CHAPTER 1:

INTRODUCTION
Herpes simplex virus-type 1 (HSV-1) is one of the most common human pathogens, infecting 40-80% of people worldwide. Although most clinical disease due to HSV-1 is relatively mild, it can sometimes cause meningoencephalitis in adults, or disseminated infection in neonates, both of which are frequently fatal if untreated. Furthermore, mucocutaneous infection with HSV-1, especially in the genital region, causes significant morbidity, and reduced quality of life, for many people in the community.

One of the characteristic features of HSV-1 is its ability to infect neurones, or nerve cells, from where it is able to cause recurrent infections. 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 latent infection for the lifetime of the host. Periodic reactivation results in HSV-1 being transported in an anterograde direction to nerve terminals, where it causes either recurrent clinical disease, or asymptomatic viral shedding. Because of the very long distances that need to be traversed by HSV-1 in neuronal axons, its transport must be an active process, and is thought to utilise cellular molecular motors, such as dynein and kinesin. The way in which HSV-1 attaches to the dynein motor during retrograde transport of HSV-1 is not known, and is the main focus of this thesis.

HSV-1 is a double stranded DNA virus; the prototype alphaherpesvirus. The viral DNA is contained in an icosahedral protein capsid, surrounded by a less structured protein tegument layer, in turn surrounded by a lipid envelope containing glycoprotein spikes. Interestingly, if the major capsid proteins VP5, VP19C, VP23 and VP26 are expressed in vitro, using recombinant baculoviruses, they spontaneously assemble into capsids, in the absence of other viral proteins.
During infection of neurones or other cells, 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. 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.

There is recent evidence that the rapid ‘retrograde’ transport of this complex to the cell nucleus proceeds along cellular microtubules, and is mediated by the minus-end-directed molecular motor cytoplasmic dynein (Dohner et al., 2002). Cytoplasmic dynein is a large molecular complex, in which the heavy chain subunits provide motive force, while cellular cargo attaches to the motor via the intermediate and light chains. Proteins from other viruses that use dynein for intracellular transport, such as rabies virus and African swine fever virus, have also been shown to bind to dynein light chains, and these interactions are thought to be important for viral transport.

It is not known which HSV-1 proteins interact with the cytoplasmic dynein motor, but the most likely candidates are those located in the outer capsid or inner tegument layers of the virion, since the outer tegument and envelope proteins are lost during cell entry. Despite a recently reported interaction between dynein intermediate chain (DIC) and HSV-1 protein UL34 (Ye et al., 2000), its role in retrograde transport has yet to be confirmed. The protein product of UL34 is not essential for HSV-1 replication in vitro (Roller et al., 2000), and is an unlikely candidate to mediate retrograde transport of HSV-1, since it is not found in mature HSV-1 virions (Reynolds et al., 2002).

Previous work in our laboratory has concentrated on anterograde axonal transport of HSV-1, as occurs following reactivation of latent HSV-1 infection. Using protein-protein interaction assays, our group has shown that the HSV-1 tegument protein US11 interacts with the ubiquitous heavy chain of the kinesin motor KIF5B.
(Diefenbach et al., 2002b), and is likely to play an important role in anterograde axonal transport.

In this thesis, using a similar approach for retrograde transport, we report an interaction between the HSV-1 capsid protein VP26 and the 14 kDa dynein light chains rp3 and Tctex1, which we recently published (Douglas et al., 2004). These dynein light chains are known to mediate binding of other cellular and viral cargoes, and are 55% homologous at the amino acid level.

We initially detected interactions between VP26 and rp3 or Tctex1 using a yeast two-hybrid system, and subsequently went on to confirm them with several in vitro protein binding assays. In separate experiments, using green fluorescent protein (GFP)-labelled virus and confocal microscopy, we confirmed the dissociation of the inner tegument protein UL37, along with other tegument proteins, from incoming HSV-1 capsids. This suggests that VP26, on the outer aspect of the HSV-1 capsid, is likely to be accessible to bind to the cytoplasmic dynein motor in vivo. Finally, we developed a novel experimental model in which we micro-injected recombinant HSV-1 capsids into individual cells, and examined their transport by confocal microscopy. In these experiments we observed co-localisation of HSV-1 capsids containing VP26 with microtubules and with dynein light chains rp3 and Tctex1. Most importantly, we were able to show that HSV-1 capsids containing VP26 were transported towards the cell nucleus, while capsids that did not contain VP26 were not.

In conclusion, we propose a new model for retrograde transport of HSV-1, in which the capsid protein VP26 mediates binding of the HSV-1 nucleocapsid to cytoplasmic dynein, via interactions with rp3, and probably Tctex1. We believe that this interaction is important during retrograde axonal transport of HSV-1 in neurones, as well as during infection of non-neuronal cells.
The long-term aim of our research is to develop a synthetic gene therapy vector, based on the HSV-1 capsid, to deliver therapeutic genes to neuronal cells. It is hoped that such a vector would circumvent antiviral immunity, and allow the delivery of genes to treat neurological diseases such as Friedreich’s Ataxia, chronic neuropathic pain, and possibly even spinal cord injury.
CHAPTER 2:

LITERATURE REVIEW
2.1 Herpes Simplex Virus Type 1

2.1.1 Epidemiology and Clinical Features of HSV-1 Infection

Herpes simplex virus-type 1 (HSV-1) is the prototype alphaherpesvirus. It is a large, neurotropic, double-stranded DNA virus that predominantly infects humans. It most commonly causes mucocutaneous infection, resulting clinically in recurrent orolabial or genital lesions. In rare cases it can cause meningo-encephalitis in older adults, or severe disseminated infection in neonates, which are frequently fatal if untreated. The closely-related serotype herpes simplex virus type 2 (HSV-2) accounts for a higher proportion of genital herpes in adults, and can also cause pharyngeal or disseminated infection.

The clinical features and epidemiology of HSV infection have been the subjects of several recent reviews (Whitley and Roizman, 2001; Smith and Robinson, 2002). Rates of acquiring HSV-1 vary widely, and depend on many demographic factors. In developing countries up to 99% of people have been infected with HSV-1 by adulthood, while in more developed countries between 40 and 80% of people are seropositive by the age of 20-40 years. HSV-2 is usually sexually transmitted, and exposure rates vary according to levels of sexual activity, with up to 75% of female prostitutes and 83% of male homosexuals seropositive. An increasing proportion of genital HSV infection is now due to HSV-1, 20% in one recent study (Lafferty et al., 2000), which may reflect changing sexual practices. Genital infection due to HSV-1 is generally less severe, and reactivates less frequently than infection with HSV-2.

An Australian study published in 1993 found 14.5% of antenatal clinical patients and 40% of STD clinic patients were seropositive for HSV-2 (Cunningham et al.,
This compares with an overall HSV-2 seroprevalence rate of 25% in the USA (Fleming et al., 1997). A study of 2616 pregnant women at Westmead Hospital, Sydney between 1995 and 1998 found antibodies for HSV-2 in 12.5% of subjects (Mindel et al., 2000). Recent unpublished studies of a randomly sampled Australian population showed a seroprevalence of 13.2% in adults, with seroprevalence slightly higher in urban than rural settings (Cunningham and Mindel, personal communication).

A study of 300 homosexual men (mean age 37 years) in a primary care setting in Melbourne in 1999/2000 found that 73% were seropositive for HSV-1 (Russell et al., 2001). In the same population seroprevalence for HSV-2 was 20% in human immunodeficiency virus (HIV)-seronegative men and 61% in HIV-seropositive men. The high rate of HSV-2 in HIV-infected people is clinically important, given the emerging evidence of interactions between the two viruses (Celum, 2004). It has long been recognised that immunodeficiency due to HIV infection increases the frequency and severity of HSV recurrence. However there is also recent evidence that genital HSV-2 infection doubles the risk of acquiring HIV, and that HSV infection per se may upregulate HIV replication.

Perinatal infection with HSV is uncommon, with a reported incidence in Australia of 3.4/100 000 live births, and is predominantly caused by HSV-1 (Jones et al., 2001). Of the 43 cases reported in Australia between 1997 and 2001, 19 (44%) were due to HSV-1, 15 (35%) to HSV-2, and in 9 cases (21%) the virus type was unknown or not recorded. Despite the availability of antiviral therapy, mortality remains high, and was 23% in this series.
2.1.2 Structure of the HSV-1 Virion

HSV-1 is a large virus, with whole virions averaging approximately 225 nm in diameter (Grunewald et al., 2003). The current model of HSV-1 has been reviewed by Roizman (Roizman and Knipe, 2001) and Rixon (Rixon, 1993), but recent cryo-electron tomography studies have added further insight into the structure of the intact virion (Grunewald et al., 2003). The HSV-1 DNA genome is enclosed in an icosahedral protein capsid shell surrounded by a less structured protein tegument layer, in turn surrounded by a lipid envelope. Most virions are approximately round in shape, but they are pleomorphic, with significant departures from sphericity in many cases (Figure 1). It was reported recently that the position of the nucleocapsid within the envelope can be eccentric (Grunewald et al., 2003). In this model the capsid is in close proximity to the envelope at the proximal pole, but separated from it by 30 to 35 nm of tegument at the distal pole (Figure 2).

2.1.2.1 Genome

The double-stranded DNA HSV-1 genome is 152 kbp in length, with a high G+C content (68% for HSV-1, 69% for HSV-2) (Roizman and Knipe, 2001). It consists of two covalently linked unique sections, designated UL (unique long) and US (unique short), flanked by inverted repeat regions. There are around 90 unique transcriptional units in the HSV-1 genome: at least 84 of these genes encode proteins, with approximately 40 structural proteins incorporated into the virion. At least 47 of the 90 gene products are not essential for viral replication in cultured cells (Roizman and Knipe, 2001). Where their function is known, these non-essential proteins either increase viral virulence in vivo, or play regulatory roles. For example, the non-
essential protein ICP34.5 has been shown to play a role in neuro-invasive disease (Bower et al., 1999).

Figure 1. HSV-1 virions viewed by cryo-electron tomography.
(A) Zero-tilt projection from a tilt series. Black dots are 10-nm gold particles used as fiducial markers. (B) Gallery of parallel slices, 15.5 nm apart and 5.2 nm thick, through the virion framed in (A). Each slice represents the average over seven planes. Red arrowheads mark filaments in the tegument. Scale bars, 100 nm (reproduced with permission from Grunewald et al., 2003).

Figure 2. Segmented surface rendering of a single virion tomogram after de-noising.
(A) Outer surface showing the distribution of glycoprotein spikes (yellow) protruding from the membrane (blue). (B) Cutaway view of the virion interior, showing the capsid (light blue) and the tegument “cap” (orange) inside the envelope (blue and yellow). pp, proximal pole; dp, distal pole. Scale bar, 100 nm (reproduced with permission from Grunewald et al., 2003).
2.1.2.2 Nucleocapsid

The central component of the HSV-1 virion is the nucleocapsid: 1250 Å in diameter, with a mass of 0.2 billion Daltons (Zhou et al., 2000). HSV-1 DNA forms the electron-dense core of the nucleocapsid, and has a toroidal appearance by traditional transmission electron microscopy (Furlong et al., 1972). In a more recent study using cryo-electron microscopy the DNA appears more like a spool, and is arranged in regularly spaced concentric layers (Zhou et al., 1999).

Surrounding the DNA core is a protein capsid shell, icosadeltahedral in mature virions, which contains 162 capsomeres. The outer layer has a T=16 symmetry, while the intermediate layer is arranged in a T=4 lattice (Roizman and Knipe, 2001). The major capsid proteins are VP5 (UL19, 149 kDa), VP26 (UL35, 12 kDa), VP23 (UL18, 34 kDa) and VP19C (UL38, 50 kDa). VP5 is the major component of the capsid, and is arranged either as pentamers at the capsid vertices (the “pentons”) or elsewhere as hexamers (the “hexons”) (Newcomb et al., 1993). Each VP5 molecule in hexons, but not pentons, is associated with a VP26 molecule, located at its tip. Between the hexons are the “triplexes”, each comprising one molecule of VP19C and two molecules of VP23 (Rixon et al., 1990). In summary, each mature HSV-1 capsid contains 12 pentons (5 × VP5); 150 hexons (6 × VP5, 6 × VP26); and 320 triplexes (1 × VP19C, 2 × VP23). This proposed capsid structure has been confirmed by cryo-electron microscopy to a resolution of 8.5 Å (Figure 3) (Zhou et al., 2000). In addition, over 30 putative α helices were identified in this analysis, some of which are in domains that undergo conformational changes during capsid assembly and DNA packaging.

The crystal structure of the upper domain of VP5 has recently been solved (Bowman et al., 2003), and is consistent with the structure predicted by structural bioinformatics,
Figure 3. Structure of the HSV-1 capsid to a resolution of 8.5 Å.

This figure represents the structure of the HSV-1 capsid shell, based on cryo-electron microscopy data. In the whole capsid view, hexons are shown in blue, pentons in red and triplices in green. The higher magnification views below demonstrate the location of the other major capsid proteins, relative to VP5. Capsid protein VP26 is located on the tips of the hexons, bound to the upper domain of VP5. The triplex proteins VP19C and VP23 are located between the capsomereres, interacting with the floor domain of VP5 (reproduced with permission from Zhou et al., 2000).
based on cryo-electron microscopy (Baker et al., 2003). Analysis of the crystal structure suggests that the different biochemical properties of pentons and hexons are due to different physical orientations of the VP5 upper domains, rather than changes in the conformation of VP5 (Bowman et al., 2003). The resulting basic nature of the larger penton channel may be important for the exit of acidic scaffolding proteins during DNA packaging (Bowman et al., 2003). Furthermore, the arrangement of VP5 upper domains in hexons creates an acidic region at the sites of contact between VP5 subunits, which is absent in the pentons (Bowman et al., 2003). This may explain why the small basic capsid protein VP26 binds specifically to hexons, but not to pentons (Wingfield et al., 1997).

Several other proteins are associated with the HSV-1 capsid, and have been designated “minor capsid proteins”. These include the products of the UL6 (Patel and MacLean, 1995), UL15 (Salmon and Baines, 1998), UL25 (McNab et al., 1998), UL28 (Taus and Baines, 1998) and UL33 genes (Beard and Baines, 2004). The UL6 protein forms the “portal complex”, a ring-like structure containing twelve UL6 molecules, which is thought to be involved in packaging DNA into capsids (Newcomb et al., 2001). Immuno-electron microscopy studies of HSV-1 B capsids have detected only one portal complex per capsid, located at one of the twelve penton vertices (Newcomb et al., 2001). The portal protein UL6 can also be incorporated into HSV-1 capsids assembled in vitro, in amounts supporting the presence of only one portal complex per capsid (Newcomb et al., 2003). The UL15 and UL28 proteins are thought to form part of the “terminase complex”, involved in cleaving in packaging viral DNA, and have both been shown to bind to UL6 (White et al., 2003). The DNA cleavage and packaging protein UL33 was also shown recently to bind to capsids, independently of UL15, UL28 and UL6 (Beard and Baines, 2004).
2.1.2.3 Tegument

Surrounding the HSV-1 nucleocapsid is the protein tegument, which occupies approximately two-thirds of the volume of the virion (Grunewald et al., 2003) and contains the majority of virion proteins (Desai et al., 2001). About 20 tegument proteins have been identified: VP1/2 (UL36), 11/12 (UL46), 13/14 (UL47), 16 (UL48), 22 (UL49), ICPs 0, 4 and the virion host shutoff protein (vhs) (UL41) plus the products of genes US2, US3, US9-11, UL11, UL13, UL14, UL16, UL17, UL21, UL37, UL51 and UL56 (Homa and Brown, 1997; Subak-Sharpe and Dargan, 1998; Mettenleiter, 2002).

The major components of the tegument include VP11/12, VP13/14, VP16, and VP22 (Desai et al., 2001). VP16 is also known as the $\alpha$-trans-inducing factor, or $\alpha$TIF. It induces expression of viral $\alpha$ genes, the first genes expressed, and its activity seems to be regulated by VP11/12 and VP13/14 (Roizman and Knipe, 2001). Another important tegument protein during early infection is the vhs protein, which is involved in early shutdown of host macromolecular synthesis (Roizman and Knipe, 2001). The function of VP22 is unknown, but it spreads from cell to cell in a monolayer following HSV-1 infection or UL49 gene transfection of a few cells (Elliott and O'Hare, 1997). The large tegument protein VP1/2 is encoded by UL36, and is believed to form the inner layer of the tegument (Mettenleiter, 2002). VP1/2 plays a role during release of the viral DNA at the nuclear pore (Batterson et al., 1983), and is essential for viral tegument assembly (Desai, 2000).

Traditionally the tegument has been described as “amorphous” in structure (Roizman and Knipe, 2001) but recent work has demonstrated some structure in the arrangement of tegument proteins (Mettenleiter, 2002). Cryo-electron microscopy has
revealed filamentous, icosahedral structures around the pentons, interacting extensively with the capsid (Zhou et al., 1999). These filaments are thought to represent VP1/2 (Mettenleiter, 2002), which has been shown to interact directly with the nucleocapsid (McNabb and Courtney, 1992a). By cryo-electron tomography, at a resolution of 7 nm, the tegument appears as a reticulum of particulate density (Grunewald et al., 2003) (Figure 1). Some thin filaments were also observed at the periphery of the tegument, apposed to the membrane. The filaments were 40 nm long, approximately 7 nm in diameter, and may represent either actin filaments or polymerised tegument proteins.

The reason why the tegument interacts at the pentons rather than the hexons was initially thought to be the absence of VP26 on pentons. However this now seems unlikely, since deletion of the VP26 gene (UL35) does not appear to alter the pattern of tegument-capsid interaction (Chen et al., 2001). Based on the crystal structure of the VP5 upper domain, it now seems likely that VP1/2 attaches specifically to pentons because of the differing orientation of VP5 upper domains. In pentons the much larger space allows VP1/2 filaments to be “threaded” between two adjacent VP5 upper domains, binding to both simultaneously (Bowman et al., 2003).

Some of the tegument proteins such as VP16 are multi-functional, providing both structural and regulatory roles. Many tegument proteins are non-essential, at least in vitro: VP11/12, VP13/14, ICP0, vhs, US2, US9, US10, US11, UL11, UL13, UL14, UL16, UL21 and UL51 (Roizman and Knipe, 2001). Although some of these proteins have an important role for viral replication in vivo, assembly of the tegument in their absence appears largely unaffected, due to the remarkable degree of redundancy in tegument protein-protein interactions (Mettenleiter, 2002).
2.1.2.4 Envelope

Mature HSV-1 virions are surrounded by a trilaminar lipid envelope, apparently derived from patches of altered cellular membrane. The envelope contains numerous protrusions or spikes, representing viral glycoproteins. The three-dimensional structure of glycoprotein gD has now been solved, and it has been shown to form homo-tetramers (Pilling et al., 1999). In a recent cryo-electron tomography analysis of HSV-1 virions, glycoprotein spikes were found to be 10 to 25 nm in length, approximately 4 nm wide, and many ended in globules that were 6 nm across (Grunewald et al., 2003). The spikes also varied in their straightness and the angle at which they protruded from the membrane, with at least one type of spike emerging at an angle of 35° to 55°. There was structural evidence of dimerisation, with at least one bifurcated spike seen. Between 600 and 750 spikes were observed in each virion, and their distribution was uneven. Spikes tended to cluster, possibly reflecting localisation to lipid rafts, and their density was much higher at the distal pole of the virion than the proximal pole (Grunewald et al., 2003). Thus the glycoproteins were clustered over the asymmetric tegument, rather than over the capsid, and this would be consistent with reports of direct protein-protein interactions between glycoprotein tails and outer tegument proteins (Mettenleiter, 2002).

Of the 12 proposed HSV-1 glycoproteins, 10 have been identified in the viral envelope: gB, gC, gD, gE, gG, gH, gI, gK, gL, gM (Roizman and Knipe, 2001). The putative glycoproteins gJ and gN have not been confirmed to be part of the virion, and their function is unclear. At least two (UL20 and US9), and possibly more (UL24, UL43) non-glycosylated proteins are also incorporated into the HSV-1 envelope (Roizman and Knipe, 2001).
2.1.3 HSV-1 Replication Cycle

The replication cycle of HSV-1 has been reviewed extensively (Roizman and Knipe, 2001), along with a more recent review into the process of tegument assembly and viral egress (Mettenleiter, 2002). Briefly, the HSV-1 virion attaches to cell surface receptors, and then enters the cell by fusion of the viral envelope with the plasma membrane. Following cell entry most of the tegument proteins dissociate, and the nucleocapsid is transported to the cell nucleus, where it docks with the nuclear pore and releases viral DNA. The expression of viral genes occurs in a regulated, sequential cascade, and is tightly regulated. Capsid assembly occurs in the nucleus, followed by packaging of newly replicated DNA. The DNA-containing capsids then pass through the nuclear membrane (primary envelopment/de-envelopment), and the inner tegument proteins are added in the cytoplasm. The outer tegument and lipid envelope are probably acquired by budding into trans-Golgi vesicles, and mature virions are released from the cell by exocytosis.

2.1.3.1 Virus Attachment and Cell Entry

Attachment and entry of HSV-1 to mammalian cells involves multiple viral surface glycoproteins, and has been the subject of recent reviews (Roizman and Knipe, 2001; Garner, 2003; Spear and Longnecker, 2003). Glycoproteins gB, gD, gH, gL, and possibly gK, are essential for viral replication in vitro, while gC, gE, gG, gI and gM are dispensable for most cell types (Roizman and Knipe, 2001). There are three stages of viral entry: attachment of the virion to the cell surface; interaction of gD with one of several cellular coreceptors; and fusion of the viral envelope with the plasma membrane. The transition from attached to penetrated virus is very rapid, occurring within minutes (Huang and Wagner, 1964; DeLuca et al., 1981).
Attachment of HSV-1 to non-polarised cells involves interactions of positively charged regions of gC, and to a lesser extent gB, with negatively charged glycosaminoglycan moieties on heparan sulphate (WuDunn and Spear, 1989; Shieh et al., 1992). Interestingly, gC is not essential for virus entry or replication in non-polarised cells, but is required for binding to the apical, but not basal, surfaces of polarised epithelial cells (Sears et al., 1991).

The second step of cell entry involves binding of viral gD to one of several cellular coreceptors, and has been the focus of much recent research (Roizman and Knipe, 2001). This process is very efficient, as mutant HSV-1 strains expressing extremely low levels of gD can still infect human keratinocytes (Huber et al., 2001). The first of these coreceptors to be recognised belongs to the tumour necrosis factor receptor family, and was initially called herpesvirus entry mediator (HVEM) (Montgomery et al., 1996), but has since been renamed HveA. It is predominantly expressed on lymphocytes, so its role in HSV-1 replication in vivo is unclear.

The second family of coreceptors belong to the immunoglobulin superfamily, act at intercellular adhesion junctions, and are called the nectins (Spear et al., 2000). These molecules include nectin-1α (HveC) (Geraghty et al., 1998), nectin-1β (herpesvirus immunoglobulin-like receptor, H1gR) (Cocchi et al., 1998) and nectin-2α (HveB) (Warner et al., 1998). Nectin-1α and nectin-1β are widely expressed in cells targeted by HSV-1 and HSV-2, and are thought to mediate cell-to-cell spread of HSV, as well as virus entry (Cocchi et al., 2000; Johnson and Huber, 2002). Nectin-1 mRNA is expressed at high levels in the human central nervous system (Cocchi et al., 1998), neuronal cell lines (Geraghty et al., 1998), and in mouse sensory, sympathetic, and parasympathetic neurones (Haarr et al., 2001). Nectin-1 protein has been found in abundance in rat sensory neurones, but not in rat motor neurones (Mata et al., 2001).
The entry of HSV-1 to primary rat sensory neurones in vitro has been confirmed to involve nectin-1 in one recent study, but not HveA, (Richart et al., 2003). Nectin-2α is thought to play a role in the entry of HSV-2, but not of wild-type HSV-1 (Warner et al., 1998).

The third family of coreceptors mediating HSV-1 binding is represented so far by a single member: 3-O-sulphated heparan sulphate (Roizman and Knipe, 2001). This molecule results from the action of D-glucosaminyl 3-O-sulfotransferase on heparan sulphate (Shukla et al., 1999). Surface expression of heparan sulphate may therefore be sufficient to allow infection with HSV-1, in the absence of other co-receptors, since unmodified heparan sulphate can bind gB and gC, while the sulphated form can bind gD. Interestingly, 3-O-sulphated heparan sulphate has been shown to mediate entry of HSV-1, but not HSV-2, which may help to explain the different tissue tropisms of these closely related viruses (Shukla et al., 1999).

In the final stage of HSV-1 cell entry, the viral envelope fuses with the cell plasma membrane, causing internalisation of the viral nucleocapsid and tegument. Several glycoproteins appear to participate in this process: gD (Ligas and Johnson, 1988), gB (Sarmiento et al., 1979), and the gH-gL hetero-dimer (Forrester et al., 1992). The exact mechanism is not known, but the binding of gD to one of its cellular coreceptors may cause a conformational change in the other glycoproteins, or a change in their distribution on the viral membrane (Roizman and Knipe, 2001).

Endocytosis of virion membrane proteins may play an important role in the life cycle of the porcine alphaherpesvirus, pseudorabies virus (PrV) (Brideau et al., 2000c). Internalisation of PrV glycoproteins by porcine monocytes may be enhanced in the presence of antibody, and has been shown to involve actin, clathrin, microtubules and dynein (Van de Walle et al., 2001).
Successful HSV-1 infection via the endocytosis pathway has also been reported, using Chinese hamster ovary cells that express gD receptors (Nicola et al., 2003). More recent studies have shown that successful infection in this model requires the HSV-1 glycoproteins gB, gD, and gH-gL (Nicola and Straus, 2004). Despite these findings, a role for endocytosis during HSV-1 infection in vivo has not yet been confirmed.

2.1.3.2 Transport to the Nucleus and Release of Viral DNA

Following HSV-1 cell entry most, but not all of the tegument proteins are lost (Ojala et al., 2000), and the nucleocapsid is transported to the cell nucleus. This transport requires intact microtubules and involves cytoplasmic dynein, as will be discussed later in more detail (Sodeik et al., 1997). It is believed that HSV-1 tegument proteins VP11/12, VP13/14, VP16 and VP22 dissociate from the virus at early stages of infection, probably following phosphorylation, while the major tegument protein VP1/2 and the minor capsid protein UL25 remain attached to incoming capsids (Morrison et al., 1998a; Morrison et al., 1998b; Elliott and O’Hare, 1999; Dohner et al., 2002; Willard, 2002; La Boissiere et al., 2004).

After arriving at the cell nucleus, the HSV-1 nucleocapsid docks at the nuclear pore complex (NPC) and rapidly releases the viral DNA. In experiments by Ojala and others, binding of the nucleocapsid to the NPC required importin-β, which was both necessary and sufficient (Ojala et al., 2000). In the same report, the contributions of various HSV-1 tegument and capsid proteins to NPC binding were also investigated. Treatment of HSV-1 virions with detergents removed the viral envelope, but not the tegument proteins VP1/2, VP13/14, VP16 and VP22. Further treatment with trypsin removed these tegument proteins, reducing binding of the capsid to the NPC by 85%.
Since VP11/12, VP13/14 and VP16 have been shown to dissociate from incoming capsids, VP1/2 was considered the most likely candidate to mediate NPC binding (Ojala et al., 2000). Another possible candidate would be the UL37 protein, since it is also located in the inner tegument, along with VP1/2 (Mettenleiter, 2002).

### 2.1.3.3 HSV-1 Gene Expression and DNA Replication

HSV-1 genes are categorised according to the time of their expression, which occurs in a tightly regulated cascade manner (Roizman and Knipe, 2001). Viral genes are transcribed in the nucleus, and all viral proteins are synthesised in the cell cytoplasm.

The first genes to be expressed are the α, or immediate-early genes, with protein synthesis peaking approximately 2 to 4 hours after infection. The virion tegument protein VP16, part of the infecting virion, helps to stimulate expression of the IE genes (Campbell et al., 1984). VP16 is carried by host cell factor to the nucleus, where it binds to the cellular protein Oct-1 and initiates viral transcription (La Boissiere et al., 1999). The six α proteins produced are ICP0, ICP4, ICP22, ICP27, ICP47 and US1.5 (Roizman and Knipe, 2001).

The viral β, or early, genes are only expressed at significant levels in the presence of the α proteins, particularly ICP4, and their expression peaks 5 to 7 hours after infection (Roizman and Knipe, 2001). The appearance of β proteins, which include viral thymidine kinase and DNA polymerase, signals the onset of viral DNA synthesis. Other notable β proteins include ICP6, the large component of viral ribonucleotide reductase (Huszar and Bacchetti, 1981), and ICP8, the major DNA binding protein (Conley et al., 1981).

Expression of the γ, or late genes is stimulated by viral DNA synthesis, and occurs later in viral replication, as part of a continuum. This group include the genes
encoding viral structural proteins and glycoproteins. Since viral protein synthesis occurs in the cell cytoplasm, targeted transport of new proteins to the appropriate cell compartment is necessary to ensure orderly virion assembly (Roizman and Knipe, 2001).

HSV-1 DNA replication can be detected 3 hours post-infection, coincident with early β gene expression, and continues for at least another 12 hours (Roizman and Knipe, 2001). Viral DNA is replicated from a circular DNA template, resulting in a continuous linear strand of new DNA, which requires cleavage prior to packaging into new virions. Seven viral proteins are required for viral DNA replication: the viral DNA polymerase (UL30) and its accessory protein (UL42); an origin-binding protein (UL9); the ICP8 ssDNA-binding protein (SSB; UL29); and the helicase-primase complex of UL5, UL8 and UL52 (Roizman and Knipe, 2001).

2.1.3.4 HSV-1 Capsid Assembly

HSV-1 capsids in an infected cell are classified according to their appearance under the electron microscope (Roizman and Knipe, 2001). A-capsids are empty protein shells, composed of VP5, VP19C, VP23 and VP26. B-capsids contain these same capsid proteins, as well as the internal “scaffolding” proteins VP22a (UL26.5), VP21 and VP24 (both cleaved from the product of the UL26 gene). C-capsids are mature capsids, which have lost the internal scaffolding proteins and contain viral DNA.

It is now believed that spherical HSV-1 procapsids mature into icosahedral capsids following cleavage of the internal scaffolding protein (Beard and Baines, 2004). In this model, C-capsids are the result of successful DNA packaging, accompanied by cleavage and expulsion of the scaffolding proteins. A-capsids and B-capsids are not thought to be precursors of C-capsids; rather they are abortive forms, resulting from...
failed attempts to package DNA (Roizman and Knipe, 2001; Beard and Baines, 2004). A-capsids are believed to arise when the scaffold is lost but DNA is not retained, whereas B capsids are formed by premature or asynchronous cleavage of scaffold proteins, with the shell undergoing rearrangement before the scaffold is degraded or expelled.

A simplified model of HSV-1 capsid assembly is summarised in Figure 4. Capsid assembly is initiated by the condensation of the major capsid protein VP5 (UL19) and the scaffolding protein pre-VP22a (UL26.5). These structures are extended to form procapsids by the addition of the triplex proteins VP19C (UL38) and VP23 (UL18), which are incorporated into the capsid as preformed complexes (Spencer et al., 1998). Small capsid-like structures will form in the absence of scaffolding protein, but a scaffold is required for the formation of complete capsids (Tatman et al., 1994; Thomsen et al., 1995).

![Figure 4. Proposed pathway for in vitro assembly of the HSV-1 capsid.](image)

Assembly begins with an early partial capsid, which enlarges to form a late partial capsid, finally closing to create the spherical closed capsid or procapsid. The procapsid then angularises to form the mature polyhedral capsid. All capsid intermediates are suggested to contain all the proteins (products of genes UL19, UL18, UL38, UL26.5 and UL26) involved in capsid assembly. Note that the transformation of procapsids to polyhedral capsids does not involve attachment of additional capsid protein molecules. Although the maturational protease (VP24) is involved in capsid assembly in vivo, no role for it is shown here because it was not present in capsids assembled in vitro (reproduced with permission from Newcomb et al., 1996).
Interestingly, HSV-1 capsids can be assembled in vitro by combining recombinant capsid proteins, produced in baculovirus-infected insect cells. Spherical procapsids will form if purified capsid proteins VP5, VP19C and VP23 are combined with a hybrid scaffolding protein (Newcomb et al., 1999). Two independent groups demonstrated that mature icosahedral capsids can be produced by expressing the seven capsid proteins, from six HSV-1 genes, in a baculovirus system (Tatman et al., 1994; Thomsen et al., 1995). Capsids thus formed were indistinguishable by electron microscopy and protein analysis from B-capsids that had been isolated from HSV-1 infected mammalian cells. The structural authenticity of these capsids has been subsequently confirmed by cryo-electron microscopy, to a resolution of 2.7 nm (Trus et al., 1995). The omission of UL26.5 (VP22a) from the baculovirus system resulted in loss of the inner core, whereas omission of UL26 (VP21 and VP24) resulted in a large-cored phenotype. Omission of both these genes resulted in partially formed or deformed capsids (Tatman et al., 1994). VP26 was not essential for capsid assembly, but its presence increased assembly efficiency in one report (Thomsen et al., 1994).

