DISSERTATION

THE ROLE OF NEURONS IN THE HERPES SIMPLEX VIRUS TYPE 1 INFECTION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SARAH M. RICHART ENTITLED *THE ROLE OF NEURONS IN THE HERPES SIMPLEX VIRUS TYPE I INFECTION* BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

THE ROLE OF NEURONS IN THE HERPES SIMPLEX VIRUS TYPE 1 INFECTION

Herpes simplex virus type 1 (HSV-1), a highly pervasive virus among humans, causes a variety of diseases, ranging in severity from cold sores to encephalitis. Initially, virus replicates at the site of inoculation at mucosal surfaces. HSV-1 then enters innervating sensory neuron termini, and travels along the axons to the nuclei in sensory ganglia where viral DNA can latently persist. Once HSV-1 establishes latency in the peripheral nervous system, the virus remains with the individual for life. From here, virus can periodically reactivate throughout the lifetime of an infected individual, causing virus shedding, as well as disease. The main questions addressed in this dissertation are: (1) what makes neurons unique such that they can support a latent HSV-1 infection, and (2) what events take place between HSV-1 and neurons during the establishment of latency?

The first interaction between virus and cell is the binding and entry step. HSV-1 entry into neurons was shown to be mediated by viral glycoprotein D. Antibody blocking experiments revealed that HSV-1 uses cellular HveC but not HveA to enter rat sensory neurons. In fact, HveA is not present on sensory neurons. In primary rat fibroblasts, however, as in other cell lines, HSV-1 uses both HveC and HveA to enter cells. In

contrast to rat sensory neurons, antibodies to HveC or HveA were not able to block HSV-1 entry into mouse sensory neurons. This may mean that rat and mouse HveC are sufficiently different such that the antibodies are not cross-reactive. It could also mean that HSV-1 uses a different molecule to enter mouse neurons than rat neurons, perhaps another glycoprotein D cellular receptor, 3-*O*-sulfated heparan sulfate. Perhaps the entry of HSV-1 in rat neurons via HveC may mediate a signal that aids in the establishment of latency.

The use of a recombinant HSV-1 virus that expresses the immediate early protein ICP0 fused to GFP demonstrated that ICP0 localized differently in neurons than in non-neuronal cells. In non-neuronal cells, ICP0 normally localizes to discrete, punctate structures in the nucleus called Nuclear Domain 10s (ND10s). In neurons, ICP0 was diffusely located throughout the cell at a low level. This suggested that perhaps ND10s were not present in sensory neurons. By examining the presence of the ND10 proteins promyelocytic leukemia protein (PML), SUMO-1 and Daxx, we showed that sensory neurons did not contain ND10 structures. In contrast, PC12 cells, a neuron-like cell line that can be differentiated with nerve growth factor, were shown to contain ND10 structures. The absence of ND10 structures in sensory neurons may in part help explain why HSV-1 establishes a latent infection in this cell-type.

While ND10 proteins were not normally present in sensory neurons, ND10 proteins were induced by interferon treatment, HSV-1 infection, or heat shock treatment. However, ND10 structures were not inducible. Interferon-induced PML and SUMO-1 were found in the cytoplasm of neurons. This suggests that the putative antiviral action of some of the ND10 proteins is not dependent on ND10 structures, but can be mediated

from the cytoplasm. Evidence demonstrated that HSV-1 did not degrade PML in neurons as seen in other cell types. Perhaps the inability of HSV-1 to overcome the antiviral response in neurons is another factor that contributes to the establishment of latency.

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CHAPTER 1

INTRODUCTION TO HERPES SIMPLEX VIRUS TYPE 1

History of herpes simplex virus type 1

Herpes simplex virus type 1 (HSV-1) enjoys a long and intimate history of association with its human host. The oldest existing scientific description of the hallmark sign of HSV-1 infection, fever blisters, was recorded by the physician Herodotus in approximately 100 A.D. (78). The word "herpes" predates this description by about 600 years, when the term that in Greek means "to creep" or "to crawl" was used to describe a number of different skin ailments (137). The most famous literary description of fever blisters comes from William Shakespeare's *Romeo and Juliet* (104):

> O, then, I see Queen Mab hath been with you.
> She is the fairies' midwife, and she comes
> In shape no bigger than an agate-stone
> On the fore-finger of an alderman . . .
> And in this state she gallops night by night . . .
> O'er ladies ' lips, who straight on kisses dream,
> Which oft the angry Mab with blisters plagues
> Because their breaths with sweetmeats tainted are.

In 1873, Vidal established that humans, not Queen Mab, were responsible for the transmission of herpes (137). A transferable infectious agent causing herpes was shown to pass through a filter (69), which, along with causing intranuclear inclusions in infected cells (20), defined the agent as a virus. Shockingly, humans were the first animals to be experimentally infected with herpes in 1875 (112), but rabbits later proved to be good

models for studying herpes encephalitis disease (32). Studying HSV-1 in rabbits led to the important finding that herpesvirus could be transported to the brain via peripheral nervous tissue (46, 47).

Pathogenesis

HSV-1 infections cause a variety of diseases, which in most healthy people may be bothersome, but are generally rather mild. What most commonly comes to mind with HSV-1 is the classical manifestation of vesicular cold sores, or herpes labialis, resulting from lytic infection of epithelial cells, either from primary infection or from reactivated virus from sensory neurons (4). HSV-1 causes oral-facial lesions (herpes labialis and herpetic gingivostomatitis, e.g.), genital herpes, cutaneous infections (herpes whitlow and herpes gladiatorum, e.g.), and herpes keratitis. Yet there are more serious diseases caused by HSV-1. In fact, keratoconjunctivitis is the leading cause of blindness in industrialized countries (82), and HSV-1 causes the most common serious encephalitis in the United States (130). HSV-1 and the closely related HSV-2 can be extremely life-threatening in neonates, causing herpes disseminated disease, which has a 57% mortality rate if untreated, and central nervous system (CNS) disease, which has a 15% mortality rate (58).

While there are many physical manifestations of HSV-1, there are also many people who are seropositive for HSV-1 who display no evidence of primary infection, presumably because of a strong immune response. Additionally, people may shed virus asymptomatically, that is, without evidence of herpetic lesions (reviewed in 36, 61).

HSV-1 is maintained in the human population by passing via close or intimate contact from a person shedding HSV-1 virus to an uninfected individual. HSV-1 enters the host at mucosal surfaces or abraded skin surfaces where it causes a primary infection. Virus replicates in these epithelial cells, and then travels up axons of enervating sensory neurons to the cell bodies in the ganglia. From here, virus will either replicate in some of the neurons (115), or establish a latent infection in other neurons where no infectious virus is being produced but viral genome is present (18). The ability of virus to enter a lytic or latent infection within the neuron may be in part due to the specific subtype of sensory neuron the virus encounters (71, 138). Those neurons harboring latent virus may be induced by various stimuli to produce infectious virus again. These stimuli include, but are not limited to, UV light, menses, fatigue, anxiety, and malnutrition (128). This phase of infection is called recrudescence or reactivation, which results in virus traveling along the neuronal axons to infect epithelial cells, again producing the characteristic vesicular lesion. Once HSV-1 sets up latency in the peripheral nervous system, the virus remains with the individual for life. From here, virus can periodically reactivate throughout the lifetime of an infected individual, causing virus shedding as well as disease.

At least one important mystery of HSV-1 pathogenesis remains: it is still unclear whether perhipheral neurons with HSV-1 genomes die after reactivation of virus, and then new neurons become latently infected from virus replicating at the epidermis, or if reactivated neurons live, and after producing productive virus, can still harbor latent genome.

Rarely, HSV-1 can infect the brain and cause serious encephalitis. Various genes contribute to the neurovirulence of HSV-1, mainly genes involved in DNA replication, as neurons do not replicate their DNA. Additionally, mutations in various glycoproteins restrict HSV-1 from entering neurons (reviewed in (132). HSV-1 can establish a latent infection in the central nervous system, and, while it is rare, can also reactivate from this state.

The advent of acycloguanosine (ACV), a nucleoside analog that terminates DNA replication, and related drugs have led to some relief from the pain and serious effects of HSV-1. ACV remains inactivated within the patient until it encounters cells with replicating HSV-1. The HSV-1 early gene, thymidine kinase, phosphorylates ACV. Cellular nucleoside kinases, however, are unable to phosphorylate ACV. Phosphorylated ACV gets incorporated into viral DNA, and because it lacks a 3'-OH for addition of the next nucleotide, causes chain termination. ACV is effective against replicating virus; however, it is unable to completely prevent latency in the mouse model (10, reviewed in 84), nor can it abolish the latent infection following reactivation (119). Recently ACV-resistant strains of HSV-1 have been identified, particularly in AIDS patients and other immunocompromised individuals (reviewed in 89), but also in immunocompetent individuals. These resistant strains necessitate the discovery of novel anti-HSV treatments, particularly treatments that can prevent or abolish latent HSV infections.

Prevalence

Because HSV-1 is not a reported infectious disease, most estimates of HSV-1 prevalence come from small-scale studies. A variety of studies that included young

people found that HSV-1 prevalence ranged from 41% of Swedish girls aged 16, to 62% of people in Cincinnati, OH aged 12-22, to 94.9% of Ugandan villagers aged 15-19 years (reviewed in 111). Typically, as age increases, so does the seroprevalence of HSV-1, which can then approach 90% in some populations (reviewed in 131).

In the United States, and in many parts of the world, most symptomatic first-episode genital herpes cases are caused by HSV-2 (100). However, in other regions, notably the United Kingdom, HSV-1 is the more common cause of genital herpes, up to 71% in some test groups (121, 124).

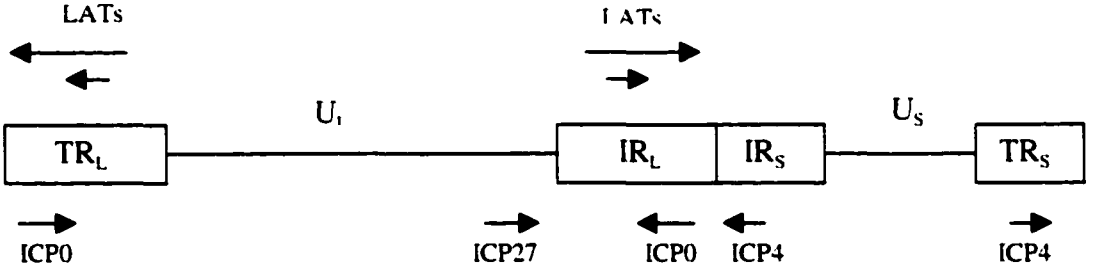

Figure 1.1 Schematic of the HSV-1 genome.

Genome structure

In the mid 1980s, the herpes simplex type 1 virus genome sequence was published. This led to an explosion in research focused on the molecular aspects of HSV-1 infection. The genome consists of 152,260 base pairs of double stranded, linear DNA,

containing at least 75 open reading frames (73-75, 87). The genome contains two unique segments, designated unique long (U_L) and unique short (U_S) that are bordered by internal repeated regions (IR_L and IR_S) and terminal repeated regions (TR_L and TR_S) (72, 93) (Figure 1). These repeats probably recombine to form the replicative rolling circle form of the genome (reviewed in 9).

Virion structure

HSV-1 virus is made up of double-stranded, linear DNA. Around the genome is an icosahedral capsid that is surrounded by a tegument. The tegument is a layer of viral proteins that comes in with the virion, but are not part of the capsid, and includes VP16, a transactivator of immediate early viral genes, and virion host shutoff (VHS) protein, which causes the degradation of host mRNA. Surrounding these elements is a lipid envelope containing viral glycoproteins, used for virus entry, along with two other non-glycosylated viral proteins (reviewed in 94). Evidence suggests that at least 39 of the 75 or more genes encode proteins that make up the virion (56).

HSV-1 gene expression

HSV-1 genes are generally divided into 3 classes, based on their temporal expression: immediate early (α genes), early (β genes) and late (γ genes). Generally, the immediate early proteins control the expression of viral genes, early genes encode DNA replication machinery, and late genes encode structural proteins for the virion, such as capsid, tegument, and envelope proteins.

There are 5 immediate early genes which are defined as (i) having regulatory sequences in their promoters that are stimulated by the virion-associated late protein, VP16 (3, 14, 19, 88), and (ii) being transcribed in the absence of *de novo* viral protein synthesis (1, 17, 57).

Immediate early gene expression begins when viral VP16 protein, a late gene that is part of the virion tegument, binds to cellular Oct-1 (9) and a host cell factor (HCF), also called C1 (114). This complex then specifically binds to the TAAGARAT sequences (2) in the immediate early gene promoters to initiate transcription.

Three immediate early proteins are important for HSV-1 viral gene transcription: ICP0, ICP4, and ICP27. All of these proteins are phosphorylated and nucleotidylated (5, 6). The other immediate early proteins are involved in immune system avoidance (ICP47) (25), and also marginally involved in transcription (ICP22) (90).

ICP0

One of the 5 immediate early proteins, ICP0, has been shown to be important in the establishment of HSV-1 latency in sensory neurons (135) and in the reactivation of virus from explanted trigeminal ganglia (11, 16, 53, 65), yet the role ICP0 plays in the latent infection in sensory neurons is as yet unknown. ICP0 is defined as non-essential for normal viral gene expression and replication in transformed cell lines. However, when ICP0 is inactivated, a defect in the onset of viral gene expression occurs that is cell type-, cell cycle- and multiplicity-dependent (12, 37, 96, 117).

For a protein that is not absolutely critical to virus replication, it performs an astounding variety of functions within infected cells. ICP0 transactivates all three classes

of viral genes (13, 40, 45, 83), inhibits the progression of mitosis in dividing cells (67), and somehow overcomes the anti-viral effects of interferon-α/β (IFNα/β) (66, 81).

ICP4

ICP4, a protein that is absolutely necessary for viral replication, is an important viral gene transactivator. ICP4 is essential for regulating early and late genes. ICP4 has been shown to directly bind to DNA (29, 41), where it recruits cellular TATA-binding protein (TBP) and TFIID (15, 51) to begin transcription. It functions as a transrepressor of viral promoters, including its own (28, 43, 44, 91). ICP4 can function alone or work cooperatively with ICP0 to enhance transcription (40, 103).

ICP27

ICP27 affects viral gene expression, most notably in concert with ICP0 and ICP4, causing an increase or decrease in gene expression, depending on the viral promoter (103). It associates with HSV-1 mRNA by shuttling it into the cytoplasm late in infection which mediates the expression of late genes (97, 107). Outside of the context of the whole virus, ICP27 can relocalize ICP4 and ICP0 to the cytoplasm of transfected cells (141-143). Within infected cells, ICP27 has been shown to cause a decrease in cellular mRNA during HSV-1 infection (54) by interfering with host mRNA splicing (55, 98). The role that ICP27 plays in the HSV-1 infection of neurons has not received much attention, even though ICP27 is intimately involved with ICP0 and ICP4 in controlling gene expression

Latency

A unique feature of the *Herpesviridae* family is the ability of its members to persist in their hosts in a latent form. HSV-1 goes latent specifically in sensory and sympathetic neurons of the peripheral nervous system.

HSV-1 latency in sensory neurons is typically defined by (i) the absence of detectable infectious virus (21, 25, 116), (ii) the maintenance of the viral genome in an endless, or episomal, form (35, 77, 105), and (iii) the cessation of viral gene transcription, except several forms of the enigmatic latency-associated transcript (LAT) (116).

The factors that affect the establishment of latency in sensory neurons are not clear, but two viral processes are most likely downregulated: immediate early gene expression and DNA replication. One current theory is that the level of ICP0 expressed in cells determines the lytic or latent infection (38, 39). Without sufficient levels of ICP0 expression, cellular factors are able to repress the HSV-1 genome, importantly, the immediate early genes. If ICP0 is expressed at a high enough level, it can overcome the repressive cellular factors, perhaps by causing their proteasome-dependent degradation. DNA replication is also downregulated during latency. In fact, latency has been established *in vitro* with viruses lacking ICP4 (102, 135), and ICP27 (60), two genes critical for virus replication, and latency has been established in the presence of acyclovir and other inhibitors of DNA polymerase (133). These data demonstrate that virus replication is not necessary for the establishment of latency.

The cellular factors that affect latency are also unknown. It has been postulated that the low level expression of certain transcription factors in neurons, such as c-jun, c-fos and Oct-1, may result in decreased levels of immediate early genes (reviewed in 79).

Another theory explores the finding that a cellular homologue of viral VP16, luman, when expressed in cells, causes the localization of HCF (also called C1) to shift from the nucleus to the cytoplasm in a cell (68). The expression of luman was also shown to render cells resistant to HSV-1 replication. This is intriguing in light of the findings that HCF is normally found in the cytoplasm of sensory neurons, not in the nuclei, as is the case with most cells (63). Perhaps the localization of HCF in neurons prevents VP16 from transactivating immediate early genes, leading to latency.

The latency associated transcripts are a family of messages that are differentially spliced to produce transcripts of sizes 8.3 kb, 2.0 kb, 1.5 kb, 1.45 kb, and 0.7 kb (92, 108, 109, 140, 144). The 8.3 kb message, or the minor LAT, and a strain-specific 0.7 kb are the only transcripts that shows evidence of polyadenylation (30, 140, 144). The other transcripts are thought to be stable introns from either the splicing of the minor LAT (2.0 kb), or from the splicing of the 2.0 kb LAT (1.5 and 1.45 kb) (27, 42, 62, 129). The 2.0 kb transcript is expressed during lytic and latent infection (108), while the 1.5 kb and 1.45 transcripts appear to be present only during latent infection (109). The expression of protein encoded by the LAT transcript has been reported by two different groups (31, 123), but it has not been a focus of investigation in the field.

While LAT has been determined to be the only transcriptionally active region of the HSV-1 genome during latency, only a small number of neurons that harbor latent HSV-1 genome express LAT *in vivo* (33, 34, 70, 76). Based on this finding and other studies showing that LAT mutants establish latency, many investigators initially believed that LAT plays no role in the establishment or maintenance of latency (8, 101, 113).

More recently, however, evidence has been published that suggests that LAT does help establish latency (86, 126).

One proposed mechanism for LAT's contribution to the maintenance of latency is that it regulates the expression of ICP0. The stable LAT species overlap with the 3' end of ICP0 transcripts, thus potentially inhibiting ICP0 protein expression in an antisense manner by causing the degradation of the resulting double-stranded RNA (reviewed in 79). Evidence also suggests that LAT promotes neuronal survival (125), perhaps by blocking apoptosis in neurons (85, 127). Analysis of the LAT promoter (LAP) suggests that there are *cis*-acting elements that allow for specific regulation of LAT in neurons (reviewed in 80). While LAT may contribute to the maintenance of latency, neither LAT nor any other viral gene tested so far has been shown to be absolutely necessary for the maintenance of latency.

Reactivation

An astonishing variety of stimuli can cause latently-infected sensory neurons to reactivate and produce infectious HSV-1. The next section explains some of the specific reactivation triggers used in the different HSV-1 experimental models. Suffice it to say, these stimuli lead to many different cell signaling events involving various biochemical pathways.

Some viral stimuli have been shown to reactivate latent virus, including delivery of adenoviruses expressing ICP0, ICP4, VP16 (52), or ICP27 (unpublished observation), and superinfection with HSV-2 (122). Another viral gene believed by some to be

important in reactivation of virus is LAT, as LAT mutant viruses displayed poor reactivation kinetics (8, 113).

The changes that occur in neurons to induce HSV-1 production can probably be summarized as either (1) inducing a transcription factor that upregulates immediate early proteins to high enough levels to set the lytic program in motion, or (2) somehow de-repressing repressed immediate early promoters, and possibly also other regions of the virus genome.

In light of the wide array of possible HSV-1 reactivation stimuli, one approach to studying the changes that these stimuli induce in neurons is to look at panels of changes in RNA expression. This has been done using differential-display RT-PCR on explanted trigeminal ganglia (120), and currently our group is collaborating with a company that is performing microarray analysis on nerve growth factor deprived dorsal root ganglion neurons harboring latent HSV-1. Hopefully, these analyses, and others, will help to establish patterns of gene regulation common to all methods of reactivation.

Experimental models of latency and reactivation

HSV-1 can infect a wide variety of cells from a broad range of hosts. However, because humans are the only true reservoirs for HSV-1 in nature, the infection in an experimental model of HSV-1 is likely to have some different features than the infection in a human. Each experimental model has its own strengths and weaknesses, and each has made contributions to the field of study.

The mouse and rabbit ocular models are the two most common *in vivo* experimental animal models of HSV-1. Virus is inoculated via the cornea, and virus then

travels to the enervating trigeminal ganglia where it goes latent. Reactivation of HSV-1 can be achieved in the mouse model by explanting ganglia (18) or by exposing mice to hyperthermic conditions (99). Reactivation can be induced in the rabbit model by the iontophoresis of epinephrine to the cornea (64), or by injection of sterile saline into the eye stroma (48).

A non-neuronal tissue culture model was developed using human fetal lung (HFL) fibroblasts (59). In this system, a mutant virus deficient in VP16 and ICP0 expression, *in*1820, is used to infect HFL at either a very low multiplicity of infection, or with interferon-α pre-treatment. Genomes can be found circularized in cells, but virus can only be reactivated upon superinfection of cells with HSV-1, in this case a temperature-sensitive mutant, *ts*K.

PC12 cells, a cell line derived from a rat pheochromocytoma, have been reported to behave like sensory neurons when differentiated with nerve growth factor (NGF) by displaying the following characteristics: responding to NGF, ceasing cell division, extending neural processes that support action potentials, and displaying other biochemical properties of peripheral nervous system neurons (49, 50). Differentiated PC12 cells have been described as an *in vitro* model for a "quiescent" HSV-1 infection (7), where endless HSV-1 genome is detectable, but a low level of infectious virus is still present. In this system, NGF withdrawal or superinfection with a second distinguishable HSV-1 mutant (118) led to an increase in virus production. More recently, another PC12 *in vitro* system of "quiescence" was developed where serum depletion and growth on collagen led to a state where virus was undetectable, and virus could then be detected after forskolin treatment or heat shock, but not NGF withdrawal (23, 24).

Previously, we developed a model of HSV-1 latency in primary rat sensory neurons *in vitro* (136). Latent HSV-1 infections can be established in essentially all of the neurons in a culture. This model is shown to reproduce many of the characteristic features of natural human HSV-1 latent infections, including restricted viral gene expression (31), circularization of the viral genome, and reactivation with forskolin or phorbol 12-myristate 13-acetate (PMA) treatment, heat shock, or NGF withdrawal can produce infectious virus (106, 134, 136). It is this model that is used in the following research.

Vaccines

While HSV-1 and HSV-2 vaccine design has resulted in more than 35 different vaccines (26), very few have shown real promise. The most current HSV-2 vaccine tested in humans has shown effectiveness only in women who do not already have antibodies to HSV-1 or HSV-2 (110). The design of a HSV-1 vaccine poses unique challenges because HSV-1 persists in neurons, where it can go undetected by the immune system (22). Some researchers are hopeful that the use of better adjuvants, as well as more recent vaccine approaches, will improve the chances of an effective HSV-1 vaccine. One such hopeful is the use of DNA vaccines carrying minigenes, which encode for small antigenic peptides from viral proteins glycoprotein B, glycoprotein D, and ICP27 (95, 139).

Hypotheses

The main hypotheses investigated in this dissertation are: (1) There are differences between sensory neurons and other cell types that allow for HSV-1 to establish a latent or lytic infection in sensory neurons, but only a lytic infection in non-neuronal cell types. (2) The early events that occur between HSV-1 and sensory neurons contribute to the establishment of latency. With these hypotheses, we aimed to investigate some of the specific differences between sensory neurons and non-neuronal cells, as well as the specific early interactions between virus and neurons.

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CHAPTER 2

ANALYSIS OF THREE RECOMBINANT HSV-1 VIRUSES THAT EXPRESS GREEN FLUORESCENT PROTEIN FUSED TO AN IMMEDIATE EARLY PROTEIN

Recombinant HSV-1 viruses were constructed by Joseph Morroni (HSVEGFP0 and HSVEGFP27) and Dr. Donald Traul (HSVEGFP4). Dr. Christine Wilcox provided the primary rat dorsal root ganglion neurons. These viruses were used for studies in the following chapters of this dissertation. The work from this chapter was presented at the following meetings:

Richart S., Morroni J., Gogain J., Traul D., and Wilcox C. (1998) Analysis of function of EGFP fusions of the HSV-1 immediate early proteins ICP0 and ICP4. 17th Annual Meeting of American Society for Virology.

Richart S., Traul D., Morroni J., Smith R., and Wilcox C. (1999) Analysis of three recombinant HSV-1 viruses that each express EGFP fused to an immediate early protein. 24th International Herpesvirus Workshop.

2.1 ABSTRACT

The Herpes simplex virus type-1 (HSV-1) immediate early proteins ICP0, ICP4 and ICP27 have a multitude of functions during the productive viral life cycle. They are best known for their function as transcriptional regulators of HSV-1 gene expression. Their activities during HSV-1 gene expression in neurons, however, are not as well characterized. We have constructed recombinant HSV-1 viruses expressing the ICP0, ICP4 or ICP27 fused to the humanized green fluorescent protein (HSVEGFP0, HSVEGFP4, HSVEGFP27, respectively) to allow us to visualize the expression patterns of these proteins in live neurons. The recombinant viruses all had similar growth curves as the wild-type virus. In HeLa cells, expression of the GFP-ICP0, or GFP-ICP4, or GFP-ICP27 fusion proteins from recombinant herpesviruses displayed the characteristic nuclear localization of ICP4 and ICP27, and nuclear punctate localization of ICP0 that has been described for the non-fused proteins. Recombinant viruses displayed wild-type characteristics of fusion protein expression over time, replication rate in cell lines, and establishment of latency. Infection of primary dorsal root ganglia neurons with the recombinant herpesviruses showed that the EGFP-ICP4 or EGFP-ICP27 proteins localized to the nucleus, while the EGFP-ICP0 gave low-level diffuse nuclear and cytoplasmic localization. These studies show that fusion of the HSV-1 immediate early proteins to green fluorescent protein retained wild-type function. These recombinant viruses offer great potential for studying HSV-1 latency and reactivation in neurons.

2.2 INTRODUCTION

The green fluorescent protein (GFP), isolated from the jellyfish *Aequorea victoria* (4), has become a valuable marker protein for many biological disciplines. Many viruses have been made to express GFP as a means of tracking the virus in live cells or animals. We have constructed three different recombinant herpes simplex virus type 1 (HSV-1) viruses to express ICP0, ICP4, and ICP27 fused to GFP (HSVEGFP0, HSVEGFP4, and HSVEGFP 27, respectively). The enhanced GFP, EGFP, a red-shifted mutant that excites maximally at 488 nm (6), was fused it to 5' end of the immediate early genes IE1, IE2, and IE3, encoding ICP0, ICP27, and ICP4, respectively. These three immediate early gene products were chosen because they are important in that they serve as transcriptional regulators of HSV-1 gene expression, and may be important during establishment and reactivation of latent HSV-1.

