti-HA mAb was used (Fig. 4, lane 3). Therefore, gD can indeed be co-immunoprecipitated with VP22, indicating that these two proteins interact in vivo.

![Fig. 2. VP22 binds to the gD tail in the absence of other viral proteins. 35S-labelled VP22 was generated using a rabbit reticulocyte lysate in vitro transcription/translation system. gDc- or GST-coated glutathione–Sepharose beads were incubated with radiolabelled VP22 in duplicate and subjected to SDS-PAGE, fixed and exposed to film for 1–2 days. Input corresponds to the amount of in vitro-translated product included in the binding reaction.](image-url)
Binding of capsids to the gD tail in the presence of tegument proteins

To examine the interaction of the glycoprotein tail and capsids, we prepared cell extracts containing capsids in which the viral genome was labelled with $^3$H thymidine, as described in Methods. The packaged, radiolabelled viral genome enabled us to monitor the presence of HSV capsids by scintillation counting. It should be noted that, since this was a relatively crude extract, tegument polypeptides such as vhs, VP22 and VP16 were also present, as determined by Western blotting (Fig. 5a). This extract was then incubated with glutathione–Sepharose beads pre-coated with equivalent amounts of GST, gDc or gHc as previously described. After washing, the beads were incubated with DNase I under conditions previously shown to destroy any DNA that has not been packaged within the icosahedral capsid (Church et al., 1998; Dasgupta & Wilson, 1999; Harley et al., 2001). Any surviving full-length, previously packaged DNA was then collected by TCA precipitation and subjected to scintillation counting. As shown in Fig. 5(b), only low levels of protected, encapsidated DNA became bound to beads coated with GST or gHc. In contrast, much higher levels of binding were apparent when capsids were incubated with bead-bound gDc.

If the capsids are binding to a specific site on the gD tail then, for any constant amount of gDc fusion protein bound to the beads, capsid binding should be saturable. Fig. 5(c) shows the result of an experiment in which increasing amounts of radiolabelled capsid-containing extract were incubated with GST- or gDc-coated glutathione–Sepharose beads and scored for capsid binding as described above. Under these conditions, binding of capsids to the gD tail appeared to be saturable at between 500 and 750 mg cytosolic protein (Fig. 5c). In contrast, binding to GST was at a much lower level and increased linearly in relation to the quantity of extract added, consistent with the lack of a saturable binding site. This indicated that capsids were binding specifically to the gD tail, most likely indirectly, via tegument proteins.

Visualization of HSV-1 capsids bound to gDc-coated glutathione–Sepharose beads

Although rapid and convenient, monitoring the c.p.m. of labelled packaged viral DNA is an indirect method of testing for the presence of capsids. As a direct test of gD tail–capsid interaction, GST- or gDc-coated glutathione–Sepharose beads were incubated with the capsid preparation, fixed and then processed for ultrastructural analysis. Fig. 6 shows representative images of capsids bound to gDc-coated beads. For gDc-coated beads, it was relatively easy to identify icosahedral capsids. Capsids were never found within beads, but always at the perimeter, suggesting they were not non-specifically trapped within the glutathione–Sepharose matrix.
In this study we did not attempt to discriminate between B and C capsids, since they are believed to have similar surface compositions (Newcomb et al., 1996; Trus et al., 1996) and both B and C capsids are capable of becoming enveloped (Baines et al., 1997). Interestingly, all capsids scored had some internal structure, either a DNA core or a scaffold, suggesting that under these conditions very few A capsids were present (data not shown). Table 1 shows quantification of the number of capsids bound to the GST- or gDc-coated beads.

**DISCUSSION**

The cytoplasmic tails of HSV-1 glycoproteins have been shown to interact with various tegument polypeptides (Brack et al., 2000; Fuchs et al., 2002; Gross et al., 2003; Zhu & Courtney, 1994), which may facilitate capsid recruitment to the site of envelopment. So far, dissecting the molecular mechanism of assembly using a genetic approach has been complicated by redundancy among the viral glycoproteins: deletion of a single glycoprotein does not generally lead to apparent defects in cytoplasmic envelopment; instead, multiple simultaneous deletions are necessary. In this study we took a biochemical approach towards analysing protein–protein interactions that might be important during virus assembly.

We generated a fusion protein between GST and the cytoplasmic tail of gD in order to test its interaction with HSV-1 tegument proteins. Our results showed that the gD tail specifically bound the tegument protein VP22 but did not interact with the abundant cellular protein α-tubulin nor with the capsid scaffold proteins ICP35 and Pra. Furthermore, co-immunoprecipitation experiments confirmed that the interaction between gD and VP22 could be detected in infected cells. Additionally, alanine-scanning mutagenesis of the gD tail identified two amino acid residues, arginine and lysine (from the amino-terminal end of the tail) at...
positions 5 and 6, respectively, that were critical for VP22 binding.