The maturation of spherical procapsids into mature icosahedral capsids in vivo requires the release of capsid proteins from underlying scaffold by the viral protease VP24, the absence of which results in the intranuclear accumulation of immature procapsids (Newcomb et al., 2000). In contrast, capsids formed in vitro using the baculovirus system, in the absence of viral protease, are icosahedral and resemble HSV-1 B-capsids (Tatman et al., 1994; Thomsen et al., 1995). The reason for this difference is not understood, but manipulation of the system has increased our understanding of the maturation process. In one recent study, spherical procapsids were purified from mammalian cells that had been infected with protease-deficient HSV-1, and were then allowed to mature slowly in vitro. By comparing cryo-electron
micrographs of capsids at different stages of maturation, an elegant “time-lapse” overview of capsid maturation was obtained (Heymann et al., 2003).

In mammalian cells infected with HSV-1, newly synthesised capsid proteins must be transported from the cytoplasm to the cell nucleus, since this is where capsid assembly occurs (Roizman and Knipe, 2001). In vitro studies in mammalian cells have shown that the HSV-1 capsid proteins VP5, VP23 and VP26, if expressed in isolation, do not localise to the cell nucleus. Early experiments showed that the scaffolding protein VP22a was able to relocate VP5, but not VP23, to the nucleus in co-transfected cells (Nicholson et al., 1994). Subsequent work has confirmed that scaffolding protein preVP22a can relocate VP5 to the nucleus, while VP19C is able to relocate both VP5 and VP23 separately (Rixon et al., 1996). The small capsid protein VP26 must complex with VP5 before it can be relocated to the nucleus, either by VP19C or pre-VP22a (Rixon et al., 1996).

The portal complex, consisting of twelve UL6 molecules, is located at a unique vertex of mature capsids, although the time of portal incorporation during capsid production is not known (Newcomb et al., 2001). There is evidence that UL6 is incorporated into growing capsids as an intact portal, as it has been shown to form stable, soluble complexes with the scaffolding protein UL26.5. In the presence of the UL26.5 protein UL6 is also incorporated into viral capsids produced in vitro (Newcomb et al., 2003).

### 2.1.3.5 Packaging of Viral DNA

The assembly of full capsids containing DNA requires multiple proteins, including at least the products of the UL6, UL15, UL25, UL28, UL32, and UL33 genes (Lamberti and Weller, 1996; Patel et al., 1996; Lamberti and Weller, 1998; McNab et
al., 1998; Roizman and Knipe, 2001). Once thought to be essential for DNA cleavage/packaging, UL37 is now known to play a role later during virion maturation, and is essential for the addition of tegument (Desai et al., 2001). Packaging of HSV-1 DNA into capsids occurs in specialised nuclear structures called “replication compartments” (Roizman and Knipe, 2001). Targeting of newly formed capsids to these replication compartments is thought to involve the viral protein UL32 (Lamberti and Weller, 1998), and possibly UL17 (Taus et al., 1998).

The newly-replicated viral DNA is initially present as concatamers, which must be cleaved into unit-length molecules during packaging (Roizman and Knipe, 2001). Cleavage occurs at specific sites, termed DR1 sequences, within the terminal a sequence of the viral genome (Mocarski and Roizman, 1982). The exact packaging process is not well understood, but it is likely that the viral DNA is fed into the capsid at the same time as the scaffolding proteins are being displaced (Roizman and Knipe, 2001). It has been suggested that the necessary dissociation of VP22a molecules in the scaffold may be triggered by a drop in pH, resulting from the entry into the capsid of viral DNA (McClelland et al., 2002). It is thought that the viral DNA enters the capsid at a unique penton vertex, through the portal complex containing the UL6 protein (Newcomb et al., 2001). Two other minor capsid proteins, UL15 and UL28, associate with the UL6 portal, and form part of the terminase complex (White et al., 2003). The viral signals necessary for cleavage and packaging are located within the Uₜ and Uₖ domains of the terminal a sequence (Varmuza and Smiley, 1985). These DNA sequences have now been mapped precisely and designated DNA packaging elements pac1 and pac2 (Deiss et al., 1986). More recent experiments have confirmed that the deletion of either or both of these packaging elements inhibits the packaging of viral DNA (Hodge and Stow, 2001).
2.1.3.6 Where Does HSV-1 Acquire its Lipid Envelope?

The maturation process from DNA-filled capsids to infectious virions is incompletely understood and has been an area of much controversy. It is generally accepted that after being packaged with viral DNA, HSV-1 C-capsids acquire an envelope at the inner nuclear membrane, and that mature virions in the extracellular space are also enveloped. Until recently the favoured model was that enveloped virions were transported from the nucleus to the extracellular space either in vesicles or via cisternae of the endoplasmic reticulum (ER) (Roizman and Knipe, 2001). As early as 1969 an alternate model was proposed, involving de-envelopment of the nucleocapsid at the outer nuclear membrane, release of naked capsids into the cell cytoplasm, and subsequent re-envelopment in another cellular compartment (Stackpole, 1969).

The intracellular location of another major tegument protein, VP22, has contributed to the controversy over the years. VP22 has the unusual property of being able to traffic to surrounding cells, either from cells infected with HSV-1 or transfected cells expressing VP22 (Elliott and O'Hare, 1997). In early studies VP22 had been observed exclusively in the cytoplasm of infected cells, suggesting extranuclear addition of tegument to naked viral capsids (Elliott et al., 1995). In contrast, work by another group observed phosphorylated VP22 in cell nuclei, suggesting the possible acquisition of tegument in the nucleus (Pomeranz and Blaho, 1999). Live cell imaging of transfected cells over-expressing GFP-tagged VP22 showed that the protein had a cytoplasmic distribution during interphase, moving to the nucleus only during mitosis (Elliott and O'Hare, 2000). The authors concluded that their results supported the re-envelopment model, since early during HSV-1 infection, or in cells such as neurones that do not divide, VP22 must be added to virions in the cytoplasm. However, recent
Our studies from our group have detected VP22 in the nucleus of neurones infected with HSV-1 (Miranda-Saksena et al., 2002). Thus, this issue still has not been resolved, and although maturing HSV-1 virions probably acquire most of their VP22 in the cytoplasm, some addition in the nucleus cannot be excluded.

Many other lines of evidence now support the newer, so-called re-envelopment model, which has gained increasing favour in recent years. It was shown that when cells were infected by a strain of HSV-1 with a mutation of the UL37 gene, DNA-filled capsids acquired a primary envelope at the inner nuclear membrane, were de-enveloped at the outer nuclear membrane, then accumulated in the perinuclear region, unable to mature further (Desai et al., 2001). This suggests that at least some of the tegument is added after exiting the nucleus, and that UL37 is required for egress and re-envelopment. The UL37 gene product is also known to be involved in secondary envelopment in the closely-related porcine herpesvirus PrV (Klupp et al., 2001b). Further observations to support this model include studies of neurones infected with HSV-1, where nucleocapsids in axons were associated with some tegument proteins, but were not enveloped (Penfold et al., 1994; Holland et al., 1999; Miranda-Saksena et al., 2000). This suggests that the viral envelope is added at a more distal site, presumably the axon terminus, rather than at the nuclear membrane (see below for more detailed discussion of these results).

The most recent studies to support the re-envelopment model looked at the addition of surface glycoproteins to maturing virions. In one set of experiments, cells were infected with a strain of HSV-1 encoding a mutant form of gH, containing an ER-retention signal, restricting its expression to the inner nuclear membrane-ER. Normal numbers of extracellular virions were released from infected cells, but they did not contain gH (an essential glycoprotein), and were thus not infectious (Browne et al.,
This suggests that gH is added to maturing virions in a post-ER compartment. In similar experiments, when an ER-retention or ER-retrieval signal was inserted into gD, 10-fold to 100-fold less infectious virus was produced, compared with normal amounts of infectious virus when a Golgi-retention or Golgi-retrieval signal was inserted (Whiteley et al., 1999). This result implies that gD is also added to virions in a post-ER compartment, probably part of the Golgi apparatus. Recent experiments by the same group have looked in more detail at the effects of inserting an ER-retrieval signal into gD. In cells infected with this mutant virus, gD was observed in the nuclear membranes of infected cells and on virions in the perinuclear space, but very little gD was detected in the viral envelope of mature virions, suggesting re-envelopment in a post-ER cellular compartment (Skepper et al., 2001).

The re-envelopment model is further supported by nuclear egress studies, using the closely related alphaherpesvirus PrV (Mettenleiter, 2002). These studies showed that the UL31 and UL34 proteins were present in virus in the perinuclear space, following primary envelopment, but were lost following de-envelopment and were not present in mature virions. In contrast, tegument proteins UL46, UL47 and UL49 were present in mature virions, but not in perinuclear enveloped virions. These results are discussed below in more detail.

2.1.3.7 HSV-1 Tegument Addition and Viral Egress

The re-envelopment model of HSV-1 maturation is now generally accepted, as above, and is discussed in detail in a recent review (Mettenleiter, 2002). Minor revisions to the model have now been proposed, based on the asymmetrical appearance of the HSV-1 tegument by cryo-electron tomography (Grunewald et al., 2003). This revised model is summarised in Figure 5.
Figure 5. Egress pathway of HSV-1 nucleocapsids.
Precursor procapsids (pc) assemble in the nucleus, are then packaged with DNA to produced mature nucleocapsids (mc) that exit the nucleus by envelopment at the inner nuclear membrane to be released into the cytoplasam by de-envelopment at the outer nuclear membrane. Viral glycoproteins accumulate in a post-Golgi compartment (pGc). Secondary envelopment is triggered by interaction of the nucleocapsid and/or attached tegument proteins with glycoprotein endodomains at a specialised site, perhaps involving lipid rafts (lr). Tegument proteins are recruited on to an extending scaffold between the capsid and the membrane in a process that may involve actin microfilaments (ac). The tegumented nucleocapsid is finally internalised into the pGc by pinching off at its proximal pole. Finally, virions are released into extracellular space by fusion of their outer, pGc-derived membrane with the plasma membrane. (Model based on the proposal by Mettenleiter, 2002. Figure reproduced with permission from supplementary online material, Grunewald et al., 2003).
The first step in virion maturation, after the capsid has been packaged with DNA, is budding of the capsid through the inner nuclear membrane. The membrane-associated, tail-anchored UL34 protein (Klupp et al., 2000; Roller et al., 2000; Shiba et al., 2000), as well as the nuclear phosphoprotein UL31 (Reynolds et al., 2001; Fuchs et al., 2002b), are both necessary for efficient capsid egress. The HSV-1 kinase US3 is also thought to play an important, but non-essential role during nuclear egress (Reynolds et al., 2002; Ryckman and Roller, 2004), possibly by targeting UL34 to the inner nuclear membrane (Klupp et al., 2001a; Reynolds et al., 2001). It is thought that the UL34 protein represents a primary envelope protein, while UL31 represents a primary tegument protein (Mettenleiter, 2002). Importantly, UL34 protein is not present in mature HSV-1 or PrV virions (Klupp et al., 2000; Reynolds et al., 2002), consistent with the re-envelopment model. Similarly, the major tegument protein UL49 (Klupp et al., 2000), as well as tegument proteins UL46 and UL47 (Kopp et al., 2002), are present in mature intracytoplasmic and extracellular PrV virions, but not in perinuclear enveloped virions.

HSV-1 tegument proteins are acquired in the cytoplasm (inner tegument), or while budding into trans-Golgi vesicles (outer tegument) (Mettenleiter, 2002). The large tegument protein VP1/2 is thought to form the innermost layer of the tegument, as it interacts with the major capsid protein VP5 (McNabb and Courtney, 1992a), and deletion of the gene encoding for VP1/2, UL36, results in the perinuclear accumulation of unenveloped, DNA-filled capsids with no tegument layer (Desai, 2000). The second layer of the tegument is thought to be the UL37 protein, since deletion of UL37 in either HSV-1 or PrV results in the perinuclear accumulation of capsids coated in VP1/2 (Desai et al., 2001; Klupp et al., 2001b). UL37 is located in the tegument of mature HSV-1 virions (Schmitz et al., 1995), and its abundance is
tightly controlled, since increasing the amount of UL37 in infected cells does not increase the amount of UL37 protein incorporated into virions (McLauchlan, 1997). The PrV UL37 protein has been shown to interact directly with the UL36 protein (VP1/2 equivalent), both by co-immunoprecipitation and by yeast two-hybrid assay (Klupp et al., 2002).

The addition of further tegument proteins is less well defined, and complex, with multiple redundancies in protein-protein interactions (Mettenleiter, 2002). Unlike the UL36 and UL37 gene products, which are essential for the subsequent addition of tegument to capsids, most other tegument proteins are non-essential, although their absence may decrease the efficiency of virion maturation. The current model suggests that the inner tegument proteins UL36 and UL37 are added directly to capsids, and that the outer tegument proteins associate with the cytoplasmic tails of glycoproteins on trans-Golgi vesicles. The capsid acquires its outer tegument by budding into the trans-Golgi vesicles, thus forming mature virions. The virions are then released from the cell by exocytosis (Mettenleiter, 2002).

One of the tegument proteins thought to be crucial in “bridging” the inner and outer tegument is VP16, the UL48 gene product. In experiments where UL48 was deleted from PrV, unenveloped capsids accumulated in the cytoplasm, coated with the inner tegument proteins UL36 and UL37 (Fuchs et al., 2002a). “Light particles”, membrane-bound particles containing the outer tegument proteins UL46, UL47 and UL49, but without a capsid, were produced and released from infected cells in abundance (Fuchs et al., 2002a). In contrast, when light particles are produced in cells infected with wild-type virus, they also contain the UL37 and UL48 proteins (McLauchlan, 1997; Fuchs et al., 2002a), supporting an important role for UL48 in
tegument assembly. Furthermore, HSV-1 VP16 was recently reported to interact with the cytoplasmic tail of gH, both in vitro and in vivo (Gross et al., 2003).

Many other protein-protein interactions have been reported among HSV-1 tegument proteins, and the current knowledge is summarised in Figure 6 (Mettenleiter, 2002).

**Figure 6. Interactions of HSV-1 and PrV gene products in virion formation.**
Solid lines or direct contacts between the rectangles representing the designated gene products indicate physical interaction, whereas arrows indicate functional effects. Dotted lines and question marks denote suggested, but not firmly established, interactions or involvement of proteins. Between glycoproteins, only direct contacts resulting in complex formation are depicted. Steps: 1, primary envelopment; 2, de-envelopment; 3, secondary (final) envelopment. NUC, nucleus; CYT, cytoplasm (reproduced with permission from a review by Mettenleiter, 2002).
2.2 Intracellular Transport Mechanisms

Mammalian cells contain complex intracellular structures, resulting in slow rates of diffusion within the cell. Cells have therefore developed active intracellular transport systems to move material around, and direct it to the correct cellular compartment. There has been much research into intracellular transport systems in recent years, which is summarised in a recent comprehensive review (Kamal and Goldstein, 2000).

The most common method for transporting molecules around a cell is in membrane-bound vesicles, carried by molecular motors attached to the cytoskeleton. Long-range vesicle transport occurs along microtubules using kinesin or dynein motors, while short-range transport is mediated by myosin motors along actin filaments. Neurones in particular require active long-range transport to carry nutrients, neurotransmitters and other molecules between the neuronal cell body and nerve synapse, frequently along very long axonal processes. This process utilises microtubules, and mutations in motor proteins or their receptors can cause defects in axonal transport, resulting in significant neurological disease (Goldstein, 2001).

In non-polarised cells, microtubules extend radially from their “minus” end, at the perinuclear microtubule organising centre (MTOC), to the actively growing “plus” end, near the cell periphery. In neurones, this arrangement extends into the axons, with the microtubule minus ends at the cell body, and the plus ends near the synapse (Topp et al., 1994). The arrangement of microtubules is more complex in dendrites, rod photoreceptors and polarised epithelial cells. In polarised cells the microtubule minus ends usually point toward the apical surface, with the plus ends extending through the cell body towards the basolateral surface (Figure 7) (Goldstein and Yang, 2000).
The main molecular motors associated with microtubules are kinesin and dynein. Kinesins are a family of (mostly) plus-end-directed motors (anterograde transport in neuronal axons and non-polarised cells), while dyneins are minus-end-directed motors (retrograde transport in axons and transport toward the nucleus in non-polarised cells). Defects in each pathway have been associated with human disease.

There is recent evidence that “slow” neuronal transport, such as that responsible for neurofilament transport, may occur through infrequent brief episodes of “fast” transport mediated by kinesins, rather than a separate transport system (Shea and Flanagan, 2001; Brown, 2003).


2.2.1 Kinesins

Fast anterograde transport in neuronal axons is mediated by kinesin moving along microtubules in a plus-ended direction (Goldstein and Yang, 2000). There are 10 major subfamilies of kinesins, and over 30 different kinesins can be found in a single species (Susalka et al., 2000). At least 45 kinesins have been identified to date, and they are classified according to the position of their motor domain: NH$_2$-terminal motor domain type (N-type), middle motor domain type (M-type), and COOH-terminal motor domain type (C-type) (Hirokawa, 1998). The 40 kDa motor domain of kinesin heavy chain (KHC) is highly conserved, but there is significant variability in the stalk and tail domains of KHC, allowing specific binding to a diverse range of cellular cargoes (Figure 8).

Conventional kinesin (kinesin-I or KIF5), is an N-type hetero-tetramer, comprising two identical KHCs and two identical kinesin light chains (KLCs). KLC binds to KHC via the C-terminal region of the KHC stalk domain, and interacts via heptad repeats (Diefenbach et al., 1998). Kinesin binds to cellular vesicles via receptors or “adapters”, with different receptors providing binding specificity and allowing effective sorting and directing of cargo (Goldstein and Yang, 2000). At least six forms of conventional kinesin are encoded by the mammalian genome, with three known genes for KHC (KIF5A, KIF5B and KIF5C) and at least three for KLC (KLC1, KLC2 and KLC3) (Goldstein and Yang, 2000). KIF5B appears to be expressed ubiquitously, while KIF5A and KIF5C seem to be expressed only in neurones. Similarly, KLC1 expression is enhanced in neurones; KLC2 expression is more uniform throughout different tissues; and KLC3 expression has not been detected to date (Goldstein and Yang, 2000).
**Figure 8. Principles of kinesin and dynein diversification.**

Kinesins appear to have evolved by gene duplication and divergence such that a conserved motor (shaded ovals) has become harnessed to a wide array of diverse tail domains. The location of the motor domain in the heavy chain polypeptide allows kinesin motors to be divided into several groups: an N-terminal group, a central group, and a C-terminal group. In addition, N-terminal kinesins have been found as homo-dimers, hetero-dimers, homotetramers, and monomers. Cytoplasmic dyneins appear to have conserved the bulk of their principle motor polypeptide and have generated many fewer divergent genes. Accessory-chain diversification may be the principal mechanism for functional specialisation (reproduced with permission from Goldstein and Yang, 2000).

The proteins that link kinesin to its membranous cargo, and the relative contributions of KHC and KLC to cargo binding, are not currently known. The first kinesin binding receptor to be identified was the transmembrane protein kinectin, which binds to KHC (Toyoshima *et al.*). Since then, many other binding partners for KHC have been identified, including the neuronal small nuclear GTPase RanBP2, which interacts with KIF5B and KIF5C, but not with KIF5A (Cai *et al.*). It has been suggested recently that
direct binding of cargoes may co-ordinate the sorting of polarised motors to different cellular compartments (Gunawardena and Goldstein, 2004). An example is GluR2-interacting protein (GRIP1), which binds to KIF5B and may help to “steer” kinesin and its attached cargo into dendrites, rather than into axons (Setou et al., 2002).

Some recent studies in our laboratory have focused on proteins that bind to conventional KHC KIF5B. We have demonstrated binding of the C-terminal tail domain of KIF5B to HSV-1 tegument protein US11 (Diefenbach et al., 2002b), synaptosome-associated proteins of 25 and 23 kDa (SNAP25 and SNAP23) (Diefenbach et al., 2002a), and most recently to the ribosome receptor, p180 (Diefenbach et al., 2004). Binding of US11 to KIF5B is probably important for anterograde transport of HSV-1 in sensory neuronal axons, and will be discussed later in more detail.

A cellular protein that binds to KLC, and may act as an adapter for membranous cargo, is the product of the gene *sunday driver* or *syd*. The name was chosen because mutations in the gene resulted in “traffic jams” of vesicles and membranous organelles in narrow axons, which caused lethal paralysis in *Drosophila* larvae (Bowman et al., 2000). The membrane-associated protein product of *syd* has been shown to interact directly with KLC (Bowman et al., 2000), suggesting a possible role as a kinesin adapter protein. Since similar phenotypes are observed following mutations of the *syd*, KHC, KLC, dynein or dynactin genes (see below), a likely common pathology is disruption of axonal transport (Goldstein, 2001). Interestingly, a recent screen for proteins that interact with the C-terminal portion of KLC identified three scaffolding proteins from the c-jun NH2-terminal kinase (JNK) signalling pathway called JNK-interacting proteins (JIPs) JIP-1, JIP-2 and JIP-3 (Verhey et al., 2001). Their precise
role in axonal transport is yet to be determined, but JIP-3 is considered to be the mammalian equivalent of \textit{syd} (Goldstein, 2001).

Amyloid precursor protein (APP), encoded on chromosome 21, is another protein that has been shown recently to bind to KLC (Kamal \textit{et al.}, 2000). Proteolytic fragments of APP are abundant in plaques found throughout the brains of people with Alzheimer’s Disease, and it has been suggested that defective axonal transport of APP may play a role in this disease (Goldstein, 2001). Mis-sense mutants of APP cause some forms of familial Alzheimer’s disease, while humans with trisomy 21 (Down syndrome) often develop premature Alzheimer’s disease.

Mutation of another member of the kinesin superfamily, KIF1B, appears to be the underlying defect in the autosomal dominant axonal peripheral neuropathy, Charcot-Marie-Tooth Disease Type 2A (Zhao \textit{et al.}, 2001). The KIF1B motor is involved in mitochondrial transport, and mutations cause defects in the transport of synaptic vesicle precursors, resulting clinically in peripheral neuropathy.

Kinesin-II, which contains KHC motor subunit KIF3A or KIF3B, is thought to be involved in the movement of proteinaceous lipid rafts, and may be important in slow axonal transport. Mutations in KIF3 affect ciliary movement, and may be related to Kartagener’s syndrome (\textit{situs inversus}) in humans, and polycystic kidney disease in mice (Goldstein, 2001).

Although most kinesin motors move along microtubules in a plus-ended direction, some can travel in a minus-ended direction, especially in polarised epithelial cells. An example is the kinesin superfamily motor KIFC3, which has been shown to transport cargo to the apical membrane of renal tubular epithelial cells (Noda \textit{et al.}, 2001).
Despite this finding, kinesin motors are not thought to contribute significantly to retrograde transport in neuronal axons, a task performed predominantly by dynein.

### 2.2.2 Cytoplasmic Dynein

The dyneins are massive molecular motor complexes, involved in cellular transport, which utilise ATP to generate force towards the minus end of microtubules. Flagellar dyneins provide motive forces in eukaryotic cells, while cytoplasmic dyneins are involved in intracellular motility, including during mitosis, maintenance of the Golgi apparatus and trafficking of intracellular particles (King, 2000). Cytoplasmic dynein also mediates transport of vesicular tubular complexes from the ER to the Golgi apparatus (Murshid and Presley, 2004). Cytoplasmic dynein is the main molecular motor involved in retrograde transport of vesicles and other particles in neuronal axons (Goldstein and Yang, 2000). Its structure is complex, and has been the focus of several recent reviews (Milisav, 1998; Vallee and Gee, 1998; King, 2000; Susalka et al., 2000; Vallee et al., 2004).

Cytoplasmic dynein is a large (1.2 MDa) complex containing heavy chains, intermediate chains, light-intermediate chains and light chains (King, 2000; Susalka et al., 2000) (Figure 9). In contrast to KHC, dynein heavy chain (DHC) is highly conserved in all domains. Cargo binding specificity seems to rely on various combinations of intermediate and light chains, the expression of which varies in different tissues (Figure 8).

The most common form of cytoplasmic dynein, termed cytoplasmic dynein 1, is widely expressed in cells, and is responsible for many intracellular functions. It is involved in the transport of a variety of membranous organelles, several aspects of chromosome behaviour, mitotic spindle orientation, nuclear migration, and cell
migration (Vallee et al., 2004). The less common form of cytoplasmic dynein, called dynein 1b or dynein 2, is much more restricted in its cellular distribution and range of functions. Dynein 2 is limited to ciliated cells, and in lower eukaryotes may play a role in transport within axonemal structures, that is, cilia and flagella. In vertebrate cells, the expression of dynein 2 is restricted to ciliated cells, and to a variety of ciliated structures, including the modified connecting cilia of sensory neurones (Vallee et al., 2004). Dynein 2 has also been identified in association with the Golgi apparatus in vertebrate cells (Vaisberg et al., 1996).

Figure 9. Cytoplasmic dynein complex.
This is a diagrammatic view of the cytoplasmic dynein complex, showing the proposed arrangement of dynein subunits. Note that the 14 kDa light chains rp3 and Tctex1 form homodimers, meaning that two copies of either rp3 or Tctex1 are present in each cytoplasmic dynein complex.
2.2.2.1 **Dynein Heavy Chain**

DHC has a molecular weight of approximately 530 kDa and provides motive force for the dynein complex. It consists of an N-terminal stem domain (160 kDa), which interacts with other heavy chains, as well as with intermediate and light-intermediate chains (Tynan *et al.*, 2000a). The 350 kDa globular head domain has a highly conserved central region (100 kDa), containing four nucleotide binding sites, and a slender stalk containing the microtubule binding domain. One of the nucleotide binding sites is involved in ATP hydrolysis; the role of the other domains is unclear (King, 2000).

Two isoforms of DHC have been identified to date. The most common form of dynein, dynein 1, contains the DHC1 isoform, while dynein 2 contains DHC2 (Vaisberg *et al.*, 1996; Vallee *et al.*, 2004). It is not known exactly how the motor activity of DHC is regulated. Possible mechanisms include direct phosphorylation of DHC (Lin *et al.*, 1994), phosphorylation of other dynein subunits, conformational change of subunits following cargo binding, interaction with unknown proteins, or change in redox poise (King, 2000).

2.2.2.2 **Dynein Intermediate Chains**

Cytoplasmic dynein intermediate chains (DICs) are 74 kDa proteins, which are thought to contribute to cargo binding, as well as forming the scaffold of the dynein complex (King, 2000). The importance of DIC in determining dynein function is supported by a genetic analysis of dynein function (Boylan *et al.*, 2000). There are two known genes (DIC1 and DIC2) that encode for DIC in mammals, sharing 76% homology. Each gene has two regions of alternate splicing, allowing up to six possible splicing variants in total. Five of these six potential DIC isoforms have been identified
in rats: DIC1A and 1B; DIC2A, 2B and 2C (Vaughan and Vallee, 1995). In mice, sequences have been determined for DIC isoforms 1a, 1c, 1d, 1e and 2. In humans, only isoforms 1a and 1c have been identified to date (Crackower et al., 1999).

Figure 10. Domain organisation of dynein intermediate chain (DIC).
This figure shows the different structural and domains of DIC, including proposed binding sites for the dynactin subunit p150Glued, dynein light chains Tctex1/rp3, LC8 and roadblock; and DHC. Note the alternate splicing sites, which allow for the expression of different DIC isoforms. The unspliced isoform shown here is DIC-1a (adapted from Mok et al., 2001).

As the central structural component of the cytoplasmic dynein complex, DIC has been shown to bind to several other subunits of the dynein and dynactin complexes, and its binding domains are summarised in Figure 10. The N-terminal domain of DIC contains a coiled-coil region made up of heptad repeats, which is responsible for dimerisation of DIC, as well as for the interaction between DIC and the p150Glued component of the dynactin complex (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). This region is followed by two regions of alternative splicing, separated by a conserved, serine-rich region (Vaughan and Vallee, 1995). Following the second region of alternative splicing are the binding domains for the dynein light chains: the first domain binds one of the 14 kDa light chains (Tctex1 or rp3); the second domain binds the 8kDa light chain, LC8 (Mok et al., 2001; Makokha et al., 2002). The region
of DIC where the roadblock (LC7) light chains bind has been identified recently, and is a little further towards the C-terminal (Susalka et al., 2002). Other cellular proteins that have been reported to bind directly to DIC include the cell junction proteins β-catenin (Ligon et al., 2001) and PLAC-24 (Karki et al., 2002), although their binding sites on DIC are not known.

The C-terminal domain of DIC contains highly conserved “WD40” repeats, which are proposed to bind to DHC (Mok et al., 2001). In this region, seven 40-residue modules containing tryptophan (W) and aspartic acid (D) at defined positions interact to form a β-propeller structure. Each repeat forms a four strand β-sheet, which in turn forms a blade in the propeller (ter Haar et al., 2000; Holm et al., 2001).

Expression of different isoforms of DIC appears to be tissue specific, and presumably reflects differing roles in cargo binding and transport (Susalka et al., 2000). In rats, DIC2C is ubiquitous, and in glial cells is the only isoform present. DIC2B is common, while DIC1B is found mainly in testis and some cultured cell lines. DIC1A and 2A appear to be specific to neurones. Adult rat brain neurones express all five isoforms.

DIC isoform expression also varies at different stages of development. Embryonic rat brains initially contain only DIC2C, but express other isoforms later in development. Similarly phaeochromocytoma cell lines, which normally express only DIC2C, can be induced to produce other isoforms of DIC by the addition of nerve growth factor, which induces differentiation and the formation of axon-like processes (Susalka et al., 2000).

As well as determining the cargo carried by cytoplasmic dynein, isoform variation may also influence the distribution of dynein complexes within the cell. For example,
after carrying cargo to the cell nucleus, dynein complexes need to be returned to the cell periphery, and are thought to utilise anterograde transport systems. Experiments in adult rat neurones have shown that 10% of cytoplasmic dynein complexes contain DIC2C, and that these complexes are transported in axons via the fast anterograde transport system, which utilises kinesin. The other isoforms are transported by slow neuronal transport systems (Susalka et al., 2000). The authors propose that the DIC2C isoforms are predominantly responsible for rapid retrograde transport of membranous cargo. Other isoforms, being firmly attached to other parts of the cytoskeleton, may be involved in anterograde propulsion of microtubules into developing axons. This model is consistent with the observation by Dillman et al. that there are different pools of dynein in axons undergoing anterograde transport, each with distinct IC isoform compositions (Dillman et al., 1996).

### 2.2.2.3 Dynein Light Intermediate Chains

There are two families of dynein light intermediate chains (LICs), with molecular weights between 50 and 60 kDa. These molecules contain ATP binding sites, undergo post-translational phosphorylation, and may be involved in controlling dynein activity (Gill et al., 1994; Hughes et al., 1995). LICs bind directly to DHC, like DIC, whereas the other light chains bind to DIC. Although the binding site for LIC on DHC overlaps with the binding site for DIC, DIC and LIC have been confirmed to be present in cytoplasmic dynein complexes at the same time (Tynan et al., 2000a).

LIC has been shown to bind to the centrosomal protein pericentrin (Purohit et al., 1999; Tynan et al., 2000b), which uses dynein for transport to and assembly at the centrosome during formation of the mitotic spindle (Young et al., 2000). Consistent with an important role during cell division, mutation of dynein LIC genes in
Caenorhabditis elegans results in abnormal mitosis, and failed zygote development (Yoder and Han, 2001).

A novel dynein LIC was identified recently, which forms part of the dynein 2 complex, and binds to DHC2 (Perrone et al., 2003). This LIC chain was up-regulated in response to de-flagellation, and may be important in the regulation of retrograde intra-flagellar transport.