ICP0, encoded by immediate early gene IE1, is a 110 kDa protein that plays a significant regulatory role in efficient viral replication in cultured cells and in the mouse model of latency. ICP0 has been shown to be non-essential for normal viral gene expression and replication. However, when ICP0 is inactivated, a defect in the onset of viral gene expression occurs that is cell type-, cell cycle- and multiplicity-dependent (2, 9, 25, 33). In transient transfection assays, ICP0 has been shown to increase the expression of all temporal classes of HSV-1 promoters (10, 11, 18), albeit by a poorly understood mechanism. In productive HSV-1 infections, as well as in transient transfections, ICP0 demonstrates distinct patterns of punctate, nuclear localization. Indeed, by the use of various ICP0 mutant constructs, a nuclear localization signal for ICP0 has been described and mapped to the C-terminus of the protein (8, 17).

ICP4 (or Vmw175) is a 175 kDa immediate early protein encoded by the IE3 gene. ICP4 is vital to virus replication (21), playing a critical role in transcriptional activation of viral genes (reviewed in (23). Like ICP0, ICP4 is localized primarily to the nuclei of infected or transfected cells. However, in contrast to ICP0, ICP4 exhibits diffuse nuclear staining. A nuclear localization signal has been mapped to a region of the C-terminus of the protein (17).

ICP27 (or Vmw63) is a 63 kDa immediate early protein encoded by the IE2 gene. Like ICP4, ICP27 is essential for virus replication (24). ICP27 is involved in both upregulation and downregulation of viral genes, host protein shutoff, and host RNA splicing inhibition (reviewed in 27). In ICP27, the nuclear localization site is found closer to the N-terminus of the protein between amino acid residues 110-137 (15).

The results of this study showed that recombinant HSV-1 viruses HSVEGF0, HSVEGFP4, and HSVEGFP27 behave in wild-type manners. One of the main goals in the use of these recombinant viruses is to study the timecourse of HSV-1 immediate early gene expression during latency and reactivation.

2.3 MATERIALS AND METHODS

Viruses and cells. HSV-1 (17^+) was the wild-type strain used in these studies. The ICP0 minus HSV-1 mutant virus, dl1403 (33), was used to construct the recombinant virus expressing EGFP fused to ICP0. The ICP4 minus HSV-1 mutant virus, D30EBA (20), was used to construct the recombinant virus expressing EGFP fused to ICP4. The 17^+ wild-type virus was used to construct the recombinant virus expressing EGFP fused to ICP27. All virus stocks were grown and quantified on Vero cells (American Type

Culture Collection [ATCC], Rockville, MD), except D30EBA, which was grown and quantified on E5 cells, a Vero derived cell line that expresses ICP4 (7).

Vero, HeLa, and E5 cells (ATCC) were maintained in Dulbecco's modified eagle medium (DMEM) (Invitrogen Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (FBS).

Neuron culture. Neuronal cultures were prepared from dorsal root ganglia neurons (DRG) from day 15 embryonic rats as previously described (34, 38). HSV-1 latency was established in neurons as described (38). Briefly, 14 days after neurons were plated at approximately 10^3 cells per well in a 24-well plate, neurons were treated with 50μM acyclovir (ACV) at least 12 hours prior to HSV-1 infection. Neurons were infected with a multiplicity of 5-10 pfu/cell. Cultures were maintained in acyclovir for at least one week, then switched to maintenance medium lacking acyclovir for the duration of latency. Maintenance medium contained 10% calf serum (Invitrogen Life Technologies) and 100 ng/ml 2.5 S mouse nerve growth factor (Harlan Bioproducts for Science, Indianapolis, IN) in F12/DMEM medium (Invitrogen Life Technologies).

***In situ* hybridizations and probes.** Neuronal cultures were latently infected with wild-type or recombinant viruses, and latency was established for 3 weeks. Neurons were then fixed in phosphate-buffered 4% paraformaldehyde for 12 hours at 4° C, dehydrated in graded ethanol, and stored at -20° C until used. Detection of the latency-associated transcript (LAT) by *in situ* hybridization was performed as described (31, 36). A digoxigenin-labeled (DIG) riboprobe anti-sense to the 5' end of LAT (pLAT) (31) was used. This probe does not overlap any other known genes in the LAT region (14). DIG

was detected with anti-DIG antibody labeled with alkaline phophatase, and the substrate was NBT/BCIP (Roche Molecular Biochemicals, Indianapolis, IN).

HSV-1 reactivation. Neuron cultures were latently infected with HSV-1, and latency was established for 2 weeks. Neurons were switched to ACV-free medium at least 24 hours prior to reactivation, and treated with 100 μM forskolin (Sigma, St. Louis, MO) as previously described (32). Four days post-forskolin treatment, neurons were freeze-thawed twice. Virus was titered on Vero cells.

Comparison of virus replication. Vero cells were seeded in 24-well plates, and infected with recombinant viruses HSVEGFP0, HSVEGFP4, HSVEGFP27, dl1403, wild-type 17^+ strain at a multiplicity of infection of 0.1 plaque forming unit (PFU) per cell, or mock infected. HSVEGFP27 was also used at multiplicity of 1 PFU per cell. Virus was allowed to replicate at various times, then culture plates were frozen. Cells and virus were thawed once and titered by plaque-assay on Vero cells.

Campenot chambers. DRG neurons grown in Campenot chambers were plated at approximately 200-400 cells per chamber, and set up as previously described (3). For establishment of latency, neurites and cell bodies were grown in medium containing 50 μM ACV. For reactivation, both neurites and cell bodies were treated with forskolin.

Viral DNA isolation. For each virus type, Vero cells were infected with virus at a multiplicity of infection of 1-10 (E5 cells were used for the D30EBA virus growth) and incubated for 48 hours at 37°C. Cells were harvested, washed once in ice-cold phosphate buffered saline (PBS, pH 7.4), and resuspended in 2 ml lysis buffer (100 mM Tris-HCl pH 8, 0.5% SDS, 20mM EDTA). The lysed cells were incubated with 0.5 mg/ml Proteinase K (Roche Molecular Biochemicals) at 37°C overnight. Cell lysates were

phenol, phenol: chloroform, phenol: chloroform isoamyl alcohol extracted, and the DNA was precipitated with 1/10th volume sodium acetate (3M, pH 5.2) and 2 volumes 100 % ethanol.

Construction of HSVEGFP0: The plasmid containing EGFP fused to the N-terminus of ICP0 was constructed by inserting the *Sst* I to *Hpa* I ICP0 coding region from pJR3 (33) (a gift from Roger Everett, MRC, Glasgow, Scotland) into the *Sst* I and *Sma* I sites of the pEGFPC1 (Clontech, Palo Alto, CA). The resultant plasmid was then cut with *Nco* I and *Bgl* II, filled-in using the klenow enzyme and dNTPs and re-ligated with T4 DNA ligase (pCMVEGFP-0). Part of ICP0 was cut out of pCMVEGFP-0 using *Nco* I (partial digest) and *Xho* I, and inserted into the *Nco* I and *Xho* I sites of pJR3 to put the EGFP-ICP0 fusion under the control of the native ICP0 promoter. This construct, pJR3EGFP-0, was then co-transfected into Vero cells with dl1403 DNA (33), a HSV-1 mutant lacking both copies of the ICP0 gene. A single fluorescent plaque was picked and plaque purified three times in Vero cells.

Construction of HSVEGFP4: The plasmid containing EGFP fused to the N-terminus of ICP4 was constructed by cloning the *Xba* I to *Pst* I fragment containing the ICP4 coding region minus the last 7 amino acids and stop codon from pXK350 (39) (gift from Kent Wilcox, Medical College of Wisconsin, Milwaukee) into *Xba* I and *Xho* I digested pEGFP-C1 to produce pEGFP-1535. To maintain the reading frame, pEGFP-1535 was then cut with *Xba* I and *Xho* I, filled in with Klenow and religated. To replace the last 7 amino acids and stop codon, the *Nru* I-*Sal* I fragment of pXK966 (gift from K. Wilcox), containing the entire ICP4 coding region, was sub-cloned into the *Nru* I and *Sal* I sites of pCMVEGFP-1535 to produce pCMVEGFP-4. This plasmid contains the entire

ICP4 gene fused in-frame to the 3' end of the EGFP gene. The plasmid pGX58, which contains the genes for ICP4 and part of ICP0 (10), was digested with *Xho* I and *Pst* I, and the ICP4 containing piece was then subcloned into the *Xho* I and *Pst* I sites of the pBSKS vector, resulting in plasmid pBSKS/pGX58. pBSKS/pGX58 was then digested with *Sal* I and filled in with Klenow and dNTPs before digesting with *Bsr* GI. pCMVEGFP-4 was partially digested with *Bsr* GI, then digested with *Eco47* III to produce the GFP-ICP4 fragment lacking the CMV promoter which was sub-cloned into the pBSKS/pGX58 plasmid to finally produce pBSGXEGFP-4, which contains EGFP-ICP4 driven from the native 17^+ ICP4 promoter. This construct was co-transfected into Vero cells with D30EBA DNA (20), a replication incompetent HSV-1 mutant lacking both copies of ICP4 gene. A single fluorescent plaque was picked and plaque-purified on Vero cells.

Construction of HSVEGFP27: The EGFP was also fused in frame to the N-terminus of the ICP27 immediate early protein. pCMVEGFP-ICP27 was constructed by sub-cloning the *Age* I to *Eco* RI fragments from pGEM-2 (gift from Rozanne Sandri-Goldin, University of California, Irvine [29]), which contains the entire ICP27 coding region into the *Age* I to *Eco* RI sites of the pEGFP-C1 expression plasmid. The new plasmid was then cut with *Eco47* III and *Eco* RI and subcloned into pBSKS (Stratagene, LaJolla, CA) from *Hinc* II to *Eco* RI. This plasmid was then cut with *Kpn* I and *Eco* RI and subcloned into the pEGFP-C1 plasmid to create the in frame fusion of the EGFP and ICP27 coding regions. To place the EGFP into the 17^+ background, the pCMVEGFP-27 plasmid was digested with *Age* I and the fragment containing the EGFP was subcloned into the *Age* I site of pGXS12 (gift of R. Everett) to create the in frame fusion of the EGFP and the ICP27 coding regions. Clones were checked for orientation of the inserted

EGFP fragment. The new plasmid pGXS12EGFP-ICP27 has the fusion protein under the control of its native promoter. This construct was co-transfected into Vero cells with 17^+ DNA, the wild-type HSV-1 used in these studies. A single fluorescent plaque was picked and plaque-purified on Vero cells.

Plasmids to express non-fused proteins and controls included: the pEGFP-C1 plasmid expressing EGFP, pXK350 expressing the wild-type ICP4 protein, pCMV-ICP0 expressing the wild-type ICP0, and pCMV-ICP27 expressing the wild-type ICP27 protein. The pCMV-ICP0 plasmid was constructed by restriction digesting the EGFP-ICP0 plasmid with *Eco47* III and Bsp EI, filling in with Klenow and dNTPs and re-ligating with T4 DNA ligase. pCMV-ICP27 was constructed by restriction digesting with *Age* I and re-ligating with T4 DNA ligase. All of the plasmids described above used the CMV IE promoter for expression in mammalian cells.

Recombinant virus production. Recombinant viruses were produced by homologous recombination between viral genomic DNA and plasmid DNA carrying the GFP-immediate early protein fusion. Vero cells were grown to 50% confluency in 100 mm plates. Medium was changed 4 hours prior to transfection. Cells were treated for DNA uptake by using the calcium phosphate method (26). 40 μg each of both viral DNA and plasmid DNA containing the gene fused to EGFP were used for each transfection. Medium containing DNA was incubated for 4 hours on the cells at 37° C, then taken off and saved. Cells were glycerol shocked for 1 minute (20% glycerol in 10%FBS/DMEM), then washed twice with PBS. The saved medium containing DNA was then replaced and cells were incubated for 6 hours at 37°C, after which fresh media

added to cells. Fluorescent plaques were picked, and plaque-purified on Vero cells at least three times.

Southern blot analysis. Viral DNA was obtained from Vero cells infected with either 17^+, HSVEGFP0, HSVEGFP4, or HSVEGFP27 virus. For HSVEGFP0 analysis, 17^+ and HSVEGFP0 DNA were digested with *Eco47* III, *Ssp* I and *Ase* I. For HSVEGFP4 analysis, 17^+ and HSVEGFP4 DNA was digested with *Xho* I. For HSVEGFP27, 17+ and HSVEGFP27 DNA were digested with *Bam* HI and *Stu* I. The EGFP-C1 plasmid (Clontech) control used in Southern blots for all recombinant viruses was digested with *Bam* HI. The fragments were separated by electrophoresis on a 0.8% agarose gel, transferred to a nylon membrane (Hybond-N, Amersham) by the salt transfer method, and UV cross-linked. ICP0, ICP4, ICP27 or GFP probes were hybridized in solution containing 6× SSC, 5× Denhardt's Solution, 50% formamide, 0.5% SDS and 100 μg/ml sheared Salmon Testes DNA (Sigma). Hybridizations were performed at 42°C for 16 hours. Stringent washes, streptavidin-alkaline phosphatase conjugation, and substrate incubation were performed according to the manufacturer's protocol (CDP-Star™ Chemiluminescence nucleic acid detection kit, NEN, Boston, MA). Images were photographed on BioMax film (Kodak Corporation), and scanned into Adobe Photoshop 5.0.

Preparation of biotin-labeled DNA probes. The template for ICP0 probe was the *Nco* I to *Xho* I 1.0 kb fragment cut from the pJR3 plasmid (33), which contains the entire ICP0 gene. The ICP4 probe was made from the 1.9 kb *Bam* HI fragment of pCW-1, a plasmid containing the ICP4 coding region. The ICP27 probe was made from the 1.28 kb *Age* I to *Stu* I fragment of pGEM-2. The EGFP probe was made from the *Eco47*

III to *Bgl* II 0.8 kb fragment cut from the pEGFP-C1 plasmid (Clontech). Biotin-14-dCTP labeled probes were generated following manufacturer's protocol (BioPrime DNA labeling system, Invitrogen Life Technologies).

Transient transfection assays. 5×10^5 Vero cells were seeded on 60 mm dishes for 24 hours. Medium was removed and 3 ml Opti-MEM media (Invitrogen Life Technologies) was added one hour prior to transfection. For each dish, 30 µl Lipofectamine (Invitrogen Life Technologies) and 20 µl Opti-MEM medium were added together in a microfuge tube. In another microfuge tube, 10 or 20 µg DNA was added to Opti-MEM media to a volume of 50 µl. The lipid and DNA mixtures were then added together and immediately added to cells. Dishes were placed in 37° C incubator for 4 hours, then 3 ml DMEM-10% FBS were added to dishes, returned to incubator, and analyzed later for stable transfection. Photomicrographs were taken using Kodak Elite II 400 ISO slide film and scanned into Adobe Photoshop 5.0.

Western blot analysis. 1×10^6 Vero cells were added to 60 mm dishes 16 hours prior to infection. Each dish was then infected with 1×10^7 PFU of virus, and harvested at various times post infection. Cells were either rinsed twice in cold PBS, scraped into PBS, pelleted and lysed in RIPA buffer with protease inhibitors, or if cells were no longer adherent, they were collected, pelleted, rinsed twice in PBS, then lysed in RIPA buffer with protease inhibitors. Protein quantification was performed using the BCA assay (Pierce, Rockford, IL). 10 µg protein samples were run on 7.5% SDS-PAGE gels. Protein was transferred to nitrocellulose membrane (Amersham Pharmacia, Piscataway, NJ). Renaissance Western Blot Chemiluminescence Reagent Plus substrate (NEN, Boston, MA) was used, and manufacturer's protocol was followed, except for the

following changes: membrane was blocked overnight at 4° C in 1 × Uniblock (Analytical Genetic Testing Centers, Denver, CO) and PBS-Tween 20 (0.1%), the primary and secondary antibodies were diluted in Uniblock-PBS-Tween 20, and the chemiluminescent substrate was diluted 1:5 in water as previously described (19). Primary antibodies used were monoclonal antibodies anti-ICP4 (1101), anti-ICP0 (1112), and anti-ICP27 (1113) (Rumbaugh-Goodwin Institute for Cancer Research, Plantation, FL) (anti-ICP27 was a generous gift from R. Sandri-Goldin). Monoclonal anti-GFP (Clontech) was also used. Secondary antibody used was anti-mouse IgG antibody conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA). Kodak BioMax film was used for visualizing protein bands, then film was scanned into Adobe Photoshop 5.0.

2.4 RESULTS

Southern blot analyses of HSVEGFP0, HSVEGFP4, and HSVEGFP27 confirmed the presence of each immediate early gene fused to the GFP gene. Both IE1 and IE3 genes occur in duplicate in the HSV-1 genome (14). Because recombinant virus clones were selected based on a green plaque phenotype, it was possible that for either HSVEGFP0 or HSVEGFP4 recombinant virus selected, either one or two copies of EGFP fused to the immediate early genes could have recombined. Therefore, it was necessary to determine if the viruses were diploid for the fused genes by Southern blot analysis. The IE2 gene occurs only singly in the viral genome, but the presence of the gene fused to EGFP was also confirmed in the HSVEGFP27 virus.

Digestion of viral DNA with *Ase I*, *Eco47* III, and *Ssp I* should generate two fragments of different sizes that each contains the entire gene encoding ICP0 or GFP-ICP0. Figure 2.1 shows the results of hybridizing the viral DNA with a DNA probe

specific for IE1 (*top left panel*). The HSVEGFP0 virus was shown to contains two copies of genes encoding GFP-ICP0, with a corresponding 800 bp shift from wild-type HSV-1 DNA lacking GFP. When the same blot was hybridized with a GFP DNA probe, the same bands containing GFP-ICP0 genes were visualized, while the GFP probe did not bind to the wild-type virus DNA (Figure 2.1, *bottom left panel*).

Digestion of viral DNA with *Xho I* should result in two fragments of different sizes that contain either the entire gene encoding ICP4 or GFP-ICP4. Figure 2.1 depicts the Southern blot of digested HSV-1 and HSVEGFP4 DNA hybridized with a DNA probe specific to IE3 (*middle top panel*). HSVEGFP4 was shown to contain two copies of GFP-ICP4, each fragment approximately 800 bp larger than the wild-type ICP4 fragments. When a GFP probe was hybridized, the same bands that hybridized to the ICP4 probe were visualized for HSVEGFP4 DNA, while the probe did not bind to the wild-type HSV-1 DNA (Figure 2.1, *bottom middle panel*).

Restriction enzyme digestion of the wild-type HSV-1 and HSVEGFP27 viral DNA with *Bam* HI and *Stu* I should produce one band containing the genes for ICP27 or GFP-ICP27. Hybridization of the probe specific for IE2 with wild-type and HSVEGFP27 DNA resulted in one band for each virus (Figure 2.1, *top right panel*). When the same membrane was stripped and reprobed with the GFP probe, the same IE2 band from HSVEGFP27 was visible (Figure 2.1, *bottom right panel*), demonstrating that the HSVEGFP27 contained one copy of the GFP-ICP27 gene.

Temporal expression of EGFP-ICP0, EGFP-ICP4, and EGFP-ICP27 were similar to ICP0, ICP4, and ICP27 during the course of infection. The regulation of HSV-1 genes is tightly controlled and, because the addition of the EGFP could

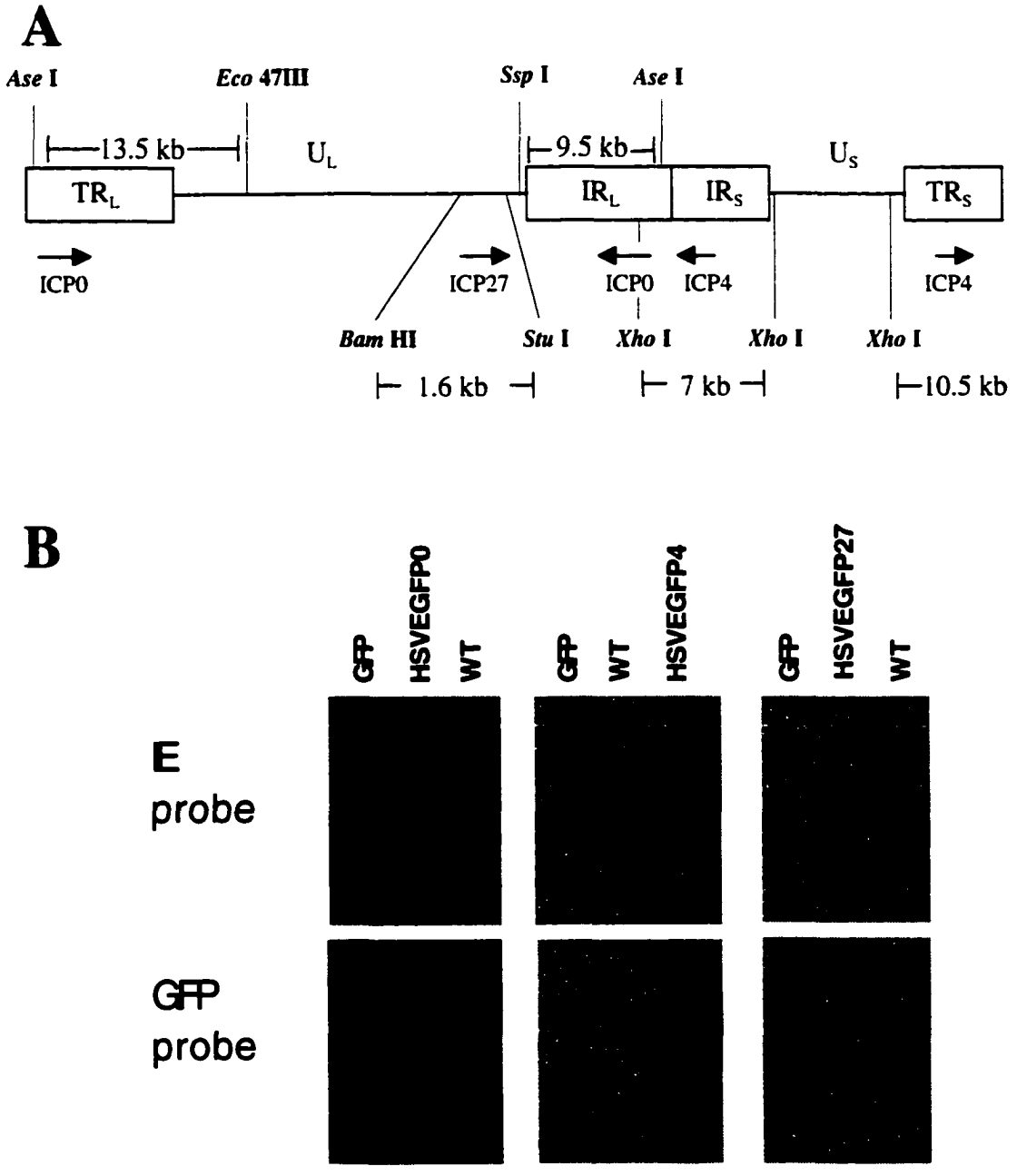

Figure 2.1 Southern blot analysis of recombinant viruses HSVEGFP0, HSVEGFP4 and HSVEGFP27. (**A**) Schematic drawing of restriction enzyme digestion of wild-type HSV-1 DNA used to generate bands on Southern blots. (**B**) Purified DNA from GFP plasmid pEGFP-C1 (**GFP**), recombinant viruses, or 17^+ wild-type (**WT**) was digested with appropriate restriction enzymes and separated by gel electrophoresis before transfer to nylon membrane. Blots were hybridized with the appropriate immediate early (**IE**) probe (top panels), then stripped and re-probed with a GFP probe (bottom panels). IE1 corresponds to ICP0, IE3 corresponds to ICP4, and IE2 corresponds to ICP27.

potentially alter the half-lives of the proteins to which it was joined, the possibility existed that the fusion proteins may not regulate the HSV-1 genes with wild-type kinetics. Therefore, it was necessary to compare the levels of protein expression between the wild-type 17^{+} and recombinant viruses. We infected Vero cells and at various times post-infection, harvested cells and performed immunoblots on the separated proteins. Cells were infected at a multiplicity of 10 pfu/cell in order to infect every cell in the culture. This was done to prevent multiple rounds of replication that would make it difficult to follow one time-course of infection.

Wild-type ICP0 displayed a peak in expression between 9 and 15 hours post-infection, with a decrease in expression after 15 hours, when detected using a monoclonal antibody to ICP0 (Figure 2.2A). We observed the same peak and decline of GFP-ICP0 fusion protein at the same timepoints in the recombinant HSVEGFP0 virus. This suggests that the addition of GFP to the ICP0 protein did not significantly alter its half-life when compared to wild-type ICP0. Additionally, the immunoblot displays the 27 kDa increase in size of GFP-ICP0 from the addition of GFP, confirming that the fusion protein was being correctly made.

ICP4 expression was seen at very early times post-infection, 5 hours, and continued to remain at high levels throughout the course of infection (Figure 2.2B). The expression of GFP-ICP4 corresponded to the expression of the wild-type ICP4 in that it was expressed at high levels by 5 hours, and continueed to be expressed strongly throughout infection. By 48 hours, nearly all cells showed CPE, and ICP4 and GFP-ICP4 expression remained high. The addition of GFP had no detectable effect on the expression levels of ICP4.

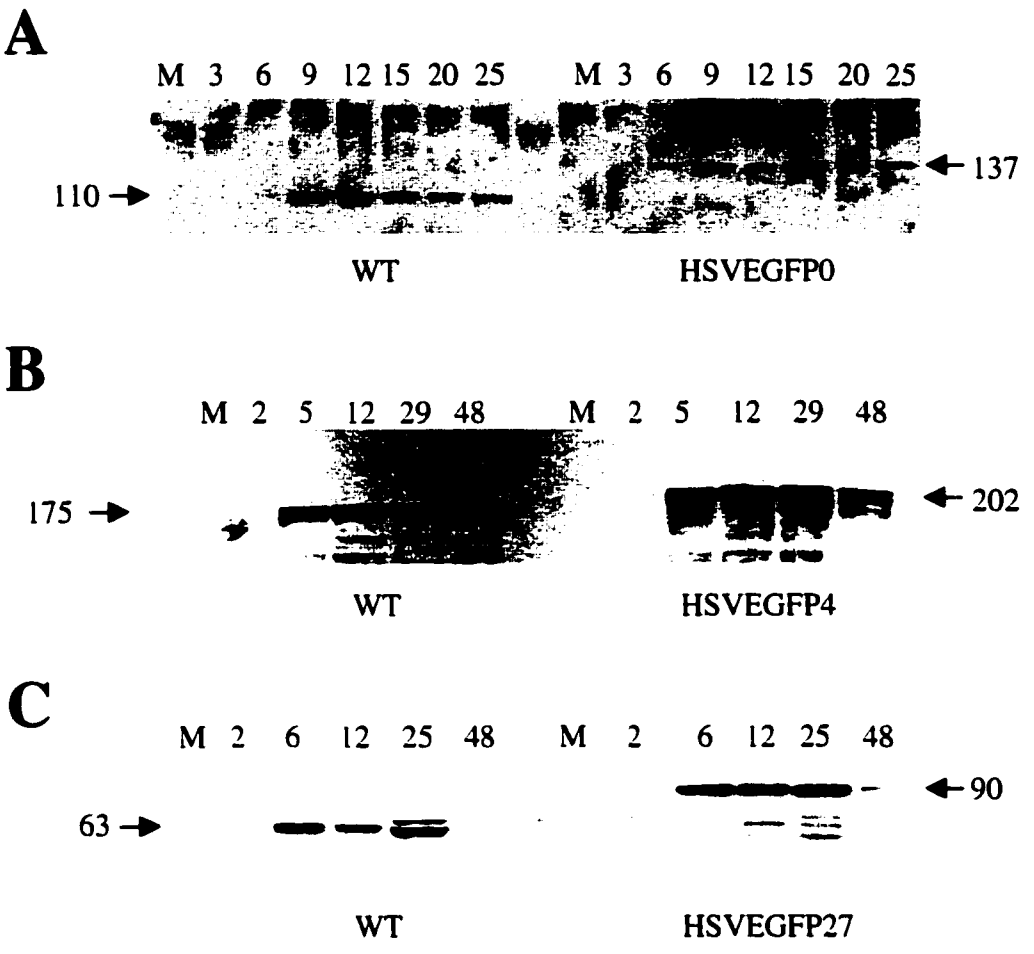

Figure 2.2 Western blot analysis of expression of immediate early proteins ICP0, ICP4 and ICP27 from wild-type virus with EGFP fused proteins from recombinant viruses during the course of productive infection. Vero cells were either mock-infected (**M**) or infected with wild-type virus (**WT**) or recombinant viruses as indicated at a multiplicity of 10 PFU per cell. Cells were harvested at various hours post-infection, as indicated by the number above the lane. Cell lysates were loaded 10μg per lane and were separated on a 7.5% acrylamide gel using SDS-PAGE. The samples were analyzed by Western blot analysis using monoclonal antibodies to ICP0 (**A**), ICP4 (**B**) or ICP27 (**C**). Numbers to left and right indicate molecular weights of proteins in kilodaltons.

