Published in final edited form as:

J Neurovirol. 2013 February ; 19(1): 32–41. doi:10.1007/s13365-012-0137-7.

C-Terminal trans-activation sub-region of VP16 is uniquely required for forskolin-induced herpes simplex virus type 1 reactivation from quiescently infected-PC12 cells but not for replication in neuronally differentiated-PC12 cells

Robert J. Danaher^{1,2}, Ross K. Cook¹, Chunmei Wang¹, Steven J. Triezenberg³, Robert J. Jacob^{1,2}, and Craig S. Miller^{*,1,2}

¹Department of Oral Health Practice, Division of Oral Medicine, Center for Oral Health Research, College of Dentistry, University of Kentucky, Lexington, KY

²Department of Microbiology, Immunology and Molecular Genetics, College of Medicine, University of Kentucky, Lexington, KY

³Van Andel Research Institute, Grand Rapids, MI

Abstract

The HSV-1 tegument protein VP16 contains a trans-activation domain (TAD) that is required for induction of immediate early (IE) genes during lytic infection and induced reactivation from latency. Here we report the differential contributions of the two sub-regions of the TAD in neuronal and non-neuronal cells during activation of IE gene expression, virus replication and reactivation from quiescently infected (QIF)-PC12 cells. Our studies show that VP16- and chemical (hexamethylenesacetamide)-induced IE gene activation is attenuated in neuronal cells. Irrespective of neuronal or non-neuronal cell backgrounds, IE gene activation demonstrated a greater requirement for the N-terminal sub-region of VP16 TAD (VP16N) than the C-terminal sub-region (VP16C). In surprising contrast to these findings, a recombinant virus (RP4) containing the VP16N deletion was capable of modest forskolin-induced reactivation whereas a recombinant (RP3) containing a deletion of VP16C was incapable of stress-induced reactivation from QIF-PC12 cells. These unique process-dependent functions of the VP16 TAD sub-regions may be important during particular stages of the virus life cycle (lytic, entrance and maintenance of a quiescent state and reactivation) when viral DNA would be expected to be differentially modified.

Keywords

herpes simplex virus; viral latency and quiescence; replication; reactivation; VP16; QIF-PC12 cells

Herpes simplex virus type 1 (HSV-1) encodes about 90 unique transcriptional units that encode at least 84 proteins with one or more functions (Roizman and Knipe 2001). Several genes and unique functional domains of multifunctional proteins are dispensable for virus replication in cell culture. The requirement of specific genes and/or particular protein functions often depends on the species and cell type of the infected cell and on the biological process being examined. Determination of the unique viral factors essential for individual

*Corresponding author: Dr. Craig S. Miller, Oral Medicine Section, MN 324, University of Kentucky College of Dentistry, 800 Rose Street, Lexington, KY 40536-0297 Tel: 859-323-5598 (office), Fax: 859-323-9136, cmiller@uky.edu.

Conflict of interest: The authors report no conflicts of interest related to this study.

stages of HSV latency in peripheral neurons (i.e. establishment, maintenance and reactivation) is complicated by the influence of these factors on multiple stages of the virus life cycle. Of particular interest is the essential tegument protein VP16 which, through its interactions with the host cell proteins HCF and Oct-1, is a major transactivator of viral immediate early (IE) gene expression during lytic infection (Weinheimer et al. 1992; Tal-Singer et al. 1999) and is required for early stages of reactivation from latency (Thompson et al. 2009; Sawtell et al. 2011). In addition to unique domains required for its interactions with HCF and Oct-1, VP16 also contains a potent transcriptional activation domain (TAD) located in its last 80 C-terminal amino acids (Triezenberg et al. 1988; Cousens et al. 1989). VP16 is also required at later stages of the replicative cycle for virion assembly (Poon and Roizman 1995), and interacts with and inhibits the virion host shutoff (vhs) protein (Smibert et al. 1994; Lam et al. 1996; Schmelter et al. 1996) preventing vhs-mediated destruction of viral mRNA and translational arrest.