### 2.2.2.4 Dynein LC8

Dynein light chain LC8 has a molecular weight of 10 kDa, and each cytoplasmic dynein complex contains one LC8 dimer (King, 2000). Binding of LC8 to DIC causes a significant conformational change in DIC, which may be important for the stability of the cytoplasmic dynein complex (Makokha et al., 2002). The LC8 protein is highly conserved across species, and is thought to perform several different intracellular functions. As well as forming part of the cytoplasmic dynein complex, LC8 has been identified in myosin V complexes (Espindola et al., 2000), although its role is unclear. LC8 is also thought to be involved in several enzyme pathways, as it interacts directly with neuronal nitric oxide synthase (nNOS), IkBα, and Bim, a Bcl-2 family protein involved in apoptosis (King, 2000). Mutations of LC8 (ddlc1) in Drosophila are lethal, causing embryonic morphogenetic defects and apoptosis (Dick et al., 1996).

Two recurrent binding motifs have been identified in proteins that bind to LC8: G(I/V)QVD and (K/R)XTQT (Lo et al., 2001; Rodriguez-Crespo et al., 2001). The Gln (Q) in both sequences has been shown to occupy an invariant position in the binding cleft of LC8, establishing strong hydrogen bonds with Glu35 and Lys36 in the side chain of LC8, thus forming a novel β-strand (Liang et al., 1999; Fan et al., 2001). The motif GIQVD is present in the cellular proteins nNOS, DNA cytosine methyl
transferase, and guanylate kinase domain-associated protein (GKAP). Cellular proteins that share the KSTQT motif include DHC, Bim, Kid-1, protein 4 and the product of the *Drosophila swallow* gene (Rodriguez-Crespo *et al.*, 2001). This motif is also present in several viral proteins that have been shown to interact with LC8 (see below).

### 2.2.2.5 14 kDa Dynein Light Chains (Tctex1, rp3)

*Tctex1* (*t*-complex *testis*-expressed-1) is a gene that was initially mapped to the *t*-complex of mouse chromosome 17, a mutated multi-gene region that causes a non-Mendelian inheritance phenomenon called “transmission ratio distortion”. It was observed that male mice who were heterozygous for the *t*-complex haplotype transmitted the mutant chromosome to their offspring at a frequency of >99%, while male *t/t* homozygotes were completely sterile (Lader *et al.*, 1989). The mutation in the *t*-complex resulted in over-expression of the Tctex1 protein (Mok *et al.*, 2001), which has subsequently been identified as a light chain component of both flagellar axonemal dyneins and cytoplasmic dynein (King *et al.*, 1996). Sperm bearing the mutant chromosome had superior mobility to sperm bearing the wild type chromosome, resulting in this unusual inheritance pattern. The human analogue of Tctex1 is sometimes called Tctel (*Tctex1*-like), but to avoid confusion it will simply be referred to as “Tctex1” throughout this thesis.

Each cytoplasmic dynein complex contains two copies of a 14 kDa light chain from the *Tctex1* family, either Tctex1 or rp3, which share 55% amino acid homology (King, 2000). Tctex1 shows considerable structural and target binding similarities with dynein light chain LC8, but shares no amino acid sequence homology (Mok *et al.*, 2001). Rp3 and Tctex1 usually form homo-dimers, and are thought to be mutually
exclusive in cytoplasmic dynein complexes (Tai et al., 2001). Despite their similarities, Tctex1 and rp3 exhibit significant differences in cellular and tissue distributions, as well as in their binding specificities.

Rp3 is expressed ubiquitously, with the highest levels found in liver and brain, significant levels in oesophagus, heart, liver, lung, muscle and spleen, and lowest levels in testis. In contrast, expression of Tctex1 is lowest in liver and adult brain, but highest in testis (King et al., 1998). Interestingly, levels of Tctex1 expression in neuronal tissue change during development, with abundant Tctex1 mRNA expression in foetal brain, but down-regulation in adult brain (Kai et al., 1997). Conversely, levels of rp3 mRNA are higher in adult than foetal neuronal tissue (Roux et al., 1994). Expression of Tctex1 or rp3 throughout the brain also seems to reflect the maturity of different neurone populations. In a study of rat hippocampus, Tctex1 was most prevalent in newly-generated granule cells, but scant in mature granule and pyramidal cells. The staining pattern for rp3 was the reverse (Chuang et al., 2001).

A recently identified role for Tctex1 during the cellular response to neurotrophins may explain its higher level of expression in developing neurones. Nerve growth factor (NGF) binds to tyrosine kinase TrkA receptors in the distal axons of developing neurones, and is taken up into vesicles. The neurotrophin-TrkA complexes are then transported to the neuronal cell body, where they stimulate nerve growth and promote cell survival (Tsui-Pierchala and Ginty, 1999). This retrograde axonal transport has been shown to be rapid, microtubule-dependent (Watson et al., 1999), and to involve cytoplasmic dynein (Bhattacharyya et al., 2002). A direct interaction between Tctex1 and TrkA was identified recently, and is now thought to mediate binding of the neurotrophin-TrkA complex to cytoplasmic dynein (Yano et al., 2001).
It has been proposed that Tctex1 is the dominant 14 kDa light chain found in cytoplasmic dynein complexes in presynaptic nerve terminals, while rp3 is more important in postsynaptic neurones (Chuang et al., 2001). Ultra-structural analysis of the dentate gyrus in rat brains demonstrated that Tctex1 was preferentially associated with the ER, and vesicles in axon terminals, while rp3 was more apparent in dendritic spines, the Golgi apparatus and trans-Golgi network (Chuang et al., 2001). Consistent with its probable role in pre-synaptic nerve termini, Tctex1 has been shown to interact directly with Doc-2α a neuronal protein involved in neurotransmitter release (Nagano et al., 1998).

In addition to their different intracellular distributions and levels of expression in various tissues, Tctex1 and rp3 differ in their binding specificities. Tctex1, but not rp3, binds to the retinal protein rhodopsin (Tai et al., 1999), as well as to the synaptic vesicle-associated protein Doc-2, (Nagano et al., 1998). Significantly, Tctex1 and rp3 have been shown to compete for binding sites on DIC, and over-expression of rp3 in MDCK cells can displace Tctex1 from cytoplasmic dynein complexes, thereby disrupting transport of rhodopsin (Tai et al., 2001).

2.2.2.6 Lissencephaly and the Lis1 Protein

Another neurological disease related to dynein function is lissencephaly, or “smooth brain”. It is caused by a haplo-insufficiency of the Lis1 gene product, and results from a lack of migration of neuronal cells from the paraventricular zone to the cerebral cortex, with loss of normal cortical folds (Morris, 2000). The exact mechanism of this defect is still unknown, but there is increasing evidence that it involves dynein and/or dynactin.
The Lis1 protein has been shown to colocalise with dynein and dynactin at kinetochores, and alterations in its level of expression or activity affects cell division (Faulkner et al., 2000). Lis1 has been shown to associate with DHC, and to upregulate translocation of microtubule segments by dynein (Sasaki et al., 2000; Smith et al., 2000). Total absence of Lis1 is lethal, but a heterozygous defect preferentially affects the brain, suggesting a higher requirement for Lis1 during brain development. Levels of Lis1 are relatively enriched in neurones, while overexpression of Lis1 in non-neuronal cells has been shown to cause an increase in the size of dynein/dynactin complexes, with increased dynein activity (Smith et al., 2000). Studies in Drosophila have shown that mutations in Lis1 result in abnormal neuronal dendrite formation, and defective axonal transport, similar to those observed following mutations in DHC (Liu et al., 2000).

### 2.2.3 Dynactin

The dynactin complex is another large, multi-molecular complex involved in rapid, microtubule-associated transport. It is essential for mitosis in multicellular organisms, and thus for viability, and is intimately associated with dynein (Schroer, 2004). It has been shown recently that dynactin binds directly to microtubules and enhances the average length of dynein-driven movements, without affecting the ATPase activity or microtubule-binding ability of dynein (Kamal and Goldstein, 2000; King and Schroer, 2000).

Dynactin subunits range in size from 22 to 150 kDa (Holleran et al., 1998), and their likely arrangement within the complex is summarised in Figure 11. The dynactin complex is asymmetrical, and contains two morphologically distinct structural domains (Schafer et al., 1994a). The main part of the complex is a 10 × 40 nm rod,
which is believed to bind to cellular cargo. Projecting from the rod is a 25-50 nm arm, terminating in two globular microtubule-binding domains (Schroer, 2004).

![Figure 11. Structure of dynactin.](image)

This schematic illustration shows the location and approximate structural features of the subunits in the dynactin complex. The overall structure of dynactin was inferred from electron microscopy studies, while the arrangement of individual subunits is a summary of published results (reproduced with permission from Schroer, 2004).

The 40 nm rod is formed by an octameric polymer of the 45 kDa subunit actin-related protein 1 (Arp1), or centractin. One end of the Arp1 filament is capped with the conventional actin-capping protein CapZ (Schafer et al., 1994a; Schafer et al., 1994b). The other end of the rod terminates in a second actin-related protein, Arp11 (Eckley et al.), which is associated with the dynactin subunit p62, along with subunits p25 and p27 (Schafer et al., 1994a).
The flexible arm projecting from the Arp1 rod is formed by a dimer of the $p150^{\text{Glued}}$ subunit, which self-associates via coiled-coil domains. Four copies of the p50 subunit dynamitin are found at the shoulder of the $p150^{\text{Glued}}$ dimer, in association with two p24/p22 hetero-dimers. It is believed that dynamitin and p24/p22 are important in anchoring the $p150^{\text{Glued}}$ arm to the Arp1 rod (Schroer, 2004).

### 2.2.3.1 Arp1-Spectrin Interactions

Spectrin, a protein found on axonal vesicles, has been shown to play an essential role during retrograde axonal transport of vesicles (Muresan et al., 2001). The dynactin subunit Arp1 has been shown recently to interact directly with $\beta$III spectrin, the isoform of spectrin that associates with the Golgi complex (Holleran et al., 2001). The authors propose that the interaction between $\beta$III spectrin and Arp1 recruits dynein and dynactin to intracellular membranes, and provides a direct link between the microtubule motor complex and its membrane-bounded cargo.

### 2.2.3.2 $p150^{\text{Glued}}$-Microtubule Interactions

$p150^{\text{Glued}}$ is the largest subunit of dynactin, and each dynactin complex contains two copies, present as a dimer. $p150^{\text{Glued}}$ was first identified as a protein that coprecipitated with the cytoplasmic dynein complex (Collins and Vallee, 1989). The initial isolate was from rat brain, and was named $p150^{\text{Glued}}$ because of its homology with the product of the *Drosophila* gene *Glued* (Holleran et al., 1998). The human gene was sequenced in 1996, and mutations were proposed to be responsible for limb-girdle muscular dystrophy type GGMD2B (Tokito et al., 1996). The genomic structure of $p150^{\text{Glued}}$ was determined in 1998 by two independent groups, one of which was investigating possible genetic links to Alström Syndrome (Collin et al., 1998; Tokito and Holzbaur, 1998).
The globular head of p150\textsuperscript{Glued}, which forms the tip of the dynactin arm, has been shown to bind to microtubules both \textit{in vitro} (Waterman-Storer \textit{et al.}, 1995) and \textit{in vivo} (Vaughan \textit{et al.}, 2002). Microtubule binding is thought to be mediated by a conserved N-terminal CAP-Gly motif (Riehemann and Sorg), with amino acids 39-150 both necessary and sufficient for binding (Vaughan and Vallee, 1995; Waterman-Storer \textit{et al.}, 1995).

A 135 kDa splice variant of p150\textsuperscript{Glued}, present at high levels in mammalian neurones, lacks this microtubule binding domain, does not decorate microtubules, and has instead been proposed to play a role in slow neuronal transport (Tokito \textit{et al.}, 1996; Holleran \textit{et al.}, 1998). It has been suggested that this smaller isoform may link cytoplasmic dynein to the \textit{slow component b} transport complex, without binding to microtubules, thus leaving dynein free to mediate anterograde microtubule “gliding” (Dillman \textit{et al.}, 1996).

\textbf{2.2.3.3 \textit{p150}\textsuperscript{Glued}-Dynein Interactions}

A direct interaction between p150\textsuperscript{Glued} and DIC was demonstrated initially \textit{in vitro} using affinity chromatography (Karki and Holzbaur, 1995), reciprocal blot overlays and immunoprecipitation (Vaughan and Vallee, 1995). Binding was localised to amino acids 200-811 of p150\textsuperscript{Glued} and the N-terminal portion (amino acids 1-123) of DIC, and was proposed to involve coiled-coil interactions (Vaughan and Vallee, 1995). A recent study of dynein-dynactin binding confirmed the interaction between p150\textsuperscript{Glued} and DIC \textit{in vivo}, as well as \textit{in vitro} (King \textit{et al.}, 2003). This more detailed analysis found that amino acids 217-548 of p150\textsuperscript{Glued}, which are predicted to form a coiled-coil \(\alpha\)-helix, are sufficient for dynein binding. It also showed that, although the N-terminal 106 amino acids of DIC were sufficient for binding, a fragment containing amino
acids 1-237 bound more efficiently, suggesting that other interactions may also contribute positively to binding (King et al., 2003).

The interaction between p150<sup>Glued</sup> and DIC is required for fast axonal transport. In a squid axon model, the addition of antibodies directed against p150<sup>Glued</sup> disrupted dynein-dynactin binding, prevented dynein binding to membranes, and significantly slowed transport of organelles along microtubules. In contrast, it did not affect the binding of dynactin to microtubules, nor dynein-mediated microtubule gliding (Waterman-Storer et al., 1997). In a separate report, addition of anti-DIC antibody to Xenopus extracts blocked DIC-p150<sup>Glued</sup> interaction, resulting in the removal of DIC from cellular membranes, impaired formation of the ER network, and disruption of organelle transport along microtubules (Steffen et al., 1997).

The regulation of cytoplasmic dynein binding to membranous cargo is incompletely understood, but is thought to involve dynactin. Phosphorylation of p150<sup>Glued</sup> has been shown to increase organelle motility, possibly through changes in either the association of dynactin with microtubules or the binding affinity of p150<sup>Glued</sup> for dynein (Holleran et al., 1998). An alternate model was proposed in a recent paper (Kumar et al., 2001), after the binding of both dynein and dynactin to cellular membranes was shown to be co-dependent (i.e. neither was able to bind membranes in the absence of the other). Dynein-dynactin complexes seem to exist in two discrete states in the cell: one capable of binding to membranes and the other not. In the proposed model, the C-terminal coiled-coil and basic domains of p150<sup>Glued</sup> interact with Arp1 (centractin), sterically blocking access of Arp1 to spectrin on membranous cargo. Deletion of this region caused constitutive binding of dynactin to membranes. The authors proposed that binding of dynactin to membranes requires a folding back of the C-terminal domain of p150<sup>Glued</sup> to expose Arp1, thereby allowing binding to
spectrin on membranous cargo (Kumar et al., 2001). The control mechanisms for such a transformation are not known.

It has been shown that movement of ER membranes in *Xenopus* egg extracts is disrupted during apoptosis, after cleavage of both DIC and p150Glued by cellular caspases (Lane et al., 2001). Membrane movement was restored by the addition of intact dynein-dynactin, further supporting a model of dynamic interaction between molecular motors and their cargo.

### 2.2.3.4 p50 Dynamitin

p50 is the second most abundant subunit in dynactin, with four copies present per complex. It is highly conserved, with 96% homology between rat and human, and contains three regions with a high probability of forming α-helices (Schroer, 2004).

Overexpression of dynamitin in prometaphase mammalian cells affects mitosis by disrupting mitotic spindle formation (Echeverri et al., 1996). Overexpression of dynamitin in interphase cells disrupts the transport of endosomes and lysosomes towards the nucleus, causing them to be redistributed towards the periphery of the cell (Burkhardt et al., 1997). Both these results suggest that dynamitin is critical in dynactin assembly.

Interestingly, overexpression of dynamitin in postnatal motor neurones of transgenic mice results in late-onset, progressive motor neurone degeneration that is clinically similar to amyotrophic lateral sclerosis in humans (LaMonte et al., 2002). This confirms the biological importance of dynactin in axonal transport, and suggests that a similar transport defect may be present in people with that disease.
2.2.4 Regulation of Anterograde and Retrograde Transport

One of the crucial aspects of microtubule-associated transport is how the apparently competing plus-end (kinesin) and minus-end (dynein) directed motors are regulated, to allow effective net transport of intracellular cargo. It is now accepted that a single cargo may have multiple motors attached to it at the same time, which helps to explain frequent observations of bidirectional movement (Waterman-Storer et al., 1997; Suomalainen et al., 1999; Smith et al., 2001; Suomalainen et al., 2001).

Since molecular motors, like most cellular proteins, are synthesised in the ER of the cell body, cytoplasmic dynein must be transported to the periphery of the cell, including to axon termini in neurones, to mediate retrograde transport. There is evidence that cytoplasmic dynein travels in both directions in axons: actively in the retrograde direction, and passively in the anterograde direction. In nerve ligation experiments, dynein and dynactin have been shown to accumulate on both the proximal and distal sides of the blockade (Hirokawa et al., 1990). In contrast, kinesin is produced in the cell body, travels in an anterograde direction to the cell periphery, but does not return (Hirokawa et al., 1991; Kondo et al., 1994; Yamazaki et al., 1995; Yang and Goldstein, 1998).

Assuming that during anterograde transport both kinesin and dynein are attached to the same cargo, net transport in the anterograde direction can occur by one of three mechanisms (Figure 12). Of these three proposed models, there is increasing evidence to support model (C), in which the relative activities of dynein and kinesin are regulated, rather than there being a “tug of war”, as proposed in model (B).
Figure 12. Working models for regulation of transport direction.

(A) Kinesin is activated when dynein is repressed, leading to anterograde movement. On kinesin destruction or inactivation, dynein is activated, leading to retrograde transport. (B) Kinesin and dynein are both active during anterograde transport, but the greater processivity of kinesin leads to net anterograde movement. On kinesin destruction or inactivation, dynein can generate retrograde movement. (C) Active regulation and coordination leads to alternating kinesin and dynein activation and repression. The balance controls direction of transport (reproduced with permission from Goldstein and Yang, 2000).

The mechanism of the coordination of kinesin and dynein function is not fully understood, but two recent papers have shed some light on the subject (Gross, 2003). A study of lipid droplet transport in *Drosophila* embryos provided strong evidence for the regulation model (C), since mutations in either DHC or dynactin not only disrupted retrograde transport, but also anterograde transport (Gross *et al.*, 2002). If the “tug of war” model (B) were true, then disruption of dynein activity would be expected to increase, rather than decrease, kinesin-dependent anterograde transport. Unlike previous studies, aberrant “locking up” of dynein motors was discounted as the explanation for reduced anterograde movement, since minus-end-directed stalling forces were shown to be normal in some circumstances (Gross *et al.*, 2002).
Furthermore, since only droplets not interacting with other cargos were studied, the interruption of anterograde transport was not due to a “traffic jam”, as had been proposed to explain results from earlier axonal transport studies (Martin et al., 1999).

It has been proposed that the regulation of dynein and kinesin activity is coordinated by dynactin (Gross et al., 2002), an hypothesis that is supported by more recent data from a *Xenopus laevis* melanophore model (Deacon et al., 2003). In this study the authors demonstrated a direct interaction between the *Xenopus* kinesin II-associate protein (XKAP) and the p150Glued subunit of dynactin. This is the first time that a direct interaction has been demonstrated between dynactin and kinesin, providing strong evidence for the role of dynactin in coordinating both kinesin and dynein motors on the same cargo (Gross, 2003). Intriguingly, a recent paper has demonstrated a direct interaction of both KLC1 and KLC2 with DIC, possibly adding another level of complexity to the coordination of these motors (Ligon et al., 2004).
2.3 Intracellular Transport of Viruses

The mechanisms involved in transporting viruses within cells are beginning to be understood, and have been the subject of several recent reviews (Sodeik, 2000; Garner, 2003). The size and complexity of eukaryotic cells effectively restricts free intracellular diffusion for molecules larger than 500 kDa, requiring cells to have organised transport systems. It has been estimated that a HSV-1 capsid would take approximately 231 years to travel 1 cm through cytoplasm by diffusion alone (Sodeik, 2000). During infection of flat cells in vitro, it may be possible for HSV-1 capsids to reach the nucleus by diffusion, since the nucleus is only a few μm away from the cytoplasm at the apical surface (Garner, 2003). However, the slow rate of diffusion is clearly not compatible with viruses travelling very long distances along sensory neurones to establish infection in humans.

As outlined in the previous section, complex intracellular transport systems have evolved to allow efficient transport and targeting of vesicles and other molecules within cells. Some viruses, such as paramyxoviruses and picornaviridae, replicate in the cytoplasm, and thus have lesser requirements for intracellular transport. In contrast, herpesviruses and retroviruses require access to the cell nucleus to replicate. Early in the infection cycle, these viruses need to move efficiently from the cell membrane to the nucleus to introduce their DNA or RNA. Having established a productive infection, newly-synthesised virions must be transported back to the cell periphery to egress into the extracellular space and complete the infection cycle. In many cases it appears that viruses utilise existing intracellular transport systems to achieve these goals (Sodeik, 2000).
2.3.1 Retrograde Viral Transport

Viruses that enter the cell by receptor-mediated endocytosis, such as poliovirus, influenza virus and Semliki Forest virus, are transported within the cell inside endocytic organelles. As discussed above, rapid retrograde transport of cellular vesicles is mediated by the microtubule-associated, minus-end-directed motor cytoplasmic dynein (King, 2000; Vallee et al., 2004). Attachment of the dynein complex to its membranous cargo is usually mediated by the dynactin complex, and probably involves a direct interaction between the dynactin subunit Arp1 and cellular proteins such as spectrin (Holleran et al., 2001; Muresan et al., 2001). Interestingly, a direct interaction was recently reported between the cytoplasmic domain of the human poliovirus receptor (CD155) and dynein light chain Tctex1 (Ohka et al., 2004). This interaction was shown to be essential in vivo for the rapid retrograde transport of poliovirus-containing vesicles along microtubules (Ohka et al., 2004). Experimentally, retrograde transport of canine parvovirus in vesicles can be blocked by injecting anti-dynein antibodies into cells, further supporting a role for cytoplasmic dynein in viral transport (Suikkanen et al., 2002).

Retrograde transport of adenoviruses is active, and also involves microtubules and cytoplasmic dynein (Suomalainen et al., 1999; Kelkar et al., 2004). Movement of adenoviruses within cells has been observed to be bidirectional and dynamic, with apparent competition between plus-end and minus-end directed molecular motors (Suomalainen et al., 1999). Adenovirus has been shown to interact with dynein and dynactin complexes by coimmunoprecipitation (Kelkar et al., 2004), and its retrograde transport can be disrupted by cellular overexpression of the p50 (dynamitin) subunit of dynactin (Suomalainen et al., 1999). Interestingly, one of the adenoviral proteins, Ad E3-14.7K, has been shown to bind to the human 14 kDa dynein light chain TcTex1,
but only in the presence of a GTP-ase, FIP-1 (Lukashok et al., 2000). Recent work suggests that integrins may be important in initiating retrograde transport of adenoviruses, and that the protein kinase A and p38/MAP pathways may also be involved (Suomalainen et al., 2001).

Proteins from several other microtubule-associating viruses have been shown to interact directly with dynein light chain LC8, via the typical G(I/V)QVD or (K/R)XTQT motifs. These proteins include phosphoprotein P from lyssavirus and the rabies-related Mokola virus (Jacob et al., 2000; Raux et al., 2000); p54 from African swine fever virus (Alonso et al., 2001); the U19 gene product from human herpesvirus 6B; and HSV-1 helicase (UL9) (Martinez-Moreno et al., 2003). Deletion of the LC8 binding motif from rabies virus P protein has been shown to dramatically reduce viral pathogenicity, which appears to be specific for retrograde axonal transport (Mebatsion, 2001). Recently, a direct interaction was also demonstrated between LC8 and the human immunodeficiency virus type 1 (HIV-1) gag protein, although neither of the known LC8 binding motifs is present in gag (Petit et al., 2003).

2.3.2 Retrograde Transport of HSV-1

Like adenoviruses, transport of HSV-1 to the cell nucleus is thought to proceed along microtubules (Topp et al., 1994; Sodeik et al., 1997). However, unlike viruses that enter the cell by endocytosis, and are transported in vesicles, herpesviruses and retroviruses infect cells by pH-independent fusion of the viral envelope with the plasma membrane (Spear and Longnecker, 2003).

Once inside the cell, most of the HSV-1 tegument proteins are lost, leaving the nucleocapsid, with some associated tegument, to be transported to the cell nucleus to release its DNA (Ojala et al., 2000). Before it can access the microtubule transport
system, the de-enveloped viral particle must first traverse the actin-rich cortex of the
host cell (Garner, 2003). This process is poorly understood, but actin microfilaments
are likely to play an active role, since treatment of cells with cytochalasin B reduces or
slows down viral entry (Wittels and Spear, 1991). Viral entry is accompanied by
phosphorylation of host cell proteins, which can be blocked with tyrosine kinase
inhibitors (Qie et al., 1999), although it is not clear whether or not this
phosphorylation stimulates actin transport.

It is clear that retrograde transport of HSV-1 requires intact microtubules, since
disruption of the microtubule network by colchicine, vinblastine or nocodazole
inhibits retrograde transport of HSV-1 in both non-polarised cells (Sodeik et al., 1997)
and sensory neurones (Topp et al., 1994). Initially it was demonstrated by electron
microscopy that incoming HSV-1 capsids associate with microtubules, and also with
cytoplasmic dynein (Sodeik et al., 1997). Subsequently, co-localisation of HSV-1
capsids with both cytoplasmic dynein and dynactin was confirmed using confocal
microscopy (Dohner et al., 2002). Most importantly, retrograde transport of HSV-1
capsids to the cell nucleus has been shown to be dynein-dependent, since it can be
blocked by over-expression of the dynactin subunit p50 (dynamitin) (Dohner et al.,
2002).

Active retrograde transport of HSV-1 in the giant squid axon model has been
visualised using time-lapse fluorescent microscopy (Bearer et al., 2000). A green
fluorescent protein (GFP)-labelled strain of HSV-1 was constructed by fusing GFP to
the tegument protein VP16. Detergent was used to remove the envelopes from HSV-1
virions and the capsid-tegument complexes were injected into live squid axons. Under
ultraviolet light, fluorescent virus particles were seen to move along the axons, in a
retrograde direction, at a mean velocity of $2.2 \pm 0.26 \, \mu\text{ms}^{-1}$, consistent with dynein-mediated transport.

In summary, retrograde intracellular transport of the HSV-1 capsid-tegument is mediated by cytoplasmic dynein, and requires intact microtubules, but it is not known which HSV-1 proteins are involved. Since the HSV-1 virion loses its envelope and most of the tegument proteins following cell entry, the most likely viral proteins to interact with dynein or dynactin are those located in the outer capsid or inner tegument.

A direct interaction has been reported between the HSV-1 UL34 protein and the N-terminal fragment of DIC-1a (Ye et al., 2000). In this paper, recombinant DIC-1a was expressed in bacteria as a fusion protein, with a glutathione-S-transferase (GST) tag. Vero or Hep-2 cells were infected with HSV-1 (F strain), labelled with $[^{35}\text{S}]$methionine, then lysed and the cellular fragments removed. A pull-down assay using GST-DIC-1a found several HSV-1 proteins that appeared to bind to DIC-1a. After immuno-blotting and reciprocal pull-down assays, the authors concluded that UL34 bound directly to DIC-1a. They also showed that UL31 bound to UL34 and was pulled down as part of a complex, along with VP5, which seemed to bind non-specifically. Subsequent work by the same group confirmed a direct interaction between UL31 and UL34, and showed that UL31 depends on UL34 expression for its stability (Ye and Roizman, 2000).

The HSV-1 viral helicase, encoded by the UL9 gene, has been noted to contain the typical LC8 binding motif, KSTQT (Rodriguez-Crespo et al., 2001). Subsequent in vitro studies, using the pepscan technique, have confirmed that UL9 peptides bind to dynein light chain LC8 (Martinez-Moreno et al., 2003).
Despite these results, neither UL34 nor UL9 is likely to play a significant role during retrograde transport of the HSV-1 capsid-tegument complex. The UL34 protein is membrane-associated (Shiba et al., 2000), and plays an important role during nuclear egress and primary envelopment (Klupp et al., 2000; Fuchs et al., 2002b; Reynolds et al., 2002). However, deletion of the UL34 gene does not prevent infection of cells by HSV-1 (Roller et al., 2000), and most importantly the UL34 protein is absent from mature virions in HSV-1 (Reynolds et al., 2002) and PrV (Klupp et al., 2000; Klupp et al., 2001b; Fuchs et al., 2002b). Similarly the UL9 protein does not form part of the mature virion (Roizman and Knipe, 2001), so is unlikely to mediate transport of incoming viral particles.

2.3.3 Anterograde Viral Transport

Anterograde transport of newly-produced viruses, from the perinuclear region to the cell periphery, is mediated by the plus-end directed kinesin family of motors (Goldstein and Yang, 2000; Tomishima et al., 2001; Garner, 2003). Transport of HSV-1 along microtubules, in a plus-end direction, was demonstrated initially in human retinal pigment epithelial cells (Topp et al., 1996). Axonal transport of the closely related porcine alphaherpesvirus, PrV, has been observed directly in chick embryo neurones, using GFP-labelled capsids (Smith et al., 2001). The authors of this report observed a combination of anterograde and retrograde motion in axons, with predominant movement in the anterograde direction. The average velocity of anterograde runs was $1.97 \pm 0.06 \mu \text{ms}^{-1}$. Many capsids moving in the anterograde direction reversed direction for a period of time, before resuming anterograde movement; a few underwent predominantly retrograde movement, at an average
velocity of 1.28 μm s⁻¹. Some capsids were seen to pause briefly, and then move again; others stalled, remaining immobile for a significant period of time.

Anterograde transport of HSV-1 in giant squid axons has also been visualised, using virions containing a fluorescent tegument protein, GFP-VP16 (Satpute-Krishnan et al., 2003). In this report, HSV-1 virions travelled at an average rate of 0.9 ± 0.3 μm s⁻¹, with a maximum observed velocity of 1.23 μm s⁻¹. Interestingly, transported virions were found to contain large amounts of APP, which has been shown to bind to kinesin via the KLC (Kamal et al., 2000). The significance of these findings for HSV-1 transport in vivo is unclear.

Our group recently reported a direct interaction between the heavy chain of ubiquitous kinesin (KIF5A) and the HSV-1 tegument protein US11, using in vitro binding assays (Diefenbach et al., 2002b). In a separate report, US11 was shown to associate with the KLC-related protein PAT1 (Benboudjema et al., 2003). Taken together, these results suggest a probable role for US11 in mediating anterograde transport of HSV-1 in neuronal axons, and work is in progress to confirm its functional significance in vivo.

Recent studies suggest that HSV-1 and PrV are transported in neuronal axons as separate viral components, rather than as intact virions. Our group reported initially that unenveloped HSV-1 nucleocapsids were present in the axons of explanted human sensory neurones, and were associated with microtubules (Penfold et al., 1994). Subsequent work in our laboratory, using immuno-electron microscopy, confirmed that the microtubule-associated capsids contained only nucleocapsid (VP5) and tegument (VP16) proteins, whereas the glycoproteins (gB, gC and gD) were more diffusely distributed throughout the axons, probably in membranous vesicles (Holland et al., 1999).
These findings, along with more detailed kinetics of anterograde transport, have been confirmed subsequently in dissociated rat neurones, using confocal microscopy (Miranda-Saksena et al., 2000). Treatment of HSV-1 infected cells with nocodazole, an inhibitor of microtubule polymerisation, inhibited the transport of nucleocapsids, tegument and glycoproteins. In contrast, treatment with Brefeldin A, which inhibits transport through the Golgi, reduced transport of glycoproteins, but not of nucleocapsids, confirming that each is transported along a separate pathway (Miranda-Saksena et al., 2000).