ICP27 was expressed strongly by 6 hours post-infection and remained detectable by immunoblot at this level beyond 25 hours (Figure 2.2C). By 48 hours, ICP27 was not detected. The GFP-ICP27 fusion protein was expressed at high levels by 6 hours, as well, and maintained the same level of detection beyond 25 hours. However, a small amount of GFP-ICP27 was still detectable at 48 hours post-infection. This suggests that the addition of GFP may alter the half-life of ICP27.

Additionally, all of the GFP-immediate early fusion proteins could be detected using a monoclonal antibody to GFP (data not shown).

GFP-immediate early fusion protein localized correctly in HeLa cells. To confirm that the fusion of GFP did not impair immediate early protein localization, human HeLa cells were infected with the recombinant viruses and viewed at early times post-infection (Figure 2.3). All three GFP-fusion proteins localized to the nuclei of infected cells, as seen by 4',6-diamidino-2-phenylindole (DAPI) staining of nuclei. GFP-ICP4 and GFP-ICP27 were found throughout the nuclei, while GFP-ICP0 was found in discrete, punctate arrangements in the nuclei. These data confirm that the fusion proteins were not impaired in their ability to localize to their predicted sites during infection.

ICP27 shifted the localization of both GFP-ICP0 and GFP-ICP4 from the nuclei to the cytoplasm of cells in transient transfections. It has been previously shown that ICP27, an essential HSV-1 immediate early protein, affects the localization of both ICP0 (40) and ICP4 (41) in transient transfection assays, in a concentration-dependent manner. Alone, both ICP0 and ICP4 localized to the nuclei of transfected cells, but the addition of ICP27 induced cytoplasmic localization of ICP0 and ICP4 in the absence of other HSV-1 proteins. In order to test the functionality of our EGFP fusion,

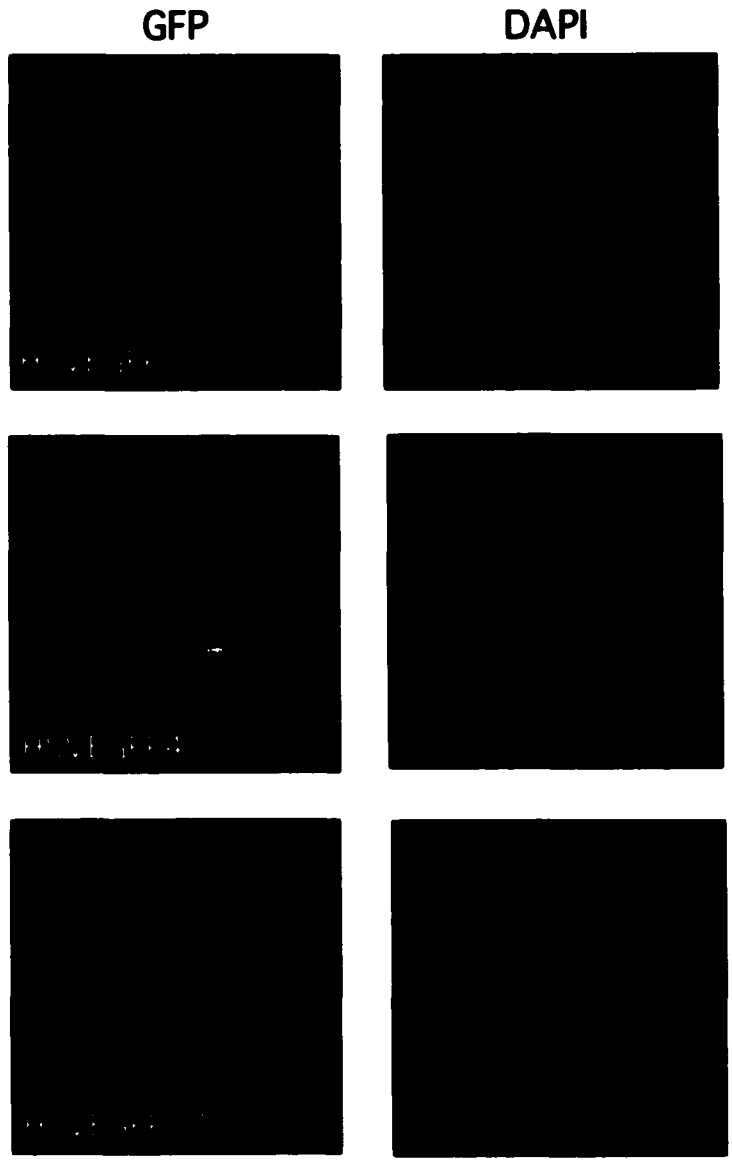

Figure 2.3 Recombinant viruses express GFP-ICP0, GFP-ICP4, and GFP-ICP27. HeLa cells were infected with recombinant viruses and photographed at 8 hours (HSVEGFP0) or 6 hours (HSVEGFP4 and HSVEGFP27) post-infection. Left panels show immediate early proteins fused to GFP. Right panels are same fields with nuclei stained with 4',6-diamidino-2-phenylindole (DAPI).

we sought to determine if the same shift in localization could be seen when each was co-transfected with a plasmid expressing ICP27.

In this experiment, three plasmid constructs were used, all driven by the human cytomegalovirus immediate early promoter (CMV): pCMV27, which contains the gene encoding wild-type ICP27; pCMV-GFP4, which contains the gene encoding GFP-ICP4; and pCMV-GFP0, which contains the gene encoding GFP-ICP0. The CMV promoter was used to drive the expression of each protein because it gives high levels of expression. When GFP-ICP0 was expressed alone in Vero cells, a punctate nuclear arrangement of the protein was seen (Figure 2.4A), while the expression of both ICP27 and GFP-ICP0 resulted in a shift of GFP-ICP0 protein to the cytoplasm (Figure 2.4B), as expected. In the same manner, GFP-ICP4 expressed alone in Vero cells resulted in a diffuse nuclear signal (Figure 2.4C), yet the protein shifted to the cytoplasm of cells that also expressed ICP27 (Figure 2.4D). The results of this experiment qualitatively demonstrate that the presence of GFP at the N-terminal regions of both ICP0 and ICP4 neither affects the nuclear localization patterns previously described for each wild-type protein, nor does it affect the interaction of each fusion protein with wild-type ICP27 in transient transfection assays.

Replication rates of recombinant viruses were similar to that of wild-type virus. In assessing the functionality of the GFP fusion proteins, it became necessary to examine the fusion proteins in the context of a normal viral infection. Thus, we compared replication rates of the recombinant viruses with wild-type HSV-1 virus to determine if the recombinant viruses exhibited wild-type or near-wild-type replication kinetics.

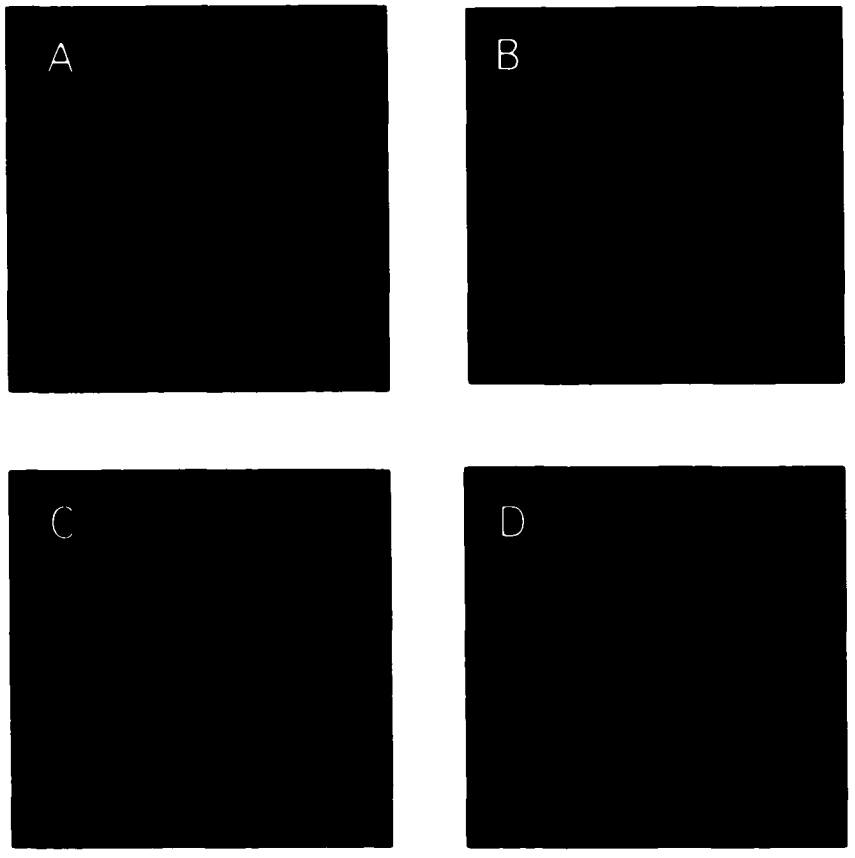

Figure 2.4 ICP27 shifts the nuclear localization of GFP-ICP4 and GFP-ICP0 to the cytoplasm. Vero cells were transfected with plasmids expressing (**A**) GFP-ICP0 alone, (**B**) GFP-ICP0 and ICP27, (**C**) GFP-ICP4 alone or (**D**) GFP-ICP4 and ICP27. All plasmids contained genes driven by the CMV promoter. Cells were photographed 48 hours after transfection using fluorescent microscopy. Pictures C and D were taken by J. Morroni.

ICP0 is not an essential protein for viral replication. However, it has been shown that at low multiplicities of infection, ICP0 minus mutant viruses replicate at considerably lower rates than wild-type virus (9, 25, 33). Because of this, a low multiplicity of infection (0.1 pfu/cell) was chosen for these studies. Figure 2.5 shows the results of the comparison of the recombinant viruses with wild-type HSV-1. At any given time-point, the titers of the recombinant viruses HSVEGFP0, HSVEGFP4, and HSVEGFP27 are never more than one log unit less than the wild-type virus. Thus, the recombinant viruses were able to replicate to titers similar to those of the wild-type virus, even at low multiplicities of infection. Additionally, the plaque sizes of wild-type, HSVEGFP0, HSVEGFP4, and HSVEGFP27 were similar (data not shown), indicating that the replication rates of the recombinant viruses are similar to that of wild-type HSV-1.

HSVEGFP0 displayed a slightly lower replication rate than the wild-type virus. However it was not statistically significantly different by Student's t test. To ensure that HSVEGPF0 replicated more efficiently than its ICP0-minus virus, dl1403, the replication rate of dl1403 was also compared. Figure 2.5A shows that dl1403 replicated to a titer of approximately 1.5 logs less than HSVEGFP0, showing that the addition of GFP-ICP0 restored wild-type-like replication kinetics. It was not possible to compare HSVEGFP4 or HSVEGFP27 with viruses lacking either ICP4 or ICP27, as such viruses are replication incompetent.

Recombinant viruses established latency similar to wild-type virus. Sensory neurons in culture have been used to study the latent phase of HSV-1 infection (34, 35, 38). One way to assess the latent infection in this system is to detect the latency-

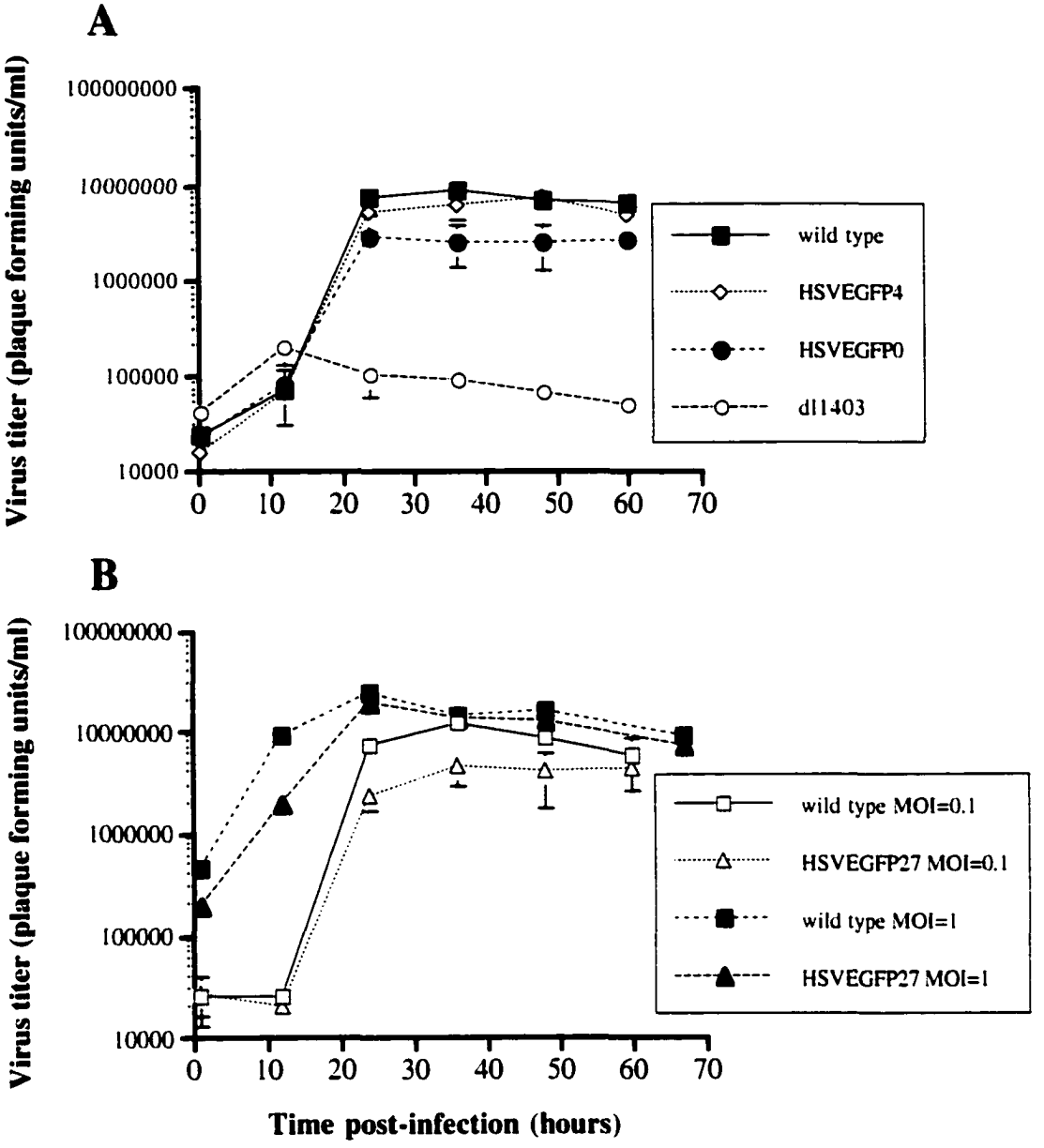

Figure 2.5 Comparison of replication rates of recombinant viruses with wild-type virus. Vero cells were infected with viruses as indicated at a multiplicity of infection (MOI) of (**A**) 0.1 PFU per cell, or (**B**) 0.1 PFU per cell and 1 PFU per cell. Dl1403 is the ICP0 minus mutant virus. Culture plates were frozen at times post-infection as indicated. Cells and virus were thawed once and titered by plaque-assay on Vero cells. Mock-infected cells at each time point showed titers of zero (not shown). The experiments were performed three times, and each graph shows the results of one experiment. Each point on the graph represents the mean titer of four cultures. Error bars indicate standard deviation.

associated transcript (LAT) by *in situ* hybridization (30). To determine if the recombinant HSV-1 viruses constructed were able to establish latency *in vitro* in a wild-type manner, latency was established in dorsal root ganglion neurons for 3 weeks with wild-type 17^+ and each recombinant virus. The number of LAT-positive neurons from each group of infected neurons was counted and compared. Results in Figure 2.6 showed that all recombinant viruses were able to establish latency in neurons at wild-type levels. Additionally, all viruses were able to reactivate upon NGF deprivation (data not shown).

Recombinant viruses expressed GFP-fusion proteins in neurons. The ultimate aim of making recombinant viruses expressing GFP fused to immediate early proteins ICP0, ICP4 and ICP27 is to study the expression of these proteins during the establishment of latency, latency, and reactivation of HSV-1 in neurons. Recombinant viruses were initially used to infect dorsal root ganglion neurons at multiplicities that would cause a lytic infection (Figure 2.7). Our observations were that in comparing all of the recombinant viruses, HSVEGFP27 expressed GFP-ICP27 at the highest level, even at low multiplicities of infection (1 pfu/cell). HSVEGFP4, like HSVEGFP27, had a high level of fusion protein expression in neurons. HSVEGFP0, however, expressed GFP-ICP0 at surprisingly low levels. Neurons had to be infected at a high multiplicity of infection (100 pfu/cell or more) to clearly see GFP-ICP0. Even with a high multiplicity of infection, the expression of GFP-ICP0 was not detected until later times in infection (24 hours or later).

HSVEGFP4 and HSVEGFP47 fusion proteins localized diffusely in the nuclei of neurons, as expected and seen in HeLa cells (Figure 2.3). However, GFP-ICP0 was found diffusely distributed in the nuclei and cytoplasm of neurons, rather than in punctate

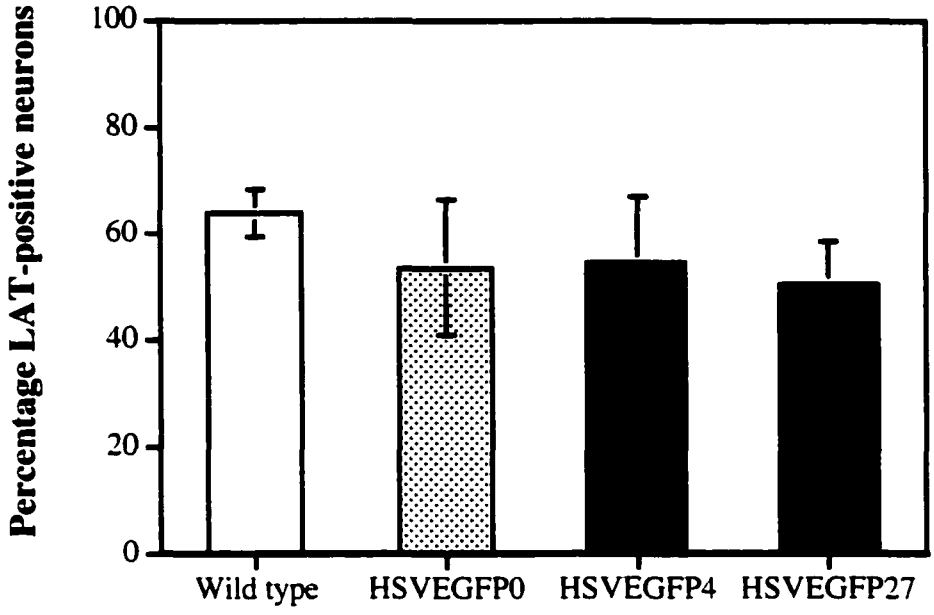

Figure 2.6 Comparison of the establishment of latency of recombinant viruses HSVEGFP0, HSVEGFP4 and HSVEGFP27 with wild-type HSV-1 in neurons by *in situ* hybridization. Dorsal root ganglion neurons were infected with wild-type or recombinant HSV-1 viruses in the presence of acyclovir to establish latency. Cultures were maintained for two weeks, then fixed for *in situ* hybridization. Cells were hybridized with a digoxigenin-labeled riboprobe specific for the latency-associated transcript (LAT). Total numbers of neurons and LAT-positive neurons were counted in 5 fields for each sample. Bars represent the mean percentage of LAT positive neurons where n=4. Error bars represent standard deviation. Experiment was performed twice, and graph represents the results of one experiment.

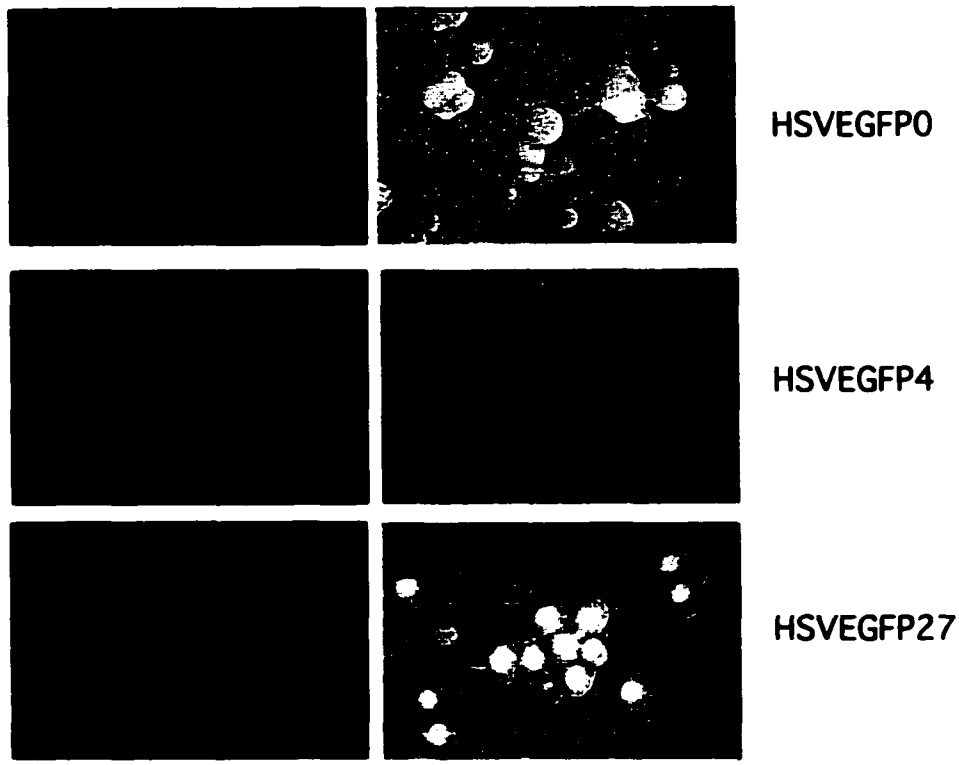

Figure 2.7 Expression of recombinant viruses HSVEGFP0, HSVEGFP4 and HSVEGFP27 in primary neurons. Neurons were infected with HSVEGFP0 at a multiplicity of infection (MOI) of 100 plaque forming units per cell (pfu/cell) and viewed at 36 hours post-infection (top panels). Neurons infected with HSVEGFP4 at MOI of 50 pfu/cell were viewed at 72 hours post-infection (middle panels). Neurons infected with HSVEGFP27 at MOI of 1 pfu/cell were viewed at 24 hours post-infection. Panels on the left are viewed with a GFP cube, while panels on the right are the same fields viewed with a FITC cube under both white light and fluorescent light.

arrangements, as seen in HeLa cells (Figure 2.3). This was a surprising observation about the behavior of ICP0 in neurons.

HSV-1 infection in Campenot chambers. In a host animal, HSV-1 replicates at mucosal surfaces and then gains access to the neuron by traveling along the axon to the cell body. In our culture system, both neurites and cell bodies are available for virus entry. In order to mimic an infection that is more like an *in vivo* infection, neurons were grown in Campenot chambers (schematic diagram shown in Figure 2.8). Campenot chambers contain a middle compartment that interacts with the surrounding medium. The two side compartments are closed off to the surrounding medium. When neurons are placed into the middle compartment after dissection, the neuronal processes extend into the closed side compartments by following the scored collagen. After two weeks in culture, many processes extend into the side compartments. Because neurites and cell bodies are physically separated in Campenot chambers, neurites alone can be infected without virus entering the neurons via the cell bodies.

When neurons were grown in Campenot chambers, and wild-type virus was added only to neurites, a latent infection was established such that when cells were reactivated with forskolin, infectious virus was produced (Table 2.1). Neurons that were latently infected but not treated with forskolin produced no infectious virus. This demonstrates that in Campenot chambers, HSV-1 infection via neurites alone results in a latent infection. This virus can then reactivate upon treatment with forskolin.

Another interesting and potentially important observation was made when the recombinant viruses HSVEGFP0, HSVEGFP4, and HSVEGFP27 were used to infect neurites but not cell bodies of DRG neurons in Campenot chambers. After 4 days of

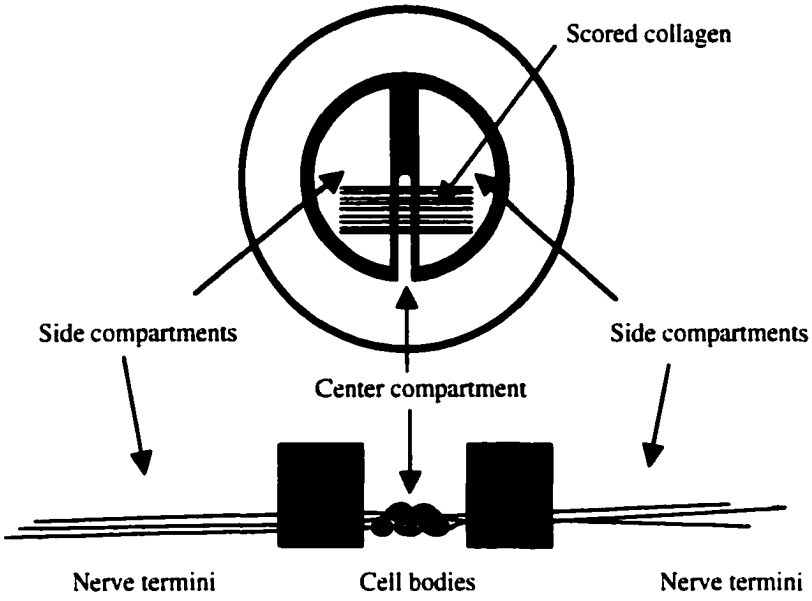

Figure 2.8 Schematic drawing of Campenot chamber. Campenot chambers physically separate nerve termini and cell bodies of neurons. Neurons are added to the center compartment where the scored collagen guides nerve termini growth into side compartments, while cell bodies remain in the center compartment.