The contributions of VP16-mediated activation of IE genes during reactivation from viral latency are not well understood. On the one hand, recombinant virus *inl814* has a greatly reduced ability to activate IE gene expression due to a 4 amino acid insertion in the region of VP16 that interacts with HCF-1 and Oct1 to form the VP16-induced complex (VIC, see Fig. 1) (Ace et al. 1989; Wysocka and Herr 2003). Nonetheless, *inl814* reactivates efficiently in both animal (Steiner et al. 1990; Valynagy et al. 1991) and cell culture (Miller et al. 2006) models of HSV-1 latency. In contrast, the recombinant virus RP5, which is completely crippled in its ability to activate IE gene expression due to the deletion of most of the VP16 TAD, is incapable of explant-induced reactivation (Tal-Singer et al. 1999). However, the inability of RP5 to reactivate in vivo could be interpreted to be due to its inability to establish an efficient state in the peripheral nervous systems of immunocompetent mice. More recent data with V422, a mutant similar to RP5, showed it is also incapable of reactivation in the quiescently infected (QIF)-PC12 model, when equivalent amounts of viral copies of mutant and wild-type strain are readily established during latency (Miller et al. 2006). Together these data support the diverse and unexplained requirements of VP16 during reactivation from latency.

A potentially key function of VP16 during reactivation from latency is trans-activation of IE gene expression. The VP16 TAD consists of two sub-regions (VP16N and VP16C, see Fig. 1), each with variable potentials for activating IE gene expression (Triezenberg et al. 1988; Regier et al. 1993; Walker et al. 1993). In previous reports, the VP16N sub-region was found to be more important than the VP16C sub-region for activation of transiently transfected IE promoters (Tal-Singer et al. 1999) and for efficient activation of IE gene expression during lytic infection in HeLa cells (Yang et al. 2002). However, the relative importance of these two sub-regions in the reactivation of HSV-1 from a quiescent state is not known. Here we used the well characterized QIF-PC12 model of HSV-1 latency (Danaher et al. 1999a, 1999b), that is similar but distinct from the PC12 model described by Block et al. (Block et al. 1994), for the advantage of establishing similar levels of viral latency from which the role of this region in reactivation could be dissected in vitro. We hypothesized that VP16N would be required for efficient reactivation due to its greater importance in activating IE gene expression as previously reported (Tal-Singer et al. 1999; Yang et al. 2002).

Materials and Methods

Cells and viruses

Rat pheochromocytoma (PC12) and African green monkey kidney (Vero) cells were grown as previously described (Danaher et al. 1999b). Human osteosarcoma (U2OS) and rat lung fibroblast (RFL-6) cells were grown in McCoy's 5A medium supplemented with 10% FBS

and F-12K medium supplemented with 20% FBS, respectively. All cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained at 37°C in 5% CO₂. Recombinant HSV-1 strains bearing deletions of all or parts of the VP16 TAD have been previously described (Tal-Singer et al. 1999). These include strains RP1 (intact TAD), RP3 (lacking amino acids 456–490, VP16C), RP4 (lacking amino acids 413–452, VP16N) and RP5 (lacking amino acids 413–490) (Fig. 1). Propagation and titration of virus stocks was done in Vero and U2OS cells, respectively, in media supplemented with 3 μM N,N'-hexamethylenebisacetamide (HMBA). In all experiments, cells were inoculated at the indicated MOIs as determined on U2OS cells in the presence of HMBA.

Neuronal differentiation

PC12 cells were dissociated by passage through a 22-gauge needle and plated in RPMI 1640 containing 0.1% fraction V bovine serum albumin (BSA) in tissue culture dishes coated with rat tail collagen type 1 (Becton Dickinson, Franklin Lakes, NJ) at 2.2×10^5 cells/well for 12-well dishes or 5.5×10^5 cells/well for 6-well dishes. Cells were neuronally differentiated (ND) and maintained in RPMI 1640 supplemented with 0.1% BSA and 50 ng/ml of 2.5S mouse nerve growth factor (NGF) (Becton Dickinson) (maintenance medium) beginning on the day of plating. Essentially 100% of the cells showed morphological indicators of differentiation as indicated by microscopic visualization of dendritic processes.