Transport studies using PrV provide further evidence for the separate transport of viral components in axons. In chick embryo neurones, PrV membrane glycoproteins gB, gC, gE, and gI have been observed in peripheral axon termini, not in association with capsids (Smith et al., 2001). Interestingly, the PrV tegument protein US9, although not essential for viral replication in vitro, is necessary for anterograde spread of virus in the rat central nervous system (Brideau et al., 2000a), and its action requires an acidic motif (Brideau et al., 2000b). This result can be explained by recent experiments in cultured rat neurones, which showed that deletion of the PrV US9 gene specifically inhibited axonal transport of viral glycoproteins, but did not inhibit transport of capsids or tegument proteins (Tomishima and Enquist, 2001). Viral replication can still proceed in vitro, in the absence of US9, since axonal transport is not required for the production of infectious virus, which can egress directly from neuronal cell bodies. However, deletion of US9 inhibits transport of viral glycoproteins to axon termini in vivo, and therefore prevents the assembly of mature virions at these peripheral sites, which is necessary for distal neuronal spread.
2.4 Summary

During primary HSV-1 infection in humans, viral nucleocapsids with some associated tegument proteins are transported along axons in a retrograde direction to neuronal cell bodies, prior to establishing latent infection. This is an active process, involving movement of viral particles along microtubules by cytoplasmic dynein, probably in association with dynactin. Although the mechanism by which HSV-1 binds to the dynein complex is not known, many cellular and viral proteins have been shown to bind to dynein via the intermediate and light chains. It is likely, therefore, that these dynein subunits are involved in binding to the HSV-1 virion.

The only HSV-1 proteins that have been reported previously to interact with dynein are UL34 (with DIC), and UL9 (with LC8). However, both these interactions have only been demonstrated in vitro, with no proven biological significance. Furthermore, neither protein is found in mature HSV-1 virions, so they are unlikely to play a significant role during retrograde viral transport of incoming virus. Since HSV-1 loses its envelope and most of the tegument proteins following cell entry, viral proteins from the outer capsid or inner tegument are the most likely candidates to interact with cytoplasmic dynein.

In this thesis, we report an interaction between the HSV-1 outer capsid protein VP26 and dynein light chains Tctex1 and rp3 (Douglas et al., 2004). The interaction was detected initially in a yeast two-hybrid system, and confirmed using in vitro binding studies. Recombinant capsids containing VP26 bound to dynein, whereas capsids without VP26 did not. To confirm that this interaction was biologically significant, a novel experimental model was developed, where recombinant HSV-1 capsids were introduced directly into live cells using micro-injection techniques. Capsids that
contained VP26 were shown to co-localise with dynein light chains rp3 and Tctex1, as well as with microtubules. Most importantly, after several hours the capsids containing VP26 had moved closer to the cell nucleus, whereas capsids without VP26 had not.

Thus, we propose that retrograde transport of HSV-1 in mammalian cells, including neurones, is mediated by an interaction between the HSV-1 outer capsid protein VP26 and dynein light chains rp3 and Tctex1.
CHAPTER 3:

GST-DYNEIN PULL-DOWN ASSAYS
3.1 Introduction

As discussed in Chapter 2, retrograde transport of HSV-1 along microtubules is thought to be mediated by cytoplasmic dynein (Sodeik et al., 1997). The only interaction to have been reported between a HSV-1 protein and dynein is an interaction in vitro between the HSV-1 UL34 protein and dynein intermediate chain (DIC) (Ye et al., 2000). In these experiments, a N-terminal fragment of DIC-1a, comprising codons 1-228, was expressed in bacteria as a GST-tagged fusion protein, bound to glutathione-agarose beads, and used in pull-down assays (See Figure 13). Vero or Hep-2 cells were infected with HSV-1, and the viral proteins radio-labelled with $^{35}$S-methionine. The infected cells were lysed, and the lysates were incubated with the GST-DIC-1a fusion protein, bound to beads. Several proteins, ranging in size from 30 kDa to 150 kDa, bound to GST-DIC-1a, but not to GST. Two of these proteins were identified as the HSV-1 UL31 and UL34 proteins by Western blot, while the largest protein was thought to be the major capsid protein VP5, based on its apparent molecular weight. The other proteins were not identified. In reciprocal pull-down experiments, DIC-1a (both full-length DIC and an N-terminal fragment including 258 amino acids) was shown to bind in vitro to GST-UL34. The authors finally showed that UL34 and UL31 formed a complex in vitro, which also associated with VP5.

Despite this reported interaction between UL34 and DIC in vitro, other lines of evidence suggest that UL34 is unlikely to play an important role during retrograde transport of HSV-1. The UL34 protein is not present in mature HSV-1 virions (Reynolds et al., 2002), and deletion of the UL34 gene does not prevent infection of
cells by HSV-1 (Roller et al., 2000). For these reasons, we wanted to identify other HSV-1 proteins that bound to dynein.

For our initial experiments we performed similar in vitro binding assays, using GST-tagged dynein light chains, as well as GST-DIC. We prepared lysates from cells infected with HSV-1, incubated them with the GST-tagged fusion proteins, and examined the bound complexes for viral proteins by Western blotting. Our results demonstrate binding of several HSV-1 glycoproteins to DIC, including gD, gC and probably gB. There was no consistent evidence of HSV-1 proteins binding to dynein light chains LC8, rp3 or Tctex1 in this model. The region of DIC involved in binding to the HSV-1 proteins lies between amino acids 143 and 267, as a GST-tagged fusion of this fragment was shown to bind the same HSV-1 proteins. It is not clear whether one or more of these glycoproteins bind directly to DIC, or whether binding is mediated by another, as yet unidentified protein. Subsequent experiments confirmed that HSV-1 glycoproteins isolated from mature HSV-1 virions bound to DIC, as well as glycoproteins from cell lysates. This suggests that the protein which binds to DIC is present in HSV-1 virions, and is likely to be a membrane-associated viral protein.
3.2 Materials and Methods

3.2.1 Dynein Expression Constructs

The entire ORF for each dynein or dynactin gene was amplified by PCR and cloned into recombinant expression vectors. Oligonucleotide primers, incorporating EcoRI and/or XhoI restriction endonuclease sites, were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) (Rozen and Skaletsky, 2000) and BioManager by ANGIS (http://www.angis.org.au) (Table 1). The gene for human dynein intermediate chain (DIC-1c isoform) was amplified from a pBluescript plasmid, kindly provided by Dr. Lap-Chee Tsui, Dept Genetics, University of Toronto (Crackower et al., 1999). Genes for dynein light chains Tctex1, rp3, LC8, and Roadblock1, as well as dynactin subunit p150Glued, were amplified from a human brain cDNA library (Display Biosystems Biotech). For DIC-1c, EcoRI sites were engineered at both the 5’ and 3’ ends. For dynein light chain genes and p150Glued, a 5’ EcoRI site and 3’ XhoI site allowed directional cloning into expression vectors.

All genes were amplified by PCR using Geneamp® XL PCR Kit (PE Applied Biosystems), according to the manufacturer’s recommendations. The optimal concentration for each reagent was determined, and used for all subsequent reactions (Appendix 1). Thermal cycling was performed on a Touchdown thermal cycler (Hybaid), with longer annealing times in later cycles, as recommended (Appendix 1). Melt temperatures for each primer were calculated using the nearest-neighbour formula (Breslauer et al., 1986) and an appropriate anneal/extend temperature chosen for each reaction (Table 1).
Table 1. Oligonucleotide Primers for PCR of Dynein and Dynactin Genes.

<table>
<thead>
<tr>
<th>Genes</th>
<th>5’/3’</th>
<th>Insertion</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Tm*</th>
<th>TAE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIC-1c</td>
<td>5’</td>
<td>EcoRI</td>
<td>DIC1A5</td>
<td>ttccggaattcatgctgac aaaaagtgaatattaa ttcggaaatctgagcataataactcaagcgttc</td>
<td>55.0</td>
<td>54.0</td>
</tr>
<tr>
<td>DIC-1c</td>
<td>3’</td>
<td>EcoRI</td>
<td>DIC1A3</td>
<td></td>
<td>58.2</td>
<td></td>
</tr>
<tr>
<td>DIC-1c (1-142)</td>
<td>5’</td>
<td>EcoRI</td>
<td>DIC1A5</td>
<td>ttccggaattcatgctgac aaaaagtgaatattaa gcggccgtggtggctaca gataggctcaagcgttc</td>
<td>55.0</td>
<td>54.0</td>
</tr>
<tr>
<td>DIC-1c (143-628)</td>
<td>3’</td>
<td>XhoI</td>
<td>HDIC1_1-142</td>
<td>aaaaagtgaatattaa gcggccgtggtggctaca gataggctcaagcgttc</td>
<td>58.9</td>
<td></td>
</tr>
<tr>
<td>DIC-1c (143-267)</td>
<td>5’</td>
<td>EcoRI</td>
<td>HDIC1_143-267</td>
<td>gcgggaattcaccagag aaaaagtgaatattaa gcggccgtggtggctaca gataggctcaagcgttc</td>
<td>54.4</td>
<td>50.0</td>
</tr>
<tr>
<td>DIC-1c (268-628)</td>
<td>3’</td>
<td>XhoI</td>
<td>HDIC1_268-628</td>
<td>gcgggaattcaccagag aaaaagtgaatattaa gcggccgtggtggctaca gataggctcaagcgttc</td>
<td>50.2</td>
<td></td>
</tr>
<tr>
<td>LC8</td>
<td>5’</td>
<td>EcoRI</td>
<td>hDLC1_5’</td>
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<td>68.8</td>
<td>50.0</td>
</tr>
<tr>
<td>rp3</td>
<td>3’</td>
<td>XhoI</td>
<td>HsRP3_3’</td>
<td>gcgggaattcaccagag aaaaagtgaatattaa gcggccgtggtggctaca gataggctcaagcgttc</td>
<td>55.9</td>
<td>54.0</td>
</tr>
<tr>
<td>Tctex1</td>
<td>5’</td>
<td>EcoRI</td>
<td>hsTctel1_5’</td>
<td>gcgggaattcaccagag aaaaagtgaatattaa gcggccgtggtggctaca gataggctcaagcgttc</td>
<td>59.3</td>
<td>54.0</td>
</tr>
<tr>
<td>Roadblock1</td>
<td>3’</td>
<td>XhoI</td>
<td>hsTctel1_3’</td>
<td>gcgggaattcaccagag aaaaagtgaatattaa gcggccgtggtggctaca gataggctcaagcgttc</td>
<td>55.4</td>
<td></td>
</tr>
<tr>
<td>p150Glued</td>
<td>5’</td>
<td>EcoRI</td>
<td>Hp150glued.5</td>
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<td>57.4</td>
<td>56.0</td>
</tr>
<tr>
<td></td>
<td>3’</td>
<td>XhoI</td>
<td>Hp150glued.3</td>
<td></td>
<td>56.5</td>
<td></td>
</tr>
</tbody>
</table>

# Predicted melt temperature
* Anneal/extend temperature for thermal cycling
PCR products were digested with appropriate endonucleases, and inserted into digested expression plasmids using a Clonables™ Kit (Novagen). For recombinant expression in *E. coli* with N-terminal fusion tags, genes were cloned into pGEX-5X-1 (Amersham-Pharmacia Biotech) for glutathione-S-transferase (GST) tag fusion protein expression. All constructs were sequenced to confirm gene sequence and correct reading frame for recombinant protein expression.

### 3.2.2 Expression of GST-tagged Fusion Proteins

For expression of dynein subunits with GST fusion tags, *E. coli* strain BL21 cells were transformed with the pGEX-5X-1 plasmid constructs. Briefly, 100 μL of competent *E. coli* cells (Novagen) were incubated with approximately 200 ng of plasmid DNA at 4 °C for 30 min, placed in a 42 °C water bath for 90 sec, and then returned to 4 °C for 2 min. 900 μL of Luria-Bertani (LB) broth (Sigma) was added to each sample, and tubes were incubated for 1 h at 37 °C, shaking at 250 rpm. 100 μL of cells was inoculated onto LB agar plates, containing 100 μg/mL of ampicillin (Progen), spread evenly with a sterile glass rod, and incubated overnight at 37 °C.

Transformed cells, growing on the agar plate, were inoculated into 3 mL of LB broth with 100 μg/mL of ampicillin and incubated overnight at 37 °C, shaking at 250 rpm. The following morning cultures were diluted to 100 mL, in LB broth with 100 μg/mL of ampicillin, grown for 3 to 4 hours to mid-log phase, at 37 °C, shaking at 250 rpm. Protein expression was then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37 °C. Induced bacterial cultures were harvested by centrifugation at 2500 × g for 10 min, in pre-chilled tubes, at 4 °C. Supernatants were removed, and the bacterial cell pellets were promptly placed in a -20 °C freezer for storage.
3.2.3 Preparation of HSV-1 Virus Stocks

To prepare virus stocks, Hep2 cells were grown to 80% confluence in 75 cm$^2$ Falcon$^\text{®}$ tissue culture flasks (Becton Dickinson), in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen), supplemented with 9% (v/v) foetal calf serum (FCS) (JRH Biosciences), at 37 °C, in 5% CO$_2$. Cells were infected with 0.01 plaque forming units (PFU) per cell of a low-passage clinical strain of HSV-1 (MC1, passage 4), and incubated in DMEM with 1% (v/v) FCS. Virus was added to cells initially in 5 mL of media, to encourage virus binding, and after 1 h at 37 °C a further 15 mL of media was added to each flask, prior to prolonged incubation. Once 80% of the cells displayed cytopathic effect (48-72 h), cells were removed using a cell scraper, frozen and thawed 3 times, subjected to sonication (3 × 7 sec, Branson Sonicator, 100% duty cycle), and cellular debris removed by centrifugation at 800 × g for 30 min, at 4 °C. The supernatant was filtered through a disposable 0.22 μm Millex$^\text{®}$-GP filter (Millipore), divided into 1 mL aliquots, and stored at -80 °C.

To determine HSV-1 titre, a viral plaque assay was performed. Human lung embryo fibroblasts (passage 4, CSL) were grown to confluence in 24-well Falcon$^\text{®}$ tissue culture plates (Becton Dickinson), in DMEM with 9% FCS. Serial dilutions of HSV-1 (range $10^1$-$10^{10}$) were prepared by diluting an aliquot of virus (1:10) in DMEM with 1% FCS. Fibroblasts were infected with 200 μL of HSV-1 (2 wells for each dilution), for 1 h at 37 °C, and the supernatant removed. Infected cells were incubated at 37 °C, in 0.5 mL of DMEM supplemented with 1% (v/v) FCS and 1.2% (w/v) carboxy methylcellulose (400-800 cps, Sigma). After 48 h the media was removed, cells were washed twice with phosphate-buffered saline (PBS), and then incubated for 15 min in 100% methanol, at room temperature. After fixation, cells were washed again in PBS.
and stained with 0.1% (w/v) crystal violet, dissolved in 20% (v/v) ethanol. After 2 to 3 minutes the crystal violet was removed, the cells were allowed to air dry, and viral plaques were counted using an inverted phase contrast microscope (Olympus).

3.2.4 Preparation of HSV-1 Cell Lysates

Hep2 cells were grown to 80% confluence in 75 cm$^2$ Falcon tissue culture flasks, in DMEM supplemented with 9% (v/v) FCS, at 37 °C, in 5% CO$_2$. Cells were infected, as described above, with a low passage clinical strain of HSV-1 (MC1, passage 5). The exact method used to harvest HSV-1 proteins from infected cells was changed over time, in an attempt to increase the yield of viral proteins.

In early experiments, cells were infected at a multiplicity of 0.01 (0.01 PFU per cell), and were removed after 72 h (80% cytopathic effect) using a cell scraper, frozen and thawed 3 times, and subjected to sonication (3 × 7 sec, 100% duty cycle). The cell pellet was collected by centrifugation at 800 × g for 30 min, at 4 °C, the supernatant was removed, and the pellet stored at -20 °C. Viral proteins were extracted by resuspending frozen cell pellets in 1 mL of lysis buffer, containing PBS, 1% Triton X-100 non-ionic surfactant (ICN Biomedical), and a protease inhibitor cocktail (diluted 1:100) (Cat # P8849, Sigma). Each sample was mixed by vortexing three times, for 15 sec, keeping on ice for 30 sec in between. The lysates were incubated for 30 min at 4 °C, and insoluble proteins were removed by centrifuging at 10,000 × g for 30 min, at 4 °C. The resulting supernatants were used the same day for binding experiments.

In later experiments, cells were infected at a multiplicity of 1, incubated for 20 h, and lysed directly in the tissue culture flask. After 20 h incubation, supernatant was removed, and the cells were washed with 5 mL of PBS. 1 mL of lysis buffer (as above) was added to cells in each 75 cm$^2$ flask, and they were detached using a cell
scraper. The cells were then resuspended in the lysis buffer, and transferred to a microfuge tube. The suspension was passed vigorously back and forth twice through a 21-gauge needle, using a 1 mL syringe, to shear cellular DNA and reduce sample viscosity. The cells were mixed by vortexing for 20 sec, and incubated on ice for 60-90 min. Cellular debris was then removed by centrifuging at 10,000 × g for 20 min, at 4 °C, and the supernatant was removed for use in binding experiments.

### 3.2.5 Preparation of HSV-1 Virion Protein Fractions

Hep2 cells were grown to 80% confluence in 6 × 150 cm² Falcon® tissue culture flasks, in DMEM supplemented with 9% FCS. Cells were infected at 0.5 PFU per cell with a low passage clinical strain of HSV-1 (MC1, passage 5), and incubated in DMEM with 1% (v/v) FCS. Virus was added to each flask in a volume of 10 mL of media, incubated for 1 h at 37 °C, in 5% CO₂, and then a further 25 mL of media was added to each flask. After 48-72 h (80% cytopathic effect), cells were resuspended into the culture media, using a cell scraper, and transferred to 50 mL Falcon® centrifuge tubes (Becton Dickinson). The cell suspensions were frozen and thawed three times, subjected to sonication in a cup-horn water bath (3 × 20 sec, 100% duty cycle) at 4 °C, and cellular debris was removed by centrifugation for 15 min at 15,800 × g in a Sorvall® SS-34 rotor, at 4 °C, in a Sorvall® RC 26 Plus centrifuge.

The supernatants were transferred to six thick walled 38 mL Polycarbonate Centrifuge Tubes (Cat # 355631, Beckman), and virions were pelleted by centrifugation for 2 h at 34,000 × g in a Beckman SW-28 rotor, at 4 °C, using an Optima XL-100K Ultracentrifuge (Beckman Coulter). The supernatants were discarded, and the virus pellets were resuspended and pooled in 3 mL PBS with 1% Nonidet P-40 (NP40, ICN Biomedicals). Samples were mixed by vortexing, and
incubated for 15 min at 37 °C. Specimens were transferred to 1.5 mL Polyallomer Microfuge® tubes (Cat # 357448, Beckman), and subjected to centrifugation at 34,000 × g in a Beckman TLA-45 rotor for 2 h, at 4 °C, using an Optima™ MAX Ultracentrifuge (Beckman Coulter).

The supernatants, containing HSV-1 envelope proteins, were removed and placed at 4 °C. The viral pellets were pooled and resuspended in 450 µL of PBS with 1% NP40 and 1M NaCl. The sample was mixed by vortexing, subjected to sonication briefly in a cup-horn water bath (Branson), incubated for 15 min at 37 °C and then separated by centrifugation at 34,000 × g in a Beckman TLA-45 rotor for 2 h, at 4 °C. The supernatant, containing HSV-1 tegument proteins, was diluted in 2.55 mL of PBS with 1% NP40, to give a final volume of 3 mL and final NaCl concentration of 150 mM, and placed at 4 °C. The pellet, containing HSV-1 capsids, was resuspended in 3 mL PBS with 1% NP40, mixed by vortexing and subjected briefly to sonication in a cup-horn water bath. Large aggregates were removed by centrifuging at 10,000 × g for 5 min, and the supernatant (containing HSV-1 capsids) was set aside, at 4 °C, for binding experiments.

3.2.6 In Vitro Pull-down Assays

To purify GST-tagged fusion proteins, bacterial cell pellets were resuspended in 5 mL of lysis buffer containing PBS, 5mM dithiothreitol (DTT), 0.1% Triton X-100, and a protease inhibitor cocktail (diluted 1:100). Cells were subjected to sonication at 4 °C, using an immersed micro-tip probe (Branson, 3 × 20 sec, output 20%, 100% duty cycle). Insoluble material was removed by centrifugation at 10,000 × g, at 4 °C, and the soluble fractions were used for binding assays.
Soluble bacterial lysates (1 mL) containing GST fusion proteins were incubated with 50 μl of glutathione-sepharose beads (Amersham Pharmacia Biotech) for 3 h with rocking at 4 °C. The beads were washed three times with lysis buffer (minus protease inhibitors) before addition of lysates from HSV-1 infected cells, or protein fractions from dissociated HSV-1 virions. Beads were incubated overnight at 4 °C with rocking before washing five times, as described above. Protein complexes were then eluted using 1 bed volume of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione) with rocking at 4 °C for 2 h.

3.2.7 Analysis of Protein Complexes

Protein complexes were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins identified by immuno-blotting as described previously (Diefenbach et al., 1998). Protein samples were separated by 10%, 12% or 14% SDS-PAGE (Laemmli, 1970) under reducing conditions. The total protein content of a sample was determined by staining the gel with Coomassie Brilliant Blue-R250 stain (Sigma) or SYPRO Ruby Protein Gel Stain (Bio-Rad). For immunoblotting (Towbin et al., 1979), proteins were electro-blotted to a Hybond-ECL nitrocellulose membrane (Amersham) at 50 mA overnight. Pre-stained BlueRanger molecular weight markers (Pierce, Rockford, IL, USA) were used to identify protein size.

After transfer, the membrane was blocked for 1 h at room temperature with 5% (w/v) skim milk powder (Diplomat) in Tris-buffered saline (TBS), with 0.1% (v/v) Tween 20 (polyethylene sorbitan monolaurate, ICN Biomedical). The membrane was then washed several times with TBS-0.1% Tween 20, and incubated for 1 h with primary antibody, diluted with 1% (w/v) skim milk powder in TBS-0.1% Tween 20. The blot was washed again with TBS-0.1% Tween 20, prior to application of the
secondary antibody, diluted with 1% (w/v) skim milk powder in TBS-0.1% Tween 20. Incubation and washes were the same as for primary antibody. Antibody binding was detected on X-ray film as chemiluminescence, generated with Supersignal™ substrate (Pierce).

### 3.2.8 Antibodies

To screen for a broad range of HSV-1 proteins, a rabbit polyclonal antibody against HSV-1 was used (Code No. B0114, Dako). For detection of HSV-1 capsid proteins, rabbit polyclonal antibodies directed against VP5 (NC1), VP19C (NC2) and VP23 (NC5) were kindly provided by Dr. G. Cohen and Dr. R. Eisenberg (University of Pennsylvania, Philadelphia, USA) (Cohen et al., 1980). A mouse monoclonal IgG₁ directed against VP5 (6F10) (Santa Cruz Biotechnology) was used in some experiments. The outer capsid protein VP26 was detected with a polyclonal rabbit IgG antibody (diluted 1:5000), a kind gift of Dr. R. Courtney (Louisiana State University Medical Centre) (McNabb and Courtney, 1992b). The minor capsid protein UL6 was detected using the rabbit polyclonal antibody YE583, the kind gift of Dr. Arvind Patel (MRC Virology Unit, Glasgow, UK) (Patel et al., 1996).

The HSV-1 tegument protein VP16 was detected using a mouse monoclonal IgG₁ antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA 95060, USA). A mouse monoclonal antibody against tegument protein US11 (diluted 1:50), as well as polyclonal antibodies against the UL31 and UL34 proteins, were the kind gift of Dr. Bernard Roizman (University of Chicago, USA) (Purves et al., 1992; Roller and Roizman, 1992; Chang and Roizman, 1993). Tegument protein VP13/14 was detected using the mono-specific rabbit polyclonal antibody R220 (diluted 1:1000), kindly provided by Dr. David Meredith (University of Leeds, UK) (Whittaker et al., 1991).
Tegument protein VP22 was detected using a rabbit polyclonal antibody AGV32 (diluted 1:5000), the kind gift of Dr. Peter O’Hare (Marie Curie Research Institute, Surrey, UK). The major tegument protein VP1/2 was detected using a rabbit polyclonal antibody (diluted 1:2000), the kind gift of Dr. R. Courtney (Louisiana State University USA) (McNabb and Courtney, 1992a).

Glycoproteins gC and gD were detected using mouse monoclonal antibodies (diluted 1:500 and 1:1000 respectively) (Cat # 1125,1121; Goodwin Institute for Cancer Research). Glycoproteins gG and gH (diluted 1:1000) were detected using mouse monoclonal antibodies LP10 and LP11, respectively, the kind gift of Dr. Anthony Minson (University of Cambridge, UK) (Buckmaster et al., 1984; Richman et al., 1986).

Secondary antibodies used were Peroxidase-conjugated ImmunoPure® Goat anti-Mouse (Cat # 31430) or Goat anti-Rabbit (Cat # 31460) IgG (Pierce), diluted 1:100,000.
3.3 Results

3.3.1 In Vitro Pull-down Binding Assays

The main experimental model used for this set of experiments is the in vitro pull-down assay. A protein of interest is expressed in bacteria as a GST-tagged fusion construct, and purified by covalent binding to glutathione-Sepharose beads (Figure 13). The beads are incubated with a mixture of other proteins (such as a cell lysate), allowing interacting proteins to bind to the GST-fusion proteins. The beads are washed, to remove unbound proteins, and the bound complexes are then eluted from the beads by incubating with reduced glutathione. Proteins in the eluted complexes can be separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and identified with specific antibodies (western blot).

3.3.2 HSV-1 Proteins Bind to DIC (aa 143-267)

Full length DIC-1c was expressed in bacteria as a GST-tagged fusion protein, and purified on glutathione-Sepharose beads. Hep-2 cells were infected with a clinical strain of HSV-1, removed from the flask and lysed, to extract soluble HSV-1 proteins. The cell lysates were then incubated with GST-DIC, bound to beads, and incubated overnight at 4°C. After thorough washing, the bound complexes were eluted from the beads, the proteins were separated by SDS-PAGE, and analysed by Western blot.

HSV-1 proteins were detected initially using a polyclonal antibody raised against purified HSV-1. A protein of approximate molecular mass 55 kDa, detectable in the HSV-1 infected cell lysates, bound to GST-DIC in multiple experiments (Figure 14A).
Figure 13. In vitro pull-down assay.
This figure summarises the principle of an in vitro pull-down protein binding assay. (A) Dynein subunits are expressed as GST-fusion proteins in bacteria, the bacteria are lysed, and the lysates are added to glutathione-Sepharose beads. (B) The GST fusion proteins bind covalently to the beads, and unbound proteins are washed away. (C) A mixture of HSV-1 proteins, either from infected cells or lysed virions, is incubated with GST-tagged dynein subunits, bound to beads. (D) The beads are washed, leaving HSV-1 proteins that interact with dynein bound to the GST-tagged dynein subunits on the beads. (E) The bound protein complexes are eluted from the beads by incubating with reduced glutathione. (F) Protein complexes are denatured by heating under reducing conditions, separated by SDS-PAGE, and analysed by western Blot.
Figure 14. HSV-1 proteins bind to DIC (aa 143-267).

In pull-down experiments, lysates from Hep2 cells infected with HSV-1, or mock-infected, were incubated with GST-tagged fusion proteins, bound to glutathione-Sepharose beads. (A) HSV-1 proteins were detected on western blots using a polyclonal antibody against HSV-1. Several HSV-1 proteins were present in a complex bound to GST-DIC, but not to the GST control, with a prominent band around 55 kDa. (B) Coomassie Blue stain confirmed good expression and elution of the GST negative control, as well as of GST-DIC. Note the significant proteolysis of full-length GST-DIC. (C) A 55 kDa HSV-1 protein (detected with anti-HSV-1 Ab) bound to a DIC fragment spanning aa 143-267, but not to fragments spanning aa 1-142 or aa 268-628. There was no evidence of binding to dynein light chains LC8, Tctex1 or rp3. (D) Expression of GST-tagged fusion constructs was confirmed by Coomassie Blue stain. Note that GST-DIC 268-628 was expressed relatively poorly, but was present in sufficient quantities to exclude significant binding.
This binding was not mediated by the GST tag, since the HSV-1 proteins did not bind to the GST tag alone, expression of which was confirmed by Coomassie Blue stain (Figure 14B). Larger proteins that labelled with the anti-HSV-1 antibody, of approximate molecular mass 65 kDa and 80 kDa, were also present in the bound complex (Figure 14A, Long Exposure). These proteins, which were detectable in HSV-1 infected cell lysates, were apparently present in bound complexes in smaller amounts than the 55 kDa protein.

To determine the minimal binding domain of DIC required to bind HSV-1 proteins, fragments of DIC were expressed as GST-tagged fusion proteins, and used in similar pull-down assays. The 55 kDa protein bound to a fragment of DIC spanning amino acids 143-267 (DIC 143-267), but not to fragments spanning amino acids 1-142 or 268-628 (Figure 14C). Binding was also confirmed using DIC fragments spanning amino acids 1-268 and 143-628, both of which include the proposed binding regions (data not shown). Expression of GST-tagged DIC fragments was confirmed by Coomassie Blue stain (Figure 14D).

Dynein light chains LC8, rp3 and Tctex1 were expressed as GST-tagged fusion proteins in bacteria, purified on glutathione-Sepharose beads, and incubated with lysates from cells infected with HSV-1, as above. Elutions from the beads were examined by western blot, using a polyclonal antibody against HSV-1. There was no evidence that the 55 kDa HSV-1 protein, or any other proteins, bound to any of these dynein light chains (Figure 14C). Expression of all GST-tagged fusion proteins was confirmed by Coomassie Blue stain (Figure 14D).

A low molecular weight band was noted in the GST-rp3 lane, but its significance is uncertain. It was not present in other experiments, and the only HSV-1 protein of similar molecular weight that is detected by the anti-HSV-1 antibody is the tegument
protein US11 (unpublished data). Although we cannot exclude the presence of US11 in this sample, US11 did not bind to rp3 in subsequent yeast two-hybrid experiments (see Chapter 4). More importantly, US11 was not present in the DIC lane, so US11 cannot mediate binding of the complex containing the 55 kDa band.

### 3.3.3 Identification of HSV-1 Proteins Bound to DIC

To identify the HSV-1 proteins present in the complex bound to DIC, eluted proteins from pull-down experiments were separated by SDS-PAGE, blotted onto nitrocellulose membrane, and probed with specific antibodies directed against various HSV-1 proteins.

The HSV-1 glycoprotein gD was shown to bind specifically to GST-DIC (Figure 15A and B), as well as to a fragment of DIC spanning aa 143-267 (Figure 16A and B). Glycoprotein gC was also shown to bind specifically to DIC 143-267 (Figure 16C).

There was no evidence that HSV-1 tegument proteins, VP16 (Figure 15C) or US11 (Figure 15D), were present in complexes bound to GST-tagged full length DIC, although they were detected in infected cell lysates. Glycoproteins gG (Figure 16E) and gH (Figure 16F) were also detected in HSV-1 cell lysates, but like VP16 (Figure 16D) did not bind to DIC 143-267, LC8 or Tctex1. HSV-1 proteins VP19C, VP26, UL6, VP1/2 and VP13/14, as well as the UL31 and UL34 proteins, were not detected in lysates from infected cells or in protein complexes bound to DIC (data not shown). Therefore, results for these proteins could not be interpreted.

Since VP26 was subsequently shown to interact with dynein light chains rp3 and Tctex1 (see Chapters 4 and 5), it should be noted that VP26 is not detected by the anti-HSV-1 antibody (data not shown). Furthermore, a more sensitive anti-VP26 antibody,
which was used in later experiments (see Chapter 5), was not available at the time when these experiments were being performed.

Figure 15. Identification of HSV-1 proteins bound to DIC.
(A) HSV-1 protein complexes bound to GST-DIC were detected by western blot, using anti-HSV-1 antibodies, as shown previously in Figure 14A. (B) HSV-1 envelope glycoprotein gD, detected in the HSV-1 cell lysate, bound to GST-DIC, but not to GST alone. (C, D) Tegument proteins VP16 and US11 were detected in the infected cell lysate, but did not bind to GST-DIC.
Figure 16. Identification of HSV-1 proteins bound to DIC 143-267.