Table 2.1 Reactivation of wild-type HSV-1 from neurons grown in Campenot chambers and infected via the neurites

treatment	sample number[a]	plaque forming units/ml[b]
untreated	1	0
forskolin[c]	1	3,000
	2	500
	3	15
	4	140,000

[a] Samples indicate individual Campenot chambers. Neurites were infected at a mutiplicity of 20 pfu/cell, assuming 200 cells per chamber, in the presence of ACV. Neurons were maintained in culture for 16 days before forskolin treatment.

[b] Neurons were freeze-thawed twice and titered on Vero cells.

[c] Neurons were grown in the presence of 100 μM forskolin for four days and then frozen for titering.

viewing cells at many different intervals, no evidence of GFP fluorescence was observed for any of the viruses (data not shown). While there was no fluorescence viewed during latency, and thus no detectable immediate early protein expression, neurons treated with forskolin were able to produce infectious virus (data not shown). This demonstrates that the establishment of latency can occur without the detectable presence of immediate early proteins ICP0, ICP4 and ICP27.

2.5 DISCUSSION

These studies have shown that the three recombinant HSV-1 viruses expressing EGFP fused to immediate early proteins ICP0, ICP4 and ICP27 behave in an essentially wild-type manner. They express GFP-fusion proteins at similar levels to wild-type proteins, and the GFP-fusion proteins are found in appropriate cellular locales in non-neuronal cells. They replicate with wild-type kinetics, and establish latency in our *in vitro* model of latency similarly to wild-type HSV-1.

These recombinant viruses are useful tools in studying HSV-1. Wild-type ICP0 and GFP-ICP0 localize to punctate regions of nuclei in many types of cells studied. However, in sensory neurons infected with HSVEGFP0, GFP-ICP0 localizes to nuclei of cells, but in a diffuse pattern and at very low levels. This surprising difference in expression based on cell type may be explained several ways: (i) the protein makeup and/or structural makeup of the neuronal nuclei is different than a dividing cell, such that ICP0 does not interact with neuronal structure or proteins to produce a punctate arrangement. (ii) Neurons degrade ICP0 at a higher level than dividing cells, as previously suggested (5). (iii) Neurons express proteins that repress the transcription of

ICP0, as some have suggested (reviewed in 22). An attempt to answer this question is reported in Chapter 4 of this dissertation.

By using these recombinant viruses, we have also observed a difference in immediate early protein expression based on route of entry into neurons. Neurons in culture expressed GFP-ICP4 and GFP-ICP27, but very little GFP-ICP0, when recombinant viruses were applied to both neurites and cell bodies. However, when recombinant viruses were inoculated onto neurites alone using Campenot chambers, there was no visible expression of GFP-ICP0, GFP-ICP4 or GFP-ICP27 in cell bodies. This shows that the route of inoculation may determine the expression of immediate early proteins. Perhaps, as virus travels up the axon, it may not bring the tegument proteins with the nucleocapsid into the nucleus. If VP16, the viral tegument protein responsible for upregulation of immediate early genes, does not enter the nucleus, this may result in a lack of immediate early protein expression.

Currently, there are no reports as to whether VP16 arrives in the nucleus following HSV-1 entry *in vivo* or *in vitro*. If VP16 does not enter the nucleus, and there is no expression of immediate early proteins ICP0, ICP4 and ICP27 during the establishment of latency, perhaps the addition of VP16, via an adenovirus, would result in ICP4 and ICP27 expression. This is supported by the finding that a mutant virus lacking both ICP0 and functional VP16 cannot express immediate early genes or replicate in Vero and HEL cells (16). In contrast to this, another study was conducted where a mutant HSV-1 virus that contained an additional VP16 gene driven by the metallothionein promoter was used to infect mice. Mice were injected with cadmium to express the VP16 in infected cells. The results of the study showed that the expression of

VP16 had no significant effect on abolishing latency or causing reactivation in mice (28). However, in this study, VP16 was expressed after the virus entered cells, and the way the virus truly functions is to bring VP16 protein in the virion with it rather than express VP16 as an immediate early protein. This factor may be critical, for example, because before VP16 can be made, cellular factors bind to immediate early gene promoters and repress them. To test this, it would be necessary to express VP16 in cells before HSV-1 arrives.

ICP4 is critical to replication, so viruses that lack ICP4 fail to reactivate (13). However, ICP4 is not critical to the establishment of latency in the *in vitro* neuronal model (36). ICP0 has been previously shown to be important in establishing latency in the *in vitro* neuronal model (37), and important in reactivation in mouse models and trigeminal ganglia *in vitro* (1, 12, 13). HSVEFGFP0, HSVEFGFP4 and HSVEGFP27 will be useful means to dissect the various contributions that the immediate early proteins make to latency and reactivation. They will be particularly useful in the *in vitro* model of latency where GFP expression can be analyzed over time in the same cells.

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CHAPTER 3

HVEC IS THE MAJOR RECEPTOR FOR ENTRY OF HSV-1 IN NEURONS

The work in this chapter was a result of collaborative effort with others. Dr Christine Wilcox performed the recombinant HveA and HveC receptor and anti-HveA and anti-HveC experiments in neurons. Scott Simpson helped in generating the recombinant HveA and HveC dose response curve in neurons. C. Wilcox was also responsible for the dissection of mouse and rat neurons. Reagents were generously provided by Dr Roselyn Eisenberg and Dr Gary Cohen. Dr. Lewis Pizer was instrumental as a liason as well as an intellectual contributer. Part of the work in this chapter will be submitted to Journal of Virology:

Richart, S., Simpson, S., Pizer, L., Eisenberg, R., Cohen, G., and C. Wilcox. HveC is the major entry receptor used by HSV-1 to enter rat neurons (in preparation)

Part of the work in this chapter was presented at the following meetings:

Simpson S., Richart S., Wilcox C., Pizer L., Krummenacher C., Whitbeck C., Cohen G., Eisenberg R., and P. Spear. (1999) Entry mediators of HSV-1 infection of the neuron. 24th International Herpesvirus Workshop.

Richart S., Simpson S., Pizer L., Krummenacher C., Whitbeck C., Cohen G., Eisenberg R., and C. Wilcox. (2000) HveC mediates entry of HSV-1 into dorsal root ganglion neurons. 25th International Herpesvirus Workshop.

3.1 ABSTRACT

Herpes simplex virus type 1 (HSV-1) has the ability to establish latent infections in neurons of the peripheral sensory nervous system. Reactivation of latent HSV-1 produces disease and transmissible virus. HSV-1 can infect many cell types via at least several cellular receptors. In addition to the heparan sulfate receptor, several co-receptors, including herpesvirus entry mediator A (HveA) and C (HveC), have recently been shown to interact with the viral glycoprotein D (gD). The receptors involved in HSV-1 infection of sensory neurons have not previously been identified.

Primary rat or mouse sensory neurons in cultures were used to examine HSV-1 entry into normal, terminally differentiated neurons following direct inoculation of the cells with HSV-1. Recombinant HSV-1 expressing the green fluorescent protein was used to detect virus entry into neurons. Soluble, truncated forms of HveC, but not HveA, prevented the entry of virus into rat and mouse neurons. Antibodies against HveC, but not HveA, blocked the infection of rat neurons. Neither anti-HveC nor anti-HveA blocked the infection of mouse neurons. One soluble gD mutant, gD(Δ290-299) was able to block some HSV-1 entry in rat neurons, but not the soluble form gD(306t). Western blot analyses indicted the absence of HveA in rat neuronal cultures. These results indicate that HveC serves as a major entry molecule for HSV-1 infection of rat sensory neurons *in vitro*, which may have important implications for understanding critical aspects of HSV-1 pathogenesis. The difference seen in the ability of HveC antiserum to block HSV-1 entry in rat and mouse neurons is discussed.

3.2 INTRODUCTION

Herpes simplex virus type 1 (HSV-1) has a broad host range. In addition, HSV-1 is able to infect many different cell types. Recent studies have identified a number of cell surface proteins that can serve as receptors for HSV-1 entry. HSV-1 binds initially to heparan sulfate via viral glycoproteins gC or gB (reviewed in 25). However, this interaction is not sufficient to mediate viral entry. The binding of viral glycoprotein D (gD) to additional cellular receptors is necessary for the entry. Glycoprotein D has been shown to bind to three structurally unrelated cellular receptors: HveA (16, 17, 29), HveC (6, 9) (or a splice-variant of HveC, termed HIgR or nectin-1β (5)), and 3-*O*-sulfated heparan sulfate (22). Additionally, HveA and HveC have each been shown to bind to different regions of gD (9).

HveA, also known as HVEM, TR2 and ATAR, is a member of the tumor necrosis factor receptor superfamily of receptors. Its cellular ligands include the pro-inflammatory cytokine lymphotoxin-α, and the membrane-associated protein LIGHT (13). HveA receptors are shown to be important for entry of free virus into cells, as well as for cell-to-cell spread of HSV-1 (19, 28).

HveC, also called poliovirus receptor-related protein-1 (Prr-1) or nectin-1α, is a member of the immunoglobulin superfamily and is involved in cell-cell adhesion (20, 27). Recently, mutations in HveC genes have been shown to cause an autosomal cleft lip/palate syndrome in humans (26). HveC also plays a critical role in cell-cell spread of wild-type HSV-1 (4).

Based on the high level of HveC mRNA expression in the human central nervous system (5) as well as in neuronal cell lines (6) and mouse brains (21), it has been

suggested that HveC may be the neuronal receptor for HSV-1. More recently, HveC protein was found abundantly in sensory neurons, but not in motor neurons (12), and HveC transcripts were found in sensory, sympathetic, and parasympathetic neurons, as well as many specific locations in the central nervous system (7). In contrast, HveA mRNA is expressed weakly in human brain tissue (10), and its expression in the peripheral nervous system is unknown. Since peripheral sensory neurons are the major sites for latent HSV-1, the mechanism of entry into sensory neurons is important to the understanding of the pathogenesis of HSV-1.

HSV-1 entry into primary rat and mouse sensory neurons *in vitro* was investigated using soluble recombinant forms of HveA and HveC, glycoprotein D, as well as antibodies against the receptors. Our results indicate that HveC is the major cellular receptor used by HSV-1 to enter rat sensory neurons.

3.3 MATERIALS AND METHODS

Cells and viruses. Vero cells and HeLa cells (American Type Culture Collection [ATCC], Rockville, MD) and primary rat fibroblasts were cultured in Dulbecco's modified eagle medium (DMEM) plus 5% fetal bovine serum (FBS) (Invitrogen Life Technologies, Rockville, MD). Clone 9 cells (ATCC) were cultured in Ham's F12K medium (Invitrogen Life Technologies) plus 10% FBS. Neuronal cultures were prepared from dorsal root ganglia (DRG) of embryonic day 15 rats and maintained as previously described (30, 31). DRG neurons were plated at approximately 5×10^3 cells per well in 24 well plates.

The virus used in these studies is HSVEGFP4, a recombinant HSV-1 (strain 17^+) expressing green fluorescent protein (GFP) fused to the C-terminus of the immediate early gene product, ICP4 (see Chapter 2).

Antibodies. Primary rabbit antisera raised against HveC (R166 and R155), HveA (R140) (17), and glycoproteins D (R45), and mouse anti-HveC (CK41) (8) (all provided by Roselyn Eisenberg and Gary Cohen, University of Pennsylvania, Philadelphia) were used in these studies. Secondary antibodies were peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (Vector laboratories, Burlingame, CA).

Blocking of HSVEGFP4 entry into DRG neurons and rat primary fibroblasts by soluble HveA, HveC, or anti-gD antiserum. Recombinant HveA(200t) or HveC(346t), which are both truncated, soluble forms of the receptors HveA and HveC, were made from a baculovirus expression system, as previously described (9, 29). A multiplicity of infection of 100 pfu/cell was used to achieve a productive infection, and 2.7×10^{-6} μg/pfu of each receptor was added to the virus suspension. Soluble receptors were incubated with virus for 2 hours prior to infection at 4° C. Alternatively, R45 anti-gD antiserum was added to virus suspension at 100μl/ml. After 2 hour, virus plus soluble receptor or virus plus anti-gD antiserum were added to neurons and incubated for 1 hour at 35° C. Virus medium was then taken off, and fresh medium was added to neurons. Virus was allowed to grow for 18 hours. Infected cells were then visualized and analyzed by fluorescent microscopy. Data are expressed as percent GFP-positive cells ((number of GFP-positive cells in field / number of cells in field) × 100).

The study with control primary rat fibroblasts was similar to above, except that cells were plated at 2×10^4 cells per well. A multiplicity of 10 pfu/cell was used, and

approximately 3×10^{-6} µg/pfu of each receptor was added to the virus suspension. Virus was allowed to grow for 8 hours at 37°C, then cells were fixed in 4% paraformaldehyde for 45 minutes at 4° C prior to analysis.

Blocking of HSVEGFP4 entry into DRG neurons and rat primary fibroblasts by antibodies to HveA and HveC. Cells were incubated at 35° C with antiserum to HveA (R140) or HveC (R166) for 1 hour prior to infection. 100µl antiserum was added per 1 ml of medium, and 200 µl of this mixture was added per well of cells. After incubation with antisera, HSVEGFP4 virus was added at a multiplicity of 100 pfu/cell. Virus incubated with cells for 1 hour, then was taken off and replaced with fresh medium. Virus grew in neurons for 18 hours at 35° C, then infected cells were visualized and analyzed by fluorescent microscopy. Data are expressed as percent GFP-positive cells ((number of GFP-positive cells in field / number of cells in field) × 100).

The same study was performed in primary rat fibroblasts with the following differences: cells were plated at 2×10^4 cells per well on 24 well plates, cells were incubated with 50 µl antiserum per 1 ml of medium prior to infection. Cells were then infected with HSVEGFP4 virus at a multiplicity of 10 pfu/ml. After 8 hours at 37° C, cells were fixed in 4% paraformaldehyde for 45 minutes at 4° C prior to visualization and analysis.

Blocking of HSVEGFP4 entry into DRG neurons with soluble glycoprotein D. Two soluble forms of viral glycoprotein D (gD), gD(306t) (24) and gD(Δ290-299t) (29), were used that were created using the baculovirus system (provided by R. Eisenberg and G. Cohen). gD(306t) contains the same sequence as viral gD protein from the KOS strain of HSV-1 up to amino acid 306. gD(Δ290-299t) is the same as gD(306t), except

amino acids 290 to 299 are deleted and replaced by 4 different amino acid residues (3, 18). Neurons were preincubated with either form of soluble gD at various concentrations for 90 minutes on ice before being infected with 100 pfu/ml HSVEGFP4 virus. Virus adsorbed to cells for 90 minutes on ice, then cells were rinsed once with medium and grown at 35°C. 24 hours after virus infection, cells were visualized by fluorescent microscopy and analyzed. Data are expressed as percent GFP-positive cells ((number of GFP-positive cells in field / number of cells in field) × 100).

Western blot analysis. Cells were harvested in RIPA buffer plus protease inhibitors (100 µg/ml PMSF, 1 µg/ml leupeptin, 20 µg/ml aprotinin and 1µg/ml pepstatin). Proteins were resolved on SDS-polyacrylamide gels, and transferred to nitrocellulose (Pro-bind, Amersham Pharmacia, Piscataway, NJ) for immunodetection. Nitrocellulose blots were blocked overnight at 4° C in 1× Uniblock blocking reagent (Analytical Genetic Testing Center, Inc., Denver, CO), diluted in phosphate-buffered saline (PBS) plus 0.1% Tween-20, before antibodies were added. Antibodies were diluted in blocking buffer and incubated with membranes. The Renaissance Western Blot Chemiluminescence Reagent Plus substrate for peroxidase (NEN Life Science, Boston, MA) was used according to the manufacturers' protocol. Kodak BioMax film was used to visualize protein bands, and film was scanned into Adobe Photoshop 6.0.

3.4 RESULTS

Protein sequence alignment of human, mouse and rat HveC. Previously, mouse and human HveC were reported to be 95% identical in amino acid sequence (21). While the rat HveC gene has only been partially sequenced on its 3' end, an alignment of the carboxy-terminal end of all three protein sequences was created (Figure 3.1). The last

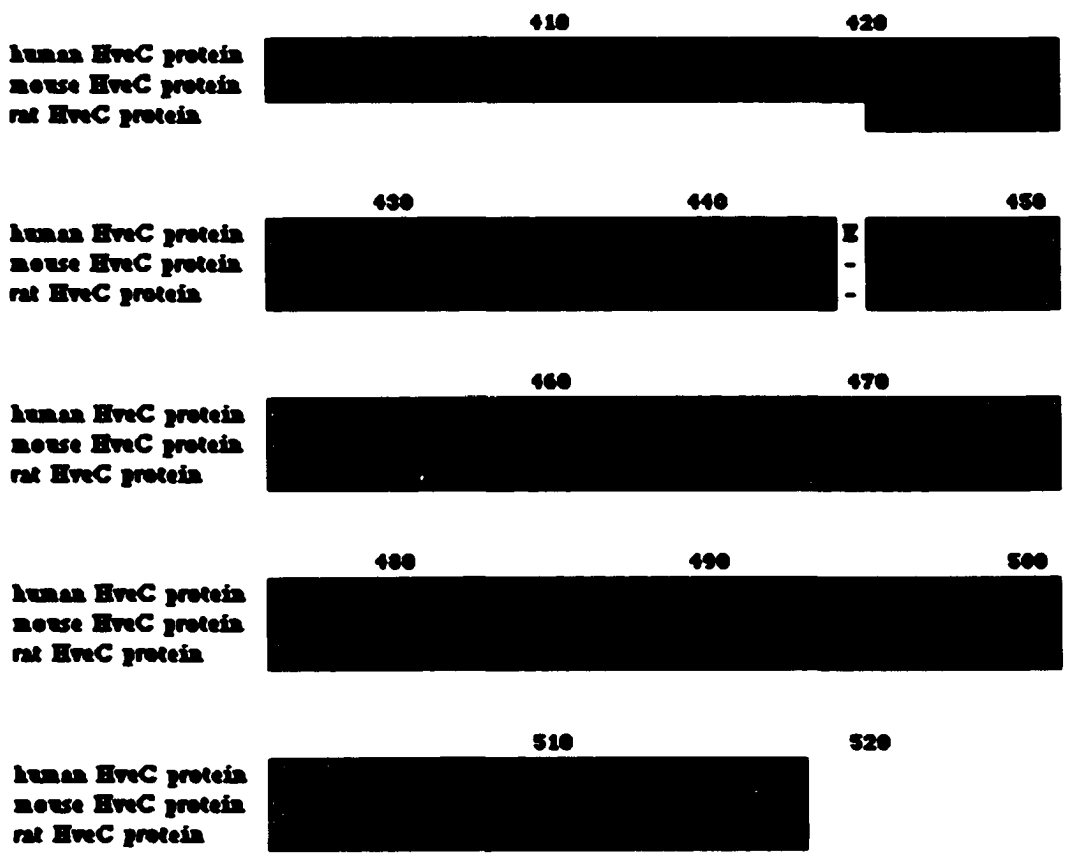

Figure 3.1 Partial protein sequence alignment of the C-terminual end of HveC from human, mouse and rat. Protein sequences were obtained from GenBank, accession numbers ACC23798 (human), AAF76195 (mouse), and AAD26534 (rat). Only the partial protein sequence was available for rat HveC. Sequences aligned using ClustalW alignment on MacVector software.

97 amino acids of rat and mouse HveC are 100% identical, and human HveC contains the identical sequence, with the exception of one additional amino acid.

Effects of soluble HveA or HveC receptors on HSV-1 entry into primary rat fibroblasts and primary rat and mouse sensory neurons. Previous studies have demonstrated that soluble forms of HveA and HveC receptors can block HSV-1 entry into cell lines, as well as cells stably transfected with native forms of HveA or HveC (6, 16). Using similar experimental conditions, we examined the ability of soluble HveA and HveC to inhibit HSV-1 infections of primary sensory neurons in culture. To compare virus entry, cells were inoculated with a recombinant HSV-1 virus expressing green fluorescent protein (GFP) fused to ICP4, which allowed visualization of infected cells. Additionally, infected cells were maintained in media containing acyclovir. This was to ensure that all GFP expressed was from the original inoculum of virus, not as a result of virus replication and subsequent infection.

Rat neurons infected with recombinant HSVEGFP4 at a MOI of 100 pfu/ml resulted in 61% GFP-positive cells at 24 hours post-infection (Figure 3.2A). Incubation of virus with recombinant HveA before addition to rat neurons resulted in 71% GFP-positive cells, slightly higher than with virus alone. However, the addition of HveC to virus reduced the percentage of GFP-positive rat neurons to 17%, an approximate 3-fold reduction in viral entry. Similar results were obtained using primary rat fibroblasts (Figure 3.2B), where addition of recombinant HveC, but not HveA, to virus blocked viral entry at 8 hours post-infection. Similarly, in the mouse neuronal cultures recombinant HveC blocked HSV-1 entry, whereas recombinant HveA resulted in a somewhat higher

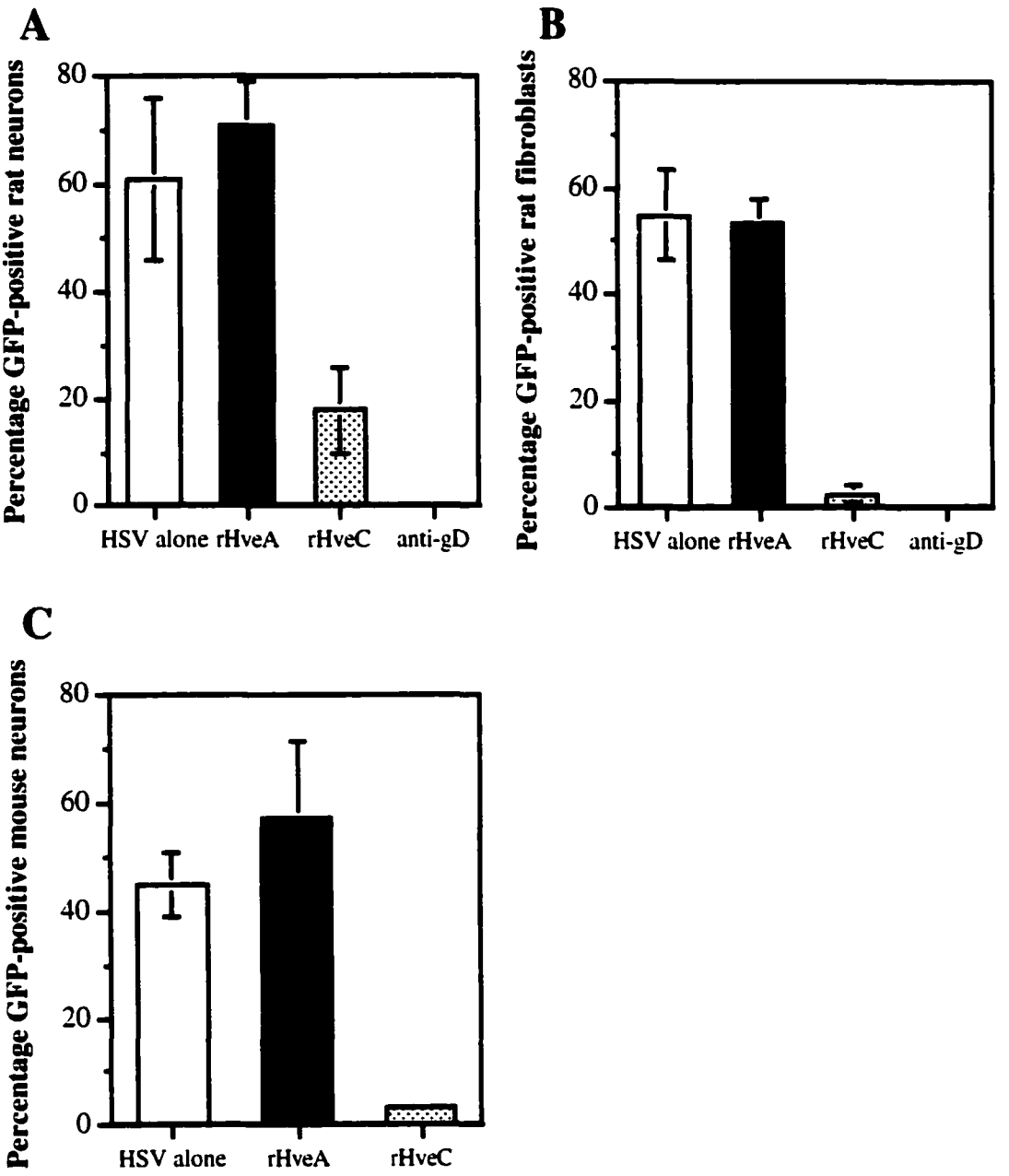

Figure 3.2 Comparison of the effects of soluble recombinant HveA or HveC on viral entry into primary rat and mouse neurons, and primary rat fibroblasts. A recombinant HSV-1 virus that expresses ICP4 fused to GFP, HSVEGFP4, was incubated with either recombinant HveA, HveC, or anti-gD antibodies (**A** and **B** only) for 2 hours prior to inoculation of sensory neurons or rat fibroblasts. A MOI of 100 PFU per cell was used for (**A**) rat sensory neurons and (**C**) mouse sensory neurons. A MOI of 10 PFU per cell was used for (**B**) rat fibroblasts. Neurons require a higher MOI than many other cell types in order to infect the same percentage of cells. For each study, the amount of recombinant receptor added was 2.7×10^{-6} µg receptor per PFU, or 100µl anti-gD antiserum per ml. Each bar represents the average percentage of GFP-positive cells per sample. Error bars indicate standard deviation

percentage of GFP-positive neurons (Figure 3.2C). Recombinant HveA was unable to significantly block virus entry into all cells tested.

To ensure that the GFP-ICP4 fusion protein expressed from the recombinant HSV-1 virus HSVEGFP4 did not somehow affect viral entry, another recombinant virus that expresses GFP fused to ICP27 was also tested (HSVEGFP27). Recombinant HveA and HveC incubated with HSVEGFP27 resulted in similar levels of GFP-positive rat neurons (data not shown).

To test the possibility that the amount of HveA or HveC used was insufficient to block viral entry, additional concentrations were used to treat virus. Incubations with a range of concentrations of HveA did not show any effects of recombinant HveA on viral entry of the rat sensory neurons (Figure 3.3A). In contrast, incubation of virus with a range of concentrations of recombinant HveC showed a dose-dependent decrease in HSV-1 entry into the neurons (Figure 3.3B). These data show that incubation of virus with soluble recombinant HveC, but not HveA, resulted in decreased HSV-1 entry into primary rat and mouse sensory neurons, as well as primary rat fibroblasts.