Quantification of relative gene activity

PC12 cells were seeded to 6-well collagen-coated dishes and neuronally differentiated for one week prior to infection. Vero, U2OS and RFL-6 cells were seeded to 6-well tissue culture dishes at 5.5×10^5 cells/well the day before infections. Cells were inoculated with HSV-1 at a multiplicity of infection (MOI) of 1. Following 2 h incubation at room temperature, cells were rinsed twice with respective culture media and incubated at 37°C for 4 h in the presence and absence of HMBA (3 μM). Total RNA was purified from HSV-1 infected cultures using the RNeasy Mini Kit (Qiagen, Valencia, CA) and quantified spectrophotometrically. RNA (2.5 μg) was treated with DNase using DNA-free (Ambion, Austin, TX) as recommended by the manufacturer. DNase-treated RNA was reverse transcribed using random primers and SuperScript II (Invitrogen). The quantity of cDNA synthesized for selected host and viral transcripts was determined by real-time PCR as previously described (Danaher et al. 2005). Genes assessed included α0 (encodes ICP0), α4 (encodes ICP4) and α27 (encodes ICP27). Primers and probes are as described (Cohrs et al. 2000) for α27 and (Danaher et al. 2005) for α4 and α0. All PCR assays of negative control (–RTase) and experimental (+RTase) samples were performed as previously reported (Danaher et al. 2005) individually and in triplicate, respectively, from duplicate samples, unless otherwise indicated. Each real time PCR assay included five standards examined in triplicate.

Establishment of a quiescent infection and reactivation of HSV-1

Quiescent HSV-1 infections were established in ND-PC12 cells in 12-well plates at an MOI of 1 as previously described (Danaher et al. 1999b; Miller et al. 2006). Briefly, ND-PC12 cells were cultured in maintenance medium for 4 days post-plating. On days 4 through 6, cells were cultured in maintenance medium supplemented with 10% horse serum and 5% FBS instead of BSA. On day 6, cells were cultured in maintenance medium supplemented with 100 M acycloguanosine (ACV) (Sigma, St. Louis, MO) and then inoculated with virus (12, 12-well plates/strain) the following day. Infected cells were cultured in maintenance medium supplemented with ACV for 10 days. After ACV withdrawal, a quiescent state was maintained for 7 days prior to experimental stress treatment (4, 12-well plates/strain/treatment group). Cultures that were free of detectable infectious virus as determined in culture supernatants were stress treated to activate virus on day 17 post-infection (pi) by

subjecting the cells to heat stress (HS) (43°C for 3 h) or to maintenance medium supplemented with 50 μ M forskolin (Sigma) as previously described (Danaher et al. 1999a, 1999b). QIF-PC12 cell culture supernatants were monitored for virus production by plaque-forming assay performed on monolayers of U2OS cells in medium supplemented with HMBA and pooled human IgG. Cultures were scored reactivation positive on the first day one or more plaques were detected on indicator cells.

Viral growth analysis

PC12 cells were seeded to 12-well collagen-coated dishes and neuronally differentiated as described above for the quantification of relative gene activity. One week post-plating, cells were inoculated with HSV-1 at MOI's of 1 and 10. Following 2 h incubation at 37°C, cells were rinsed once with citrate buffer, pH 3, twice with maintenance medium and then incubated in maintenance medium at 37°C. Cultures were frozen at various times post-inoculation, and virus titers were determined in duplicate by plaque assay with U2OS cells in medium supplemented with HMBA and pooled human IgG.

Results

IE gene activation is compromised in neuronal cells

At present, the contribution of the neuronal phenotype in the ability of VP16 to activate IE gene expression is not well understood. We initially addressed this by comparing IE gene expression in neuronal and non-neuronal cells infected with a recombinant virus lacking VP16 TAD (RP5) or with a recombinant virus containing a restored VP16 TAD (RP1). In these experiments, IE gene expression levels following infection with RP5 were designated “basal” levels and were compared to identically infected rat lung (RFL-6), monkey kidney (Vero) and human osteosarcoma (U2OS) cells. These three permissive cell lines served as controls for comparison of host species and neuronal cell effects. In all cases, total RNA was purified from cultures 4 h pi, and cDNA, synthesized from equivalent amounts of total RNA, was quantified by quantitative real-time PCR (qPCR). Following RP5 infections, similar levels of basal IE gene expression, based on the relative number of cDNA copies generated per ng total RNA, were observed in ND-PC12, Vero and RFL6 cells, but were at least 10-fold higher in U2OS cells (Fig. 2a). Expression levels of IE genes (ICP0, ICP4 and ICP27) were 10 to 100-fold higher in RP1- than RP5-infected ND-PC12 cells (Fig. 2b). This level of induction was at least 10-fold lower than observed in Vero and RFL-6 infected cells, indicating a compromise in IE gene activation function of VP16 in the neuronal cells. Also, the similar IE gene activation levels observed in RFL-6 cells compared to Vero cells indicates that the reduced activation in the neuronal cells is not merely due to their rat origin. As expected, the magnitude of IE gene activation in U2OS cells was lower than that in Vero and RFL6 cells, likely due to the greater level of basal IE expression observed in RP5-infected U2OS cells.