(A) HSV-1 protein complexes that bound to a GST-tagged DIC fragment (spanning aa 143-267) were detected on western blot using anti-HSV-1 antibody, as shown previously in Figure 14C. (B) The envelope glycoprotein gD bound to GST-DIC 143-267, but did not bind to other DIC fragments or dynein light chains. (C) Glycoprotein gC also bound specifically to GST-DIC 143-267, but not to the other fusion constructs tested. (D-F) Tegument protein VP16 and glycoproteins gG and gH were detected in the infected cell lysate, but did not bind to any of the GST-tagged fusion proteins. Note some non-specific labelling of GST-DIC 143-267 with the anti-gH antibody.
3.3.4 Binding Experiments Using HSV-1 Virion Proteins

It has been reported previously that treating HSV-1 virions with non-ionic detergent removes the viral envelope and membrane glycoproteins, while treatment with concentrated salt solution removes most of the tegument proteins (Meredith et al., 1991; Schmitz et al., 1995; McLauchlan, 1997). We used these techniques to separate HSV-1 virion proteins into separate fractions, and then used the fractions in binding experiments, to confirm which viral proteins bound to dynein in vitro.

Freshly isolated HSV-1 virions were treated with PBS containing 1% NP40 (a non-ionic detergent), to dissolve the viral envelope and glycoproteins, resulting in a soluble “outer” fraction. The insoluble viral capsid-tegument complexes were then treated with PBS containing 1M NaCl, as well as 1% NP40, to dissolve tegument proteins, resulting in a soluble “middle” fraction. The remaining HSV-1 capsids were resuspended in PBS, to give an “inner” fraction.

HSV-1 protein fractions were incubated with GST-tagged dynein subunits DIC, LC8 and Tctex1, bound to glutathione-Sepharose beads, in pull-down experiments (Figure 17). After extensive washing, the bound protein complexes were eluted from the beads, separated by SDS-PAGE, and analysed by Western blot, as in previous experiments.

There was significant separation of viral proteins into fractions, as shown using a polyclonal antibody against HSV-1 (Figure 17A). Glycoproteins gC and gD were present mostly in the outer fraction, and bound to GST-DIC, but not to GST-LC8 or GST-Tctex1 (Figure 17C and D). There was no evidence of binding between US11, VP16 or VP23 and any of the GST-tagged dynein subunits tested (Figure 17B,E,F).
Figure 17. HSV-1 virion glycoproteins gD and gC bind to DIC.

HSV-1 virion proteins were separated into fractions and added to GST-tagged dynein subunits, bound to glutathione-Sepharose beads, in a pull-down assay. (A) There was clear separation of viral proteins into fractions, detected here with a polyclonal antibody against HSV-1. (B-F) Glycoproteins gD and gC bound to GST-DIC, but not to GST-LC8 or GST-Tctex1. There was no binding of tegument proteins US11 or VP16, or of capsid protein VP23, to any of the dynein subunits tested.
US11 was detected in the middle fraction; VP16 was detected most strongly in the outer fraction, while VP23 was detected in both the inner and outer fractions. The presence of the capsid protein VP23 in the outer fraction is difficult to explain, but may have been an artefact, as it was not observed in other experiments. Attempts to detect capsid proteins VP5 and VP26, as well as tegument protein VP13/14, were unsuccessful with available antibodies.

### 3.3.5 Yeast Two Hybrid Analysis

The cytoplasmic tail of glycoprotein gD (aa 364-395) was cloned into Bait vector and tested in a LexA yeast two-hybrid system for interactions with dynein subunits (including Roadblock1) (see Chapter 4 for methods). The gD fusion protein was expressed well, but there was no evidence of a direct interaction between gD 364-395 and DIC, LC8, rp3, Tctex1 or Roadblock1 (Figure 18).
Figure 18. The cytoplasmic tail of gD does not interact with dynein subunits.

The cytoplasmic tail of gD (US6) (aa 364-395) was cloned into Bait vector, and tested in a LexA yeast two-hybrid system against dynein subunits in Target vector (see Chapter 4 for more details). (A) Negative control (against Target with no insert), showing no colour change. (G) Positive control (DIC in Bait against LC8 in Target), demonstrating good yeast growth and strong blue colour change. (B-F) There was no evidence of interaction between gD 364-395 and dynein subunits DIC, LC8, rp3, Tctex1, or Roadblock 1.
3.4 Discussion

3.4.1 Summary

The only previous evidence of a direct interaction between a HSV-1 protein and cytoplasmic dynein was the reported interaction between the UL34 protein and DIC (Ye et al., 2000). In their paper, the authors detected a complex of HSV-1 proteins bound to a GST-tagged fragment of DIC-1a, spanning amino acids 1-228 (equivalent to DIC-1c 1-211). Two prominent proteins in the complex were identified as UL31 and UL34 by western blot, and another protein was presumed to be VP5, based on molecular weight. Several other unidentified proteins were also present in the bound complex, including proteins with molecular masses between 50 kDa and 80 kDa.

In similar experiments, we detected several HSV-1 proteins that bound to full-length DIC-1c, and the minimal binding domain of DIC was identified as the region spanning amino acids 143-267. The only two proteins in the complex that were identified consistently by Western blot were glycoproteins gC and gD. There was no evidence of any HSV-1 tegument or capsid proteins, the most likely candidates to mediate retrograde viral transport.

3.4.2 Minimal Binding Region of DIC

Dynein intermediate chain (DIC) forms the “backbone” of the cytoplasmic dynein complex, as discussed in Chapter 2. The arrangement of recognised functional domains is summarised in Figure 19. The N-terminal region (aa 1-66) contains coiled-coil domains, which interact with the dynactin subunit p150\textsuperscript{Glued} (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). There are two alternate splicing regions, the first of which (DIC-1a aa 76-92) is not present in DIC-1c, so subsequent numbering will refer
to amino acid positions in the DIC-1c isoform. There is a conserved, serine-rich region (aa 80-98), followed by the second alternate splicing region (aa 104-123) (Vaughan and Vallee, 1995). The 14 kDa light chains rp3 and Tctex1 compete for the same binding site (aa 124-142) (Mok et al., 2001; Tai et al., 2001), followed closely by the LC8 binding region (aa 148-157) (Lo et al., 2001; Makokha et al., 2002). The binding site for the roadblock light chains (aa 226-265) has been identified more recently (Susalka et al., 2002), and had not been reported when most of the experiments reported in this chapter were being performed. The C-terminal domain of DIC (aa 268-628), which binds to DHC (Mok et al., 2001), contains multiple WD40 repeats and forms a β-propeller structure (ter Haar et al., 2000; Holm et al., 2001).

**Figure 19. Binding regions of DIC.**

This figure summarises the fragments of DIC used in our binding experiments, relative to known binding domains. Note how the fragment that bound to a complex of HSV-1 proteins, including gC and gD, contains the proposed binding regions for light chains LC8 and Roadblock, and overlaps with the proposed binding site for the UL34 protein (Ye et al., 2000). The unspliced isoform shown here is DIC-1a, but the numbering of amino acids corresponds to the DIC-1c isoform, used in our experiments, which lacks the 17 amino acids in the first region of alternate splicing (figure adapted from Mok et al., 2001).
The fragment of DIC-1a reported to bind to the UL34 protein (aa 1-228) (Ye et al., 2000) corresponds to DIC-1c aa 1-211, and thus included the binding domains for p150Glued, rp3/Tctex1 and LC8, as well as the serine rich region and two regions of alternate splicing (the first of which is absent in DIC-1c).

In our experiments we demonstrated binding of a protein complex, containing glycoprotein gD, to full-length DIC-1c, as well as to fragments spanning amino acids 1-267 and 143-267. We did not detect binding of any HSV-1 proteins to DIC-1c fragments spanning amino acids 1-142 or 268-628. The region of DIC-1c spanning amino acids 143-267 includes the reported binding sites for dynein light chains LC8 and Roadblock, but not the binding sites for rp3/Tctex1, p150Glued, or DHC. It seems likely that the region of DIC responsible for HSV-1 binding in our experiments is the same one that was identified by Ye et al. In fact, those authors noted the presence of several other HSV-1 proteins in the complex bound to DIC, including one of approximately 55 kDa, but were unable to identify them. In light of our results, the 55 kDa protein may have been gD, which would further narrow down the minimal binding domain on DIC to the region between amino acids 143 and 211. This region is known to contain the LC8 binding domain, but its other functions have not been identified to date.

Our results suggest that the complex of HSV-1 proteins containing gD bound directly to DIC, rather than indirect binding via dynein light chains. In several pull-down experiments we included GST-tagged dynein light chain LC8, as well as Tctex1 and rp3. There was no consistent evidence of HSV-1 proteins binding to any of these light chains, in contrast to DIC. In Chapter 4 we identify a direct interaction between the HSV-1 outer capsid protein VP26 and dynein light chains rp3 and Tctex1, using a LexA yeast two-hybrid system. Unfortunately we were not able to detect VP26 in these
pull-down experiments, in either the infected cell lysates or the bound complexes, with the available anti-VP26 antibody.

We cannot exclude from our results alone that binding of the HSV-1 protein complex to DIC is mediated via Roadblock. However, if we include the results of Ye et al., the most likely region of DIC responsible for binding spans aa 143-211, a region that does not include the Roadblock binding site. Further experiments, using GST-tagged Roadblock in similar pull-down assays, would be required to address this question directly.

3.4.3 Identification of HSV-1 Proteins Binding to DIC

Using lysates from cells infected with HSV-1, in pull-down experiments, we showed that full-length DIC-1c, and fragments including aa 143-267, bound to a complex of HSV-1 proteins. Several proteins in the complex were detected with a polyclonal antibody against HSV-1, ranging in size from approximately 50 kDa to 80 kDa. The protein that was most consistently detected in pull-down assays had an apparent molecular mass of approximately 55 kDa, based on its gel mobility by SDS-PAGE.

There are approximately 31 HSV-1 proteins with a predicted molecular mass between 40 kDa and 80 kDa (http://www.stdgen.lanl.gov). Of these proteins, many are non-structural, so are unlikely to play a functional role in retrograde transport of HSV-1. The proteins in this size range that are most likely to mediate retrograde transport are: capsid proteins VP19C (UL38, 50.3 kDa), UL25 (62.7 kDa) and UL6 (74.1 kDa); tegument proteins UL16 (40.4 kDa), US3 (52.8 kDa), VP16 (UL48, 54.3 kDa), vhs protein (UL41, 54.9 kDa), UL13 (57.2 kDa), VP13/14 (UL47, 73.8 kDa), UL17 (74.6 kDa), and VP11/12 (UL46, 78.2 kDa). Other HSV-1 virion proteins in the same size range include the membrane glycoproteins gD (US6, 43.3 kDa) and gC (UL44, 55.0
kDa). Note that molecular masses are predicted from the protein sequence, and do not include post-translational modifications, such as phosphorylation or glycosylation. For this reason, the actual molecular mass of HSV-1 proteins present in infected cell lysates or virions is likely to be larger than the predicted molecular mass.

We attempted to identify the HSV-1 proteins in the bound complex by western blot, using specific antibodies. Our initial experiments focused on HSV-1 capsid and tegument proteins, since we felt that these were the most likely candidates to bind to dynein and mediate retrograde viral transport. With the available antibodies, we were able to detect tegument proteins VP16, VP22 and US11 in cell lysates, and to exclude that these proteins were present, in significant amounts, in complexes bound to DIC. We also tried antibodies directed against capsid proteins VP5, VP19C, VP23, VP26 and UL6; tegument proteins VP1/2 and VP13/14; and the UL31 and UL34 proteins, but were unable to consistently detect these proteins in the infected cell lysates (the positive control). For this reason, we were unable to exclude that they were present in the bound complexes.

As another approach to identifying the proteins in the bound complex, we attempted to analyse them by mass spectrometry. However, the HSV-1 proteins pulled down from infected cell lysates by DIC were not present in sufficient quantities to detect with protein stains (Coomassie Blue, SyproRuby, or silver stain), and were thus not suitable for analysis by mass spectrometry.

To obtain larger amounts of viral protein, we isolated large numbers of HSV-1 virions from cell cultures, and extracted the viral proteins into different fractions. Treatment of intact HSV-1 virions with detergent disrupts hydrophobic protein-protein interactions, thus removing the viral envelope, membrane-associated proteins, and some of the outer tegument proteins. Treatment with 1 M NaCl disrupts electrostatic
protein-protein interactions, and removes most of the tegument proteins, leaving viral capsids with some residual tegument (Meredith et al., 1991; Schmitz et al., 1995; McLauchlan, 1997).

In pull-down experiment performed on fractionated HSV-1 proteins, we found that the majority of viral proteins pulled down by DIC were from the outer fraction, rather than the middle or inner fractions. Specific antibodies against HSV-1 glycoproteins gD and gC confirmed that both these glycoproteins were present in the complex of proteins pulled down by GST-tagged DIC or DIC 143-267, with gD present in larger amounts. Re-analysis of western blots from earlier experiments using the anti-gD antibody confirmed that gD was also present in the complex of proteins pulled down from infected cell lysates by GST-tagged DIC, and DIC fragments spanning aa 143-267. Glycoproteins gG and gH, although detected in the outer fraction of HSV-1 proteins, were not present in significant amounts in the complex bound to DIC. It was not possible to detect gB in these experiments, as no suitable antibody was available.

Comparing these results with those obtained using the polyclonal anti-HSV-1 antibody, it appears that the 55 kDa band detected with the polyclonal antibody is likely to be gD, while the 80 kDa band is likely to be gC. It is unclear why the 80 kDa band was not always detected with the anti-HSV-1 antibody, when it was detectable with the anti-gC antibody, but this may relate to differing antibody sensitivities.

### 3.4.4 Significance of Results

We have shown in these experiments that glycoproteins gD and gC are present in a complex pulled down by DIC, and defined the region of DIC required for binding. Our results suggest that binding to the dynein complex is mediated by a direct interaction with DIC, rather than via dynein light chains, as discussed above. The fact that we
were able to pull down HSV-1 glycoproteins from fractionated extra-cellular virions, as well as from infected cell lysates, suggests that the binding partner of DIC is present in HSV-1 virions, and is most likely a viral protein.

The presence of multiple glycoproteins in the bound complexes implies a probable interaction between the glycoproteins, both in HSV-1 virions and in infected cells. Cross-linking studies have shown that gB, gC and gD in HSV-1 virions form the hetero-dimers gB-gC, gC-gD, and gD-gB, as well as forming homo-dimers (Handler et al., 1996a; Handler et al., 1996b). Thus these glycoproteins must be within 11.4 Å of each other in the virion envelope, and may be part of a large functional complex, as proposed in a model of membrane fusion (White, 1992). The presence of the same hetero-dimers has been confirmed by other groups (Rodger et al., 2001), although the interactions between gB, gD and gH-gL were found to be not important for their incorporation into virions. It is further possible that these glycoproteins are found in lipid rafts, as has been shown recently for PrV glycoproteins gB, gC, gD and gE (Favoreel et al., 2004). The HSV-1 glycoprotein gB has been shown to associate with lipid rafts in cells (Bender et al., 2003), but whether or not this also occurs in HSV-1 virions remains to be confirmed.

It is not clear which glycoprotein(s) in the complex, if any, bound directly to DIC, or what the functional significance of this interaction might be in vivo. Since HSV-1 loses its envelope during cell entry, it is unlikely that glycoproteins play a role in retrograde transport of HSV-1 virions to the nucleus. However, an interaction with dynein may be important for intracellular trafficking of glycoproteins during viral assembly, or for endocytosis of vesicles containing viral glycoproteins.

It was recognised recently that cytoplasmic dynein plays a role in the transport of newly synthesised proteins from the ER, along microtubules, to the Golgi apparatus
(Murshid and Presley, 2004). Thus any direct interaction between HSV-1 glycoproteins and dynein may be involved in targeting glycoproteins to the Golgi complex, prior to HSV-1 envelopment. Because of the arrangement of glycoproteins in the membrane, the cytoplasmic tail is the most likely part of the protein to be accessible to bind to dynein, and to direct trafficking. In support of this, the cytoplasmic tail and trans-membrane region of gD have been shown to target gD and other chimeric proteins to the nuclear membrane, endoplasmic reticulum (ER) and Golgi compartments (Ghosh and Ghosh, 1999). In this paper the authors also provide evidence that gD may be transported from the Golgi compartment to the nuclear envelope in transport vesicles, a process that may also involve dynein.

In our experiments, glycoprotein gD was the most prominent HSV-1 protein in complexes bound to DIC, as detected by western blot. We therefore tested the cytoplasmic tail of gD (aa 364-395) for a possible direct interaction with dynein subunits DIC, LC8, Tctex1, rp3 or Roadblock1, using a yeast two-hybrid system. No interaction was detected. One possible explanation for the large amount of gD in complexes bound to DIC, in the absence of direct gD binding, is the relatively high abundance of gD in HSV-1 virions, compared with gB (10×) and gC (at least 5×) (Handler et al., 1996b). The absence of direct binding between gD and dynein is consistent with reports that the cytoplasmic tail of gD can be deleted without affecting viral infectivity (Feenstra et al., 1990; Muggeridge et al., 1990).

Another attractive candidate for binding to DIC is glycoprotein gB, since truncation of the cytoplasmic tail of HSV-1 gB has been shown to disrupt gB transport in rat hippocampal neurones infected with HSV-1 (Potel et al., 2003). In this model, insertion of green fluorescent protein (GFP) into the cytoplasmic tail of gB resulted in retention of immature gB in the ER, and prevented its transport into axons. In contrast,
insertion of GFP into the extra-cellular domain of gB did not affect its maturation, and vesicles containing glycosylated GFP-gB were transported into axons, separate from HSV-1 nucleocapsids (Potel et al., 2002; Potel et al., 2003). The authors propose that the cytoplasmic tail of gB is required for its transport from the ER to the Golgi complex, as has been shown for gB homologues in Varicella-Zoster Virus (Heineman et al., 2000; Heineman and Hall, 2002) and human Cytomegalovirus (Meyer and Radsak, 2000). More recent studies further support this idea (Beitia Ortiz de Zarate et al., 2004). The cytoplasmic tail of gB is also required for gB endocytosis, in both HSV-1 (Fan et al., 2002), and the closely related porcine alphaherpesvirus PrV (Van Minnebruggen et al., 2004). Endocytosis of PrV gB involves a YQRL motif, which binds to the Clathrin-Associated AP-2 Adaptor Complex (Van Minnebruggen et al., 2004). A corresponding motif in HSV-1 has not been identified to date. We did not have access to an anti-gB antibody for our experiments, so although we were unable to show that it was present in complexes bound to DIC, we were also unable to exclude it.

Although our results provide indirect evidence for an interaction between a HSV-1 glycoprotein and DIC, we have not confirmed a direct protein-protein interaction. Therefore we cannot rule out the involvement of an intermediate cellular protein, nor of another membrane-associated HSV-1 protein that was not detected by our polyclonal antibody. In particular, we were unable to rule out the presence of UL34 or UL31 in the bound complexes, because the available antibodies were unable to detect these proteins specifically in the lysate from HSV-1 infected cells. Despite the reported interaction between UL34 and DIC (Ye et al., 2000), our results cannot be explained on the basis of this interaction, since we were able to pull down HSV-1 glycoprotein complexes from fractionated extra-cellular virions, as well as from cell
lysates, and UL34 is not present in mature HSV-1 virions (Reynolds et al., 2002). There were no HSV-1 capsid or tegument proteins detected in the complex, so binding cannot be explained by our subsequently identified interaction between the capsid protein VP26 and dynein light chains rp3 and Tctex1 (see Chapter 4).

Although intriguing, any interaction between HSV-1 glycoproteins and cytoplasmic dynein is unlikely to play a role in retrograde transport of virus during infection, for the reasons discussed already. Therefore it was decided that identification of the protein-protein interaction responsible for our results was not a high priority for this project. However, further yeast two-hybrid experiments are planned in the future, to test for possible direct interactions between the cytoplasmic tails of gB or gC and cytoplasmic dynein subunits. Confirmation of a direct interaction between these proteins may have implications for other phases of the HSV-1 life cycle, particularly during viral assembly and egress.

If these planned experiments fail to show a direct interaction between gB or gC and dynein, it may be possible to test other membrane-associated HSV-1 proteins for interactions with dynein, using the yeast two-hybrid system. The poor solubility of membrane-associated proteins may require testing of the soluble cytoplasmic domains of these proteins, details of which may not be available for all candidate proteins. If an intermediate cellular protein is involved in the interaction between DIC and HSV-1 glycoproteins, this would be even more difficult to identify. One approach would be to screen a cellular cDNA library, using the yeast two-hybrid system, for proteins that interact with DIC and/or the cytoplasmic tails of HSV-1 glycoproteins. Such experiments are not planned at this stage.
CHAPTER 4:

YEAST TWO-HYBRID SCREEN
4.1 Introduction

In order to screen for possible interactions between HSV-1 capsid or tegument proteins and dynein subunits, we needed a system that detects interactions between individual proteins. We elected to use the LexA yeast two-hybrid system, because it can test multiple protein combinations for potential binary interactions in eukaryotic cells.

In the yeast two-hybrid system *Saccharomyces cerevisiae* cells are co-transfected with three expression plasmids: Bait, Target and Reporter. Proteins of interest are expressed in the yeast as fusion constructs with an N-terminal fusion tag: either a DNA binding domain (the *E. coli* LexA protein in the Bait construct), or an activation domain (the *E. coli* B42 peptide in the Target construct). The Bait protein binds via the LexA tag to LexA operators on the reporter plasmid, upstream of a minimal promoter region for the reporter gene (Figure 20) Following induction of Target protein expression, any interaction of the Target protein with the Bait protein brings the B42 activation domain into close physical proximity to the minimal promoter region, and initiates transcription of the reporter gene.

Yeast two-hybrid systems are a sensitive way to detect relatively weak and transient protein-protein interactions, that although critical in complex biological systems may not be detectable using standard biochemical assays (Guarente, 1993; Estojak et al., 1995). The LexA system is particularly sensitive, allowing detection of protein-protein interactions with a dissociation constant (K\textsubscript{d}) as low as 10\textsuperscript{-6} M (Golemis et al., 1996). The incorporation of two different reporter genes (*LEU2* in the yeast chromosome and *LacZ* in the reporter plasmid) increases specificity of the system, thus eliminating many false positives.
Yeast two-hybrid systems are most frequently used to screen a single protein against large cDNA libraries for potential interaction partners. However, in this project we wanted to test a pre-defined number of HSV-1 and dynein proteins for possible interactions, in various combinations. Therefore we chose a pair-wise matrix approach, where individual protein pairs were tested for interaction one at a time.
Figure 20. The *LexA* yeast two-hybrid system.

The yeast two-hybrid assay is used to test for protein-protein interactions. Yeast cells are co-transformed with three plasmid vectors: Bait, Target and Reporter. Proteins of interest are cloned into Bait or Target vectors, and expressed as recombinant proteins, fused to either a LexA DNA binding domain (Bait vector), or an activation domain (Target vector). If the two proteins interact, the activation domain is brought into close physical proximity with the minimal promoter region on the Reporter plasmid. This induces expression of the *LacZ* reporter gene, which codes for β-galactosidase. When grown on appropriate media, yeast containing interacting proteins express β-galactosidase, and form blue colonies. Blue colonies are selected, and interaction is confirmed by quantitative assay.
4.2 Materials and Methods

4.2.1 Expression Constructs

All HSV-1 genes were amplified by PCR using the Geneamp® XL PCR Kit (PE Applied Biosystems), as described in Chapter 3. The entire ORF for each gene was amplified and cloned into recombinant expression vectors in all cases except for HSV-1 UL36, where only the N-terminal two-thirds of the gene (amino acids 1-1874) could be cloned.

Oligonucleotide primers, incorporating EcoRI and/or XhoI restriction endonuclease sites, were designed using Primer3 software (Rozen and Skaletsky, 2000) (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and BioManager by ANGIS (http://www.angis.org.au) (Table 2). In genes with no internal EcoRI or XhoI sites, a 5’ EcoRI site and 3’ XhoI site allowed directional cloning into expression vectors. In genes with internal EcoRI or XhoI sites, the other restriction site was inserted at both ends of the gene. The exception was UL36, which had a 3’ BglII site for insertion into BamH1 sites. UL32 had both internal EcoRI and XhoI sites, so NcoI sites were engineered. In the case of UL19, the full-length gene was excised from plasmid pE19 (Nicholson et al., 1994) by digestion with BglII before inserting into the BamHI site of displayBait (Display Systems Biotech).
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<tr>
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</tr>
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<td>EcoRI</td>
<td>UL36_F1_5'</td>
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</tr>
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<td></td>
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<tr>
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<td>73.9</td>
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</tr>
<tr>
<td>(gD)</td>
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<td>XhoI</td>
<td>US6_364-395_3'</td>
<td>gcgcgcgctgctgaggctgctttt</td>
<td>76.5</td>
<td></td>
</tr>
</tbody>
</table>

* Predicted melt temperature (nearest-neighbour formula) (Breslauer et al., 1986)

* Anneal/extend temperature for thermal cycling
HSV-1 genes were amplified from a series of overlapping DNA cosmids encoding the entire sequence of HSV-1 strain 17 (Cunningham and Davison, 1993). The PCR template used to obtain HSV-1 US11 was plasmid pRB4766 (Cassady et al., 1998), which contains HSV1 US11 strain F genomic DNA in pGEX-KG (kindly provided by Bernard Roizman, University of Chicago). PCR products were gel purified (Biorad or Fermentas kit), digested with appropriate restriction endonucleases and concentrated by precipitation (Pellet Paint™, Novagen). Expression plasmids were digested with corresponding restriction endonucleases, treated with calf alkaline phosphatase (Promega), gel purified as above and PCR products were inserted by ligation using a Clonables™ Kit (Novagen).

For testing in the LexA yeast two-hybrid system, genes were inserted in frame with the LexA DNA-binding domain of displayBait vector and the B42 DNA-activation domain of displayTarget vector (Display Systems Biotech). All constructs were sequenced to confirm gene sequence and correct reading frame for recombinant protein expression. Genes for dynein subunits, cloned previously (see Chapter 3), were excised from pGEX-5X-1 expression plasmids and re-ligated into displayBait and displayTarget vectors using a Clonables™ Kit (Novagen).

4.2.2 Yeast Two-Hybrid Screen

Potential interactions between dynein subunits and HSV-1 proteins were tested in a Clontech Matchmaker LexA yeast two-hybrid system, using a pair-wise matrix approach, as described previously (Diefenbach et al., 2002a).

The pair-wise screen was performed according to the manufacturer’s protocols (Clontech MATCHMAKER LexA Two-Hybrid System User Manual, #PT3040-1). Small-scale transformation procedures were based on the lithium acetate method, as
outlined in Appendix 2 (Clontech Yeast Protocols Handbook #PT3024-1). *S. cerevisiae* strain display-YEAST-H (Display Systems Biotech) was co-transformed with a *LacZ* reporter plasmid (Invitrogen pSH18-34; confers ability to grow in the absence of uracil), displayBAIT plasmid (equivalent to Clontech pLexA; confers ability to grow in the absence of histidine), and displayTARGET plasmid (equivalent to Clontech pB42AD; confers ability to grow in the absence of tryptophan).

Transformed yeast were grown on agar plates containing synthetic dropout (SD) minimal media with dextrose as the carbon source and deficient in histidine, uracil and tryptophan (SD/dextrose/-Ura/-His/-Trp). Transformants on SD/Dextrose/-His/-Ura/-Trp agar plates were patched onto the same media, as well as onto agar plates containing galactose and raffinose as the sole carbon source, deficient in histidine, uracil, tryptophan and leucine and containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and BU salts (SD/Gal/Raf/-His/-Leu/-Ura/-Trp) (Clontech Yeast Protocols handbook #PT3024-1). At least 13 colonies were replica plated for each Bait/Target construct combination. Positive interactions on SD/Gal/Raf/-His/-Leu/-Ura/-Trp gave rise to growth and a blue colour change as a result of activation of both *LEU2* and *LacZ* reporter genes.

For determination of both β-galactosidase activity (Pierce Yeast β-galactosidase kit) and protein expression (Clontech Yeast Protocols handbook #PT3024-1), patched colonies from SD/Dextrose/-His/-Ura/-Trp were used to inoculate SD/Gal/Raf/-His/-Ura/-Trp liquid media. At least three colony patches were picked from each Bait/Target combination for quantitative β-galactosidase activity determination.
4.2.3 Statistical Analysis

The statistical software package SPSS for Windows (Version 11.0, SPSS Inc.) was used to analyse the data. For comparison of quantitative β-galactosidase activity in the yeast two-hybrid system, analysis of variance was performed. The factors fitted were Target insert (dynein subunits), which had five levels, and Bait insert (HSV-1 proteins), which had three levels. There was a statistically significant interaction between the Bait insert type and Target insert effects (p<0.001). Therefore the effects of Target inserts were analysed separately for each Bait insert type. For each Bait insert, there was a highly significant overall effect due to Target inserts (p<0.001). Tukey’s correction for multiple comparisons was then used to investigate pair-wise differences between Target (dynein) inserts for each Bait (HSV) insert.
4.3 Results

4.3.1 Cloning of HSV-1 Capsid and Tegument Genes

Genes for HSV-1 major capsid proteins VP5 (UL19), VP19C (UL38), VP23 (UL18) and VP26 (UL35), as well as minor capsid proteins UL6 and UL25, were successfully cloned into both Bait and Target yeast two-hybrid vectors, as above (Table 3). Genes for HSV-1 tegument proteins UL13, UL16 and UL21, as well as for proteins UL32, UL34 and the cytoplasmic tail of gD (US6; aa 364-395) were also cloned. For the major tegument protein VP1/2 (UL36), it was only possible to clone amino acids 1-1874 into Bait plasmid, and 1-767 into Target plasmid. Attempts to clone the tegument protein US9 were unsuccessful. Genes for HSV-1 tegument proteins US11, UL11, UL14, UL16, UL17, UL36, UL37, UL41, UL46, UL47, UL48 and UL49 had previously been cloned and sequenced in the laboratory by Valerio Vittone (VV) (Vittone et al., manuscript in preparation).

Genes for dynein subunits DIC-1c, LC8, rp3, Tctex1 and Roadblock1 were cloned from bacterial expression vectors (see Chapter 3) into Bait and Target vectors for yeast two-hybrid testing.

Expression of all yeast two-hybrid constructs generated in this study was confirmed by western blot (Figure 21), using antibodies directed against either the LexA tag (for Bait constructs) or HA tag (for Target constructs). Expression of UL48 was also confirmed, as an example of constructs made by VV. Expression of other constructs generated by VV have been confirmed previously (Vittone et al., manuscript in preparation).
Table 3. HSV-1 Capsid and Tegument Proteins.

<table>
<thead>
<tr>
<th>Virion Proteins</th>
<th>MW (kDa) from ORF</th>
<th>Essential in vitro</th>
<th>Yeast Two-Hybrid Assay</th>
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<td><strong>Capsid</strong></td>
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*Auto-activates in Bait construct

*Clones constructed by Valerio Vittone

*Only available in Target

*UL36 (aa 1-1874) in Bait; UL36 (aa 1-767) in Target

*Cytoplasmic tail (aa 364-395)
Figure 21. Expression of fusion constructs in the yeast two-hybrid system.
Protein expression was confirmed in the yeast two-hybrid system. Proteins in panel A are Bait fusion constructs, detected on Western blot using anti-LexA antibody. Proteins in panel B are Target fusion constructs, detected using anti-haemagglutinin (HA) antibody. Note that UL36 in panel A and UL48 in panel B were expressed at lower levels than other proteins, so a more over-exposed image is shown for these lanes.
4.3.2 VP26 and VP11/12 Bind to rp3 and Tctex1

A yeast two-hybrid matrix approach was used to screen for interactions between HSV-1 capsid or tegument proteins and cytoplasmic dynein. A strong interaction was indicated by both growth on media lacking leucine and a blue colour change of yeast colonies within 48 h (Figure 22). Interactions were further confirmed with a quantitative β-galactosidase assay. Known interactions between DIC and dynein light chains (Mok et al., 2001; Tai et al., 2001; Makokha et al., 2002; Susalka et al., 2002), as well as LC8 dimerisation, were confirmed in our system (Table 4).

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 4). β-galactosidase activity for each protein interaction was measured using a quantitative assay, and statistically examined using analysis of variance (Figure 23). 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 (Desai and Person, 1996) was confirmed in our system.

To determine the minimal binding domain of VP26 involved in interactions with rp3 and Tctex1, deletion fragments were tested in the yeast two-hybrid system. Initial fragments tested represented the N-terminal (aa 1-52) and C-terminal (aa 53-112) parts of the protein. Neither of these proteins demonstrated any interaction with dynein light chains, suggesting that the interaction is conformationally dependent.