While these data do not firmly establish that HveC is the primary entry molecule used by HSV-1 to enter rat neurons, they do establish and confirm that viral glycoprotein D is the means by which virus gains access to rat neurons, as well as rat fibroblasts. This is shown most convincingly by the incubation of virus with antiserum against viral gD, which was capable of completely prevented infection of rat neurons and rat fibroblasts (Figure 3.2A and B).

Soluble gD(Δ291-299) but not gD(306t) inhibited virus entry into rat neurons. One way to confirm the specificity of viral gD for entry into rat neurons is to incubate

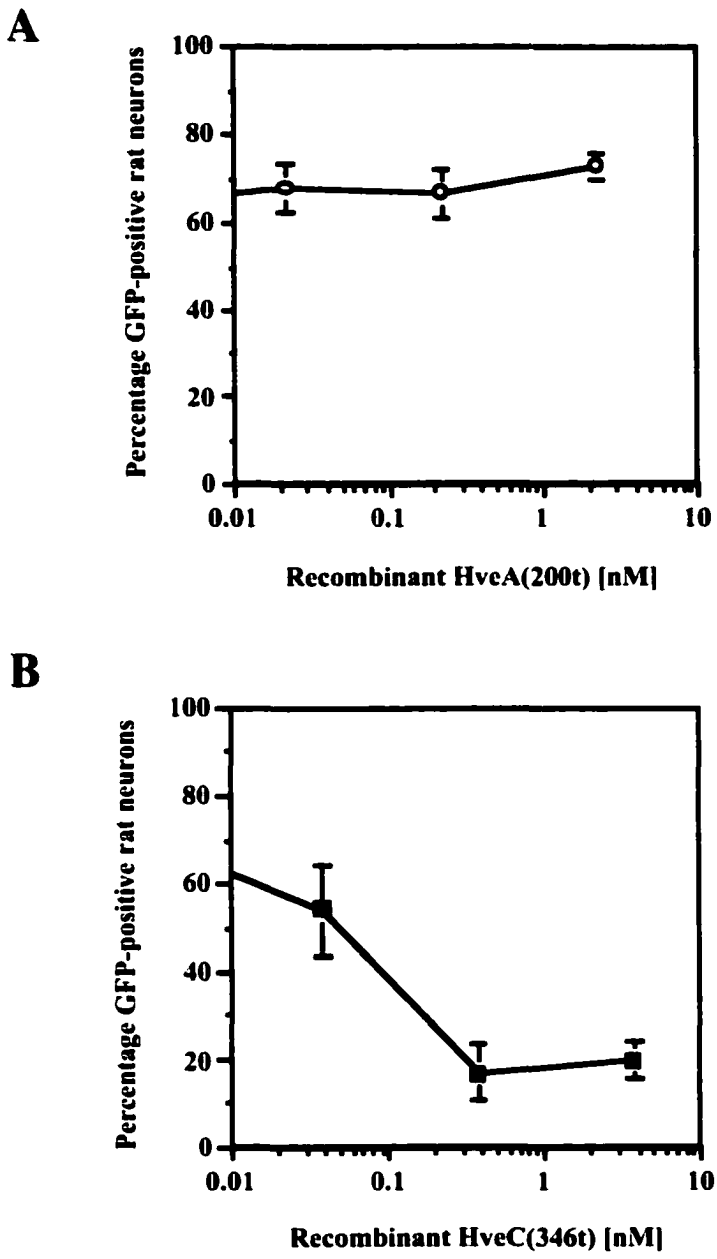

Figure 3.3 Dose-response effects of soluble recombinant HveA and HveC on viral entry into primary rat sensory neurons. HSVEGFP4 virus was incubated with soluble forms of (**A**) HveA or (**B**) HveC at the indicted concentrations for 2 hours prior to inoculation of rat sensory neurons. A MOI of 100 PFU of HSVEGFP4 per cell was used. Each bar represents the average percentage of GFP-positive cells per sample. Error bars indicate standard deviation (n=4). Experiment performed by Dr. Christine Wilcox and Scott Simpson.

neurons with soluble recombinant forms of gD. Previously, it has been reported that the expression of gD in cells blocked HSV-1 entry (2, 11), and that soluble gD incubated with cells blocked HSV-1 entry (29). In CHO cells that were stably transfected with HveA, only gD(Δ291-299) was able to block virus entry, while in Vero cells, both gD(306t) and gD(Δ291-299) were able to block virus entry (29).

In this experiment, rat neurons were incubated with both forms of the recombinant gD protein at several concentrations prior to infection with HSVEGFP4. Only gD(Δ291-299) was able to block virus entry into neurons (Figure 3.4), where the highest concentration (1 μM protein) resulted in 33.4% of neurons expressing GFP, compared to 52.9% of neurons expressing GFP in cells not treated with gD(Δ291-299). Even gD(Δ291-299) at a concentration of 100 nM resulted in 41.5% virus entry into neurons, a significant difference from untreated neurons ($p=0.04$, one-tail). We speculate that at a higher concentration of gD(Δ290-299), more virus would be blocked from entering neurons, as we did not reach a maximal blocking concentration. Recombinant gD(306t) was not able to block HSV-1 entry into rat neurons.

Effects of antibodies against HveA or HveC on HSV-1 entry into primary rat fibroblasts and primary rat and mouse sensory neurons. To determine whether HSV-1 uses cellular HveA or HveC to gain entry into cells, antibodies against HveA or HveC were incubated with cells prior to infection with HSV-1. Cells were incubated with either HveA or HveC rabbit antiserum prior to infection with HSV-1.

In rat neurons, anti-HveA treatment slightly decreased viral entry over no treatment (Figure 3.5A). However, preincubation with HveC antiserum greatly reduced the number of GFP-positive rat sensory neurons, resulting in greater than a 90%

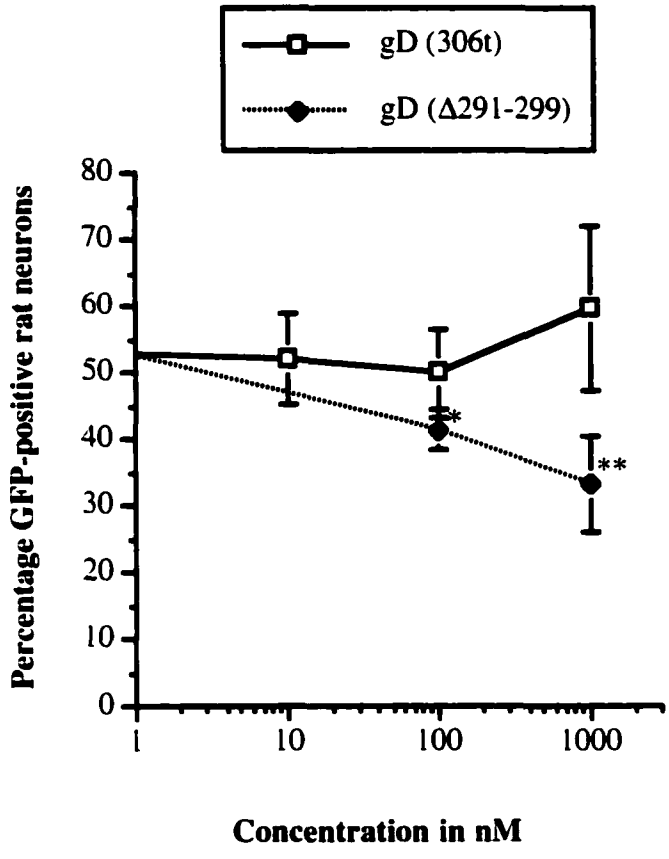

Figure 3.4 Effect of incubation of glycoprotein D with rat neurons on virus entry. Rat DRG neurons were incubated with two different recombinant glycoprotein Ds, gD (306t) and gD (Δ291-299), prior to infection with HSVEGFP4 virus. Cells were infected at a MOI of 100 pfu/cell. Each point represents the average percentage of GFP-positive cells per group. Error bars indicate standard deviation (n=3 or 4). * p=0.04, **p=0.004, one-tail by Student's t test.

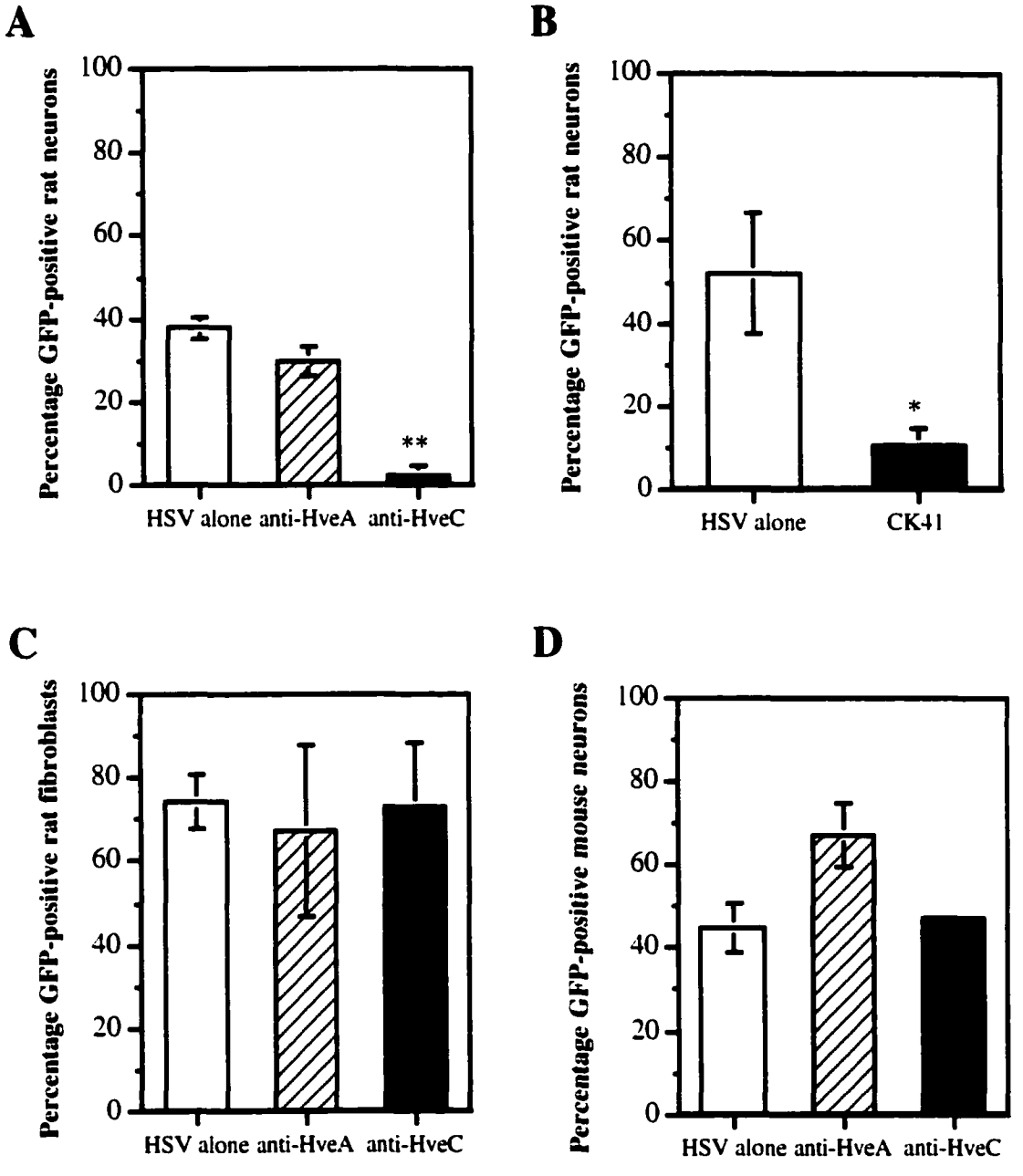

Figure 3.5 Comparison of the effects of pretreatment of cells with antibodies to HveA or HveC on viral entry into primary rat and mouse sensory neurons and primary rat fibroblasts. Cells were pretreated with antibody against either HveA or HveC for 1 hour prior to inoculation with HSVEGFP4. Anti-HveA and anti-HveC are rabbit antisera R140 and R166 respectively, while CK41 (**B**) is a monoclonal antibody raised against HveC. A MOI of 100 PFU per cell was used for (**A, B**) rat and (**D**) mouse sensory neurons. A MOI of 10 PFU per cell was used for (**C**) rat fibroblasts. Each bar represents the average percentage of GFP-positive cells per group. Error bars indicate standard deviation (n=3 or 4). * p=0.011, **p<0.001, each one-tail.

reduction in virus entry compared to untreated neuronal cultures (Figure 3.5A) ($p< 0.001$, one tail). Similarly, rat neurons were treated with a monoclonal antibody against HveC (CK41), an antibody that has been shown to block the gD binding site on cellular HveC (8). Incubation of rat neurons with this monoclonal antibody caused a 60% decrease in viral entry into rat neurons (Figure 3.5B), a significant decrease in entry ($p=0.011$, one-tail). These results indicate that HSV-1 used HveC to gain entry into rat sensory neurons.

The observed ability of anti-HveC antibodies to interfere with HSV-1 entry was restricted to the rat sensory neurons. Surprisingly, treatment with HveC antiserum did not affect HSV-1 entry into mouse sensory neurons (Figure 3.5D). The monoclonal anti-HveC antibody CK41 also did not block HSV-1 entry into mouse neurons (data not shown). Treatment of other cells, including primary rat fibroblasts (Figure 3.5C), Clone 9 or HeLa cells (data not shown), with HveC antiserum was not effective at blocking HSV-1 entry.

HveA protein was not present in rat sensory neurons. The inhibition of HSV-1 infection of rat sensory neurons by soluble HveC and anti-HveC antibodies suggested that the HveC receptor was the main functional receptor available on the rat sensory neurons. The presence of HveC on these neurons has previously been demonstrated (7, 12). However, the presence of HveA has not yet been investigated in this cell type.

We detected HveA in human HeLa cells and primary rat fibroblasts with the predicted molecular weight of about 35kDa by western blot (Figure 3.6). Since the R140 antiserum picked up the same size band in the primary rat fibroblasts as in the HeLa cells, it was clear that R140 could detect rat HveA, even though it was generated against the

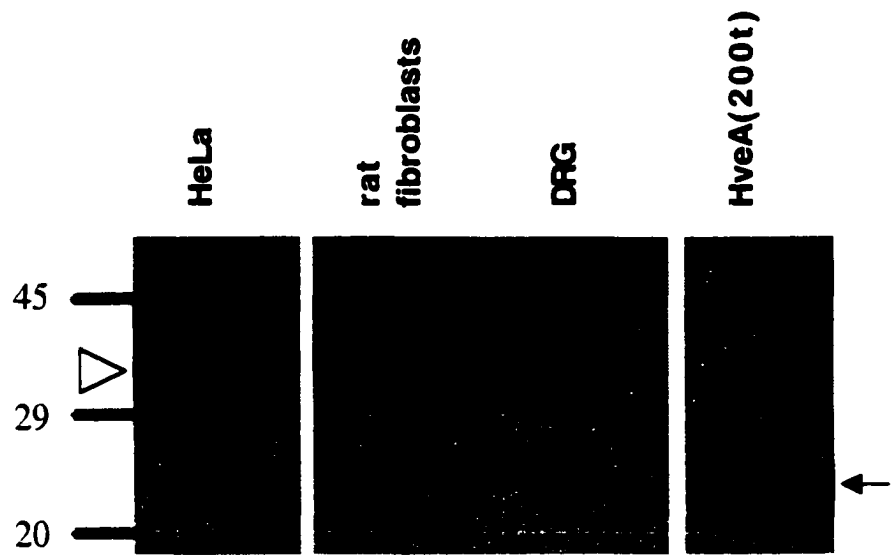

Figure 3.6 HveA is not expressed in DRG neurons. 30μg protein from whole cell lysates from HeLa cells, primary rat fibroblasts, and DRG neurons, and 2 ng of recombinant HveA(200t) protein, were separated by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit antiserum to HveA (R140). All lanes were from the same film at the same exposure. Open triangle shows cellular HveA, arrow shows recombinant truncated HveA. Numbers to the left indicate molecular weight markers in kilodaltons.

human HveA protein originally cloned from HeLa cells. In the rat neuronal cultures, there was no corresponding HveA band. Thus we conclude that HveA is not present on rat DRG neurons.

Localization of GFP-ICP4 in rat and mouse neurons. In the previous experiments, the effects of recombinant HveA, HveC, and antibodies to HveA and HveC, on the infectivity of neurons by HSVEGFP4 were studied and compared in rat vs. mouse neurons. One observation noted over the course of these experiments is that the cellular localization of the GFP-ICP4 differs between rat and mouse neurons. At 20 hours post-infection, GFP-ICP4 is arranged in punctate regions within the nuclei of rat neurons (Figure 3.7, top panels). However, in mouse neurons, GFP-ICP4 is located diffusely in the nuclei (Figure 3.7, bottom panels). The implications of this phenomenon will be discussed.

3.5 DISCUSSION

While recent studies show that HSV-1 mutants lacking glycoproteins B, D and H are unable to infect primary rat sensory neurons (1), little is known about HSV-1 entry into sensory neurons, the major site of HSV-1 latency. The studies presented here demonstrate that HveC is the functional HSV-1 viral entry receptor for primary rat sensory neurons *in vitro*, and that this entry is mediated through viral glycoprotein D. We cannot, at this time, conclude which neuronal receptor HSV-1 uses in mice.

Our results suggest that HveA was not utilized as a receptor for HSV-1 entry into either rat or mouse sensory neurons in culture. Indeed, HveA was undetectable in the rat sensory neuronal cultures by western blotting, but detectable in rat primary fibroblasts. It is still possible that the rabbit anti-HveA antibodies are not good at blocking rodent HveA

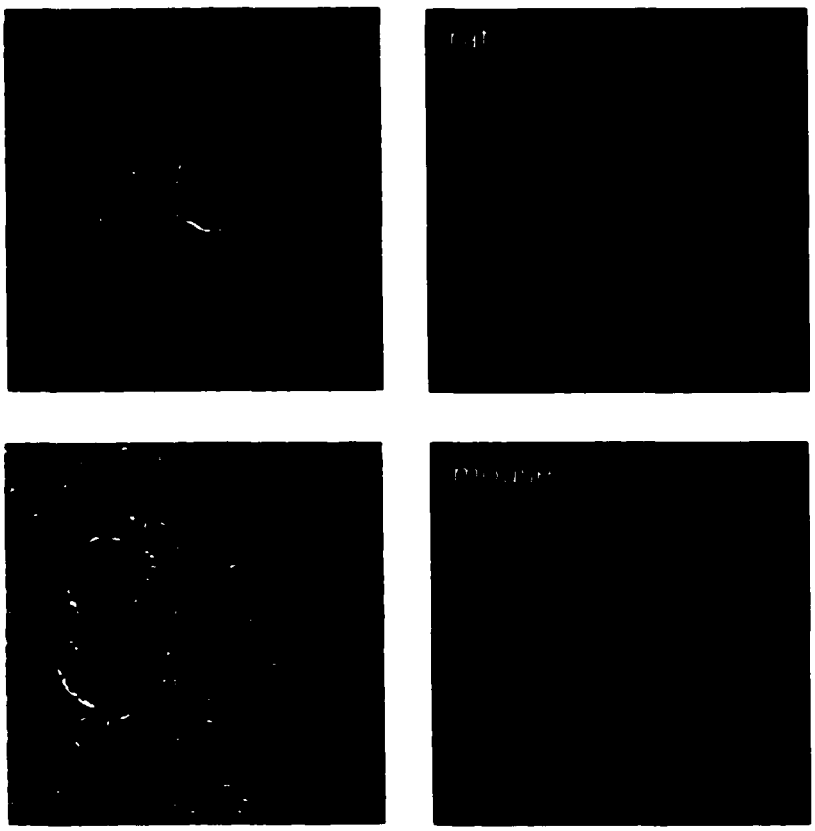

Figure 3.7 Localization of GFP-ICP4 in rat and mouse neurons. Rat and mouse dorsal root ganglion neurons were infected with HSVEGFP4 at a MOI of 100 pfu/cell and photographed 20 hours post-infection under white light (left panels). The same fields were viewed using epifluorescence to visualize GFP-ICP4 (right panels). Note the punctate nuclear localization in rat neurons, while mouse neurons have diffuse nuclear localization of GFP-ICP4.

in vitro, but may be better at binding denatured proteins by western blot. If a panel of monoclonal antibodies against HveA that block viral entry into human cells were available, this question could be further investigated. While primary rat fibroblasts expressed HveA as detected by western blot, neither soluble HveA nor anti-HveA blocked viral entry. This is not surprising, since we have been able to detectHveC protein in primary rat fibroblasts by western blot (data not shown). If rat HveA were able to be blocked by antibodies, HveC would still be available for gD binding. Thus, if both anti-HveA and anti-HveC antibodies were incubated with the primary rat fibroblasts, perhaps we would see a greater inhibition of HSV-1 entry into these cells.

Likewise, virus was still able to enter rat fibroblasts treated with antibodies to HveC, probably because HveA was still available. In contrast to virus treated with HveA, however, recombinant HveC protein incubated with virus decreased virus entry into rat fibroblasts, despite the presence of HveA on the cells, consistent with previous studies (6). This may indicate that at the levels used in these experiments, recombinant HveC blocks not only the HveC but also the HveA and/or the 3-*O*-sulfated heparan sulfate binding domains of viral gD.

Treatment with anti-gD antibodies completely prevented virus entry into all of the cell types tested. While antibodies to HveC drastically lowered viral entry into rat neurons, they were unable to completely block entry, even at the high concentrations used. This may be the result of at least several possibilities. (i) Anti-HveC antibodies may have a lower affinity for HveC than do antibodies to gD. (ii) The steric hindrance of antibodies to gD on the virion might prevent any other glycoprotein-cell interactions (glycoproteins B and C initially bind cellular heparan sulfate), while antibodies to HveC

on the cell would still allow for viral gB/gC to bind to the cell, then there would be a better chance for the low amounts of unbound HveC to interact with the virion. (iii) Without cellular HveC available, perhaps viral gD can still bind 3-*O*-sulfated heparan sulfate sufficiently to enter some cells. If, however, gD is bound by antibodies, the virion cannot use any cellular entry molecules. Transcripts of the enzyme responsible for 3-*O*-sulfate-modified heparan sulfate, D-glucosaminyl 3-*O*-sulfotransferase-3_B, have been detected very weakly in brain tissue (23), but whether they are expressed in sensory neurons remains to be determined.

Treatment with a soluble form of HveC significantly reduced HSV-1 entry into rat and mouse sensory neurons. Unexpectedly, antiserum against HveC significantly reduced HSV-1 entry into rat sensory neurons, but not mouse sensory neurons. These results may reflect subtle differences between the rat and mouse HveC molecules. The antibodies to HveC were generated using recombinant human HveC. While sequence data indicate that HveC is highly conserved among human, rat and mouse, slight differences in the proteins can significantly alter antibody affinity for specific epitopes. Critical HveC epitopes for virus entry were blocked by anti-HveC antiserum in the rat neurons, but not in the mouse neurons, which might be because of species differences in HveC. While HSV-1 gD can use murine HveC to enter cells, the interactions between gD and murine HveC are weak compared to the affinity gD has for human HveC (14, 15). However, studies have shown that gD -/- mutant virus or virus treated with anti-gD antibodies were unable to enter cells that express murine HveC as the only gD receptor (14), demonstrating that gD does use murine HveC as a cellular receptor. Pre-treating mouse neurons with the monoclonal anti-HveC antibody CK41, or perhaps other

monoclonal anti-HveC antibodies shown to block HSV-1 entry in human cells, might help indicate whether HveC is the main receptor for HSV-1 in mice.

Ideally, it would be helpful to determine if HveC is the cellular receptor used by HSV-1 to gain access to human sensory neurons. It has already been demonstrated that viral gD has a higher affinity for human HveC than for mouse HveC (14). If HveC proves to be the entry receptor on human neurons, it might be wise to rethink the mouse model of HSV-1 infection, particularly with respect to latency. If virus cannot enter mouse neurons as efficiently, due to the lower affinity gD has for mouse HveC, or if virus were able to enter the mouse via a different receptor, for instance using 3-*O*-sulfated heparan sulfate instead, the mouse infection might be very different from the human infection. One can imagine that some sort of signal might possibly be transduced through HveC in neurons, perhaps a signal that aids in the establishment of latency. If virus enters a mouse neuron through a different receptor, perhaps that signal would be bypassed and thus the infection changed. The fact that antibodies made against human HveC blocked HSV-1 entry into rat neurons certainly gives the rat *in vitro* model of latency an edge over the mouse model.

Another difference that was consistently seen between mouse and rat neurons was the localization of GFP-ICP4 in virally infected cells. GFP-ICP4 localized to punctate regions in most nuclei of rat neurons, while it was found diffusely in most nuclei of mouse neurons. This may reflect a difference in protein makeup between rats and mice, a difference in numbers of virus particles able to enter cells, or a difference in the pathway of viral entry. At this point, it is impossible to say why there was a difference in localization.

These results show that HveC serves as the major entry molecule for HSV-1 infection of rat sensory neurons *in vitro*, which may have important implications for understanding critical aspects of HSV-1 pathogenesis.

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CHAPTER 4

NUCLEAR DOMAIN 10S SENSORY NEURONS: IMPLICATIONS FOR HSV-1 LATENCY

The preparation of one of the recombinant viruses used in the work reported in this chapter, HSVEGFP0, is discussed in Chapter 2. Dr. Christine Wilcox provided the primary rat dorsal root ganglion neurons used for these studies. The confocal microscopy was viewed and photographed by Liz Hunsperger. Part of the work in this chapter will be submitted to Proceeding of the National Academy of Science.

S. Richart, L. Hunsperger, and C. Wilcox. Dorsal root ganglion neurons, but not PC12 cells, lack promyelocytic leukemia gene product (PML)-containing nuclear domain 10s (to be submitted to Proceedings from the National Academy of Science Journal).

Part of the work in this chapter was presented at the following meetings:

Richart S., and Wilcox C. (2001) Of herpes and neurons. 2nd Annual Graduate Research and Creativity Symposium, Colorado State University.

Richart S., Hunsperger L., and Wilcox C. (2001) The absence of nuclear domain 10s in sensory neurons: implications for herpes simplex type 1 latency. Cell and Molecular Biology Symposium, Colorado State University.

Richart S., Hunsperger L., and Wilcox C. (2001) The absence of nuclear domain 10s in sensory neurons: implications for herpes simplex type 1 latency. American Society for Microbiology, Spring 2001 Rocky Mountain Branch Meeting.

4.1 ABSTRACT

Nuclear Domain 10s (ND10s) are discrete, nuclear structures defined by their protein makeup. During lytic HSV-1 infection, the viral immediate early protein ICP0 localizes to these regions and disrupts them by causing the proteasome degradation of several ND10 proteins. Recent findings have demonstrated that ICP0 is found at very low abundance in trigeminal ganglion neurons (X. Chen, J. Li, M. Mata, J. Goss, D. Wolfe J. Glorioso, and D. Fink, J. Virol. 74:10132-41, 2000). When a recombinant HSV-1 virus expressing GFP fused to ICP0 used to infect dorsal root ganglion neurons at a high multiplicity of infection, the pattern of ICP0 localization was unexpected. ICP0 was found in a diffuse pattern in the nuclei of neurons, rather than the punctate pattern in nuclei reported in most other cell types. This implied that ND10s may not be present in neuronal nuclei.