IE gene activation is known to be HMBA responsive (McFarlane et al. 1992; Smiley and Duncan 1997; Yang et al. 2002) in an MOI-dependent manner. We therefore further analyzed IE gene activation under chemical stress induction of HMBA. Infections with RP5 were established in the same four cell lines described above in the presence or absence of HMBA. Here we hypothesized that HMBA would induce IE genes in neuronal cells similar to its ability to induce IE genes in permissive cell lines. Surprisingly, the VP16 TAD deletion was only poorly complemented by HMBA in ND-PC12 cells (Fig. 2c). This is in sharp contrast to 100-1,000-fold induction by HMBA that occurred in Vero, RFL-6 and U2OS cells. Thus, IE gene activation by both VP16 and chemical induction appears to be compromised in the neuronal cells used.

VP16N trans-activation sub-region is not required for forskolin-induced reactivation

Since we have previously shown that VP16 TAD is required for stress-induced reactivation of QIF-PC12 cells (Miller et al. 2006), it was of interest to determine if viruses lacking individual VP16 sub-regions would reactivate in a pattern consistent with the observed IE activation potentials. QIF-PC12 cultures were established (MOI = 1) with recombinant viruses RP3 and RP4 that lack VP16C and VP16N, respectively, and evaluated for spontaneous, HS- and forskolin-induced reactivation. As expected, low spontaneous reactivation was observed from the mock-treated QIF cultures, and RP1 reactivated at typical wild-type levels within 8 days following induction with forskolin (reactivation detected in 32 of 42 treated cultures, (32/42, 76%) and heat-stress (37/41, 90%) (Fig. 3a). In contrast, RP5 was incapable of efficient reactivation, with virus detection in only 6.3% (3/48), 17% (8/48) and 0/48 of cultures within 8 days of HS-, forskolin- and mock-treatment, respectively (Fig. 3d). Similar to RP5, RP3 (Fig. 3b) was incapable of efficient stress-induced and spontaneous reactivation as virus was only detected in 15% (7/48), 10% (5/48) and 0/48 of cultures within 8 days of HS-, forskolin- and mock-treatment, respectively. Unexpectedly however, RP4 (Fig. 3c), which is reported to be substantially more restricted in IE gene activation than RP3 (Yang et al. 2002), was capable of forskolin-induced reactivation with the detection of infectious virus in 49% (23/47) of cultures compared to only 15% (7/48) or 4.3% (2/47) in HS- or mock-treatment cultures, respectively. These data suggest that VP16C is required for forskolin-induced reactivation, whereas VP16N is not required. Moreover, VP16N is not sufficient for reactivation under either forskolin- or HS-induced conditions. These findings indicate that forskolin and HS may act via alternative means in these cells.