Although VP19C auto-activated when expressed in Bait, β-galactosidase activity increased significantly when co-expressed with rp3 or Tctex1, compared with either
the negative control, DIC or LC8 (p<0.05). To explore this further, fragments of VP19C were constructed, incorporating either the first 90 amino acids (aa 1-90), or the remaining C-terminal fragment (aa 91-465). Although VP19C (1-90) did not auto-activate in Bait, its interaction with rp3 was weaker than for the whole VP19C protein, while its interaction with Tctex1 was not significantly above background (Table 4). VP19C (91-465) auto-activated in Bait, so could not be tested further.

With dynein subunits in Bait vector, rp3 and Tctex1 strongly auto-activated so could not be tested further. No interactions were detected between DIC, LC8 or Roadblock1 in Bait vector and any of the HSV-1 proteins tested in Target vector (Table 5). The previously reported interaction between DIC and UL34 could not be confirmed because UL34 was poorly expressed when inserted in Target vector.

**Figure 22. Yeast two-hybrid screen – examples.**
(see over page)
Proteins were tested for interaction in a LexA yeast two-hybrid system, as described in Materials and Methods. Yeast were co-transformed with Bait, Target and Reporter plasmids, utilising a *LacZ* reporter gene, and grown on agar plates deficient in uracil and containing X-gal. A positive interaction between the Bait and Target proteins is detected by the ability of yeast to grow on uracil-deficient plates and a strong blue colour change. VP26 (the protein product of HSV-1 gene UL35) interacts with dynein light chains rp3 and Tctex1 (A), but not the negative control (Target fusion tag alone) (B, lower half). VP11/12 (the protein product of HSV-1 gene UL46) also interacts with dynein light chains rp3 (C) and Tctex1 (D), but not with the negative control (not shown). Dynein light chains rp3 (E) and Tctex1 (F) both interact with dynein intermediate chain (DIC), but not with the negative controls (Bait fusion tag alone).
Table 4. Yeast Two-Hybrid Screen: HSV-1 Proteins in Bait Plasmid.

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*UL36 aa 1-1874

β-galactosidase activity (arbitrary units)

+ 10-50
++ 50-300
+++ 300-1000
++++ > 1000
- No colour change at 48 hours
(-) No colour change but protein poorly expressed
AA Strong auto-activation at 48h
ND Not determined (no further testing possible due to auto-activation)

HSV-1 proteins, inserted in Bait vector, were tested for interaction with dynein subunits, inserted in Target vector, in a LexA yeast two-hybrid system. “Positive interactions”, defined as growth on leucine-deficient media and blue colour change of yeast colonies within 48 h, were confirmed using a quantitative β-galactosidase assay.
Table 5. Yeast Two-Hybrid Screen: HSV-1 Proteins in Target Plasmid.

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\(^a\)UL36 aa 1-767

\(\beta\)-galactosidase activity (arbitrary units)

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<td>No colour change but protein poorly expressed</td>
</tr>
<tr>
<td>AA</td>
<td>Strong auto-activation at 48h</td>
</tr>
<tr>
<td>ND</td>
<td>Not determined (no further testing possible due to auto-activation)</td>
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HSV-1 proteins, inserted in Target vector, were tested for interaction with dynein subunits, inserted in Bait vector, in a LexA yeast two-hybrid system. "Positive interactions", defined as growth on leucine-deficient media and blue colour change of yeast colonies within 48 h, were confirmed using a quantitative \(\beta\)-galactosidase assay.
Figure 23. VP26 and VP11/12 interact with dynein light chains rp3 and Tctex1.
Positive interactions in a yeast two-hybrid screen (Table 4) were confirmed using a quantitative β-galactosidase assay. This figure summarises the mean β-galactosidase activity for interactions with VP26 (▲) and VP11/12 (■), 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.
4.4 Discussion

Our yeast-two hybrid screen demonstrated a strong interaction of the HSV-1 outer capsid protein VP26 and the tegument protein VP11/12 with dynein light chains Tctex1 and rp3. This was initially detected by good growth of transformed yeast and strong blue colour change when grown on leucine-deficient media containing X-gal. A quantitative liquid β-galactosidase assay subsequently confirmed strong activation of the reporter gene with these proteins.

Interaction of HSV-1 proteins with light chains Tctex1 or rp3 would be a likely way to mediate binding to the cytoplasmic dynein motor. These proteins are thought to mediate binding to various cellular cargoes, and there is a precedent for viral transport, with Tctex1 having been shown to bind the poliovirus receptor CD155 (Mueller et al., 2002).

Of the two HSV-1 proteins, VP26 is more likely than VP11/12 to play a role in retrograde cellular transport of incoming virus. VP26 coats the outer aspect of the viral capsid, bound to the major capsid protein VP5 at the hexon tips (Wingfield et al., 1997). As outlined in the introduction most of the tegument dissociates from the capsid following cell entry, leaving the capsid to be transported to the cell nucleus (Ojala et al., 2000). GFP-VP11/12 has specifically been shown to dissociate from incoming capsids, remaining at the periphery of the cell (Willard, 2002).

Interestingly newly expressed GFP-VP11/12 has been observed in cells undergoing rapid directional movements in the peri-nuclear region (Willard, 2002). This movement was obliterated by nocodazole (which depolymerises microtubules), and was thought to involve molecular motors. A direct interaction between VP11/12 and
dynein, via light chains rp3 or Tctex1, could explain this phenomenon, and may play an important role during viral assembly and acquisition of tegument.

The apparent interaction of dynein subunits with the HSV-1 triplex protein VP19C is intriguing, since it is also located on the outer aspect of the viral capsid. However unlike VP26 it is located on the floor of the capsomeres, rather than on the hexon tips (Zhou et al., 2000), and may be less accessible to bind to dynein. Furthermore it was not possible to confirm an interaction in the yeast two-hybrid system due to the background level of auto-activation. In an attempt to eliminate auto-activation, fragments of VP19C were constructed, incorporating either the first 90 amino acids (aa 1-90), or the remaining C-terminal fragment (aa 91-465). These fragments were chosen because it has been shown that the first 90 amino acids of VP19C are not required for triplex formation and capsid assembly (Spencer et al., 1998), raising the possibility that the N-terminal domain may be external to the capsid, and accessible for binding. Unfortunately results were not conclusive using these fragments, although there was some evidence of an interaction between VP19C (1-90) and rp3.

There were no significant interactions detected between dynein subunits and any of the other HSV-1 capsid or tegument proteins tested. The capsid protein VP5 (UL19), tegument protein UL13 and the protein UL34 were poorly expressed in yeast, so potential interactions with these proteins could not be excluded.

In summary, yeast two-hybrid analysis identified an interaction between the HSV-1 capsid protein VP26 and the tegument protein VP11/12 with dynein light chains rp3 and Tctex1. Of these, the interaction between VP26 and dynein is more likely to be involved in retrograde transport of HSV-1 during infection. In order to confirm these protein-protein interactions, further protein binding studies were performed, as described in the following chapter.
CHAPTER 5:

INTERACTIONS BETWEEN

VP26 AND DYNEIN IN VITRO
5.1 Introduction

Since our aim was to understand the retrograde cellular transport of HSV-1, we wished to identify viral outer capsid or tegument proteins that bind to the cytoplasmic dynein motor. Our initial screen, using a LexA yeast two-hybrid system (Chapter 4) detected binding of the HSV-1 outer capsid protein VP26, and the tegument protein VP11/12, to the homologous dynein light chains Tctex1 and rp3.

In order to confirm these protein-protein interactions, we went on to perform in vitro binding assays. Proteins were expressed in bacteria as fusion proteins, with either GST or His\textsubscript{6} tags. In vitro pull-down assays were performed, using either glutathione-Sepharose beads (for GST-tagged proteins) or nickel-charged beads (for His\textsubscript{6}-tagged proteins).

We were able to confirm binding of His\textsubscript{6}-tagged VP26, but not His\textsubscript{6}-tagged VP11/12, to GST-rp3 and GST-Tctex1 in these assays. We were also able to precipitate cytoplasmic dynein complexes using His\textsubscript{6}-VP26, confirming that the dynein light chains were still able to bind to VP26 as part of a functional motor complex, in the presence of other dynein subunits.

Finally we wanted to confirm whether VP26 in its native conformation, as part of a HSV-1 capsid, was still able to bind to dynein. Recombinant HSV-1 capsids, some of which contained VP26 and some of which did not, were used in similar pull-down assays. In these experiments, VP26+ capsids bound to GST-rp3, while VP26- capsids did not.
5.2 Materials and Methods

5.2.1 Expression Constructs

All genes were amplified by PCR using the Geneamp® XL PCR Kit (PE Applied Biosystems), as described in Chapter 3. For recombinant expression in *E. coli* with N-terminal hexa-histidine (His$_6$) fusion tags, genes were cloned into pET-28a (Novagen). Genes were excised from other expression plasmids with appropriate restriction endonucleases, and inserted into digested pET-28a using a Clonables™ Kit (Novagen). All constructs were sequenced to confirm correct reading frame for recombinant protein expression. Construction of His$_6$-tagged fusions of kinesin heavy chain KIF5B (aa 771-963) (Diefenbach *et al.*, 1998) and HSV-1 tegument protein US11 (Diefenbach *et al.*, 2002b) have been described previously. Construction of GST-tagged dynein subunits was as described in Chapter 3.

5.2.2 Expression and Purification of His$_6$-tagged Proteins

His$_6$-tagged constructs were expressed in *E. coli* strain BL21 (DE3). Protein expression was as described in Chapter 3, except that 50 mg/mL of kanamycin was added to the media instead of ampicillin, and protein expression was induced with 1 mM IPTG for 3 h at 37 °C. The His$_6$-tagged constructs were harvested and lysed as described previously (Diefenbach *et al.*, 2002a). His$_6$-KIF5B was purified as described previously (Diefenbach *et al.*, 2002a). To solubilise His$_6$-VP26, inclusion bodies were resuspended in 5 mL of “denaturing buffer” (8 M urea, 100 mM NaH$_2$PO$_4$, 10 mM Tris-HCl, pH 8.0) containing 0.5 mM imidazole. After 1 h at 4 °C the solution was centrifuged at 16,000 × g for 30 min at 4 °C, the supernatant removed and centrifuged
for a further 10 min. At this point the protein concentration was approximately 5 mg/mL (Biorad Protein Assay). The supernatant was passed over a column (2.5 mL bed volume) of nickel beads (His.Bind® Kit, Novagen), prepared according to manufacturer’s instructions. The column was washed with denaturing buffer containing 20 mM imidazole. His$_6$-VP26 was then eluted with 15 mL of denaturing buffer containing 200 mM imidazole, collected as 1 mL fractions. The protein content of each fraction was estimated using both the Biorad Protein Assay and SDS-PAGE with Coomassie Blue stain. The three fractions with the highest protein concentration (approximately 5 mg/mL) were pooled and stored at 4 °C.

To refold His$_6$-VP26, 200 μL of stock His$_6$-VP26 solution (in 8 M urea) was slowly diluted 1:8 (in buffer containing 100 mM NaH$_2$PO$_4$ and 10 mM Tris-HCl, pH 8.0) to a final urea concentration of 1 M.

5.2.3 Preparation of Recombinant HSV-1 Capsids

Recombinant VP26+ or VP26- HSV-1 capsids, kindly provided by Dr. Fred Homa (Pharmacia Corp., Kalamazoo, Michigan 49001, USA), were constructed by him as described previously (Thomsen et al., 1994). 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. Capsids were purified by sucrose gradient centrifugation, and have been shown by transmission and cryo-electron microscopy to have the same structure, size and appearance as authentic HSV-1 B capsids (Thomsen et al., 1994; Trus et al., 1995). All recombinant capsids thus contained the major capsid proteins VP5, VP19C and VP23, as well as the scaffolding proteins VP21, VP22a and VP24. In addition, those formed in the presence of UL35 also contained the major capsid protein VP26.
5.2.4 **Negative Staining of Recombinant Capsids**

Negative staining of recombinant capsids and electron microscopy was performed by Jo-Ann McCraig, under the supervision of Dr. Ross Boadle, at the Electron Microscope Laboratory, Westmead Millennium Institute and Institute of Clinical Pathology and Medical Research, Westmead Hospital, New South Wales 2145, Australia.

1 µL of either VP26+ or VP26- recombinant HSV-1 capsid suspension was diluted in 100 µL of PBS. 20 µL drops of 0.1% (w/v) poly-L-lysine hydrobromide, double-distilled water and diluted capsid suspension were dispensed separately into wells on glass slides. For each specimen, a 400 mesh copper grid, coated with Formvar-Pioloform and carbon, was allowed to float briefly on the poly-L-lysine hydrobromide, then on the double-distilled water, draining in between on filter paper. The grid was then allowed to float on the diluted capsid suspension for 1 minute, and was dried by gently touching the edge with a wedge of filter paper. The grid was then allowed to float on a 10 µL drop of 2% (w/v) phosphotungstic acid (pH 7.2) on a glass slide for 30 sec, dried as above with filter paper, then allowed to air-dry for 30 minutes.

Grids were examined using a Philips CM10 transmission electron microscope, operated at 80 kV.

5.2.5 **In Vitro Pull-down Assays**

For pull-down assays of dynein complexes using His6-VP26, refolded His6-VP26 (prepared as above) was bound to nickel beads (His.Bind® Kit, Novagen,), incubated with cell lysates, then eluted from the resin, as described previously (Diefenbach *et al.*, 1998). Cell lysates containing intact dynein complexes were prepared from Hep2
cells grown to 80\% confluence, as follows. Cells were detached with trypsin, washed with phosphate-buffered saline (PBS), and then resuspended in lysis buffer containing PBS, 0.1\% (v/v) Triton X-100, and a protease inhibitor cocktail (P8849, Sigma-Aldrich). Cells were subjected to sonication in a Branson cup-horn water bath (3 × 20 sec, 100\% duty cycle), and centrifuged at 12 000 × g for 30 min at 4 °C to remove cellular debris. Pull-downs were performed by incubating with GST-tagged dynein subunits, bound to glutathione-Sepharose beads (Amersham Biosciences), as described in Chapter 3.

5.2.6 Western Blots, Antibodies

Western blots were performed as described in Chapter 3. The major capsid protein VP5 was detected using the rabbit polyclonal anti-VP5 antibody (NC1, kindly provided by Dr. G. Cohen and Dr. R. Eisenberg, University of Pennsylvania, Philadelphia, USA) (Cohen et al., 1980). VP26 was detected by a polyclonal rabbit IgG antibody, a kind gift of Dr. Stanley Person (Johns Hopkins University, Baltimore, Maryland, 21205, USA) (Desai et al., 1998). His\textsubscript{6}-tagged fusion proteins were detected using a mouse monoclonal anti-His antibody (SC-8036, Santa Cruz). Secondary antibodies used were Peroxidase-conjugated ImmunoPure\textsuperscript{®} Goat anti-Mouse (31430) or anti-Rabbit (31460) IgG, (H+L) (Pierce). Supersignal Substrate\textsuperscript{TM} (Pierce) was used for enhanced chemiluminescence, and detected using photographic film.

For detection of dynein complexes by Western blotting, the Odyssey\textsuperscript{™} Infrared Imaging System (LI-COR) was used. Labelling of nitrocellulose membranes was performed according to the manufacturer’s protocol (see Appendix 3), using Odyssey\textsuperscript{™} Blocking Buffer as blocking agent and antibody diluent. To detect dynein
complexes a mouse monoclonal antibody directed against DIC (IC74) was used (MAB 1618, Chemicon). A secondary Alexa Fluor 680-conjugated Goat anti-Mouse IgG (H+L) (A-21057, Molecular Probes) was used, at a dilution of 1:2000, and fluorescence detected using the Odyssey™ Infrared Imaging System.

5.2.7 Software for Digital Images

Photographic film (for western blots utilising chemiluminescence), and electron micrographs were scanned at 300 dots per inch, cropped using Adobe Photoshop 7.0 and stored as TIFF files. For Western blots utilising infrared fluorescence, images were acquired using Odyssey™ Software Release 1.1 (LI-COR) at 300 dots per inch, cropped and stored as TIFF files. Brightness, contrast and colour balance for all digital images was optimised using Adobe Photoshop 7.0.
5.3 Results

5.3.1 His$_6$-VP26 Binds to GST-rp3 and GST-Tctex1 In Vitro

His$_6$-tagged fusion constructs of HSV-1 proteins VP26 and VP11/12 were expressed in *E. coli*. His$_6$-VP26 formed insoluble inclusion bodies, which were solubilised by denaturing with 8 M urea, then slowly refolded by dilution to 1 M urea. VP26 expressed in bacteria, denatured then refolded has previously been shown to bind to HSV-1 capsids, in either 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonic acid (CHAPS) buffer or 1 M urea (Wingfield *et al.*, 1997; Macnab-Bain, 1999). His$_6$-VP11/12 was only expressed at very low concentrations, and could not be detected consistently in soluble or insoluble fractions. Attempts to express His$_6$-VP19C in bacteria were unsuccessful.

*In vitro* pull-down assays were performed by incubating His$_6$-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 was confirmed for each GST-dynein construct, with GST-DIC present at lower concentrations than the other constructs (Figure 24A). Refolded His$_6$-VP26 bound to GST-Tctex1 and GST-rp3, but not to GST-DIC, GST-LC8 or GST alone (Figure 24B and C). Binding was not mediated via the His$_6$ tag, since a His$_6$-tagged kinesin heavy chain fragment (His$_6$-KIF5B residues 771-963) did not bind to rp3 or Tctex1 (Figure 24B). There was no evidence of dynein binding to His$_6$-VP11/12, but this may simply reflect low expression levels.
Figure 24. His$_6$-VP26 binds to GST-rp3 and GST-Tctex1 in vitro.

Purified His$_6$-VP26 and His$_6$-KIF5B (aa 771-963, His$_6$ tag negative control) were incubated with GST-tagged dynein subunits, bound to glutathione-Sepharose beads, in a pull-down assay. His$_6$-VP26 bound to dynein light chains GST-rp3 and GST-Tctex1, but not to GST-DIC, GST-LC8 or the GST tag control. His$_6$-VP26 was detected initially with Coomassie Blue stain (A), before confirmation on western blots using anti-His (B) and anti-VP26 (C) antibodies. (A) Expression, binding and elution of all GST-dynein fusion constructs were confirmed with Coomassie Blue stain. GST-DIC was present at lower concentrations than other fusion constructs.
5.3.2 His<sub>6</sub>-VP26 Binds to Intact Dynein Complexes *In Vitro*

To confirm that VP26 binds to dynein light chains in the context of a native dynein complex, as well as to individual dynein subunits, an *in vitro* pull-down assay of cytoplasmic dynein complexes was performed. His<sub>6</sub>-fusion constructs of HSV-1 proteins VP26 (refolded as above) and US11 (negative control) were bound to nickel beads, incubated with lysates from uninfected Hep2 cells, and the eluted complexes were analysed by SDS-PAGE and western blot (Figure 25). Dynein complexes, which were detected in cell lysates, bound to His<sub>6</sub>-VP26 but not to US11 (Figure 25A). The presence of DIC in the bound complex was used as a surrogate marker for intact dynein complexes, since we have found no evidence of a direct interaction between VP26 and DIC *in vitro*. An equivalent concentration of His<sub>6</sub>-VP26 and His<sub>6</sub>-US11 in eluted fractions was confirmed by Coomassie Blue stain (Figure 25B).

![Figure 25](image)

**Figure 25.** His<sub>6</sub>-VP26 binds intact cytoplasmic dynein complexes *in vitro*. In a pull-down assay Hep2 cell lysates were incubated with His<sub>6</sub>-tagged VP26 or US11 (HSV-1 tegument protein, negative control), bound to nickel beads. Eluted complexes were analysed by SDS-PAGE and western blot, with dynein complexes detected by anti-DIC antibody. (A) Dynein complexes, detected in cell lysates, bound to His<sub>6</sub>-VP26 but not to His<sub>6</sub>-US11. (B) An equivalent concentration of His<sub>6</sub>-VP26 and His<sub>6</sub>-US11 in eluted fractions was confirmed by Coomassie Blue stain.
5.3.3 Synthesis of Recombinant HSV-1 Capsids, +/- VP26

To investigate the behaviour of capsid proteins in their usual tertiary and quaternary structure, recombinant HSV-1 capsids were constructed with or without VP26 (VP26+ or VP26-) (Trus et al., 1995). Capsids were made by expressing the major capsid proteins VP5, VP19C and VP23, along with scaffolding proteins, in insect cells using a baculovirus system, and purified on a sucrose gradient.

The capsids were examined by electron microscopy and shown to be mostly in single particle suspension (Figure 26C). As expected, their morphology was similar to HSV-1 B capsids, and there was a higher proportion of incomplete capsids in the absence of VP26 (approximately 20%, data not shown). SDS-PAGE, Coomassie Blue stain and western blot confirmed the presence of equivalent amounts of the major capsid protein VP5 in each sample, while VP26 was only present in VP26+ capsids (Figure 26A and B).

5.3.4 HSV-1 Capsids Containing VP26 Bind to GST-rp3

Recombinant capsids (+/- VP26) were used in pull-down assays with GST-tagged dynein subunits bound to beads, as above. VP26+ capsids bound to GST-rp3, but not to other dynein subunits, in replicate experiments (Figure 27). 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. Similar input amounts of VP26+ and VP26- capsids were confirmed by Western blot, using anti-VP5 (NC1) antibody.
Figure 26. Composition of recombinant HSV-1 capsids, +/-VP26.

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). (B) There was a similar content of VP5 in each capsid preparation, as detected by the NC1 polyclonal anti-VP5 antibody. Proteolytic fragments of VP5 were also present. (C) Electron microscopy confirmed icosahedral capsid morphology, similar to HSV-1 B capsids.
Figure 27. VP26+, but not VP26- capsids bind GST-rp3 in vitro.
Recombinant HSV-1 capsids were incubated with GST-tagged dynein subunits, bound to glutathione-Sepharose beads, in a pull-down assay. Capsids bound to any GST-tagged fusion proteins were detected by western blot, using NC1 polyclonal anti-VP5 antibody, in replicate experiments (A, B). VP26+ capsids bound to GST-rp3, but not to other dynein subunits. Proteolytic fragments of VP5 were also detected. VP26- capsids did not bind significantly above background.
5.4 Discussion

*In vitro* binding studies confirmed binding of the HSV-1 outer capsid protein VP26 to dynein light chains rp3 and Tctex1. In pull-down assays His<sub>6</sub>-VP26 bound to GST-rp3 and GST-Tctex1, but not to other dynein subunits. Furthermore, His<sub>6</sub>-VP26 was able to pull down intact dynein complexes from cell lysates, demonstrating that dynein light chains are still able to bind to VP26 when incorporated into dynein complexes. Finally, using recombinant HSV-1 capsids in similar pull-down assays VP26+ capsids bound to GST-rp3, whereas VP26- capsids did not. This demonstrates that VP26 in its functionally relevant conformation, incorporated into HSV-1 capsids, is still able to bind to dynein.

Despite strong interaction between VP26 and Tctex1 in the yeast two-hybrid system and *in-vitro* pull-down assays (using His<sub>6</sub>-VP26), 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. It may be that VP26 in its correct conformation, bound to capsids, binds less strongly to Tctex1 than in its soluble form. However, results in Chapter 7 demonstrate colocalisation between VP26+ capsids and Tctex1 in a cellular model, suggesting that such binding still occurs.

Binding of the tegument protein VP11/12 to rp3 and Tctex1 was detected in the yeast two-hybrid system, but could not be confirmed with pull-down assays due to poor expression of soluble His<sub>6</sub>-tagged fusion proteins in *E. coli*. The significance of these results is unclear, but any role is likely to be later in the viral replication cycle, rather than during retrograde transport. It seems unlikely that VP11/12 is involved in retrograde transport of the capsid/tegument complex, since it dissociates from
incoming capsids during cell infection (Willard, 2002), although retention of some residual VP11/12 during retrograde transport will be difficult to exclude. However, an interaction of VP11/12 with dynein may play a role during tegument assembly, since newly synthesised VP11/12 has been observed in perinuclear particles undergoing rapid, microtubule-dependent movement (Willard, 2002).

The HSV-1 capsid protein VP19C showed possible binding to rp3 and Tctex1 in the yeast two-hybrid screen, although this could not be confirmed due to auto-activation (Chapter 4). It was not possible to express this protein in bacteria with a His$_6$ tag, so it could not be tested for binding in vitro using pull-down assays. Although we cannot exclude some contribution of VP19C to dynein binding, the fact that VP26- capsids, which contain VP19C, did not bind to GST-rp3 in vitro suggests that any contribution of VP19C is minor compared with the role of VP26.

In summary, in vitro binding results support initial yeast two-hybrid findings that the HSV-1 capsid protein VP26 binds to dynein light chains rp3 and Tctex1. Pull-down experiments using recombinant HSV-1 capsids suggest that VP26, when incorporated into capsids, may bind more strongly to rp3 than Tctex1.
CHAPTER 6:

DISSOCIATION OF HSV-1

TEGUMENT PROTEINS
6.1 Introduction

Having confirmed a direct interaction in vitro between the HSV-1 outer capsid protein VP26 and dynein light chains rp3 and Tctex1, we were interested in the cellular distribution of various HSV-1 proteins during natural infection. In particular, we wanted to confirm exactly which tegument proteins remained attached to incoming capsids, and whether or not VP26 was likely to be accessible to interact with cytoplasmic dynein.

Previous studies have shown that following HSV-1 cell entry most, but not all of the tegument proteins are lost, and the nucleocapsid-tegument complex is transported to the nucleus (Ojala et al., 2000). It is believed that HSV-1 tegument proteins VP11/12, VP13/14, VP16 and VP22 dissociate from the virus at early stages of infection, probably following phosphorylation, while the major tegument protein VP1/2 and the minor capsid protein UL25 remain attached to incoming capsids (Morrison et al., 1998a; Morrison et al., 1998b; Elliott and O’Hare, 1999; Dohner et al., 2002; Willard, 2002). The fate of other tegument proteins, including the inner tegument protein UL37, is not known.

For this set of experiments, we infected neurones and other cells with HSV-1, and examined the intracellular distribution of viral proteins at early time points by confocal microscopy. To aid with detection of tegument proteins, we used a recombinant HSV-1 strain with UL37 fused to green fluorescent protein (GFP).

Our results show that the HSV-1 inner tegument protein UL37 dissociates from capsids during cell entry, supporting previous data that only tegument protein VP1/2 remains associated with incoming capsids. This suggests that the outer tegument proteins do not play a role during retrograde transport of HSV-1, and is consistent
with an important role for the outer capsid protein VP26, through interactions with cytoplasmic dynein. The distributions of dynein light chains rp3 and Tctex1 in rat DRG neurones and IMR32 neuroblastoma cells were also examined.
6.2 Materials and Methods

6.2.1 Construction of GFP-UL37 HSV-1 Virus (Rixon, F.J.)

A strain of HSV-1 with the carboxy terminus of tegument protein UL37 fused to GFP was provided as a kind gift by Dr. Frazer Rixon (MRC Virology Unit, Institute of Virology, Glasgow G11 5JR, UK). The techniques he used in constructing the virus, called GFP-UL37 HSV-1, are summarised below.

A SpeI endonuclease site was engineered into the GFP basic expression vector pGFPemd-b® (Packard Bioscience), and designated pGFPSpeI. SpeI digested pGFPSpeI was ligated with SpeI digested HSV-1 strain 17 virion DNA (HSV-1 has a single SpeI site at the 3’-end of the UL37 ORF). When pGFPSpeI was inserted into this site it regenerated the precise sequence at the C-terminus of UL37 and fused the GFP ORF in frame with the UL37 ORF.

The ligated DNA was transfected into BHK cells (in a 24-well plate) using Lipofectin (Invitrogen). After four days the culture medium was harvested and set up on 35 mm dishes of BHK cells. Green fluorescing plaques were purified through three cycles of plaque picking. One isolate was selected and grown to high titre. The presence of the GFP protein sequences was confirmed by western blotting of infected cells and purified virions.

6.2.2 Preparation of High Titre GFP-UL37 HSV-1

Hep2 cells were grown to confluence in 150 cm² Falcon® tissue culture flasks, infected with GFP-UL37 HSV-1, at 0.01 PFU per cell, and incubated in DMEM (Invitrogen™), supplemented with 1% (v/v) FCS (JRH Biosciences). Once 80% of the
cells displayed cytopathic effect (48-72 h), cells were removed using a cell scraper, frozen and thawed 3 times, subjected to sonication (3 × 7 sec, 100% duty cycle), and cellular debris was removed by centrifugation at 15,800 × g for 10 min at 4 °C. Virus was pelleted by centrifugation at 64,000 × g for 2 h at 4 °C. The virus pellet was then resuspended in a small volume of DMEM supplemented with 1% (v/v) FCS, plaqued on human foreskin fibroblasts (see Chapter 3), and stored at -80 °C.

### 6.2.3 Preparation and Infection of Neuronal Cells

DRG neurones were prepared from 4-day-old Wistar rat neonates, as described previously (Miranda-Saksena et al., 2000). DRG were dissociated in Hanks calcium and magnesium-free solution (Invitrogen™) plus 0.25% (w/v) trypsin (CSL) and 0.05% (w/v) collagenase (Worthington Biomedical Co.) for 70 min at 37 °C, washed three times by centrifugation at 700 × g and passed through a 35 to 45 % Percoll gradient (Sigma). The cell pellet was resuspended in 200 mL of DMEM, plated onto Matrigel™ (Collaborative Biomedical Products)-coated glass coverslips in 24-well plates, and cultured at 37 °C, in DMEM supplemented with 5.2 g/L of D-glucose, B27 supplement (1:50) (Invitrogen™), 4 mM L-glutamine (Invitrogen™), 20 to 100 ng/mL 7S-nerve growth factor (Collaborative Biomedical Products), and 2% (v/v) FCS.

On average there were approximately 500 neurones per well, concentrated on the Matrigel™ in the centre of the cover slip. After 72 h at 37 °C, the neurones were infected with GFP-UL37 HSV-1. Approximately 1 × 10⁸ PFU of virus were added to each well, cultures were incubated for 30 min or 2 h at 37 °C, washed three times in PBS, then fixed, permeabilised and immuno-labelled.
IMR32 cells (a human neuroblastoma cell line) were seeded onto glass coverslips (approx $1 \times 10^5$ cells per well) and forced to differentiate by culturing in serum-free Neurobasal™ medium containing B27 supplement (1:50) and 4 mM L-glutamine. After 72 h, these cells were infected with GFP-UL37 HSV-1 as above ($1 \times 10^8$ PFU per well: approximately 1000 PFU per cell).

### 6.2.4 Immunofluorescence and Confocal Microscopy

DRG neurones or IMR32 cells cultured on coverslips were fixed in 4% formaldehyde (ProSci Tech) in Sorenson’s buffer (pH 7.4) for 30 min and permeabilised in 0.1% (v/v) Triton X-100, diluted in PBS, for 10 min. Non-specific antibody binding was blocked by incubating for 15 min in Tris-buffered saline (TBS), supplemented with 10% (v/v) goat serum, 0.1% (w/v) bovine serum albumin (BSA), 0.05% (v/v) Tween-20 and 0.02 M NaNH₃. The cells were then incubated for 30 min with primary antibody, diluted 1:50 in TBS, supplemented with 1.5% (v/v) goat serum, 0.1% (w/v) BSA, 0.05% (v/v) Tween-20 and 0.02 M NaNH₃. After rinsing six times in PBS, the cells were incubated for 1 h with the appropriate fluorochrome-conjugated secondary antibody, diluted in the same solution as the primary antibody, rinsed six times in PBS, and mounted on glass slides using the glycerol component of a Pierce SlowFade® Anti-fade kit (S-2828, Pierce).

Slides were examined using a Leica Confocal System TCS SP2, attached to a Leica DMRE fluorescent microscope. A Leica PLAPO 63× oil immersion objective, numerical aperture 1.32, was used.

Primary antibodies used to detect HSV-1 capsids were a rabbit polyclonal anti-VP5 antibody, NC1 (diluted 1:2000) (kindly provided by Dr. G. Cohen and Dr. R.
Eisenberg, University of Pennsylvania, USA) (Cohen et al., 1980), and a commercial mouse monoclonal anti-VP5 antibody (diluted 1:50) (6F10, Santa Cruz).

Primary antibodies used to detect HSV-1 tegument proteins US11, VP13/14 and VP16 were as follows. US11 was detected by a mouse monoclonal antibody (diluted 1:50), the kind gift of Dr. Bernard Roizman (University of Chicago, USA) (Roller and Roizman, 1992). VP13/14 was detected using the mono-specific rabbit polyclonal antibody R220 (diluted 1:1000), kindly provided by Dr. David Meredith (University of Leeds, UK) (Whittaker et al., 1991). VP16 was detected using a commercial mouse monoclonal IgG1 (diluted 1:50) (Santa Cruz).