We determined the presence of ND10s in sensory neurons by detection of ND10 proteins PML, SUMO-1 and Daxx. We found very little protein, and no ND10 structures in these cells. We did, however, find transcripts of USP7, a ubiquitin-specific protease that interacts with ICP0 and localizes to ND10s, in sensory neurons. PC12 cells, a transformed cell line that displays neuron-like properties when differentiated with nerve growth factor, in contrast to the primary sensory neurons, displayed abundant ND10 proteins and ND10 structures. These findings demonstrate an important difference between primary sensory neurons and PC12 cells. They may also begin to explain the observed low-level expression of ICP0 in neurons, which may ultimately help unravel the mysteries of HSV-1 latency.

4.2 INTRODUCTION

Nuclear domain 10s (ND10s), also called promyelocytic leukemia protein (PML) nuclear bodies (NBs), PML oncogenic domains (PODs), or nuclear dots, are subcellular structures described in a number of cell lines and cell types. ND10s are defined by their protein components, some of which are listed in Table 4.1, and are typically arranged in 5-30 punctate dots per nucleus. The appearance of ND10s can be altered by exposure to interferons, viral infection or heat shock stress (2, 35, 43, 45, 54). ND10s are distinct from other nuclear structures such as nucleoli and coiled bodies (2). ND10 structures break down during mitosis: as cells enter metaphase, ND10s are undetectable, and then as cells progress to telophase, ND10s reform (2).

SUMO-1 (also called sentrin, SMT3C, hSMT3, PIC1, UBL1, and GMP1) is a member of a growing group of ubiquitin-related proteins involved in the post-translational modification of proteins. SUMO-1 is similar to ubiquitin in structure, although it shares only 18% sequence identity (reviewed in 43). Like ubiquitin, SUMO-1 can be covalently attached to specific lysine residues of numerous proteins. While the covalent addition of ubiquitin to a protein marks that protein for proteolytic degradation, the addition of SUMO-1 to a protein may result in various outcomes: it may preserve the protein from ubiquitin-mediated degradation by competing for ubiquitin-binding sites, as is seen with IκBα (11); it may localize the protein to the cytoplasmic side of the nuclear pore complex, as in the case of RanGAP1 (40); and it may localize the protein to ND10s, as in the case of PML protein (51).

SUMO-1 transcripts have been detected in a wide variety of human and mouse tissues, including brain (29, 39). While other proteins found in ND10s are SUMOlated,

Table 4.1 Some proteins that make up nuclear domain 10s

PROTEIN	FUNCTION	CELLULAR ROLE	SUMO-1 MODIFIED	KNOCKOUT PHENOTYPE
PML	transcription factor	upregulates MHC class I expression (74), suppresses tumors, antiviral activity	yes (33, 51)	sensitive to infections; splenomegaly (67); protected from Fas, TNF an d IFN-dependent apoptosis (68).
Sp100	transcription factor (27, 72)	unknown	yes (61)	ND
USP7	ubiquitin-dependent protease (21)	unknown		ND
Daxx	Fas-binding protein (73)	activates apoptosis via jun kinase pathway		lethal (50)
BLM (31)	DNA helicase	genome stability (75)		lethal (6)
CBP (36)	transcriptional activator/repressor	involved in a variety of transcriptional pathways		lethal (52)
p53 (24, 56)	transcription factor	regulates cell cycle progression and apoptosis	yes (24, 56)	early tumorogenesis (22)
pRB (1)	transcription factor	regulates cell cycle		embryonic lethal (8, 32, 37)
CENP-C (16)	centromere protein	involved in mitotic progression		ND
NDP-55 (2)	unknown	unknown		ND
ISG20 (23)	interferon-induced protein	unknown		ND
HP1 (38, 58)	heterochromatin protein	gene silencing		ND

such as Sp100 (61), and p53 (24), it is generally believed that there is a requirement only for SUMOlated PML in order for ND10s to form (31, 51, 77).

An all-encompassing model of ND10 function has yet to be proposed. However, ND10s are involved in many cellular activities (Table 4.1). At the center of many of these processes is the ability of ND10 proteins to activate or repress transcription (reviewed in 11, 73), as well as to participate in apoptosis (28, 55, 65, 68, 78).

While there is no unifying hypothesis addressing the specific role of ND10s in the cell, the disruption of these nuclear structures is present in some human diseases, such as acute promyelocytic leukemia (APL) (reviewed in 54), and the neurodegenerative disease, spinocerebellar ataxia type I (59). Several DNA viruses that localize to ND10s, where they begin transcription and replication, also disrupt ND10s, such as adenovirus type 5, human cytomegalovirus (HCMV), and herpes simplex virus type 1 (HSV-1) (reviewed in 38).

The immediate early HSV-1 protein ICP0 is responsible for the localization and disruption of ND10s in HSV-1 infected cells at very early times post-infection (19, 42, 43). This disruption is caused by the proteasome-dependent degradation of the ND10 proteins PML, Sp100 and CENP-C (4, 15, 17). Not only does ICP0 localize to and disrupt ND10s, but HSV-1 genomes also localize to areas adjacent to ND10s, where they form prereplicative sites in cell lines (30, 44).

Whether HSV-1 interacts with ND10s in sensory neurons is currently unknown. In fact, very little information exists regarding ND10 structure in neurons, as well as the cellular role ICP0 plays in HSV-1 infection of neurons. One study has shown that normal human fetal brain tissue contains diffuse, granular distributions of two ND10 proteins

(undefined as yet) in greater than two-thirds of cells, while in adult gray matter, less than one-third of cells showed ND10 structures at a frequency of 1-5 ND10s per stained cell (7). Also reported is the presence of PML but the surprising absence of Sp100 in differentiated NT2 cells, a central nervous system-derived neuroblastoma (41). Whether these data correlate to peripheral nervous system sensory neurons has not been ascertained, and thus this study was undertaken.

ICP0 localizes to the nuclei of productively infected cells. However, it does not preferentially accumulate in nuclei of primary sensory neurons or in differentiated rat PC12 cells (5). In fact, there is very little ICP0 protein found in these cells, even though ICP0 transcripts are readily detectable. This suggests the instability of ICP0 protein in neurons, perhaps due to a lack of ND10 domains.

In an effort to understand the early virus-cell interactions that occur in neurons, which ultimately lead to a latent HSV-1 infection, we sought first to determine whether classical ND10s exist in primary sensory neurons. Additionally, ND10s were examined in PC12 cells, a cell line that displays neurons-like qualities when differentiated with nerve growth factor (25, 26). PC12 cells are used by some investigators as models of *in vitro* HSV-1 latency (3, 10). Here we compare the ND10 makeup of primary sensory neurons to PC12 cells in an effort to compare or contrast the two *in vitro* models of HSV-1 latency.

To investigate the ND10 structures in neurons and PC12 cells, the presence of three proteins was assessed: PML, SUMO-1, and Daxx proteins. Additionally, the transcript for USP7, a ubiquitin-specific protease shown to interact with viral ICP0 in ND10s (17, 20), was examined in DRG neurons. We found that classical PML-

containing ND10s were not present in primary DRG neurons. However, components of the nuclear environment were detected in both undifferentiated and differentiated PC12 cells.

4.3 MATERIALS AND METHODS

Cells and viruses. Vero cells and HeLa cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified eagle medium (DMEM) plus 5% and 10% fetal bovine serum (FBS), respectively. Clone 9 cells, a rat cell line (ATCC), were grown in Ham's F12K medium with 10% FBS. PC12 cells (ATCC) were cultured in RPMI-1640 medium supplemented with 10% horse serum, 5% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, 0.45% dextrose (w/v), and 1.5 g/L sodium bicarbonate. To differentiate PC12 cells, cells were switched to the above medium with the following changes: 50 ng/ml 2.5S mouse nerve growth factor (NGF) (Harlan Bioproducts for Science, Indianapolis, Indiana) and only 1 % horse serum and 0.5% FBS. All media and cell culture products were purchased from Invitrogen Life Technologies, except where noted. Neuronal cultures were prepared from dorsal root ganglia (DRG) neurons from day 15 embryonic rats as previously described (69, 71). Neuronal cultures used for western blots were treated with anti-mitotics (fUDR and AraC) to deplete cultures of non-neuronal accessory cells. Cultures used for immunofluorescence studies were untreated to provide internal positive controls.

The wild-type HSV-1 strain used was 17^+. The recombinant virus HSVEGFP0 (see **Chapter 2**) was derived from a 17^+ background strain and contains two copies of the α0 gene(encoding ICP0) fused to the enhanced green fluorescent protein (GFP) gene.

Western blot analysis. Cells were rinsed and pelleted, then lysed in RIPA buffer with protease inhibitors. Protein quantification was performed using the BCA assay (Pierce, Rockford, IL). Proteins were resolved on acrylamide gels, then transferred to nitrocellulose membrane (Amersham Pharmacia, Piscataway, NJ). The Renaissance Western Blot Chemiluminescence Reagent Plus substrate (NEN, Boston, MA) for peroxidase was used, and manufacturer's protocol was followed, except for the following changes: membrane was blocked overnight at 4° C in 1× Uniblock (Analytical Genetic Testing Centers, Denver, CO) and PBS-Tween 20 (0.1%), the primary and secondary antibodies were diluted in Uniblock-PBS-Tween 20.

Immunofluorescence/Immunocytochemistry. Cell cultures were fixed and kept in ice-cold methanol at -20° C. Cells were permeablized in 1% Triton-X-100 in phosphate-buffered saline (PBS) for 10 minutes, washed twice with PBS, and blocked for one hour with 0.1% Tween-20 and 5% dried milk in PBS. Colorometric substrate for alkaline phosphatase was NBT/BCIP (Roche Molecular Biochemicals, Indianapolis, IN).

Antibodies. Primary antibodies used are mouse anti-PML (5E10, as previously described (62)) and goat anti-PML (A-20, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Daxx (M-112, Santa Cruz Biotechnology), and mouse anti-SUMO-1 (called anti-GMP1, Zymed Laboratories, San Francisco, CA). All of these antibodies recognize rat, as well as human, antigens. Secondary antibodies used were FITC-labeled anti-mouse IgG and anti-rabbit IgG, alkaline phophatase-labeled anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), and peroxidase-labeled anti-mouse IgG and anti-rabbit IgG (Vector Laboratories, Burlingame, CA).

***In Situ* Hybridization.** A non-radioactive method for *in situ* hybridization was used, where riboprobes were labeled with digoxigenin (DIG) (Roche Molecular Biochemicals), as previously described for DRG neurons (70), and detected using anti-DIG antibodies labeled with alkaline phosphatase and NBT/BCIP as a substrate (Roche Molecular Biochemicals). Anti-sense riboprobes were made to the 1.5 kb LAT transcript (60), and both sense and anti-sense riboprobes were made against the 1.5 kb *Ssp* I to *Hind* III fragment of USP7 transcript (gift of Roger Everett, MRC, Glasgow, Scotland).

4.4 RESULTS

A GFP-ICP0 fusion protein expressed from the recombinant HSV-1 virus, HSVEGFP0, did not locate to punctate regions in the nuclei of infected sensory neurons. A strange observation was made when we were unable to detect ICP0 by immunofluorescence in neurons infected with wild-type HSV-1 (data not shown). In contrast, the same cultures stained positively for ICP4 and general HSV-1 antigens (data not shown). A theme emerged once the recombinant HSVEGFP0 virus was made and used to infect neurons. HSVEGFP0 expresses ICP0 fused to GFP (see Chapter 2), and has been shown to express very little GFP-ICP0 in neurons. In order to see any GFP-ICP0, neurons had to be infected with a very high multiplicity of infection (100 plaque forming units per cell or higher) and examined many hours after infection; still the expression was weak.

The visible GFP-ICP0 was located diffusely in the nuclei and cytoplasm of infected neurons, rather than in the typical punctate nuclear regions that are seen in other cells studied, in this case HeLa cells (Figure 4.1). The typical nuclear punctate

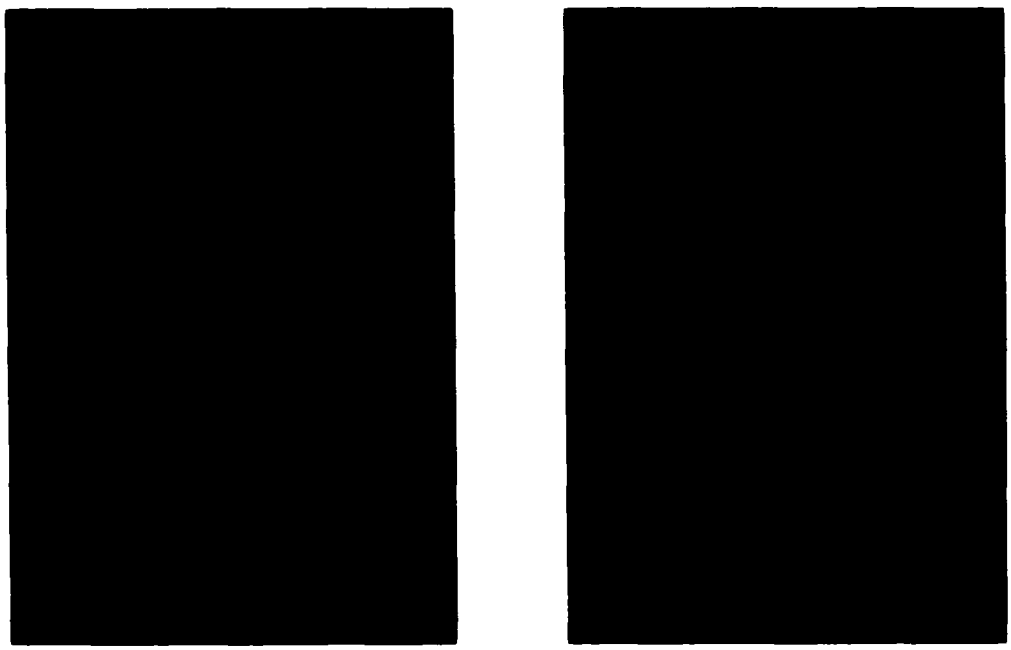

Figure 4.1 Expression of ICP0 fused to GFP in neurons and HeLa cells. HeLa cells and DRG neurons were infected with HSVEGFP0 virus and photographed at different times post-infection. HeLa cells photographed 8 hours post-infection. DRG neurons photographed 36 hours post-infection. Photomicrographs are not shown at same magnification, and cells were not infected with same multiplicity of infection.

localization of ICP0 seen in non-neuronal cells corresponded to the localization of several ND10 proteins (19, 43). Because GFP-ICP0 in neurons did not display this nuclear punctate pattern, we reasoned that perhaps neurons lack ND10s.

PML was present in PC12 cells but not in primary sensory neurons. The SUMO-1 modification of PML is generally believed to be critical to the formation of ND10s (31, 76). In order to assess the ND10 presence or composition in neurons, we first looked at PML protein profiles in primary neurons and in undifferentiated and differentiated PC12 cells (Figure 4.2). The anti-PML monoclonal antibody 5E10 recognizes three bands on the immunblot from PC12 cells, both differentiated and undifferentiated.

Immunofluorescence was performed on cultures to specifically view the cellular localization pattern of PML in PC12 cells. Primary neurons, not surprisingly, displayed no specific staining with the monoclonal 5E10 antibody (Figure 4.3). However, PML was present in both undifferentiated and differentiated PC12 cells in punctate, nuclear arrangements, which were indicative of ND10s. This illustrates a major difference in the normal cellular makeup between PC12 cells and primary sensory neurons.

SUMO-1 was detectable in PC12 cells but not in primary sensory neurons. The SUMO-1 modification of PML is generally believed to be critical to the formation of ND10s (31, 76). In order to assess the ND10 presence or composition in neurons, we looked at SUMO-1 protein profiles in neurons by western blot. Because the ND10 proteins PML, Sp100 and p53 are not the only proteins in cells that are post-translationally modified with SUMO-1, one expects to see many protein bands that contain SUMO-1 when examining whole cell lysate by immunoblotting. Surprisingly,

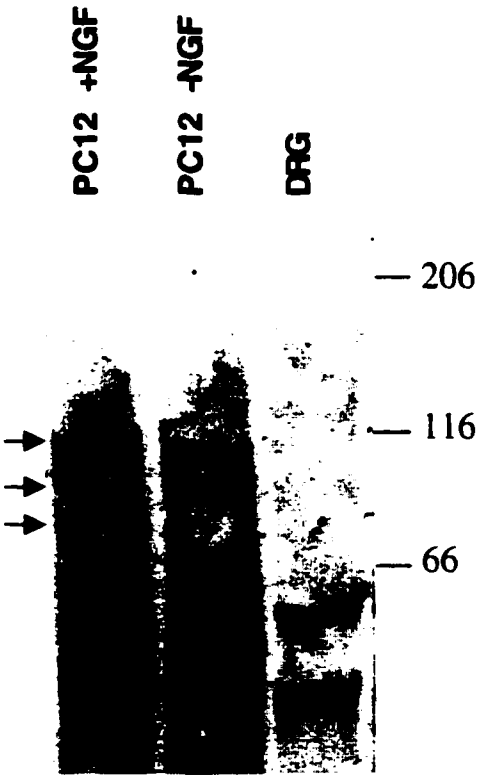

Figure 4.2 Detection of PML in PC12 cells, but not in DRG neurons. Western blot analysis of 30 µg per lane of whole cell lysate from (*l* to *r*) differentiated PC12 cells (**PC12 +NGF**), undifferentiated PC12 cells (**PC12 -NGF**), and dorsal root ganglion neurons (**DRG**). Primary antibody is monoclonal 5E10. Positions of molecular weight markers are indicated on the right in kilodaltons. Arrows denote PML isoforms.

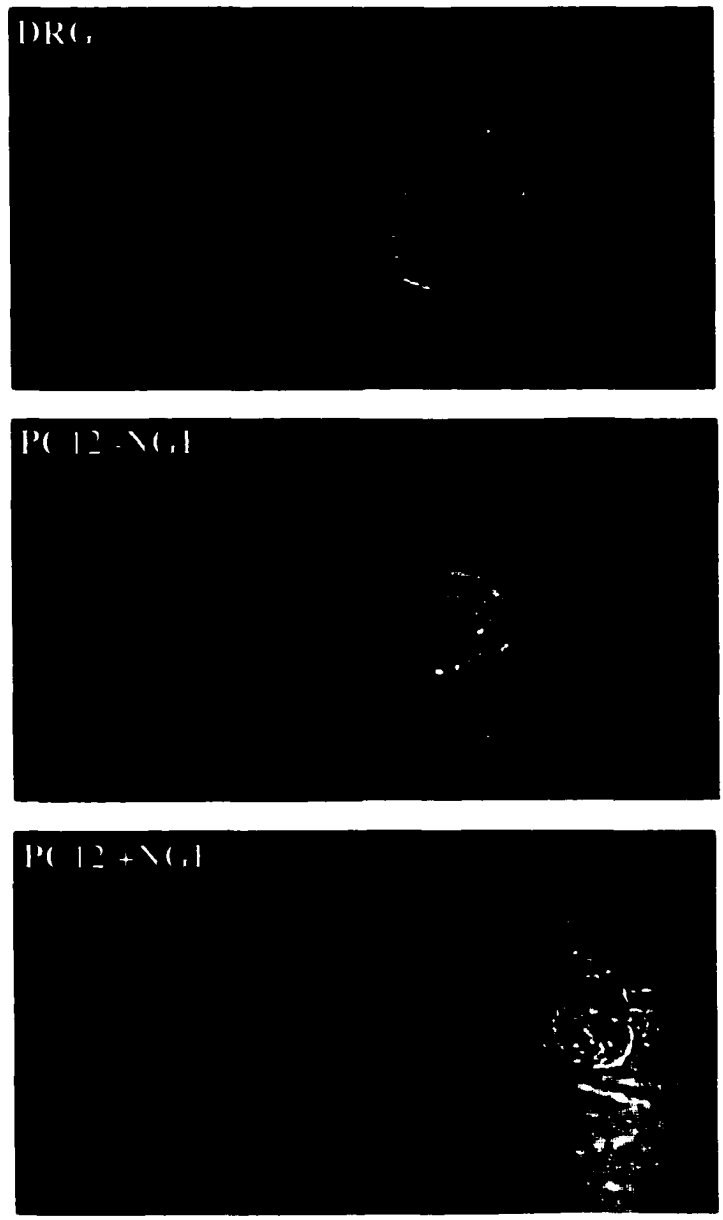

Figure 4.3 PML is present in PC12 cells but not in DRG neurons. PML staining by immunofluorescence of dorsal root ganglion neurons (**DRG**), undifferentiated PC12 cells cultured without nerve growth factor (NGF) (**PC12 - NGF**) or differentiated PC12 cells cultured with NGF (**PC12 +NGF**). Cells were fixed in cold methanol and stained with anti-PML antibody (5E10) followed by FITC-labeled secondary antibody. Left panels show PML staining and right panels are the same fields shown under phase contrast. Cells were viewed using confocal microscopy, with pictures taken at 5 μm intervals throughout the cells and then stacked. Cells are not shown at the same magnification. Photomicrographs taken by Liz Hunsperger.

SUMO-1 was present in neuronal cultures at virtually undetectable levels compared with other cell lines (Figure 4.4B). The two intense bands detected in the sample from the neuronal cultures were most likely non-specific as they appear in most western blots, regardless of primary antibody used (see Figure 4.2, DRG lane). Thus there were no major proteins with SUMO-1 modifications. Interestingly, both undifferentiated and NGF-differentiated PC12 cells appeared to contain many proteins that were SUMO-1 modified.

These findings from western blot analysis were confirmed by immunocytochemistry. Similarly, sensory neurons contained no detectable SUMO-1 (Figure 4.4A). However, undifferentiated and differentiated PC12 cells showed abundant SUMO-1 expression, particularly in the cytoplasm. These findings illustrate a major difference in the protein makeup between primary SENSORY neurons and PC12 cells, and potentially a difference in the way proteins are post-translationally modified in neurons.

Daxx was found in PC12 cells but not in primary DRG neurons. Daxx protein, which is involved in apoptosis and in transcription repression, is also found in ND10s, although not exclusively (reviewed in 46). DRG neurons and PC12 cell extracts were blotted and probed with antibodies to Daxx. The results show that the two major phosphorylated Daxx bands of 97 and 110 kDa were present in undifferentiated and differentiated PC12 cells, while those two bands were only present in DRG neuron extracts (Figure 4.5). The top band seen around 200 kDa appears to be non-specific, as a 200 kDa Daxx isoform has not been reported.

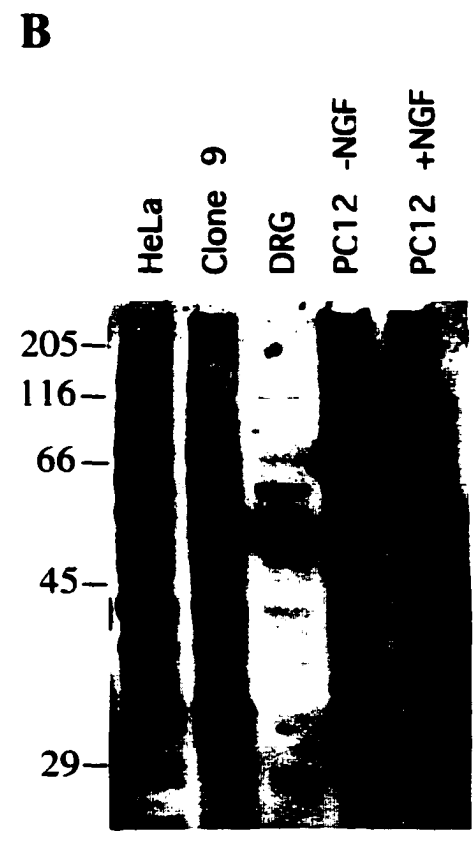

Figure 4.4 SUMO-1 was detected in PC12 cells but not in DRG neurons. (**A**) SUMO-1 staining by immunocytochemistry of undifferentiated PC12 cells (**PC12 -NGF**), differentiated PC12 cells (**PC12 +NGF**), and dorsal root ganglion neurons (**DRG**). Secondary antibody was linked to alkaline phosphatase and cultures were treated with NBT/BCIP substrate. All photomicrographs are shown at the same magnification. (**B**) Western blot analysis of 30 μg per lane of whole cell lysate from (*l to r*) HeLa cells, Clone 9 cells, dorsal root ganglion neurons (**DRG**), undifferentiated PC12 cells (**PC12 -NGF**), and differentiated PC12 cells (**PC12 +NGF**). Positions of molecular weight markers are shown on the left in kilodaltons.

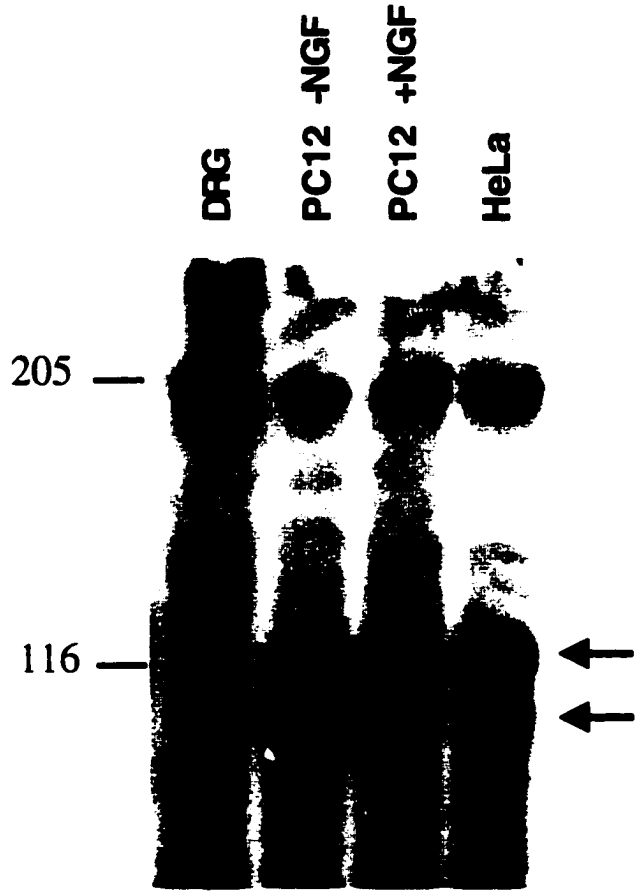

Figure 4.5 Expression of Daxx in DRG neurons and PC12 cells. Western blot analysis of 25 μg per lane of whole cell lysate from DRG neurons, undifferentiated PC12 cells (**PC12 -NGF**), differentiated PC12 cells (**PC12 +NGF**), and HeLa cells. NEN's Renaissance Chemiluminescent substrate for peroxidase was used to visualize protein bands. Positions of molecular weight markers are indicated on the left in kilodaltons. Arrows indicate the predicted sizes of the two reported Daxx isoforms.