VP16N more efficiently trans-activates IE genes than VP16C in neuronal cells

To determine if the unexpected reactivation phenotypes of RP3 and RP4 were due to TAD sub-region-specific requirements for IE gene activation specific to neuronal cells, the level of IE gene expression for these mutants were evaluated in ND-PC12 cells. Since our initial experiments showed that activation of IE gene expression was uniquely perturbed in ND-PC12 cells independent of species influences, gene expression levels were compared in ND-PC12 cells with control Vero cells. Both cell lines were infected (MOI = 1) and IE gene expression was evaluated as described above. Here the presence of VP16 sub-regions markedly influenced the level of IE gene expression observed. As expected, IE gene expression was induced by VP16 ~100- fold in RP1 infected ND-PC12 (Fig. 4a) and ~1,000-fold in Vero cells (Fig. 4b) at 4 h pi, respectively. In contrast, RP-3 induced IE gene expression ~3-fold in ND-PC12 cells (Fig. 4a) and 65 to 950 fold in Vero cells (Fig. 4b) which corresponds to approximately 10-fold or greater reduction compared to respective RP1 infected cultures. Further, IE gene expression in RP4 infected ND-PC12 and Vero cells was less than 10% of those infected with RP3 such that the levels of expression in ND-PC12 cells (Fig. 4a) was near the limit of detection. Importantly, RP4 was more highly attenuated in IE gene activation in both neuronal (Fig. 4a) and non-neuronal cells (Fig. 4b) than RP3. These findings reaffirm that VP16N is more important than VP16C for trans-activation of IE genes during lytic infection of neuronal and non-neuronal cells. Counter-intuitively, the ability of RP4 to reactivate more efficiently than RP3 when induced by forskolin is not due to a greater ability of RP4 to induce IE gene activation at early times following infection.

VP16N is required for efficient virus replication in neuronal cells

The reduced level of IE gene expression in RP4-infected cells, compared to that in RP3-infected cells, is consistent with RP4's reduced replication efficiency in Vero cells (Tal-Singer et al. 1999). To determine if the contrasting IE gene activation (RP3 > RP4) and forskolin-induced reactivation (RP4 > RP3) phenotypes of these recombinant viruses was due to neuronal-specific replication phenotypes, we evaluated the efficiency of virus

replication of both strains in ND-PC12 cells by growth curve analysis. Following one week of NGF-induced differentiation, ND-PC12 cells were infected with RP3 and RP4 at MOI's of 1 and 10. Cultures were infected with RP1 and RP5 as positive and negative controls, respectively. Consistent with their relative replication efficiencies in Vero cells (Tal-Singer et al. 1999), the kinetics of RP3 replication following infection of ND-PC12 cells at an MOI of 1, was lower than that of RP1, yet substantially greater than RP4 and RP5 (Fig. 5a). In fact, the detection of RP4 replication required infection of ND-PC12 cells at an MOI of 10 and yields still did not reach those of cultures infected with RP3 at MOI of 1 (Fig. 5b). These data indicate that the relative efficiency of virus replication in ND-PC12 cells is consistent with their reduced replication efficiencies in Vero cells (Tal-Singer et al. 1999). Also, RP3 and RP4 replication characteristics are consistent with RP3's greater IE gene activation potential in ND-PC12 and Vero cells (Fig. 4). Together, the findings indicate that the enhanced forskolin-induced reactivation phenotype of RP4 is not the result of either 1) enhanced IE gene activation or 2) efficient replication in neuronal-like cells, but instead appears to be specific to processes directly associated with reactivation of a quiescent genome.

Discussion

We used a series of VP16 mutants bearing various deletions of the TAD (Tal-Singer et al. 1999) to evaluate the importance of individual VP16 TAD sub-regions (VP16N and VP16C) for IE gene expression and stress-induced reactivation of HSV-1 from QIF-PC12. Our IE gene activation studies in different cell lines showed that neuronal cells are much less efficient in supporting VP16 TAD-dependent IE gene activation than non-neuronal cells and that deletion of the N- or C-terminal VP16 TAD sub-regions substantially reduced IE gene activation and subsequent virus replication in neuronal cells. Using the QIF-PC12 model, we observed that a recombinant virus lacking VP16C (RP3) was incapable of HS- and forskolin-induced reactivation whereas a recombinant virus lacking VP16N (RP4) reactivated modestly following induction with forskolin but was incapable of HS-induced reactivation. These findings indicate that the deletion of either the C- or N-terminus of the VP16 TAD confer unique process-dependent functions during reactivation and virus replication. In addition, the results support the interpretation that forskolin (chemical) and HS (physical) induction from quiescence involves most likely different processes in our system.