To confirm dynein expression, uninfected DRG neurones and IMR32 cells were labelled with rabbit polyclonal antibody, directed against dynein light chains (a kind gift of Dr. Stephen King, University of Connecticut Health Centre, USA). Primary serum containing antibody R5270 against rp3 (King et al., 1998) or R5205 against Tctex1 (King et al., 1996) was used, at a dilution of 1:50. During construction of the GFP-UL37 HSV-1 strain, a monoclonal antibody against recombinant GFP (Clontech) was used to detect GFP-UL37.

The secondary antibodies used were: Cy3-conjugated goat anti-mouse or goat anti-rabbit IgG (Amersham Pharmacia Biotech), diluted 1:400; and Alexa Fluor® 488-conjugated goat anti-mouse or goat anti-rabbit IgG (Molecular Probes), diluted 1:200.

6.2.5 Campenot Nerve Growth Chambers

Campenot nerve growth chambers were set up, according to recommended protocols (Campenot, 1992; Campenot, 1997). To encourage the adherence of cultured neurones, glass coverslips were coated with a layer of cell matrix. Round glass coverslips (22 mm) were placed in 6-well plates, and sterilised by heating for 16 min
in a 1200 W microwave oven. A confluent monolayer of human foreskin fibroblasts grown in a 75 cm² Falcon® tissue culture flask was dissociated using 0.25% trypsin (CSL) in Hanks Buffered Saline Solution (Invitrogen™), and seeded onto the coverslips, at a dilution of 1:20. Cells were cultured in DMEM supplemented with 9% (v/v) FCS, 100 IU/mL penicillin and 100 µg/mL streptomycin (Sigma), at 37 ºC, in 5% CO₂. After 4 days the fibroblasts had grown to confluence, and were removed from the coverslips with a cell scraper. The coverslips were air-dried, and a series of parallel scratches introduced into the cell matrix layer using a specially designed pin-rake (Tyler Research Corporation, Edmonton, Alberta, Canada), leaving tracks of cell matrix approximately 200 µm wide. These coverslips were placed in clean 6-well plates, sterilised again, and stored in the freezer until required.

Teflon CAMP10 Campenot Nerve Cell Growth Chambers (Tyler Research Corporation) were placed in a glass Petri dish, the lid secured with autoclave tape, and sterilised in an autoclave. A stainless steel syringe (Tyler Research Corporation) was filled with Dow Corning™ high-vacuum grease, secured with metal wire to prevent movement of the plunger, and autoclaved. A set of 21 cm Haemostatic Forceps, with jaws angled 90 degrees (Heiss, Cat. No. GS3174) was also cleaned and sterilised.

Culture medium was prepared by supplementing DMEM with 1% (v/v) FCS, B27 supplement (1:50), 4 mM L-glutamine, 20 to 100 ng/mL 7S-nerve growth factor, 100 IU/mL penicillin and 100 µg/mL streptomycin, and 1.6% (w/v) carboxy-methyl cellulose (400-800 cps, Sigma). In a laminar flow cabinet, a drop of culture medium was placed on the scratched region of a coverslip to wet it. A CAMP10 Teflon ring was picked up using the sterile haemostat, and inverted under the stereoscopic microscope. Silicone grease was applied to the bottom of the ring using the stainless steel syringe and 18-gauge blunt-ended needle, taking care that the regions under
which the neurites would cross received a neat “rope” of grease. The Teflon ring was then dropped (not placed) onto the coverslip, and carefully pressed around the rim to seat the grease, using the fine forceps. Care was taken not to press in the region where the neurites would cross. A dab of grease was placed at the entry of the slot, and anywhere around the diameter of the ring where there was not a complete seal. A drop of culture medium was placed in each lateral compartment, on the scratched region, to prevent drying. The process was repeated for each well of the 6-well plate, the lid was replaced, and the plate was placed in the incubator for one hour at 37 ºC, to allow settling of the rings. Culture medium was then added to fill the lateral chambers, and the chambers returned to the incubator overnight, to exclude leakage of media.

The following day, dissociated DRG neurones were prepared from 4-day old Wistar rats, as above. Approximately 100 μL of cell suspension was added to the centre compartment slot of each ring, using a 1 mL syringe and 21-gauge needle, and incubated overnight at 37 ºC, in 5% CO₂. The next day the cell-containing compartment was topped up with culture medium and returned to the incubator.

Neurones were monitored daily for growth, by viewing under an inverted phase contrast microscope (Olympus). In particular, the regions either side of the Teflon dividers were observed closely, for evidence of axonal growth. From published guidelines on the Campenot Nerve Growth Chambers, we anticipated neurite growth of approximately 1 mm per day (Campenot, 1992).

After the neurites have penetrated under the Teflon divider (approximately 6 to 7 days), media should be changed, omitting NGF from media in the central slot, where the neurone cell bodies and proximal neurites are located (Campenot, 1992; Campenot, 1997). This step inhibits development of new neurites, and encourages
further growth of distal neurites, in the side chambers, which continue to be exposed to NGF.

Once stable neuronal cultures were established, media was changed at least weekly. The side chambers were filled to the top of the Teflon divider with media, while media in the central slot and surrounding dish was kept at a low level (Campenot, 1992). This ensured that any significant leakage of media between chambers was detected, by equalisation of media levels. Such cultures were discarded.
6.3 Results

6.3.1 Distribution of Tctex1 and rp3 in Neuronal Cells

Since our protein binding results suggested a role for 14 kDa dynein light chains Tctex1 and rp3 during retrograde transport of HSV-1, we wished to confirm their presence in rat sensory neurones and IMR32 cells (a human neuronal cell line). DRG neurones were isolated and cultured, as above, on Matrigel™-coated glass coverslips. IMR32 cells were grown on plain glass coverslips, and forced to differentiate by removal of serum, as above. After 72 h the cells were fixed, labelled with antibodies directed against Tctex1 and rp3, and examined by confocal microscopy.

Cell morphology was determined using the transmission mode of the confocal microscope. DRG neurones were confirmed to have developed a network of axons (Figure 28A), while differentiated IMR32 cells had developed a network of neuritic projections (Figure 28B).

We were able to detect the presence of both Tctex1 (Figure 28C) and rp3 (Figure 28E) in neuronal cell bodies and axons, although Tctex1 was relatively more abundant in neuronal axons than rp3. Similar results were obtained with IMR32 cells (Figure 28D and F), although both Tctex1 and rp3 could be detected in neuritic projections.
Figure 28. Distribution of Tctex1 and rp3 in neuronal cells.

Rat DRG neurones (A,C,E) or differentiated IMR32 cells (B,D,F) were grown in culture, fixed, labelled with fluorescent antibodies against 14 kDa dynein light chains, and examined by confocal microscopy. Panels (A) and (B) are transmission micrographs, showing cell morphology, including neuritic processes. Panels (C) and (D) show the intracellular distribution of Tctex1, while (E) and (F) show the intracellular distribution of rp3.
6.3.2 GFP-UL37 Dissociates from Incoming HSV-1 Capsids

For VP26 to contribute to retrograde transport of HSV-1 capsids in neurones and other cells, it must be accessible to bind dynein. To confirm which tegument proteins remain associated with incoming capsids, we infected neurones with HSV-1, and used confocal microscopy to examine the intracellular distribution of capsid and tegument proteins.

In order to increase the specificity of detecting HSV-1 tegument proteins by confocal microscopy, we wanted a strain of virus that expressed a fluorescent tegument protein. Since we were most interested in the fate of the inner tegument proteins, we chose a strain of HSV-1 that expresses the inner tegument protein UL37 as a fusion product, linked to GFP.

Insertion of GFP into the UL37 protein caused no apparent effects on the biology of the virus. The UL37-GFP HSV-1 strain has been confirmed previously to have similar growth kinetics to the parent (strain 17) virus (Rixon, personal communication). In our experiments GFP-UL37 HSV-1 grew well in tissue culture, and was able to be isolated at high titres (2×10^8 PFU/mL). By growing large quantities of the virus in Hep2 cells and concentrating it (see Materials and Methods), infectious titres of up to 5×10^9 PFU/mL were obtained. Confocal microscopy confirmed that 24 h after infection, when new virus is assembling, GFP-UL37 had the expected nuclear and patchy cytoplasmic distribution, particularly in the perinuclear region (data not shown) (Schmitz et al., 1995).

To determine whether or not GFP-UL37 remained attached to HSV-1 capsids during cell entry, dissociated neonatal rat DRG neurones, or differentiated IMR32 cells, were infected with GFP-UL37 HSV-1 and examined at early time points by confocal
microscopy. At various times after infection the cells were fixed, labelled with anti-VP5 antibody to detect capsids, and examined by confocal microscopy (Figure 29). At standard multiplicities of infection (1-10), neither incoming GFP-UL37 nor VP5 could be detected (data not shown). When cells were infected at very high multiplicity of infection (over 10⁸ PFU of virus per well: a multiplicity of at least 1000), both GFP-UL37 and VP5 were detectable at early time points. The polyclonal NC1 antibody (Figure 29A and B) was much more sensitive than the commercial monoclonal anti-VP5 antibody (Figure 29C and D), and was the only antibody we tried that was able to consistently detect incoming HSV-1 proteins during infection.

In DRG neurones, at up to 2 h post-infection, GFP-UL37 remained at the periphery of the cell body, subjacent to the plasma membrane and the axolemma in the axon, with no proximal extension of UL37 antigen into the cytoplasm observed (Figure 29A and C). In contrast, the distribution of capsids, represented by staining for VP5, was mostly intracellular by 2 h post-infection. A similar protein distribution was seen when GFP-UL37 HSV-1 was used to infect differentiated IMR32 cells (Figure 29B and D), or Hep2 cells (data not shown). Specific labelling with both anti-VP5 antibodies, as well as the absence of background green fluorescence in uninfected cells, was confirmed using mock-infected cultures (Figure 29E-G).

In summary, our results demonstrate significant dissociation of the inner tegument protein UL37 from HSV-1 capsids, during infection of both sensory neurones and other cell lines.
Figure 29. GFP-UL37 dissociates from HSV-1 capsids during cell entry.
Rat DRG neurones (A,C) and IMR32 cells (B,D) were infected with a strain of HSV-1 expressing GFP-UL37 (green), or mock infected (E-G). After 2 h the cells were fixed, and viral capsids were labelled with either the NC1 polyclonal (A,B) or 6F10 monoclonal (C,D) anti-VP5 antibody (red). At that time GFP-UL37 remained at the periphery of the cell body, subjacent to the plasma membrane and the axolemma in the axon, with no proximal extension of UL37 antigen into the cytoplasm observed. In contrast the distribution of capsids, represented by staining for VP5, was mostly intracellular by 2 h post-infection, suggesting dissociation of UL37 from incoming capsids. Panels (E) to (G) demonstrate minimal background fluorescence for mock-infected cells.
6.3.3 Distribution of HSV-1 Capsid and Tegument Proteins

To confirm the fate of other HSV-1 proteins following infection, rat DRG neurones or IMR32 cells were infected with GFP-UL37 HSV-1, as above, fixed after 2 h, and labelled with antibody directed against HSV-1 capsid and tegument proteins.

At 2 h post-infection, weak but specific labelling, using mouse monoclonal antibodies, was obtained for tegument proteins US11 (Figure 30A and G) and VP16 (Figure 30B and H). US11 was distributed at the periphery of the cell, in association with the plasma membrane, while VP16 had a more diffuse cytoplasmic and nuclear distribution. The tegument protein VP13/14 appeared to have a peripheral cytoplasmic distribution at the same time point (Figure 30C and I), but there was also some non-specific background labelling with this polyclonal antibody (Figure 30L). In contrast there was essentially no non-specific labelling with the monoclonal antibodies against VP16 and US11 (Figure 30J and K).

Unfortunately it was difficult to detect incoming HSV-1 proteins consistently at these early time points, prior to the onset of viral replication. The amount of viral protein within infected cells was very low, resulting in weak fluorescent signal, which was not always above background levels. It was not possible to detect any other HSV-1 capsid or tegument proteins, by confocal microscopy, with the antibodies that were available.
Figure 30. Distribution of tegument proteins US11, VP16 and VP13/14.

DRG rat neurones were infected with GFP-UL37 HSV-1 (A-I), or mock infected (J-L). After 2 h the cells were fixed, and labelled with antibody directed against HSV-1 tegument proteins US11 (A,D,G,J) VP16 (B,E,H,K) or VP13/14 (C,F,I,L). Panels (A) to (C) show merged images of the green (GFP-UL37) and red (tegument proteins) channels. Individual channels are shown in the panels below each merged image, with mock infected cells in the bottom row.
6.4 Neuronal Two-Chamber Models

6.4.1 Background to the Neuronal Two-Chamber Model

During natural infection with HSV-1, the only way for a virus to reach the neurone cell body is via its axon, a process that requires active transport. One of the challenges when examining axonal transport in tissue culture is to experimentally isolate the axon from the rest of the neurone. When cells are infected with HSV-1 in a standard tissue culture well, virus is able to infect the neurone cell body directly through the plasma membrane, as well as via the axon. Therefore, when viral proteins are observed in the neurone cell body, close to the nucleus, it is almost impossible to know whether they have been transported there along the axon, or have merely travelled the very short distance from the apical cell membrane to the nuclear membrane, possibly by diffusion.

A published neuronal two-chamber model that addresses this problem (Lycke et al., 1984) was adapted previously in our laboratory, for studies on anterograde transport of HSV-1 (Figure 31) (Penfold et al., 1994; Holland et al., 1999). A stainless steel ring was placed on a glass slide within a tissue culture well, and silicone grease was used to obtain a watertight seal. Explanted human foetal DRG were cultured inside the stainless steel ring (the inner chamber), and a skin explant was cultured outside the ring (the outer chamber). At one point there was a small gap under the ring, which was sealed with an agarose plug. Neuronal axons were encouraged to grow through the agarose, attracted by nerve growth factors and the skin explant in the outer chamber. DRG neurones in the inner chamber were infected with HSV-1, and new viral proteins were observed during transport along axons, in an anterograde direction, to the outer chamber (Penfold et al., 1994; Holland et al., 1999).
To study the anterograde transport of HSV-1 in neuronal axons, human foetal DRG neurones were cultured within a stainless steel ring (the inner chamber), and encouraged to grow through an agarose plug to an outer chamber, where they interacted with a skin explant. After inoculating the inner chamber with HSV-1, newly produced virus was transported along axons, past the agarose plug, into the outer chamber (reproduced with permission from Holland et al., 1999).

For our current study we wished to use a similar model to study retrograde transport of HSV-1 in axons. Our experimental plan was to inoculate the outer chamber with HSV-1, and study the transport of virus along axons, in the retrograde direction, towards the neurone cell body. Similar experiments were performed by Lycke when the model was first developed, but incoming viral particles were observed by transmission electron microscopy, with no specific analysis of which proteins were present (Lycke et al., 1984). We planned to examine neurones using confocal
microscopy, to define which HSV-1 tegument proteins were transported along axons to the neurone cell body, and which proteins remained in the outer chamber.

Unfortunately it was not possible to obtain useful data from these experiments within the timeframe of the project, due to several technical difficulties. As a guide for ongoing work in this area, I will briefly outline the specific difficulties that were faced, and the progress that was made towards overcoming them.

6.4.2 Campenot Nerve Growth Chamber Model

Whereas Lycke had used rat neurones for his original experiments, subsequent work in our laboratory relied on human foetal DRG neurones, since human axons were found to penetrate the agarose plug more readily than rat axons (Holland, personal communication). For various reasons there has been a decrease in the availability of human foetal neuronal tissue since these experiments were last performed in our laboratory, making it difficult to re-establish the same model in a timely fashion.

To address the decreased availability of human neuronal tissue, a different neuronal chamber model that incorporates rat neurones was chosen (Campenot, 1992; Campenot, 1997). This model utilises a commercially available Teflon ring, and has been designed for rat neurones, specifically for the purpose of axonal transport studies (Figure 32).

To set up the model, a glass coverslip is coated with collagen, and scratched with a series of parallel tracks (see Materials and Methods). The inferior aspect of the Teflon ring, including the Teflon divider, is coated with a fine layer of silicone grease, and placed on the coverslip. By pre-wetting an area in the centre of the coverslip, the silicone forms a gasket seal under the Teflon divider, but forms a watertight seal around the outer diameter of the ring, where the coverslip is dry. When dissociated rat
neurones are plated in the central compartment of the ring, their axons grow under the gasket seal on the divider, into the lateral compartments (Figure 32B).

**Figure 32. Campenot nerve growth chamber model.**
This diagram summarises the design of a Campenot nerve growth chamber, which can be used to study axonal transport (figure reproduced with permission from Campenot, 1997).

Our planned experimental design was to add HSV-1 to one or both lateral chambers, and examine its transport along axons, in a retrograde direction, to the neurone cell body in the central compartment. A potential advantage of this model over the stainless steel ring is that different conditions can be applied to each lateral chamber, effectively allowing an experiment and its control to be performed on the same
coverslip. For instance, HSV-1 could be added to one lateral compartment, and mock or inactivated virus to the other compartment. Alternatively, retrograde transport from one lateral compartment could be selectively inhibited by adding a microtubule depolymerising agent, such as nocodazole, or a dynein inhibitor, such as ortho vanadate (Gibbons et al., 1978; Kobayashi et al., 1978).

6.4.3 Experience with Campenot Nerve Growth Chambers

Despite several attempts the Campenot chamber model could not be established within the time constraints of this project. Preliminary results were promising, however, and it is hoped that the model will prove useful for future work in our laboratory. From the experience we gained, it seemed that the following technical details are critical for success of the model:

1. Use of the correct silicone grease. The grease must be non-toxic to neuronal axons, allowing them to grow underneath it.

2. Applying the correct amount of silicone grease to the Teflon divider, to allow the passage of neuronal axons, but not to allow leakage of media or virus.

3. Obtaining the correct viscosity of culture media, to allow diffusion of growth factors, but not to allow leakage of media between compartments.

4. Appropriate coating of the glass coverslips, to allow adherence of neurones, and to direct axons along scored tracks.

6.4.3.1 Silicone Grease

Work in our laboratory on the original two-chamber model had initially utilised Dow-Corning™ grease, which allowed some passage of rat neuronal axons, as well as human axons (Holland, D.J., personal communication). For several years the Dow-
Corning™ grease was unavailable, and other brands were found to have slightly different properties. Although the newer greases were compatible with human axons penetrating an agarose plug, they were inferior when rat neurones were used, especially in the absence of an agarose plug. Therefore for the new Campenot chamber model a supply of Dow-Corning™ high-vacuum grease was obtained, as recommended in the published protocol (Campenot, 1997).

6.4.3.2 Application of Silicone Grease to Teflon Dividers

One of the main challenges in setting up the Campenot chambers was applying the correct amount of silicone grease to the Teflon dividers. If too little grease was applied, leakage of culture media occurred between the central and lateral compartments. In contrast, if too much grease was applied, neuronal axons were unable to grow under the grease, into the lateral chambers.

To allow consistent application of grease, a stainless steel syringe was purchased, and the grease was applied using an 18-gauge, blunt-ended needle. Haemostatic forceps, with a 90 degree angle in their jaws, were used to securely hold the Teflon divider in an inverted position during grease application.

6.4.3.3 Media Viscosity

Published guidelines for the Campenot chambers recommend the addition of methyl cellulose 4000 cps (Sel-Win Chemicals, Ltd., Vancouver, British Columbia) to the culture media, at a concentration of 1.5 g/500 mL (Campenot, 1997). Since this was not available in our laboratory, carboxy-methyl cellulose was substituted initially, at a concentration of 1.6% (w/v). Unfortunately culture media supplemented in this way
was too viscous, and it was not possible to add it precisely to the Teflon ring, due to the high surface tension generated.

Media without methyl cellulose was tried in the Campenot chambers, since the necessity for its addition has not been rigorously investigated (Campenot, 1997). This was unsatisfactory, however, as there was rapid leakage of media under the Teflon dividers into adjacent compartments.

Methyl cellulose 400 cps was ordered for future experiments, but due to significant delays in its availability and supply, by the time it was delivered the Campenot chamber model was no longer being used for this project. It is hoped that the availability of the recommended viscosity-increasing agent will aid future experiments using the Campenot chambers.

6.4.3.4 Collagen Coating of Coverslips

Published protocols for the Campenot chambers recommend preparing a fresh solution of rat tail collagen to coat 35 mm glass culture dishes, scoring the surface with a pin-rake, and storing the dishes in the freezer (Campenot, 1997). Previous work in our laboratory, using the old two-chamber model, had demonstrated that coating glass coverslips with cell matrix from human fibroblasts was superior to simple collagen coating (Holland, personal communication). Coverslips were therefore prepared by allowing fibroblasts to grow on them for several days, to lay down a matrix. After the cells had become confluent, they were removed with a cell scraper, the coverslips were allowed to air-dry, and the matrix was scored with a pin rake, as recommended.

The thickness of cell matrix layer prepared in this way was unfortunately quite variable, so that it was frequently difficult to obtain continuous tracks with the pin
rake. We have found previously that Matrigel™ is the best agent for encouraging adherence of rat DRG neurones to glass coverslips (Miranda-Saksena, personal communication). Unfortunately Matrigel™ is not suitable for the Campenot chambers, since the coating on the coverslip must be scored to direct axonal growth, and allowing Matrigel™ to dry out completely results in non-specific antibody labelling (data not shown).

In future experiments, the following variables could be altered, to more closely follow the recommended protocols, and possibly improve neuronal growth (Campenot, 1992):

1. Coating coverslips with rat tail collagen, to confirm whether or not it is superior to cell matrix in the Campenot chamber model. A recipe for preparing fresh rat tail collagen is described in the guidelines for the Campenot nerve growth chambers.

2. Addition of ascorbic acid to media in the central slot, to encourage neuronal growth (DMEM does not contain ascorbic acid, and the contents of B27 are not stated by the manufacturer).

3. Using NGF at higher concentrations, up to 200 ng/mL.

4. Using rat serum, rather than foetal calf serum, at concentrations of 2.5-5%.
6.5 Discussion

The aims of these experiments were to determine which HSV-1 tegument proteins remained attached to incoming capsids during infection of neurones, and which proteins dissociated. This information is important for our hypothesis, because if the interaction between outer capsid protein VP26 and dynein light chains rp3 or Tctex1 is to mediate retrograde transport of HSV-1, VP26 must be accessible to bind dynein, and should not be obscured by tegument.

To visualise the intracellular distribution of inner tegument protein UL37, neuronal cells were infected with a strain of HSV-1 expressing GFP-UL37. During infection of either rat DRG neurones or human neuronal IMR32 cells, GFP-UL37 dissociated from incoming HSV-1 capsids, remaining at the periphery of the cell.

Although the HSV-1 tegument is traditionally said to lack structure, it is becoming clear that there is in fact some order to the arrangement of tegument proteins in HSV-1 virions (Mettenleiter, 2002). When the HSV-1 UL36 gene is deleted, new capsids accumulate in the cytoplasm, without the addition of any further tegument (Desai, 2000). Deletion of the UL37 gene in HSV-1 (Desai et al., 2001), or the closely related alphaherpesvirus pseudorabies virus (PrV) (Klupp et al., 2001b) also drastically inhibits or abolishes virus maturation. In the absence of UL37, new viral capsids accumulate in the cell cytoplasm, embedded in a matrix of UL36 protein (Mettenleiter, 2002). No other tegument proteins are added to capsids, and they do not undergo secondary envelopment, suggesting that UL37 is required to link the capsid and inner tegument (UL36) to the outer tegument and viral envelope.
In preliminary results, using the yeast two-hybrid system, we have shown a direct interaction between HSV-1 VP1/2 (UL36) and the UL37 protein (data not shown). This is consistent with the published interaction between the PrV UL37 and UL36 proteins (Klupp *et al.*, 2002), and further supports the location of the UL37 protein in the inner layer of HSV-1 tegument.

Because it is located in the inner tegument, dissociation of the UL37 protein from incoming capsids during HSV-1 infection, as we have observed in these experiments, suggests that most other tegument proteins are also lost. This is consistent with EM studies of incoming HSV-1 capsids, which have shown that most of the tegument is lost following cell entry (Ojala *et al.*, 2000). Our other preliminary results suggest that HSV-1 tegument proteins US11, VP16 and VP13/14 also dissociate from incoming capsids. The diffuse cytoplasmic distribution of VP16 we observed in neurones 2 h after infection is consistent with previous results from our laboratory (Miranda-Saksena *et al.*, 2000). Our results are also consistent with published findings that tegument proteins VP16 (Morrison *et al.*, 1998a; La Boissiere *et al.*, 2004), VP13/14 (Morrison *et al.*, 1998a), VP11/12 (Willard, 2002), and VP22 (Elliott and O'Hare, 1999) all dissociate from HSV-1 capsids during infection.

The only tegument protein that has been shown to remain attached to incoming HSV-1 capsids is the major tegument protein VP1/2 (UL36) (Morrison *et al.*, 1998a), which is also thought to mediate binding of the capsid to the nuclear pore complex (Ojala *et al.*, 2000). This is further evidence to support a model where VP1/2 forms the innermost layer of the tegument (Mettenleiter, 2002). Cryo-electron microscopy studies suggest that VP1/2 attaches to HSV-1 capsids at the vertical pentons, but not at the hexons (Zhou *et al.*, 1999). In contrast, the HSV-1 outer capsid protein VP26 is
present on hexons, but not pentons (Wingfield et al., 1997), and does not interfere with capsid-tegument interaction (Chen et al., 2001).

Putting all these findings together, our results suggest that most HSV-1 tegument proteins, including UL37, dissociate from incoming nucleocapsids during cellular infection. The only remaining tegument protein is probably VP1/2, and because of its sites of attachment to the capsid at the pentons, it is unlikely to interfere with interactions between the cytoplasmic dynein complex and VP26 on the capsid hexons.

A better way to study axonal transport of virus is to use a neuronal chamber model, which prevents direct entry of virus into neurone cell bodies. Such models are technically challenging, and are susceptible to small variations in protocol. It was not possible to use the two-chamber model that had been developed previously in this laboratory (Penfold et al., 1994; Holland et al., 1999), because of the reduced availability of human foetal neurones. Therefore a new model using rat neurones was adapted, based on the Campenot nerve growth chamber (Campenot, 1992; Campenot, 1997). Unfortunately it was not possible to obtain meaningful results from this model within the time constraints of the project, due in part to limited availability of reagents. Many of the initial problems encountered with the model have now been overcome, and it is hoped that it will prove useful for future work in our laboratory.

Due to the technical problems we encountered with the neuronal chamber models, and the difficulties of detecting HSV-1 proteins at early time points, a new experimental model was developed (see Chapter 7). In this novel model, microinjection techniques were used to introduce HSV-1 capsids directly into live cells, and investigate the role of VP26 in retrograde cellular transport of viral capsids.
CHAPTER 7:

MICROINJECTION OF

HSV-1 CAPSIDS
7.1 Introduction

In earlier chapters we demonstrated that the HSV-1 outer capsid protein VP26 binds to dynein light chains rp3 and Tctex1. This was shown initially in the yeast two-hybrid system (Chapter 4) and subsequently using in vitro binding studies (Chapter 5). In Chapter 6 we confirmed that most of the tegument, including inner tegument protein UL37, dissociates from the HSV-1 capsid during natural infection. This is consistent with VP26 playing an important role during retrograde viral transport, as it would expose the outer capsid to allow dynein binding.

To confirm that the interaction between VP26 and dynein occurs in live cells, and is important for retrograde transport, we developed a model where we directly introduced recombinant HSV-1 capsids into live cells using microinjection techniques. This allowed us to bypass the usual requirements for virus binding, membrane fusion and cell entry. Furthermore we knew precisely which HSV-1 proteins were present in the recombinant capsids, unlike capsids derived from infected cells, where it is not clear which minor capsid or tegument proteins may be present on capsids.

The outer capsid protein VP26 (the product of HSV-1 gene UL35) is not required for capsid assembly (Booy et al., 1994), so it was possible to produce recombinant capsids that contained VP26 (VP26+) and capsids that did not (VP26-). By directly comparing the behaviour of VP26+ and VP26- capsids following micro-injection, we were able to confirm the importance of VP26 for retrograde intracellular transport of HSV-1.
7.2 Materials and Methods

7.2.1 Microinjection of Recombinant HSV-1 Capsids

Recombinant VP26+ or VP26- HSV-1 capsids, kindly provided by Dr. Fred Homa (Pharmacia Corp., Kalamazoo, Michigan 49001, USA), were prepared as described in Chapter 5, based on published methods (Thomsen et al., 1994). All recombinant capsids contained the major capsid proteins VP5, VP19C and VP23, as well as the scaffolding proteins VP21, VP22a and VP24. In addition, those formed in the presence of the major capsid protein VP26 also contained VP26.

To remove aggregates prior to microinjection, capsids were diluted 1:10 in 20 mM Tris-HCl buffer (pH 7.5), containing 0.5 M NaCl and 1 mM EDTA, subjected to sonication in a Branson cup-horn water bath (3 × 40 sec, 50% duty cycle), and centrifuged at 12 000 × g for 30 sec. The approximate final protein concentration, measured by the Bio-Rad Protein Assay, was 0.1 mg/mL (3 × 10⁸ capsids per µL).

Hep2 cells were seeded in 24-well plates, at a density of 1 × 10⁶ cells per well, onto CELLlocate glass coverslips (Eppendorf), scored with a 55 µm grid. Cells were cultured for 24 h in DMEM (Invitrogen) supplemented with 9% (v/v) FCS (JRH Bioscience), at 37 ºC, in 5% CO₂. During microinjection, coverslips were maintained at room temperature for approximately two to three hours in DMEM, supplemented with 2% (v/v) FCS, and buffered with 25 mM HEPES-NaOH (pH 7.4). For colocalisation experiments, PTK2 cells were seeded at a density of 0.25 × 10⁵ cells per well in MEM (Invitrogen) supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 10% (v/v) FCS.
An Eppendorf FemtoJet® microinjector and InjectMan® NI 2 micromanipulator, attached to a Nikon DM-IRB inverted microscope, were used to inject recombinant HSV-1 capsids through a Femtotip® glass micropipette into Hep2 or PTK2 cells. The injection parameters used were: compensation pressure 50 hectopascals (hPa); injection pressure 280 to 300 hPa (range 200 to 400 hPa); injection time 0.2 to 0.4 sec. Between 200 and 400 cells were injected for each experimental group or time point.

### 7.2.2 Immunofluorescence and Confocal Microscopy

Following microinjection, Hep2 cells on coverslips were fixed and permeabilised as described previously (Miranda-Saksena et al., 2000), either immediately or after 2 to 4 hours incubation at 37 °C. A commercial monoclonal anti-VP5 antibody (6F10, Santa Cruz) was tried for labelling capsids, but was not sufficiently sensitive, possibly due to limited accessibility of binding epitopes. Sensitive and specific labelling of intracytoplasmic capsids was obtained with rabbit polyclonal anti-VP5 antibody (NC1, kindly provided by Dr. G. Cohen and Dr. R. Eisenberg, University of Pennsylvania, Philadelphia, USA) (Cohen et al., 1980). Some non-specific labelling of antigens within the nucleus was observed, but did not involve the nuclear membrane or cytoplasm. Immuno-labelling was as described previously (Miranda-Saksena et al., 2000), except antibodies were diluted in Tris-buffered saline (TBS), supplemented with 1.5% (v/v) goat serum, 0.1% (w/v) BSA, Tween-20 (0.05% v/v) and NaNH$_3$ (0.02 M). Additional primary antibody used was mouse anti-bovine $\alpha$-tubulin antibody (monoclonal 236-10501, Molecular Probes), diluted 1:400. Secondary antibodies were Cy™3-conjugated goat anti-mouse IgG (Amersham Pharmacia Biotech), diluted 1:400, and Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Molecular Probes), diluted 1:200.
For colocalisation experiments, PTK2 cells were microinjected with recombinant HSV-1 capsids as above, incubated for 1 h at 37 °C, then fixed and permeabilised (Miranda-Saksena et al., 2000). Dynein light chains were detected with rabbit polyclonal antibodies directed against dynein light chains (a kind gift of Dr. Stephen King, Department of Biochemistry, University of Connecticut Health Centre, Farmington, Connecticut). Primary antibody serum R5270 against rp3 (diluted 1:50) (King et al., 1998) or R5205 against Tctex1 (diluted 1:100) (King et al., 1996) was used as above, followed by Cy™-conjugated goat anti-rabbit IgG (1:400) (Amersham Pharmacia Biotech). Microtubules were labelled using mouse monoclonal antibody against α-tubulin, as above.