To address whether the low levels of Daxx detected in the neuronal culture extract were contributed by the non-neuronal cells present in culture, immunofluorescence was performed. DRG neurons displayed no Daxx nuclear staining, while both PC12 cell groups displayed nuclear Daxx staining (Figure 4.6). Due to fixation and autofluorescent proteins in DRG neurons, there was non-specific cytoplasmic fluorescence present in all samples. However, a nuclear, punctate staining was seen in PC12 cells, which was absent in DRG neurons. Figure 4.6 photographs were taken using epifluorescence, but confocal images confirmed the lack of Daxx in DRG neurons and punctate staining of PC12 cells (data not shown).

Transcripts of USP7 protease were detected in sensory neurons. ICP0 binds directly to a ubiquitin-specific protease called USP7 (formerly called HAUSP for herpes associated ubiquitin-specific protease) (21, 47, 48), and this binding event contributes to the ability of ICP0 to stimulate gene expression and to increase viral replication (20). Antibodies to USP7 were not available, so to determine if USP7 was present in neurons, *in situ* hybridization was performed to detect mRNA. The USP7 human plasmid that was used to make probes because the rat USP7 is not available nor has it been sequenced. However, portions of mouse cDNA have been sequenced that have very high homology to human USP7 and probably correspond to USP7. Because of this, we reasoned that rats would probably be similar as well, and that riboprobes made from human USP7 might hybridize to rat USP7. To increase the chances of hybridization, riboprobes were fragmented by alkaline hydrolysis. Control hybridizations with sense USP7 riboprobe showed very low non-specific staining (Figure 4.7 A). Most neurons incubated with anti-sense USP7 riboprobe stained positively for USP7 transcripts (Figure 4.7 B). These

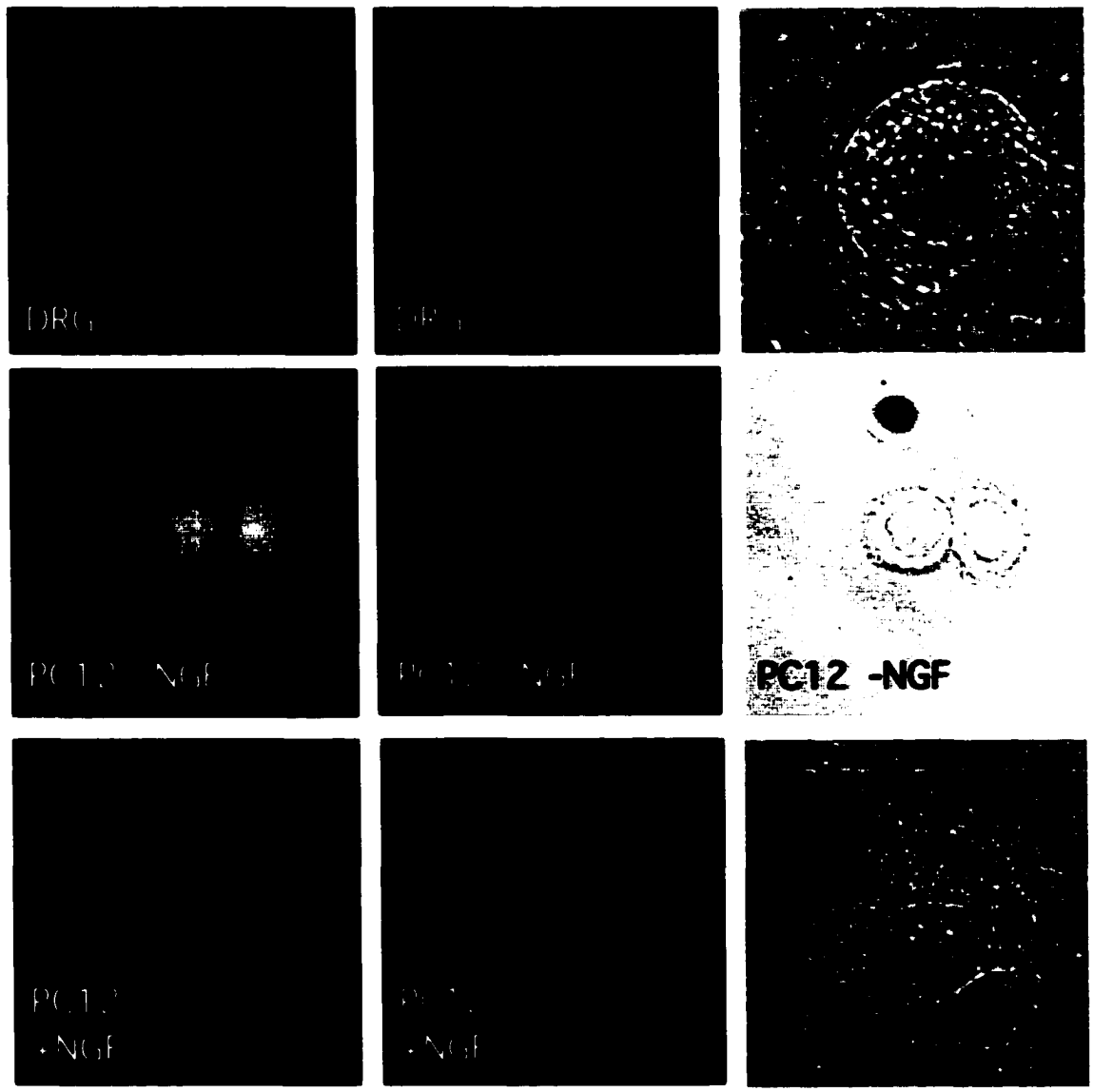

Figure 4.6 Expression of Daxx in DRG neurons and PC12 cells. Daxx staining by immunofluorescence of dorsal root ganglion neurons (**DRG**), undifferentiated PC12 cells cultured without nerve growth factor (NGF) (**PC12 -NGF**) or differentiated PC12 cells cultured with NGF (**PC12 +NGF**). Cells were fixed in cold methanol and stained with anti-Daxx antibody followed by FITC-labeled secondary antibody. Left panels show cell nuclei stained with DAPI. Middle panels show the same fields with Daxx staining. Right panels show same fields under white light. Photomicrographs taken using epifluorescence and white light. Cytoplasmic signals seen in cells stained with Daxx are non-specific due to fixation. Note the lack of nuclear signal in DRG neurons.

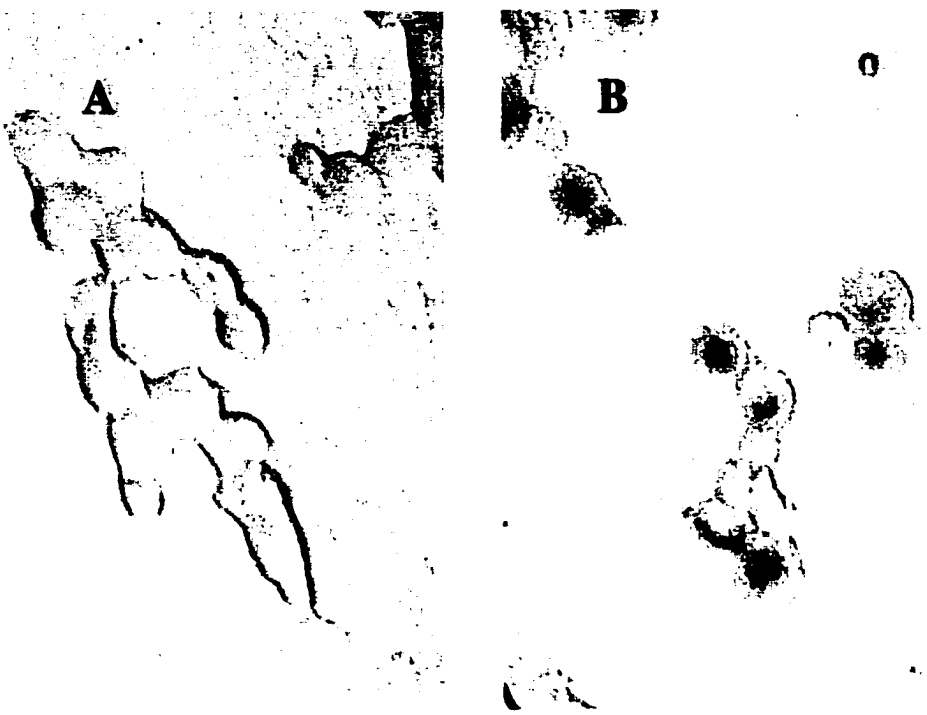

Figure 4.7 USP7 transcripts are present in DRG neurons. Neurons were grown in culture and fixed for *in situ* hybridization. Neurons were hybridized with sense (control) (**A**) or anti-sense (**B**) strand specific DIG-labeled riboprobes for the 1.5 kb *Ssp* I to *Hind* III fragment of USP7 cDNA.

results indicate that USP7 was most likely present in DRG neurons, and thus this nuclear ubiquitin-specific protease may have a function in the neuron despite the absence of ND10s.

4.5 DISCUSSION

The results of this study show that rat DRG neurons do not normally contain ND10 regions, as they were lacking both quantity and localization of PML, SUMO-1 and Daxx proteins. In contrast, both differentiated and undifferentiated PC12 cells expressed all three proteins in ND10 arrangements. We did not find it surprising that neurons lack ND10s, as previous studies have shown that ND10 arrangement is linked to cell cycle, and that at G_1 phase, ND10 structures are absent (18). Neurons, which are terminally differentiated, are arrested in G_0 phase. G_0 cells typically displayed the least amount of ND10 proteins in tissue (34, 64). By that logic, however, nerve growth factor-differentiated PC12 cells would also be predicted to lack ND10s. However, our findings confirmed the presence of ND10s in PC12 cells, and may be the result of the transformation of these cells.

Many believe that the switch between lytic or latent HSV-1 infection is influenced primarily by the levels of ICP0 protein in infected cells (reviewed in 12, 13). One critical question, then, is how ICP0 expression is regulated in neurons versus cell lines. Our recombinant HSVEGFP0 virus showed little ICP0 expression in DRG neurons. It has previously been suggested that ICP0 expression may be depressed in neurons due to specific transcriptional repression factors found in these cells (reviewed in 50). However, several lines of evidence argue against this explanation. It is reported that ICP0 transcripts are readily detected in the absence of abundant ICP0 protein in DRG neurons

(5), suggesting that ICP0 is not regulated on the level of transcription. Additionally, our group has constructed recombinant adenovirus vectors expressing immediate early proteins ICP0, ICP4 and ICP27 fused to GFP, all driven by the adenovirus E1A promoter. GFP-ICP4 and GFP-ICP27 proteins are expressed abundantly in DRG neurons, but curiously, GFP-ICP0 is not (66). Since the promoter is identical in all of these viruses, this again supports the idea that ICP0 transcription is not repressed, but rather ICP0 protein is preferentially degraded or is not translated efficiently in neurons.

In our study, USP7 was the only component of ND10s found to be present in DRG neurons. This is interesting since ICP0 is known to associate with USP7 in non-neuronal cells, and somehow this interaction contributes to the function of ICP0 in gene expression and replication (17, 20). Whether the little ICP0 expressed in neurons interacts with USP7 protein remains to be determined.

There are many other proteins that interact in ND10s that were not examined, most notably, Sp100. At this point in time, the available antibodies to ND10 proteins, either do not react with rat antigens or have not been tested in the rat. It remains possible that if different reagents were used, ND10s would be detected in neurons. However, this seems unlikely, given that SUMO-modified PML appears to be the critical determinant in ND10 formation (31, 51, 77). An indirect way of confirming the absence of ND10s would be to infect neurons with HSVEGFP0 and treat with cyclosporin A (5) to allow GFP-ICP0 protein to accumulate. Depending on where GFP-ICP0 localizes at early times post-infection would indicate the presence or absence of ND10s. Additionally, it will be interesting to observe the pattern and level of GFP-ICP0 expression during reactivation in neurons.

There are many possible ways to explain why ICP0 message is found abundantly in neurons, but not ICP0 protein. One explanation may involve the absence of ND10s in sensory neurons. In non-neuronal cells, ICP0 normally localizes very early in infection to ND10s, and then causes the proteasome-dependent degradation of some ND10 proteins. Perhaps in non-neuronal cells there are some ND10 proteins with which ICP0 interacts that stabilize ICP0 (e.g. via post-translational modification) and protect it from the normal program of degradation. Since the ND10 structures are lacking in neurons, perhaps ICP0 is not directed to interact with the hypothetical protein(s), or perhaps the said hypothetical protein(s) is absent in neurons. Thus ICP0 is degraded in this scenario. Another way to explain the presence of ICP0 transcripts but lack of ICP0 protein in neurons is the possibility that neurons regulate ICP0 on the level of translation.

This study demonstrates that PC12 cells, both differentiated and undifferentiated, contained ND10s. Since NGF-differentiated PC12 cells have been used as an experimental model of *in vitro* HSV-1 latency and reactivation, or "quiescence" (9, 10), this argues against the hypothesis that the lack of ND10s in sensory neurons contributes to HSV-1 latency. However, other investigators, including some in our group, have had difficulty in establishing this PC12 model without a low level of virus production during "quiescence." Additionally, changes occurred in the PC12 cells after growth in culture such that 16 days after infection with HSV-1, virus could not be reactivated with superinfection of HSV-1, nor was the superinfected virus able to be produced, even in PC12 cells that had no latent virus (63). Thus, as PC12 cells remain in culture, they become resistant to virus production. This suggests that PC12 cells change, perhaps they become more "differentiated," and perhaps at this timepoint, or earlier when viral gene

expression is no longer detectable, they lose ND10s. Looking at the ND10 makeup of PC12 cells when they are no longer able to produce virus might help to explain this change over time.

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CHAPTER 5

ND10 PROTEINS BUT NOT STRUCTURES ARE INDUCED BY INTERFERONS IN NEURONS: ROLE OF INTERFERONS AND PML IN HSV-1 INFECTION

The confocal photomicrographs in this chapter were taken by Liz Hunsperger. Dr. Christine Wilcox provided the dorsal root ganglion neurons. Part of the work in this chapter will be submitted for publication. It has not yet been titled. Part of the work in this chapter was presented at the following meetings:

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Richart S., Hunsperger L., and Wilcox C. (2001) The absence of nuclear domain 10s in sensory neurons: implications for herpes simplex type 1 latency. American Society for Microbiology, Spring 2001 Rocky Mountain Branch Meeting.

5.1 ABSTRACT

ND10s have been previously shown to increase in number and size in cells following interferon alpha/beta (IFN-α/β) treatment. Interferons were also able to upregulate the expression of several ND10 proteins, even in cells that did not constitutively express these proteins. This implies a role for ND10 proteins in the antiviral response. We show here that the overexpression of the ND10 promyelocytic leukemia protein (PML) directly inhibited HSV-1 replication in HeLa cells.

While dorsal root ganglion (DRG) neurons were previously shown to lack classical ND10 structures, we investigated the ability of poly (I:C), a synthetic double-stranded RNA and inducer of type I interferons, to upregulate ND10s in DRG neurons. Neither PML protein nor ND10 structures were detected in neurons using 5E10 antibody. However, using A-20 antibody, PML protein was upregulated in neurons treated with poly (I:C). We did find, however, that SUMO-1 and PML proteins (as detected by a different antibody, A-20) were upregulated and found in DRG neurons, primarily in the cytoplasm. Thus, while we saw SUMO-1 and PML protein induced, we saw no evidence for the upregulation of ND10 structures in neurons.

The PML isoforms detected by the A-20 antibody in poly (I:C) treated neurons were also detected in HSV-1 infected neurons, as well as heat shocked neurons. This indicates that (i) HSV-1 did not degrade PML as it has previously been reported to do at early times post-infection in non-neuronal cells, and (ii) interferon induction may be a response to heat shock in neurons.

5.2 INTRODUCTION

The size and number of ND10s in cells has been shown to increase with interferon alpha/beta (IFN-α/β) treatment (20). Previous studies have also demonstrated that the ND10 protein components promyelocytic leukemia protein (PML) and Sp100 proteins were upregulated by IFN-α/β, interferon gamma (IFN-γ), and retinoic acid (RA) (3, 14, 17, 29, 31). IFN-α/β upregulated ND10 protein ISG20, as well (11). Additionally, it has been shown that even cells that did not normally express PML or Sp100 at detectable levels were induced to express PML and Sp100 by treatment with IFN-α/β or -γ (13). Because these ND10 proteins are upregulated by interferons (IFNs), ND10s may be involved in the cellular antiviral response.

In fact, PML protein alone played a role in conferring resistance to some viruses, including lymphocytic choriomeningitis virus (LCMV) (9), vesicular stomatitis virus and influenza A virus, but not encephalomyocarditis virus (4). The direct effect PML has on HSV-1 replication has not been determined.

Peripheral neurons have been shown to be sensitive to IFN α/β as well. In fact, studies have indirectly shown that rat DRG neurons express IFN α/β receptors (33). Treatment of PC12 cells and rat superior cervical ganglion neurons with crude IFN prior to infection with both HSV-1 and vesicular stomatitis virus (VSV) resulted in decreased viral titers (35). Mice harboring latent HSV-1 in trigeminal ganglia were less likely to reactivate virus and had lower viral titers when ganglia were explanted in the presence of IFN (36). These results indicate that neurons can establish an interferon-mediated antiviral state that inhibits HSV-1 infection.

IFN-α/β inhibits wild-type HSV-1 replication in cell lines only at low levels (16, 18, 19). Infection of cell lines by wild-type HSV-1 typically results in only a very small increase in IFN-inducible genes (22, 27, 40). Yet clearly, HSV-1 stimulates IFN-α/β production in infected cells. In peripheral blood mononuclear cells, UV-inactivated HSV-1 as well as viral glycoprotein D alone stimulated IFN-α/β production (1), showing that IFN stimulation is an early event that happens without viral gene transcription. Non-neuronal cells that were infected with HSV-1 in the presence of protein synthesis inhibitors also upregulated five IFN-inducible genes: ISG54, IFI56, ISG15, 9-27 and MxA (27). Viruses that carry mutations in ICP0 were much more sensitive to IFN-α/β (23). Additionally, cells infected by a HSV-1 mutant lacking both ICP0 and VP16 (a virus that is unable to cause a lytic infection [23]) were able to upregulate almost all genes that are normally upregulated by treatment with IFN-α/β (22). These data demonstrate that while HSV-1 induces an interferon response in infected cells, the virus is also able to counteract the interferon response with the expression of immediate early protein ICP0.

Since ICP0 plays a role in disarming the IFN response, this may have some interesting implications for the role of IFN in establishing latency in neurons. ICP0 does not accumulate in the nuclei of HSV-1 infected primary sensory neurons in culture (5, our observations, see Chapter 2). While transcripts are made, ICP0 protein is at very low abundance. Because of the low amount of ICP0 protein found in neurons, perhaps the IFN response is not overcome by the virus in these cells, and this IFN response might contribute to the establishment of latency.

5.3 MATERIALS AND METHODS

Cells and viruses. Vero cells and HeLa cells (ATCC, Rockville, Maryland) were cultured in Dulbecco's modified eagle medium (DMEM) (Invitrogen Life Technologies, Rockville, MD) plus 5% and 10% fetal bovine serum (FBS) (Invitrogen Life Technologies), respectively. Neuronal cultures were prepared from dorsal root ganglia (DRG) neurons from day 15 embryonic rats as previously described (37, 39).

The wild type HSV-1 strain used was 17*.

Neuron culture. Neuronal cultures were prepared from dorsal root ganglia neurons (DRG) from day 15 embryonic rats as previously described (37, 39). HSV-1 latency was established in neurons as described (39). Briefly, 14 days after neurons were plated at approximately 10^3 cells per well of a 24-well plate, neurons were treated with 50μM acyclovir at least 12 hours prior to HSV-1 infection. Neurons were infected with a multiplicity of 5-10 pfu/cell. Cultures were maintained in acyclovir for at least one week, then switched to maintenance medium lacking acyclovir for the duration of latency. Maintenance medium contains 10% calf serum (Invitrogen Life Technologies) and 100 ng/ml 2.5 S mouse nerve growth factor (Harlan Bioproducts for Science, Indianapolis, IN) in DMEM.

***In situ* hybridizations and probes.** Neuronal cultures were latently infected with HSV-1, and latency was established for 3 weeks. Neurons were then fixed in phosphate-buffered 4% paraformaldehyde for 12 hours at 4° C, dehydrated in graded ethanol, and stored at -20° C until used. Detection of the latency-associated transcript (LAT) by *in situ* hybridization was performed as described (28, 38). A digoxigenin-labeled (DIG) riboprobe anti-sense to the 5' end of LAT (pLAT) (28) was used, which does not overlap

any other known genes in the LAT region (21). DIG was detected with anti-DIG antibody conjugated to alkaline phophatase, and NBT/BCIP substrate (Roche Molecular Biochemicals, Indianapolis, IN).

For compiling data, 5 coverslips for each group was hybridized with LAT riboprobe. Neurons and LAT-positive neurons from five fields for each coverslip were counted.

Immunofluorescence/Immunocytochemistry. Cell cultures were fixed and kept in ice-cold methanol at -20° C. Cells were permeablized in 1% Triton-X-100 in phosphate-buffered saline (PBS) for 10 minutes, washed twice with PBS, and blocked for one hour with 0.1% Tween-20 and 5% dried milk in PBS or TBS (for alkaline phophatase detection). Primary and secondary antibodies were diluted in blocking solution. Substrate for alkaline phosphatase was NBT/BCIP (Roche Molecular Biochemicals), and substrate for peroxidase was DAB (Vector Laboratories).

Antibodies. Primary antibodies used are mouse anti-PML (5E10, as previously described (32)) and goat anti-PML (A-20, Santa Cruz Biotechnology), rabbit anti-Daxx (M-112, Santa Cruz Biotechnology), and mouse anti-SUMO-1 (called anti-GMP1, Zymed Laboratories). Secondary antibodies used were FITC-labeled anti-mouse IgG and alkaline phosphatase-labeled anti-mouse IgG (Jackson ImmunoResearch), and peroxidase-labeled anti-goat IgG (Vector Laboratories).

Transfection of HeLa cells. HeLa cells were seeded in 24-well plates at 2 x 10^4 cells per well and transfected 24 hours later using Superfect reagent (Qiagen, Valencia, CA), according to manufacturers' protocol, with either PML plasmid (pJLT33, gift from Kent Wilcox, Medical College of Wisconsin, Milwaukee) or control pBluescript KS II

(Stratagene, La Jolla, CA) plasmid. Five hours after transfection, DNA and Superfect reagent were taken off cells and replaced with normal medium. 48 hours post-transfection, cells were infected with HSV-1 at a MOI of 0.1 plaque-forming unit per cell. Virus adsorbed to cells for 1 hour, then cells were rinsed twice with medium and fresh medium was put on cells. Virus was allowed to replicate for 20 hours, then cells and virus were freeze-thawed twice before virus was titered on Vero cells.

Poly (I:C) treatment. Cells were treated with 100μg/ml polyinosinic-polycytidylic acid (poly I:C) (Sigma, St. Louis, MO), synthetic dsRNA, to stimulate the interferon α/β response. This dose correlates to the amount of poly I:C necessary to stimulate expression of MHC class I proteins in IMR-32 cells, a neuron-like cell line (8). Cells were generally treated for 18 hours before virus infection or reactivation.

Heat shock of neurons. Cells were put in water bath at 44°C for 15 minutes, then returned to the normal temperature of 35°C. Cells were harvested at 10 hours and 21 hours post-heat shock and lysed for western blots.

HSV-1 infection for western blots. Cells were infected with HSV-1 at a MOI of 5 pfu/cell (the same amount of virus used in cultures to establish latency). Eighteen hours after infection (similar to poly (I:C) treatment), cells were harvested and lysed for western blots.

5.4 RESULTS

HSV-1 replication is decreased in HeLa cells overexpressing PML. Because PML has been shown to inhibit virus replication in many different viruses, we endeavored to explore the effects PML alone may have on repressing HSV-1 replication, first during a lytic infection. HeLa cells were transfected with either a plasmid over-

expressing PML from the CMV IE promoter or a control plasmid, and then infected with HSV-1 at a relatively low multiplicity of infection (MOI, 0.1) (Figure 5.1). ICP0 is known to degrade PML, thus a low MOI was chosen to ensure that ICP0 would not eliminate PML. Additionally, it has been shown that HSV-1 is sensitive to IFN at low MOIs Cells transfected with either plasmid showed no evidence of cell death. Cells expressing PML had an 8-fold reduction in virus titer as compared to cells transfected with the control plasmid ($p=0.0023$, one tail, Student's t test). This demonstrated that HSV-1 replication can be inhibited by PML in non-neuronal cells.

ND10 protein SUMO-1 was induced in neurons by poly (I:C), but not ND10 structures. While the results of Chapter 4 of this dissertation demonstrated that ND10s are absent in DRG neurons, we asked if ND10 structures and proteins could be induced in DRG neurons in response to type I IFN. The monoclonal anti-PML antibody recognizes ND10s in nuclei, as previously reported (32). When neurons were treated with poly (I:C) and incubated with the 5E10 anti-PML antibody, there was no increase in PML expression, nor any indication of ND10s by confocal microscopy (Figure 5.2A). The only fluorescence seen in these cells is a granular, cytoplasmic, non-specific fluorescence that is excited over a broad range of wavelengths. This phenomenon is typical of sensory neurons. The positive control for this antibody is found in Chapter 4, Figure 4.3, where PC12 cells stain positively for PML protein.

In contrast to this, SUMO-1 was shown to increase in poly (I:C) treated neurons, particularly in the cytoplasm (Figure 5.2B). It was not possible to view ND10s by using immunocytochemistry, so it is not clear whether SUMO-1 localized to punctate regions in the nuclei. However, since the PML isoforms recognized by the 5E10 antibody did not

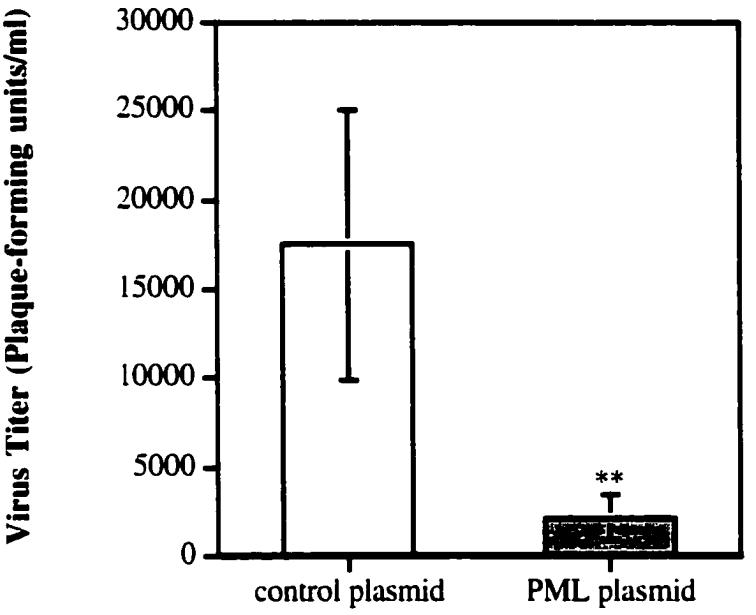

Figure 5.1 Effect of overexpression of PML on HSV-1 replication in HeLa cells. HeLa cells were transfected with a plasmid expressing PML or a control plasmid (pBluescript KS II). 48 hours post-transfection, cells were infected with HSV-1 at a multiplicity of 0.1 pfu/cell. Virus was allowed to replicate for 20 hours. Virus was titered on Vero cells. Graph indicates mean titer of 6 samples for each plasmid ± standard deviation. ** p=0.0023, one tail, by Student t-test.