Since VP22 and VP16 form a complex (Elliott et al., 1995) and we observed VP16 binding to the gD tail, we were interested to test whether VP22 bound the gD tail directly or via VP16 or other viral proteins. Therefore, we synthesized 35S-labelled VP22 using a rabbit reticulocyte lysate in vitro transcription/translation system and tested its ability to bind the gD tail. We found that in vitro-translated VP22 did bind specifically to the gD tail, showing that this binding was independent of other viral polypeptides.

### Table 1. Quantification of bead-bound capsids

<table>
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<tr>
<th>Fusion protein</th>
<th>No. of beads</th>
<th>Total edge length (μm)</th>
<th>No. of bound capsids (B and C)*</th>
</tr>
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<tr>
<td>gDc</td>
<td>96</td>
<td>6806</td>
<td>344</td>
</tr>
<tr>
<td>GST</td>
<td>107</td>
<td>7889</td>
<td>2</td>
</tr>
</tbody>
</table>

*Only icosahedral capsids immediately adjacent to bead edges were counted.

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**Fig. 6.** Visualization of bead-bound capsids by electron microscopy. Capsid-containing cell extracts were incubated with bead-bound gDc. After washing, the mixture was fixed in glutaraldehyde and processed for thin-section electron microscopy. (a–c) Representative bead perimeters. Bound capsids are indicated by arrows. (d) Sevenfold magnification of the capsid indicated in (b). Bar, 200 nm.

**Fig. 7.** Mutation of arginine and lysine residues essential for VP22 binding abolishes binding to capsids. Equivalent amounts of GST, gDc and gDR5Kc were added to the GST in vitro-binding reaction and incubated with a crude capsid preparation, as in Fig. 5(b). The level of capsid binding seen for the mutant gDR5Kc was similar to the background binding seen for the negative control of GST.
Under our conditions we also observed capsid binding to the gD tail. This binding was saturable, unlike the low level of binding seen with GST alone. We speculated that capsid binding to the gD tail was mediated by tegument components present in the crude capsid preparation. Our results showed that the gD mutant tail gDR5K6 that failed to bind VP22 also failed to bind capsids. This suggested that VP22 was mediating capsid–tail interactions. However, it is also possible that other tegument proteins mediate binding of capsids to the gD tail, utilizing the same residues as VP22. Although VP16 is present on these capsids (Fig. 5a) and can bind to the gH tail (Gross et al., 2003), it appeared unable to tether capsids to the gH tail (Fig. 5b). Additionally, we observed that VP16 was capable of binding to the gD mutant tail gDR5K6, which does not bind capsids (data not shown). This also suggested that VP16 was not involved in capsid–tail interactions under these conditions.

The association between VP22 and gD may be one of many interactions between glycoprotein tails and tegument proteins that facilitate the budding of capsids into organelles and the incorporation of tegument/glycoproteins into nascent virions. In HSV-1, deletion of the cytoplasmic tail of gD had relatively little effect on normal virus production (Farnsworth et al., 1990). However, simultaneous deletion of gD and gE showed elimination of cytoplasmic envelopment and accumulation of large numbers of unenveloped nucleocapsids within a dense layer of tegument in the cytoplasm (Farnsworth et al., 2003), indicating that gD and gE may share overlapping roles in cytoplasmic envelopment. Our data suggest that at least one of these roles may be in VP22 recruitment and predict that the cytoplasmic tail of gE may also bind VP22. Interestingly, Farnsworth et al. (2003) reported that the cytoplasmic tail of gE, when fused to GST, was indeed able to bind VP22. Thus, gD and gE may act in a redundant manner to incorporate VP22 into the newly forming virions. Alternatively, it could be argued that VP22 serves to gather gD and gE to the sites of capsid–envelope interaction.

Our findings regarding HSV-1 are somewhat similar to data reported for the alphaherpesvirus PrV. gE and gM have been shown to interact with the U1.49 protein, a homologue of HSV-1 VP22 (Fuchs et al., 2002), and, following simultaneous deletion of these two glycoproteins, the U1.49 product does not become incorporated into the PrV particle (Fuchs et al., 2002). However, in the absence of the U1.49 protein, gE and gM are incorporated into virions, implying that they may interact redundantly with other tegument proteins for this purpose.

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