For recent colocalisation experiments a new mouse monoclonal antibody against the HSV-1 major capsid protein VP5 was obtained, a kind gift of Dr. Frazer Rixon (MRC Virology Unit, Glasgow, UK). The antibody (DM165) was raised against purified VP5 (McClelland et al., 2002), and was much more sensitive than the commercial antibody used in earlier experiments (6F10, Santa Cruz). Thus for colocalisation with rp3 and Tctex1, capsids were detected using a mouse monoclonal anti-VP5 antibody (DM165) (diluted 1:100), while for colocalisation with microtubules a rabbit polyclonal anti-VP5 antibody (NC1) (diluted 1:10) was used, as above.

After labelling, coverslips were mounted on glass slides using the glycerol component of a Pierce SlowFade® Anti-fade kit (S-2828, Pierce). Cells were examined through a Leica PL APO 63× oil immersion objective (numerical aperture 1.32) or Leica PL APO 100× oil immersion objective (numerical aperture 1.40) using a Leica Confocal System TCS SP2, attached to a Leica DMRE fluorescent microscope. Confocal images were acquired using Leica Confocal Software Version 2.00 Build 0871. Dual channel images were overlaid using the Leica Software, and
colocalisation determined using the Boolean “AND” operator. For a green/red overlay, each pixel is analysed by the software: where a red and green pixel colocalise a yellow pixel is displayed; where pixels do not colocalise a black pixel is displayed.

7.2.3 Measurement of Intracellular Distribution of Capsids

To quantitatively assess the distribution of recombinant capsids following microinjection, the distances of each fluorescent particle from the cell nucleus $a$, and the nearest part of the cell membrane $b$, were measured, with the aid of image analysis software (ImageJ v1.29, NIH). The relative position of each particle was represented by a “Nuclear Migration Index”, calculated as $b/(a+b)$, such that particles near the cell periphery had values close to 0, while particles near the nucleus had values close to 1 (McDonald et al., 2002). Image analysis was performed “blinded”, to remove potential observer bias. Images were randomly coded by a person not involved in particle measurement, and the code was not broken until after all data had been collected. Fluorescent particles were confirmed to be within the cytoplasm, rather than sitting on the cell membrane, using serial $z$-sections. In areas where several fluorescent particles congregated, individual particles were visually separated by image analysis using threshold functions or $z$-sections.

7.2.4 Statistical Analysis

To quantify intracellular movement of capsids in microinjection experiments, each fluorescent particle was allocated a Nuclear Migration Index, as above. In the idealised situation represented by a circular cell cross section with the nucleus being a central point within this section, randomly distributed markers would produce a triangular shaped histogram, decreasing in height as the ratio increased. A flat or
uniform histogram would arise if the marker density was inversely proportional to the
distance from the centre of the nucleus.

The statistical software package SPSS for Windows (Version 11.0, SPSS Inc.) was
used to formally analyse the data. General linear models with experiment as a random
effect and time and VP26 status as fixed effects were used. There was no statistically
significant three-way interaction between time, VP26 status and experiment so this
term was omitted from the model (p=0.6). The model involving all main effects and
two-way interactions was then fitted. There were statistically significant interactions
between VP26 status and time (p<0.001) and between VP26 status and experiment
(p=0.003). Therefore the data from experiments with VP26+ and VP26- capsids have
been analysed separately. In neither experiment was there a significant time by
experiment interaction term (p=0.9 for VP26- capsids, p=0.6 for VP26+ capsids), so
the final model fitted contained only time and experiment main effects.
7.3 Results

7.3.1 VP26+ Capsids Colocalise with rp3, Tctex1 and Microtubules

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 labelled 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 colocalisation of capsids with dynein light chains rp3 and Tctex1 (Figure 33A), as well as with microtubules (Figure 33C). Formal image analysis using the Boolean “AND” operator confirmed colocalisation with dynein and microtubules for the majority of HSV-1 capsids (Figure 33B and D).

Figure 33. HSV-1 capsids colocalise 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, immuno-labelled and examined by confocal microscopy. Capsids were labelled 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 colocalise with dynein light chains Tctex1 (A and B), rp3 (A and B, insets) and microtubules (C and D). To look for co-localisation red and green images were initially overlaid (A and C), and co-localisation formally analysed using the confocal software (B and D). The Boolean “AND” operator was used: where a red and green pixel co-localised, a bright pixel was displayed; where there was no co-localisation a black pixel was displayed.
7.3.2 VP26+ Capsids are Transported Towards the Nucleus

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 (VP26+ or VP26-). Cells were then fixed either immediately or after 2 or 4 h (replicate experiments), labelled with anti-VP5 antibody, and the distribution of capsids within each cell determined by confocal microscopy (Figure 34A-D). Immediately following injection, both VP26- and VP26+ capsids were distributed diffusely throughout the cytoplasm (Figure 34A and C). After 2 h at 37 ºC, VP26- capsids remained in a similar distribution (Figure 34B), while VP26+ capsids had congregated near the nuclear rim in most cells (Figure 34D). Similar distributions were observed after 4 h. Rp3 and Tctex1 were confirmed to be present in the cytoplasm of Hep2 cells (data not shown).

Figure 34. VP26+, but not VP26- capsids move towards the cell nucleus.
(see over page).
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 ºC then fixed. Capsids were labelled using NC1 polyclonal anti-VP5 antibody (green), and microtubules with monoclonal anti-α-tubulin antibody (red). (A-D) Fluorescent particles (arrows) were clearly visible in the cytoplasm of injected cells, which were not present in uninjected cells (E). After 2 h, a redistribution of VP26+ (D), but not VP26- (B) capsids towards the cell nucleus was observed in these typical images. (E) Non-specific labelling of cell nuclei was observed with NC1 antibody, so intra-nuclear fluorescence was not included in our analysis. (F) 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. Relative nuclear migration for each particle was expressed as a “Nuclear Migration Index”, calculated as b/(a+b) (McDonald et al., 2002). Thus particles near the cell periphery received values close to zero, and particles near the nucleus received values close to one.
Nuclear Migration Index = \frac{b}{a+b}
7.3.3 Quantitative Analysis of Capsid Migration

To quantify the distribution of capsids at different time points, a Nuclear Migration Index was assigned to each visible fluorescent particle, such that particles near the plasma membrane received values close to zero, while particles near the cell nucleus received values close to one (Figure 34F). Over a thousand fluorescent particles were analysed, in 2 separate experiments. Fluorescent particles observed “within” the nucleus of injected and uninjected cells (Figure 34E) were due to background fluorescence, and were not included (see Materials and Methods). There was no non-specific labelling of the nuclear membrane, and fluorescent particles adjacent to the nuclear membrane could be distinguished clearly. General linear models were used to formally investigate the data from both experiments. Within experiments involving VP26+ capsids, the Nuclear Migration Index increased on average by 0.099 (SE 0.026), p<0.001, over two or four hours, confirming overall movement towards the nucleus (Figure 35). In contrast, within experiments involving VP26- capsids the Nuclear Migration Index decreased on average by 0.067 (SE 0.024), p=0.006.
Figure 35. Movement of VP26+, but not VP26- capsids is statistically significant.
Recombinant HSV-1 capsids, VP26+ (●) or VP26- (▲), were microinjected into Hep2 cells, incubated for 0, 2 or 4 h at 37°C, and examined by confocal microscopy. The Nuclear Migration Indices (Figure 34F) of over a thousand fluorescent particles were analysed, in 2 separate experiments. General linear modelling was performed for both experiments (A and B) and confirmed that VP26+, but not VP26- capsids had moved towards the cell nucleus after 2 or 4 h.
7.4 Discussion

To confirm that the interaction between VP26 and dynein is important for cellular transport, recombinant HSV-1 capsids were microinjected into living cells. After 1 h, VP26+ capsids were shown to colocalise with microtubules (α-tubulin), as well as with dynein light chains rp3 and Tctex1. These data support results from the yeast-two hybrid screen (Chapter 4) and in vitro binding studies (Chapter 5), which showed binding of the HSV-1 capsid protein VP26 to dynein light chains. They also provide further evidence of an interaction between VP26 and Tctex1, as well as with rp3.

At later time points after microinjection, on average there was significant movement of VP26+ capsids towards the cell nucleus after 2 or 4 h, which was absent for VP26- capsids. VP26- capsids in fact appeared to redistribute slightly towards the cell periphery, but this may simply reflect diffusion to a truly random distribution, where more capsid particles are located near the periphery of the cell than near the nucleus, due to the larger volume (see Materials and Methods, Statistical Analysis, page 171).

The movement of VP26+ capsids, but not VP26- capsids, towards the nucleus over time strongly suggests that the outer capsid protein VP26 mediates retrograde intracellular transport of HSV-1 capsids, through interactions with cytoplasmic dynein. In this minimal capsid system the presence of VP26 on capsids is both necessary and sufficient for retrograde transport in cells, in the absence of other capsid or tegument proteins.

As well as providing strong evidence of a biological role for the interaction between VP26 and dynein, these results suggest that the triplex protein VP19C is unlikely to play an important role during retrograde transport. Results from the yeast two-hybrid screen in Chapter 4 suggested a possible interaction of VP19C with rp3 and Tctex1,
but this could not be confirmed due to auto-activation. It was not possible to confirm a direct interaction between VP19C and dynein using \textit{in vitro} binding studies in Chapter 5, because VP19C was not expressed in bacteria. However, in other experiments in Chapter 5 recombinant VP26+, but not VP26- capsids bound to GST-rp3 \textit{in vitro}. Since VP19C was present in both VP26- and VP26+ capsids, this suggested that VP19C did not bind strongly to dynein when incorporated into HSV-1 capsids. Data from this current chapter further support this conclusion. Since VP26+, but not VP26- capsids (which also contain VP19C) are transported towards the cell nucleus following microinjection, any binding of capsid protein VP19C to dynein has minimal biological significance during retrograde transport.

These results therefore provide strong evidence of a role for the outer capsid protein VP26 in the retrograde cellular transport of HSV-1 capsids. In the absence of HSV-1 tegument proteins microinjected VP26+ capsids colocalise with dynein light chains rp3 and Tctex1, as well as with cellular microtubules. After 2 or 4 hours VP26+, but not VP26- capsids have moved towards the cell nucleus. Thus VP26 is both necessary and sufficient to mediate retrograde transport of capsids in this model. The significance of these results for our overall understanding of the retrograde transport of HSV-1 is discussed in the following chapter.
CHAPTER 8:

FINAL DISCUSSION
8.1 Study Aims

The main aim of this project was to identify the proteins involved in retrograde intracellular transport of HSV-1, particularly in neuronal axons. Although retrograde HSV-1 transport is known to be mediated by cytoplasmic dynein, and to involve microtubules, it was not known which viral proteins were involved, nor to which part of the cytoplasmic dynein complex they bound.

8.2 Significance of Findings

The results of our study suggest that, during infection with HSV-1, retrograde transport of viral capsids toward the cell nucleus is mediated by the binding of the HSV-1 outer capsid protein VP26 to dynein light chain rp3, and probably to Tctex1.

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 (King et al., 1998), compete for binding sites on DIC, and have differing binding specificities (Tai et al., 2001).

We initially demonstrated binding of VP26 to dynein light chains rp3 and Tctex1 in a yeast two-hybrid system, and 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 immobilised His6-VP26 is able to bind to intact cytoplasmic dynein complexes in cell lysates. Most importantly, we have confirmed
binding of dynein (rp3) to VP26 in its biologically relevant form, as part of a viral capsid, bound to VP5.

Thus we have confirmed specific binding of VP26 to dynein light chain rp3 in three separate assays, including as part of a HSV-1 capsid. This suggests that the binding of VP26 to rp3 utilises different VP26 epitopes from those involved in binding to VP5 in capsids (Desai et al., 2003), and is consistent with the interaction playing a significant role in vivo. Furthermore, in microinjected cells we have shown co-localisation 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 the yeast two-hybrid system and in-vitro pull-down assays (using His6-VP26), and co-localisation 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 bind more strongly to soluble VP26 than to VP26 incorporated into recombinant HSV-1 capsids. However the co-localisation 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, while Tctex1 is expressed predominantly in foetal tissue and testis
Furthermore, it has been proposed that rp3-containing dynein complexes play the predominant role during retrograde transport from post-synaptic nerve terminals (Chuang et al., 2001), the site of HSV-1 entry.

VP26, at the hexon tips, is ideally located to interact with molecular motors (Wingfield et al., 1997), since most of the tegument is lost soon after cell entry (Ojala et al., 2000). During infection of neuronal cells with GFP-UL37 HSV-1, we observed dissociation of GFP-UL37 from incoming capsids, the first time this has been reported to our knowledge. It has been shown previously that tegument proteins VP11/12, VP13/14, VP16 and VP22 dissociate from the virus at early stages of infection, leaving probably only the major tegument protein VP1/2, and the minor capsid protein UL25, attached to incoming capsids (Morrison et al., 1998a; Morrison et al., 1998b; Elliott and O'Hare, 1999; Dohner et al., 2002; Willard, 2002; La Boissiere et al., 2004). In our experiments, using neuronal cells, we observed similar dissociation of tegument protein US11 from capsids during infection, in addition to VP16 and VP13/14.

We demonstrated that the HSV-1 UL37 protein interacts directly with the UL36 protein VP1/2, in a yeast two-hybrid system, consistent with a similar report for the closely related alphaherpesvirus PrV (Klupp et al., 2002). Other recent work also suggests that UL37 forms part of the inner tegument layer in alphaherpesviruses, and is the next protein to be added to maturing virions after UL36 (Mettenleiter, 2002). Deletion of the UL37 gene from HSV-1 (Desai et al., 2001) or PrV (Klupp et al., 2001b) blocks the addition of further tegument proteins, resulting in the accumulation of DNA-filled capsids in the perinuclear region, embedded in a matrix of UL36 protein. Therefore, because of its location in the inner tegument, dissociation of UL37
from incoming capsids during infection strongly suggests that all other tegument proteins have also been lost, apart from VP1/2.

The major capsid protein VP1/2 appears to attach to capsids at the vertical pentons, but not at the hexons (Zhou et al., 1999), probably due to differing orientations of the upper domain of VP5 (Bowman et al., 2003). In contrast, VP26 is found exclusively on hexons, rather than pentons (Trus et al., 1995; Zhou et al., 1995; Wingfield et al., 1997), and does not interfere with capsid-tegument interaction (Chen et al., 2001). Therefore if VP1/2 is the only tegument protein remaining on incoming HSV-1 capsids, the location of VP26 on the hexons should leave it exposed to interact with the dynein molecular motor during retrograde transport.

Binding of the tegument protein VP11/12 to rp3 and Tctex1 was detected in the yeast two-hybrid system, but could not be confirmed with pull-down assays due to poor expression of soluble His6-tag fusion proteins in E. coli. The significance of these results is unclear, but any role is likely to be later in the viral replication cycle, rather than during retrograde transport. It seems unlikely that VP11/12 is involved in retrograde transport of the capsid/tegument complex, since it mostly dissociates from incoming capsids during cell infection (Willard, 2002), although retention of some residual VP11/12 during retrograde transport would be difficult to exclude. An interaction of VP11/12 with dynein may play a role during tegument assembly, however, since newly synthesised VP11/12 has been observed in perinuclear particles undergoing rapid, microtubule-dependent movement (Willard, 2002).

To confirm that the interaction between VP26 and dynein is important for cellular transport, recombinant viral capsids were microinjected into living cells. On average there was significant movement of VP26+ capsids towards the cell nucleus after 2 h or 4 h, which was absent for VP26- capsids. VP26- capsids in fact redistributed slightly
towards the cell periphery, but this may simply reflect diffusion to a truly random distribution. These results therefore suggest an important role for VP26 during retrograde transport, through interactions with cytoplasmic dynein.

The relative contribution of other tegument or capsid proteins to HSV-1 transport is unclear and requires further study. We cannot exclude that VP1/2, or one of the minor capsid proteins such as UL25, may also contribute to dynein binding in vivo, as suggested by others (Tomishima et al., 2001; Dohner et al., 2002). Any binding of these or other HSV-1 proteins to dynein would imply redundancy in viral proteins available for retrograde transport, and may explain why VP26 is not an essential protein for replication in vitro (Desai et al., 1998). Redundancy for such an important process may be advantageous for the virus, and there are precedents for functional redundancy in other HSV-1 and PrV proteins (Farnsworth et al., 2003; Kopp et al., 2003). In our yeast two-hybrid system there was no evidence of an 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 auto-activation.

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 (Desai et al., 1998). Deletion of VP26 decreased the amount of infectious virus in trigeminal ganglia by 100-fold, whereas titres 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 hours after ocular infection, suggesting that 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, since wild-type virus can arrive as early as 19 hours post infection (Kramer et al., 1998).

8.3 Conclusions

In this 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 (Tomishima et al., 2001).

We have demonstrated by two different assays that VP26 binds to dynein light chains rp3 and Tctex1 in vitro, and confirmed binding of VP26 to intact dynein complexes. We have demonstrated co-localisation in cells of micro-injected HSV-1 capsids with rp3, Tctex1 and microtubules. We have further shown 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. We have confirmed dissociation of the inner tegument protein UL37 from incoming HSV-1 capsids during neuronal infection, exposing VP26 to interact with dynein. 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-1 proteins mediating retrograde axonal transport in vivo, perhaps another example of redundancy for key viral functions.
8.4 Future Directions

Despite the insights gained from our results, there are still many unanswered questions about retrograde axonal transport of HSV-1. In particular, although VP26 is necessary and sufficient for retrograde transport of HSV-1 capsids in a minimal capsid model, it is unlikely to be the sole viral protein mediating retrograde viral transport in vivo. Assuming there is redundancy, with more than one HSV-1 protein available to mediate retrograde transport, the other proteins(s) have yet to be identified. Other unanswered questions include the possible role for an interaction between HSV-1 tegument protein VP11/12 and dynein light chains during viral assembly and egress; the mechanisms that control the dynein and kinesin motors during co-ordinated transport of HSV-1; and the mechanism by which HSV-1 capsids are transported from the microtubule organising centre to the nuclear pore.

There are several potential lines of future research that emerge from this project. The most immediate, and important challenge is to confirm in other models the role for VP26 in retrograde transport of HSV-1. We plan to use fluorescent labelled HSV-1 strains and real-time fluorescent microscopy to track transport of viral particles to the cell nucleus during cellular infection. Strains of HSV-1 in which GFP has been inserted into the outer capsid protein VP26 (Desai and Person, 1998) should allow visualisation of HSV-1 capsids in real time, as has been demonstrated already for PrV (Smith et al., 2001). There are other strains of HSV-1 available in which GFP has been inserted into tegument proteins such as VP22 (Brewis et al., 2000), VP11/12 (Willard, 2002), VP16 (La Boissiere et al., 2004) and VP13/14 (Donnelly and Elliott, 2001a; Donnelly and Elliott, 2001b), in addition to the GFP-UL37 strain that was used in our experiments. It is even possible to label two separate HSV-1 proteins with
different fluorescent labels, as has been reported for the tegument proteins VP22 and VP13/14 (Hutchinson et al., 2002). We hope to employ similar technology to track HSV-1 capsids and tegument proteins separately, in real time, during cell infection.

The ideal model in which to track incoming HSV-1 capsids would be a two-chamber neuronal model. In such a model, as discussed already, HSV-1 is prevented from infecting neuronal cell bodies directly, so that the only way virus is able to reach the cell body is by retrograde transport along neuronal axons. In our laboratory we already use an established *in vitro* neuronal model, employing dissociated rat DRG neurones, to study HSV-1 assembly and egress (Miranda-Saksena et al., 2000; Miranda-Saksena et al., 2002). In the near future we hope to further develop the Campenot nerve growth chamber model, using rat DRG neurones, to study retrograde transport of HSV-1.

As well as observing fluorescent-labelled HSV-1 in the neuronal chamber model, we would like to study the transport of recombinant HSV-1 capsids, to confirm the role of VP26 in retrograde transport of HSV-1 in neurones. It may be possible to incorporate GFP-labelled VP26 into the capsids, thus allowing visualisation of recombinant capsids in real time.

Due to the very small diameter of neuronal axons, it is unlikely that we would be able to directly introduce capsids into axons by micro-injection. An alternative approach is to use liposomal reagents to facilitate entry of the recombinant capsids into distal axons. Capsids introduced into cells in this way could be tracked as they were transported along the axons, in a retrograde direction, to the neurone cell body in the central chamber. Although this has not been attempted in neurones, to our knowledge, liposomes have been used to introduce HSV-1 into other cell types. In one report HSV-1 capsids, as well as whole HSV-1 virions or plasmid DNA, have been
successfully transduced into cells using liposomes, both in vitro and in vivo (Fu and Zhang, 2001).

An important result to confirm is the apparent dissociation of the inner tegument protein UL37 from incoming HSV-1 capsids. Although our results using confocal microscopy show that most GFP-UL37 remains at the cell membrane, we cannot exclude that some UL37 remains attached to capsids during infection. Therefore we plan to examine intracellular HSV-1 capsids by transmission immuno-electron microscopy, at early time points after infection, to examine this question further. Although limited by the sensitivity of the technique, if UL37 is not detected on intracellular capsids, but is detectable in extracellular virus, this would strongly support our confocal microscopy results.

The long term goal of our research is to design synthetic vectors, based around the HSV-1 capsid, for targeted gene therapy of neuronal cells (Figure 36). Potential applications for a HSV-1 based gene therapy vector in the nervous system include the treatment of Parkinson’s disease, glioblastoma multiforme, spinal cord trauma, motor neurone injury, peripheral polyneuropathy and chronic pain (Glorioso and Fink, 2004).

Replication-deficient HSV-1 vectors, expressing the endogenous opiate proenkephalin, have been used successfully in rodent models to treat chronic inflammatory pain resulting from cancer in bone and arthritis, as well as for neuropathic pain (Braz et al., 2001; Goss et al., 2001; Goss et al., 2002b; Mata et al., 2003). Clinical trials of gene therapy for chronic pain in human have now been proposed (Mata et al., 2003). HSV-1 based gene therapy may also allow targeted expression of Nerve Growth Factor (NGF) for the treatment of diabetic neuropathy (Goss et al., 2002a), as well as for neuropathic bladder dysfunction (Goins et al.,
Expression of NGF by HSV-1 vectors is even being explored for the treatment of spinal cord injury following trauma (Blesch et al., 2002).

One of the major barriers to using whole HSV-1 viral particles for gene therapy is host anti-viral immunity. Pre-existing immunity can inhibit the efficacy of HSV-1 based vectors, or else immunity can develop in naïve hosts following the first
administration, thus limiting subsequent dosing (Fu and Zhang, 2001). An advantage of using minimal HSV-1 capsids, rather than whole virions, is that they are potentially less immunogenic, since they do not contain HSV-1 surface glycoproteins. Furthermore, it has been shown that using liposomal reagents to deliver capsid vectors can protect the vectors from host antiviral immunity, allowing multiple doses with minimal loss in efficacy (Fu and Zhang, 2001; Yotnda et al., 2002).

In the near future we plan to transfet HSV-1 capsids into neurones, using liposomal reagents, to test their potential as gene therapy vectors. The results from this thesis suggest that capsids containing the outer capsid protein VP26 should be transported in a retrograde direction along axons to the nerve cell body, a prerequisite for gene expression. Our planned experiments, using a neuronal chamber model, should be able to confirm retrograde transport of capsids in neuronal axons, as well as in the cell body. Assuming that axonal transport of HSV-1 capsids can be demonstrated in vitro, experiments in an animal model are planned, to confirm that such transport occurs in vivo.

Clearly much work will need to be done before HSV-1 capsids can be used as an effective vector for gene therapy. Packaging of therapeutic DNA into HSV-1 vectors will most likely require the portal protein UL6, as well as viral proteins involved in the terminase complex, such as UL15 and UL28. It is not known which other viral proteins will be required to ensure delivery of the vector DNA into the cell nucleus, but it is likely that these will include the major tegument protein VP1/2 (Ojala et al., 2000).

Despite these future challenges, we feel that the results in this thesis represent a significant first step towards achieving our ultimate goal. One of the greatest stumbling blocks to date has been a lack of knowledge as to which HSV-1 proteins are
required for retrograde transport of the virus during infection. Our results strongly suggest that the outer capsid protein VP26 plays an important role during retrograde transport, although other viral proteins may also be involved in vivo. We hope that future experiments will further define the requirements for retrograde transport of HSV-1, and in the long term will contribute to the development of a safe, effective gene vector for the treatment of neurological diseases.
CHAPTER 9:

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APPENDICES:

EXTRA PROTOCOLS
Appendix 1 – Polymerase Chain Reaction (PCR)

PCR Reagent Mix - Geneamp® XL PCR Kit (PE Applied Biosystems)

<table>
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<tr>
<th>Component</th>
<th>Volume (µL)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>Water</td>
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</tr>
<tr>
<td>3.3× XL Buffer II</td>
<td>12</td>
<td>1×</td>
</tr>
<tr>
<td>dNTPs (10 mM)</td>
<td>8</td>
<td>800 µM (200 µM each)</td>
</tr>
<tr>
<td>Primer #1 (20µM)</td>
<td>2</td>
<td>40 pmol/rxn</td>
</tr>
<tr>
<td>Primer #2 (20µM)</td>
<td>2</td>
<td>40 pmol/rxn</td>
</tr>
<tr>
<td>Mg(OAc)$_2$ (25 mM)</td>
<td>4.8</td>
<td>1.2 mM</td>
</tr>
<tr>
<td><strong>Upper Reagent Mix (60 µL)</strong></td>
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<td></td>
</tr>
<tr>
<td>Water</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>3.3× XL Buffer II</td>
<td>18</td>
<td>1×</td>
</tr>
<tr>
<td>DNA Template</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>rTth DNA Polymerase, XL</td>
<td>2</td>
<td>4 U/rxn</td>
</tr>
</tbody>
</table>

PCR Thermal Cycling (Hot start technique)

- Wax bead (Ampliwax® PCR Gem 100, PE Applied Biosystems) added to Lower Reagent Mix in 200 µL PCR tube
- Tube heated at 80°C for 5 min to melt wax, allowed to cool (solidifies on top)
- Upper Reagent Mix added on top of solid wax layer

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>94°C</td>
<td>60 sec</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>94°C</td>
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<td>17</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$T_{\text{A/E}}$</td>
<td>5 min</td>
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</tr>
<tr>
<td>3</td>
<td>1</td>
<td>94°C</td>
<td>15 sec</td>
<td>20</td>
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<tr>
<td></td>
<td>2</td>
<td>$T_{\text{A/E}}$</td>
<td>5-12 min</td>
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<tr>
<td>4</td>
<td>1</td>
<td>72°C</td>
<td>10</td>
<td>1</td>
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</table>

*Anneal/Extend Temperature

#Time increased by 20 sec per cycle
Appendix 2 – Yeast Transformation Protocol

Small-Scale LiAc Yeast Transformation (Clontech Protocols #PT3024-1)

1. Inoculate 1 mL of YPD or SD with several colonies, 2–3 mm in diameter.
   Note: for strains previously transformed with another autonomously replicating plasmid, use the appropriate SD selection medium to maintain the plasmid.
2. Vortex vigorously for 5 min to disperse any clumps.
3. Transfer this into a flask containing 50 mL of YPD or the appropriate SD medium.
4. Incubate at 30°C for 16–18 h with shaking at 250 rpm to stationary phase (OD$_{600}$>1.5).
5. Transfer 30 mL of overnight culture to a flask containing 300 mL of YPD. Check the OD$_{600}$ of the diluted culture and, if necessary, add more of the overnight culture to bring the OD$_{600}$ up to 0.2–0.3.
6. Incubate at 30 °C for 3 h with shaking (230 rpm). Final OD$_{600}$ should be 0.4–0.6.
7. Place cells in 50 mL tubes and centrifuge at 1,000 × g for 5 min at room temperature.
8. Discard the supernatants and thoroughly resuspend the cell pellets in sterile TE or distilled H$_2$O. Pool the cells into one tube (final volume 25-50 mL).
9. Centrifuge at 1,000 × g for 5 min at room temperature.
10. Decant the supernatant.
11. Resuspend the cell pellet in 1.5 mL of freshly prepared, sterile 1× TE/1× LiAc.
12. Add 0.1 µg of plasmid DNA and 0.1 mg of herring testes carrier DNA to a fresh 1.5-mL tube and mix. Note: for simultaneous co-transformation (using two different plasmids), use 0.1 µg of each plasmid (an approximately equal molar.
ratio), in addition to the 0.1 mg of carrier DNA. For transformations to integrate a
reporter vector, use at least 1 µg of linearised plasmid DNA in addition to the
carrier DNA.

13. Add 0.1 mL of yeast competent cells to each tube and mix well by vortexing.
14. Add 0.6 mL of sterile PEG/LiAc solution to each tube and vortex at high speed
   for 10 sec to mix.
15. Incubate at 30 °C for 30 min with shaking at 200 rpm.
16. Add 70µL of dimethyl sulfoxide (DMSO). Mix well by gentle inversion. Do not
   vortex.
17. Heat shock for 15 min in a 42 °C water bath.
18. Chill cells on ice for 1-2 min.
19. Centrifuge cells for 5 sec at 14,000 rpm at room temperature. Remove
    supernatant.
20. Resuspend cells in 0.5 mL of sterile 1× TE buffer.
21. Plate 100 µL on each SD agar plate that will select for the desired transformants.
22. Incubate plates, up-side-down, at 30 °C until colonies appear (generally 2-4 days).
Appendix 3 – Odyssey™ Labelling Protocol

LI-COR, Lincoln, NE 68504, USA

Odyssey™ reagents:

- IR-labelled secondary antibodies
- Odyssey™ blocking buffer

Additional reagents needed:

- Blotted nitrocellulose membrane
- Primary antibody
- Tween-20
- PBS wash buffer
- Double distilled water

Protocol

After transfer:

1. Wet the membrane in PBS for several minutes.
2. Block each membrane in 20 mL Odyssey blocking buffer for 1 hour.

   - DO NOT add Tween-20 when blocking the membrane. The membrane should not be exposed to Tween-20 until blocking is completed, or high background will result.

   - Odyssey blocking reagent (which can be diluted 1:1 in PBS) will yield higher and more consistent sensitivity and performance than other blockers.

   - Never block with BSA or BSA-containing solutions; they cause very high background on the Odyssey imager.
3. Dilute the primary antibody in Odyssey blocker. The optimum dilution will depend on your antibody, and should be determined empirically. A suggested starting range is 1:1000 – 1:5000.

   - For nitrocellulose membranes, add 0.1-0.5% Tween-20 to the diluted antibody before incubation to lower background. The optimum Tween-20 concentration will depend on your antibody.

4. Incubate blot in primary antibody for 60 minutes or longer with gentle shaking (optimum incubation times will vary for different primary antibodies). Use enough antibody solution to completely cover the membrane.

5. Wash membrane 4 x 5 minutes in PBS + 0.1% Tween-20 with gentle shaking, using a generous amount of buffer.

6. Dilute the fluorescently-labelled secondary antibody in Odyssey blocker. Avoid prolonged exposure of antibody vial to light. Suggested dilution range is 1:2000 – 1:10,000. Add Tween-20 to the diluted antibody as you did for the primary antibody.

7. Incubate blot in secondary antibody for 60 minutes with gentle shaking. Protect from light during the incubation.

   - Allowing incubation to proceed more than 60 minutes may increase background.

8. Wash membrane 4 x 5 minutes in PBS + 0.1% Tween-20 with gentle shaking. Protect from light.

9. Rinse membrane with PBS to remove residual Tween-20

The membrane is now ready to scan.

   - Scan in the appropriate channels (700 nm for Alexa Fluor 680 antibody, 800 nm for IRDye800™ antibody).
• Protect the membrane from light until it has been scanned.

• Keep the membrane wet if you plan to strip and re-use it. Once a membrane has dried, stripping will be ineffective.

• Blots can be allowed to dry before scanning if desired. Signal strength may be enhanced on a dry membrane. The membrane can also be re-wet for scanning.

• The fluorescent signal on the membrane will remain stable for several weeks or longer if protected from light. Membranes may be stored dry or in PBS buffer at 4°C.

• If signal on membrane is too strong, re-scan the membrane at a lower intensity setting.