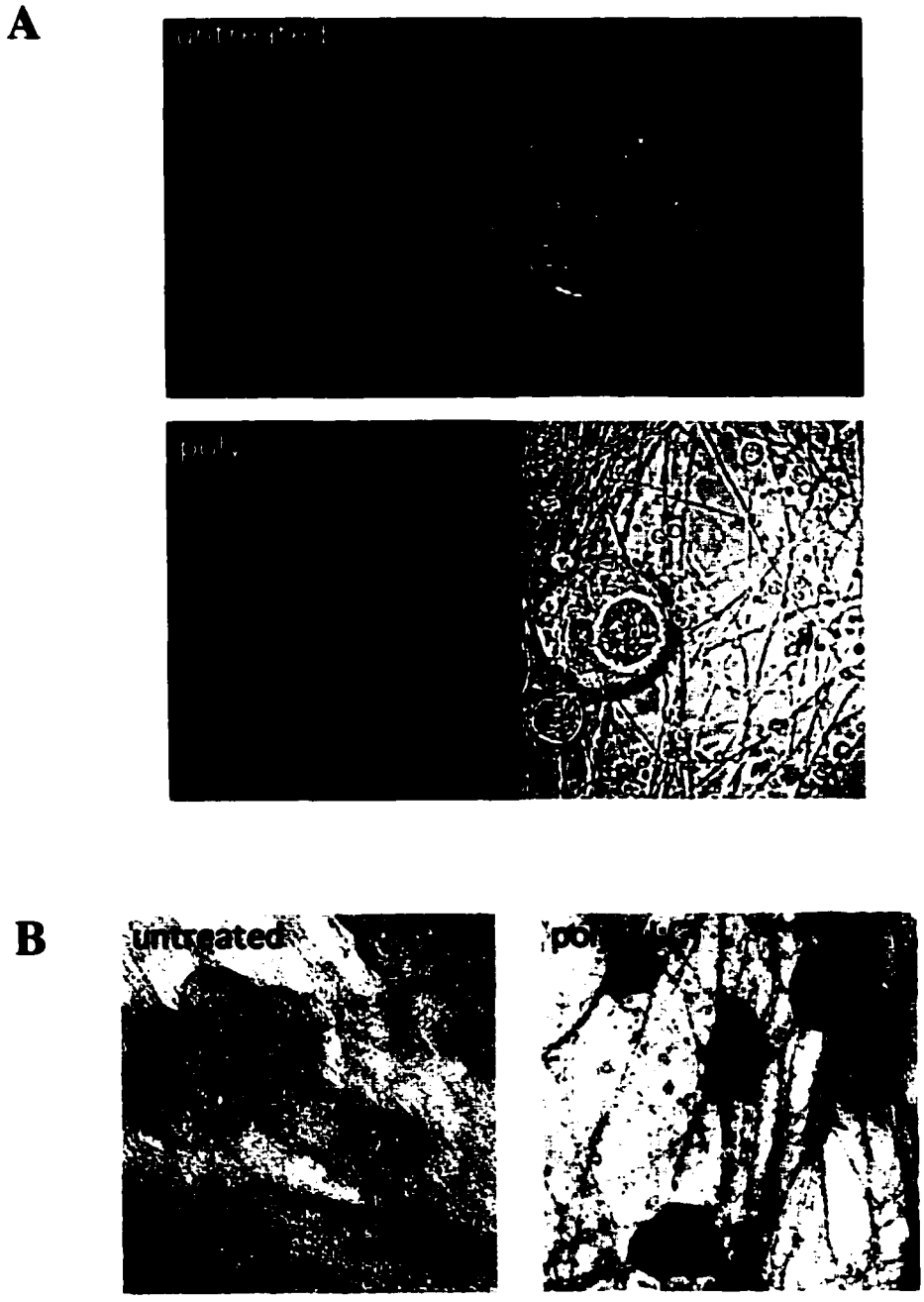

Figure 5.2 Detection of PML and SUMO-1 in DRG neurons treated with poly (I:C). Neurons were untreated or treated with 100 μg/ml poly (I:C) for 18 hours prior to fixation for immunofluorescence or immunocytochemistry. (**A**) Detection of PML (5E10 antibody) with FITC-labeled secondary antibody by confocal microscopy. (**B**) Detection of SUMO-1 with alkaline phosphatase-labeled secondary antibody, and NBT/BCIP substrate. Photomicrographs in (**A**) taken by Liz Hunsperger.

increase in neuronal nuclei when viewed using confocal microscopy, it is likely that the SUMO-1 is not localized there, either.

The increase in SUMO-1 expression was surprising. While PML and Sp100 are two ND10 proteins that are known to be upregulated upon IFN treatment, SUMO-1 has not been reported as an IFN-inducible protein.

These data also confirmed that poly (I:C) induced gene expression in neurons. IFN-α/β molecules are very species-specific, such that there is little ability for IFN from one species to act on another species. Additionally, each species has its own IFN subtypes and numbers of IFN-α and IFN-β genes. Thus, it was difficult to directly assay the ability of poly (I:C) to stimulate IFN-α/β because that would necessitate the availability of antibodies to all rat IFN subtypes, and then recombinant rat IFNs to use for these studies. This simply was not available to us. One advantage of poly (I:C) is that it is not species-specific.

PML, as recognized by A-20 antibody, was induced in neurons. Because neurons have unique ways of splicing transcripts (reviewed in 12), it was possible that different isoforms of PML were present in neurons that were not detectable by the 5E10 anti-PML antibody. Thus we assessed by western blot whether a different antibody, A-20 (Santa Cruz), which was generated from the amino terminus of PML, was able to detect PML in neurons. Initially, we found that PML was detected in poly (I:C) treated neurons (data not shown).

Because ICP0 is not expressed highly in neurons, and ICP0 has been shown to somehow counteract the IFN response in HSV-1 infected cells, we reasoned that an IFN response might not be overcome by viral proteins in neurons infected with HSV-1. If this

were true, then perhaps IFN-inducible proteins would be visibly upregulated. Neurons were infected with HSV-1 at a MOI of 5, the same MOI typically used for establishing latent HSV-1 infections *in vitro*, and harvested 18 hours later to examine PML expression. Figure 5.3A shows that a very abundant, large protein was upregulated in HSV-1 infected cells, and that it reacted with the A-20 antibody. There appeared to be at least 2 smaller, less abundant proteins that were also recognized by the antibody. These bands corresponded to the same bands that were upregulated in poly (I:C) treated neurons.

The large PML bands were also induced by heat shock. Uninfected neurons were heat shocked for 15 minutes at 44°C to induce virus reactivation conditions. One group that has performed differential display RT-PCR of explanted trigeminal ganglia found that IFN-α and β and interferon response factor-1, were upregulated as a consequence of explantation (34). Thus we reasoned that if IFN-α/β might be a response to heat shock or other neuronal stimuli that result in virus reactivation, perhaps PML might be upregulated as well. We found that PML was, indeed, upregulated in response to heat shock (Figure 5.3A).

When immunocytochemistry was performed on untreated neurons with the A-20 anti-PML antibody, there were no neurons that stained positively for PML (Figure 5.3A, *left*). The localization of PML in poly (I:C) treated neurons was unusual, though. Many cells stained positively for PML throughout the cell (Figure 5.3B, *near right*), particularly smaller neurons. There were also many non-neuronal cells that stained throughout the cell. Some of the larger neurons appeared to stain just at the cytosolic side of the plasma membrane (Figure 5.3B, *far right*). This confirmed some of our earlier findings by

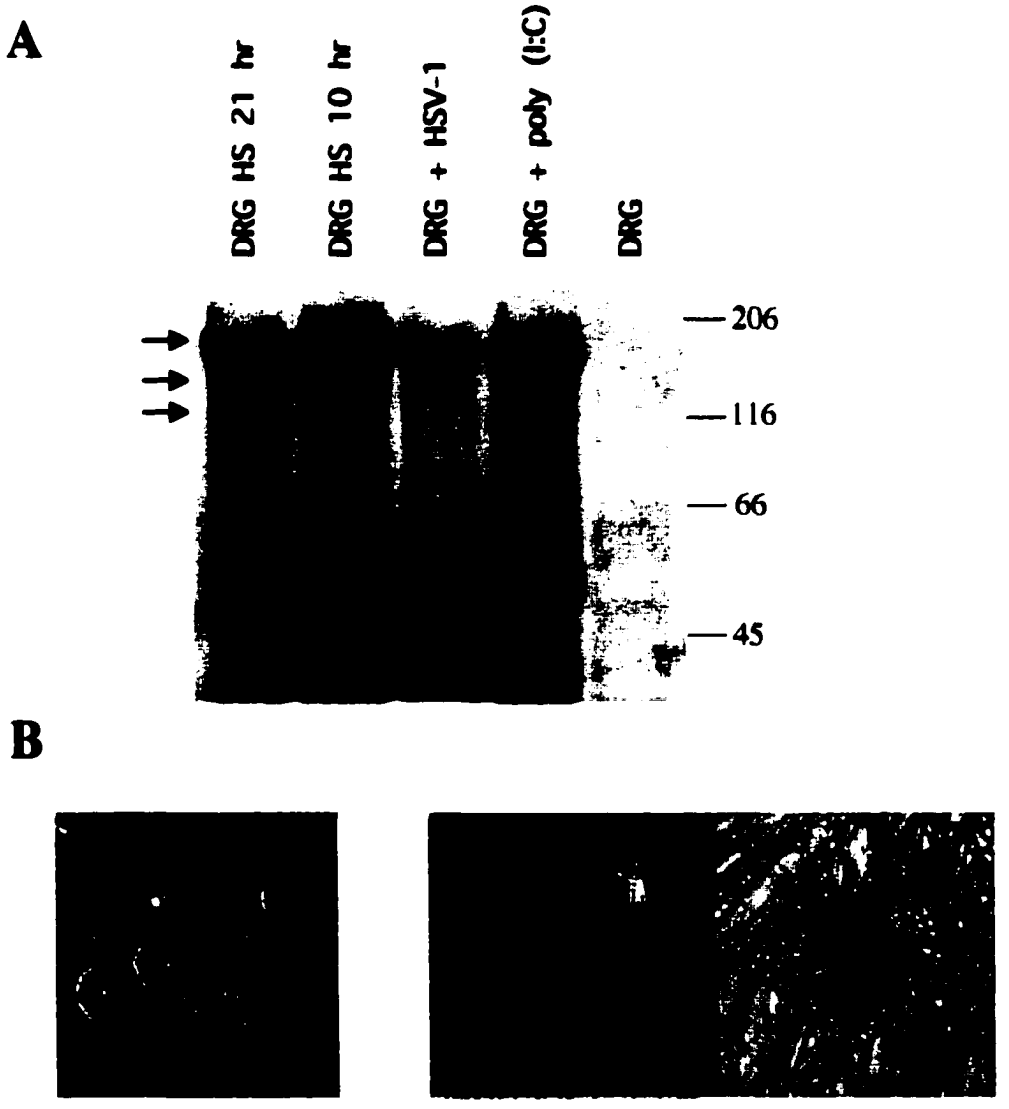

Figure 5.3 PML can be induced in neurons . (A) Detection by western blot of the large molecular weight PML isoform(s) can be induced in neurons by poly (I:C) treatment (**DRG + poly (I:C)**, infection with HSV-1 at a multiplicity of infection of 5 (**DRG + HSV-1**), and heat shocking at 44°C for 15 minutes and harvesting neurons 10 hours and 21 hours post-heat shock (**DRG HS 10 hr** and **DRG HS 21 hr**, respectively). Primary antibody used is A-20 anti-PML. 30 μg whole cell lysate loaded per lane. Positions of molecular weight markers indicated to the right in kilodaltons. Arrows indicate potential PML isoforms. (B) PML as detected by immunocytochemistry with A-20 antibody in untreated neurons (**DRG**) and two views of neurons treated with poly (I:C) (**DRG + poly (I:C)**. All photomicrographs are shown at the same magnification.

confocal microscopy that the A-20 antibody recognized proteins in the cytoplasm of neurons treated with poly (I:C), but nothing in the nuclei resembling ND10s (data not shown).

Poly (I:C) had no effect on establishment of HSV-1 latency. The model of latency proposed in the last part of this chapter would predict that interferon has no effect on the establishment of latency because ICP0, the major viral antagonist of the IFN α/β response, is at low levels in neurons. Thus treatment with poly (I:C) and HSV-1 infection have the same outcome: the expression of IFN-inducible genes. To test this, untreated and poly (I:C) treated neurons were latently infected with HSV-1, and then three weeks post-infection, they were fixed for *in situ* hybridization for detection of the LAT transcript, one marker of latency. Table 5.1 has the results of this experiment. HSV-1 established latency at similar frequencies in untreated and poly (I:C) treated neurons (49.9% vs 45.7%, respectively). Thus, poly (I:C) had no discernable effect on the establishment of HSV-1 latency in neurons.

5.5 DISCUSSION

While PML and SUMO-1 proteins were upregulated in neurons treated with poly (I:C), ND10 structures were not. Since PML was not normally expressed in neurons, but was upregulated by poly (I:C), it may function solely as an antiviral protein in this cell type. Overexpression of PML has already been shown to inhibit vesicular stomatitis virus and influenza A, and PML did not need to be localized to ND10s in order for this inhibition to occur (4). In this chapter, we confirmed that overexpression of PML inhibited HSV-1 in HeLa cells.

Table 5.1 Effect of poly (I:C) on the establishment of HSV-1 latency.

	number LAT positive cells/total cells counted[a]	percentage LAT positive cells
untreated neurons[b]	224/476	47.1%
	290/669	43.3%
	282/412	68.4%
	125/315	39.7%
	228/449	50.8%
total	1149/2321	49.9 ± 11.2%[d]
neurons + poly (I:C)[b,c]	277/668	41.5%
	192/484	39.7%
	261/633	41.2%
	291/497	58.6%
	296/626	47.3%
total	1317/2908	45.7 ± 7.8%[d]

Latency was determined in neurons by *in situ* hybridization for the detection of the latency associated transcript, LAT.

[a] for each sample, five fields were counted. Each sample number represents the sum of all LAT positive cells in all five fields, and the sum of all cells counted in all five fields.

[b] neurons were infected at a multiplicity of 5 pfu/cell, and maintained in culture in the presence of acyclovir for 3 weeks before fixation.

[c] neurons were treated with 100 µg/ml poly (I:C) 18 hours prior to HSV-1 infection, then placed in normal maintenance medium after infection.

[d] percentage ± standard deviation.

The PML species found in neurons were unexpected. They did not react with the 5E10 antibody, but only reacted with the polyclonal antibody A-20 that was generated against the amino-terminal end of PML. PML protein is normally found in many different isoforms due to post-translational modification and alternative splicing of the transcripts (10, 25, 26, 30). In fact, 4 different cDNAs from human HeLa cells encoding different PML splice-variants have been identified and found to localize to different regions within the cell (2). The 5E10 antibody was found to only react with one of these PML splice-variants (2), the one typically found in ND10s when post-translationally modified with SUMO-1. Reports state that the different PML isoforms retained the same amino terminal exons, and differed in their carboxy termini (10). This probably explains why the A-20 antibody recognized the neuronal isoforms of PML. It will be interesting to investigate the PML transcripts in poly (I:C) treated neurons and HSV-1 infected neurons by northern blot, and compare them to cell lines to see if there are unique PML species expressed in neurons.

One surprising finding of the previous study in Chapter 4 is that SUMO-1 and SUMO-modified proteins are normally absent from primary sensory neurons. This implies that neurons do not normally post-translationally modify cellular proteins with SUMO-1. However, since SUMO-1 localized to the cytoplasm in neurons treated with poly (I:C), proteins involved in SUMO-1 covalent addition (Ubc9 (7) for conjugation of SUMO-1 and SAE1/SAE2 dimer (6) for activation of SUMO-1) must either be present or able to be upregulated in neurons. To our knowledge, this is the first demonstration that SUMO-1 expression is inducible by interferons.

SUMO-1 and PML were found primarily in the cytoplasm of poly (I:C) treated neurons. Whether SUMO-1 modifies the cytoplasmic PML remains to be determined by co-localization studies. It will be interesting to investigate why SUMO-1 is upregulated in neurons treated with IFN. It is possible that it may stablilize certain proteins may play a role in stabilizing proteins that are upregulated by the IFN.

PML protein was not found in resting DRG neurons, but was induced by IFN- α/β (poly (I:C) treatment, infection with HSV-1, and heat shock. In a non-neuronal cell, the interaction between ICP0 and ND10s presumably results in the degradation of various ND10 proteins, such as Sp100 and PML (which are interferon-inducible proteins themselves). But in neurons, which lack ND10s, ICP0 is not able to cause the proteasome degradation of these proteins. One possibility is that one of the interferon-inducible ND10 proteins, such as PML, is a repressor of virus genome. In the absence of ND10s, or at low levels of ICP0, HSV-1 cannot cause the degradation of PML. PML then represses the genome to a high degree in neurons, contributing to the latent state.

We found that, as we expected, poly (I:C) had no effect on the establishment of latency in neurons. This is probably because the treatment with poly (I:C) induced a state similar to the state induced by infection of neurons with HSV-1. The low levels of expression of ICP0 in neurons or the lack of ND10 structures or both may prevent the virus from overcoming an IFN-induced antiviral state. Again, perhaps an IFN-inducible protein may repress viral gene expression.

Surprisingly, heat shock, a condition that results in virus production, also upregulated PML in neurons. Also, it has been previously demonstrated that IFN genes are upregulated during reactivation-inducing conditions in neurons (34). These data add

a confounding factor to our hypothesis that PML, or perhaps another IFN-inducible protein, contributes to the latent state. However, it is possible that during reactivation, once ICP0 is expressed, it can be stabilized in neurons. The observation that ICP0 accumulates in HSV-1 infected sensory neurons that are treated with cyclosporin A (5) demonstrated that changes in phosphorylation within a neuron can contribute to the stability of ICP0. Perhaps this also helps explain why ICP0 minus mutants reactivate so poorly (15). It will be critical to examine the expression of ICP0 protein during reactivation.

If it turns out that ICP0 protein is never expressed at high levels during reactivation, perhaps the IFN response cannot be overcome in neurons at all. However, there are probably other stimulatory signals firing in the neuron that may cause the virus to be able to replicate, but at a lower rate due to the inhibitory action of IFNs. If this is the case, it might help explain why it takes so long to recover infectious virus from explanted ganglia and NGF-deprived cultured neurons, and why the titers of virus remain so low from these cells.

To help clarify some of these questions, it would be helpful to treat latently infected neurons with poly (I:C) or recombinant IFN α/β prior to NGF-withdrawal or forskolin treatment to test whether it has an effect on reactivation. Additionally, cells could be treated with poly (I:C) in the absence of NGF-withdrawal or forskolin treatment to see if poly (I:C), or some IFN-inducible gene, can actually cause reactivation. Our group is currently making an adenovirus that overexpresses PML (the ND10 isoform), and it will be interesting to see how the delivery of this PML species might affect HSV-1. These experiments are based on an assumption that the IFN-response somehow affects

HSV-1 reactivation. It may turn out that it has little or no direct effect, but is a co-incidental phenomenon. But, if I may anthropomorphize for a moment, it would be in keeping with HSV-1's character to somehow use the host IFN response to its advantage.

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CHAPTER 6

SUMMARY AND CONCLUDING REMARKS

"There will come a time when you believe everything is finished.
That will be the beginning."

-Louis L'Amour

The questions that were investigated in this dissertation, as well as the new questions that came to light, were questions pertaining to some of the basic interactions occurring between HSV-1 and its latency host, the sensory neuron. The question that plagues HSV-1 latency aficionados is, what makes sensory neurons different from every other mammalian cell type that HSV-1 can infect such that they allow for HSV-1 to completely shut-down virus production? The use of the *in vitro* model of HSV-1 latency affords us the opportunity of studying the virus-neuron interactions on a molecular level without any confounding systems, such as an immune system.

Looking first at the infection process, we examined the viral entry process in DRG neurons. As in all cell types studied so far, viral glycoprotein D (gD) was specifically responsible for the entry of virus into neurons. When virus was incubated with antibodies to gD, or recombinant cellular HveC protein, viral entry was either abolished or severely impaired in not only rat sensory neurons, but all other cell types tested. When neurons were incubated with a recombinant gD mutant, viral entry was also diminished. We were able to take this study further and demonstrate that HveC is the rat primary neuronal entry molecule used by HSV-1 by blocking entry with anti-HveC antibodies. As rat primary neurons did not express HveA as an alternative entry molecule for HSV-1, we were able to block entry with this method. To our knowledge, this is the only cell type described thus far that expresses HveC but not HveA (excepting lab modified cell lines).

This information may have therapeutic applications. Of course, perhaps the most impractical and unethical solution to eradicating HSV-1 from the population based on this research and others would be to genetically modify humans to express viral gD to

compete with viral entry molecules. But there may be alternative, less evil solutions. For instance, could a modified strain of HSV-1 be created that has a genetically modified gD protein that recognizes HveA but not HveC? Innoculation with this strain could infect epithelial cells, stimulate an immune response, but not set up latency because it would be unable to enter sensory neurons. With a primed immune system, perhaps subsequent natural exposure with wild-type virus could be prevented from setting up latency. The immune system might be able to contain the spread of HSV-1 before it reached sensory neurons.

Additionally, it is possible that a signal is mediated through HveC, either in all cell types, or specifically in the nervous system. This is not unfeasible, as previous studies have shown that in peripheral blood mononuclear cells, recombinant viral gD alone was able to induce an interferon response (1). Whether that specific cell-signaling event occurred through HveA or HveC was not addressed. But it demonstrates that binding of viral ligand to host cells can influence the cell's behavior. If neurons are capable of signaling through the HveC receptor, and since it appears that HSV-1 can only use HveC to enter neurons, perhaps a signal through this molecule changes the environment of the neuron to repress HSV-1 and establish latency.

By using our recombinant viruses, our first important observation was that ICP0 was very weakly expressed in DRG neurons, while ICP4 and ICP27 were expressed at high levels. ICP0 did not accumulate in punctate, nuclear regions, suggesting that ND10s are not structures that are resident in neurons. ICP0 is known to localize to ND10s very early in infection (4 hours or earlier) in non-neuronal cells, and alter the makeup of these

structures by causing the proteasome degradation of the proteins. If the ND10s are not present in neurons, how does this affect the virus?

Our investigation into the ND10 makeup of neurons led us to discover that ND10s were indeed absent from primary sensory neurons. At least, the classical SUMO-1-modified PML containing ND10s were absent. These studies leave us with more questions about the virus-ND10 interactions. For instance, does ICP0 interact with ND10s in non-neuronal cells mainly as a means of counteracting the cellular antiviral response by causing interferon-inducible ND10 protein (e.g. PML, Sp100) degradation? Does ICP0 need to localize to ND10s in order to stabilize itself for its viral roles, including the upregulation of viral genes, a function that ultimately leads to lytic infection? Can ICP0 function most efficiently as a transcriptional activator in ND10 regions? Are ND10s convenient anchors for HSV-1 pre-replication compartments? And then, what are the consequences for the virus if ND10s are absent in the infected cells? If viral latency is the consequence, is it due to the neuron actively causing latency, or is it a default condition arising from the absence of these structures?

PML and SUMO-1 proteins were upregulated in primary neurons treated with poly (I:C), a synthetic double-stranded RNA molecule that turns on the type I interferon response. Presumably, this confers some advantage to the infected neuron. Previously, ND10 structures have been shown to increase in number and size as a consequence of interferon treatment (6), leading to one hypothesis that ND10s exert antiviral effects on cells. But if PML that is found in the cytoplasm of neurons is upregulated as an antiviral response, perhaps the antiviral effects of these proteins are not restricted to ND10s, or maybe they are not even exerted from the ND10s.

PC12 cells contain ND10s where primary sensory neurons do not, which perhaps is a good enough reason to re-think the PC12 latency model system. Particularly if early events in infection, such as ICP0 and ND10 interactions, can take place in PC12 cells but not in DRG neurons. The recombinant viruses HSVEGFP0, HSVEGFP4, and HSVEGFP27 did not express GFP-immediate early fusion proteins when infected via the neurites of DRG neurons in Campenot chambers, but did express fusion proteins when cell bodies and neurites were infected. This may indicate that DRG neurons grown in Campenot chambers offer a more refined *in vitro* experimental model of latency. This model preliminarily displays the assumed mode of HSV-1 establishment of latency: the cessation of immediate early gene expression.

We were somewhat surprised to see such a difference in the ability of anti-HveC antibodies to block virus entry into mouse and rat neurons. Antibodies to HveC on rat neurons were able to block virus entry, but were completely ineffectual on mouse neurons. Previous findings have demonstrated that viral gD has a much lower affinity for mouse HveC than for human HveC (7), so low as to be almost undetectable (8). While we recognize that rats are a long way away from humans, it was encouraging to see that the anti-human HveC antibodies were functional in blocking entry into rat neurons. This suggests that rat HveC is much more similar to human HveC, and reiterates that the rat sensory neuron *in vitro* model is a good model for studying the interactions betwixt HSV-1 and neurons.

The finding that recombinant GFP-ICP0 is not expressed at high levels in sensory neurons, as well as the previous finding that ICP0 transcripts, but not protein, accumulate in sensory neurons (2), underscore the point that protein levels of ICP0 during the

establishment of latency and reactivation must be examined thoroughly. In the past, immediate early gene expression during latency and reactivation has often been measured in terms of promoter activity or transcript levels (4, 5, for example). With our recombinant viruses tested and functional, we are ready to characterize the immediate early protein expression in neurons during the establishment of latency, latency and reactivation.

One question that plagues this researcher's mind is the seemingly contradictory roles that ICP0 plays in latency. On one hand, ICP0 protein is expressed at such low levels from recombinant HSVEGFP0 virus (Chapter 2), from wild-type HSV-1 (2), and from recombinant adenovirus carrying the GFP-ICP0 gene under the E1A promoter (9). On the other hand, ICP0 has been shown to be important in establishing latency in our *in vitro* model (10), as well as *in vivo* in the mouse model (3) by using ICP0 deletion viruses. Perhaps whatever function ICP0 serves in the establishment of latency may be accomplished with very little protein. Another controversial way to explain this is would be to postulate that ICP0 transcripts alone may somehow be necessary for the establishment of latency. It may not be that unfeasible, since LAT seems to play a role in HSV-1 infection as a transcript. This could be tested two ways. First, by making a mutant HSV-1 virus that is mutated in its start codon, such that the transcript can no longer be translated, and comparing the establishment of latency to wild-type as well as the ICP0 deletion virus latency. Second, by infecting neurons with the ICP0 deletion virus and microinjecting recombinant ICP0 protein.

If the research and understanding in the field of HSV-1 latency can be likened to a huge jigsaw puzzle, perhaps the current status of that puzzle is that a good portion of the

outside edge is completed. It is my hope that my research has contributed at least a piece to that puzzle that makes the overall picture more recognizable. Hopefully it is a piece upon which others can build.

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