HSV-1 demonstrates attenuated infection in neurons in many model systems and humans. The attenuated infection in vivo results from influences by the local immune response (Liu et al. 1996; Sainz and Halford 2002) as well as intraneuronal processes (Bloom and Stevens 1994). The intraneuronal events include many biochemical processes such as enhanced survival capacity (Garrido et al. 1999), lack of cellular factors required for productive infection (i.e., cdk's (Schang et al. 2002), production of intraneuronal inhibitory proteins (Zhangfei) (Akhova et al. 2005), lack of a robust DNA damage response (Lilley et al. 2005), abundant expression of LAT (Maillet et al. 2006), degradation of viral protein in neurons (Eom et al. 2004), and the unique regulation of viral gene expression in neurons (Pesola et al. 2005; Bertke et al. 2011). Our findings add to the growing body of evidence that replication in neurons is dampened by the unique functions of VP16 relative to IE gene activation in neuronal cells. We found that during infection, rat neuronal cells support substantially less IE gene expression than that observed in permissive primate cell lines. One hypothesis suggests that subtle differences in the primary structure of the host Oct-1 proteins may be responsible for this outcome (Cleary et al. 1993; Suzuki et al. 1993). However, the efficient IE gene activation observed in the rat RFL-6 cell line may argue against this notion. Furthermore, the reduced level of IE gene activation in ND-PC12 cells was not due to higher basal IE gene expression in these cells, thus VP16 appears to have reduced activity in a

neuronal milieu, similar to that observed by others (Kemp and Latchman 1989; Wheatley et al. 1990; Nichol et al. 1996; Weir 2001). A possible explanation for the reduced VP16-mediated IE gene activation in ND-PC12 cells is that both VP16 (Kim et al. 2012) and HCF-1 (Kristie et al. 1999) are preferentially located in the cytoplasm in neurons and are therefore not present at appropriate levels in the nucleus to contribute to efficient IE gene expression.

Consistent with the unique neuronal phenotype, VP16-independent IE gene activation in neuronal cells was only minimally responsive to HMBA (0.25- to 0.65-fold increase) compared to 50- to 1,000-fold increases in RFL-6 and Vero cells. This novel finding stands in contrast with HMBA's ability to activate IE genes independent of VP16 in permissive cells (McFarlane et al. 1992; Smiley and Duncan 1997; Yang et al. 2002). Although the exact mechanism of HMBA complementation of IE genes is unclear, it is possible that the lack of a cellular stress response or state of the viral DNA in neurons is contributory (Su et al. 2002; Lilley et al. 2005; Terry-Allison et al. 2007; Bloom et al. 2010). Interestingly, HMBA has been shown to enhance IE gene transcription in HSV-1 infected embryonic chicken neurons *ex vivo* (Hafezi et al. 2012). However, as these studies involved infection of trigeminal ganglia explant cultures consisting of macroglial cells and neurons in the presence of HMBA for 24 h, it is not clear if the enhanced IE expression was dependent on secondary signaling through the macroglial cells and/or the long duration of chemical exposure. Alternatively, difference between chicken and rat backgrounds may be responsible for the differences observed.

We next investigated the relative requirements of two sub-regions of VP16 TAD for reactivation from HSV-1 quiescence. The VP16N and VP16C sub-regions are able to independently activate transcription by unique mechanisms (Seipel et al. 1992; Goodrich et al. 1993; Regier et al. 1993; Sullivan et al. 1998; Ikeda et al. 2002) and their deletions do not affect the VIC domain. We used QIF-PC12 cells to investigate this region because the model allows for the establishment of a latent-like state where equivalent viral genome copy numbers of mutant and wild-type viruses can be obtained, and reactivation can be induced using physical, chemical and biological stimuli, even with mutants that have extremely poor replication properties (Danaher et al. 1999a, 1999b, 2001, 2005). Also, the absence of immune and permissive cells allows us to observe uniquely neuronal events (Liu et al. 2000, 2001; Decman et al. 2005). Recombinant viruses lacking either of the individual sub-regions VP16C (RP3) or VP16N (RP4) were markedly reduced in stress-induced reactivation from QIF-PC12 cells compared to RP1, which contains the intact VP16 TAD. Like RP5, RP3 did not reactivate significantly following induction with either HS or forskolin, indicating that the VP16C sub-region is required for efficient stress-induced reactivation. In contrast, RP4 reactivated after induction with forskolin treatment, albeit at reduced levels compared to that of RP1. Thus, these data indicate that VP16N is not required for forskolin-induced reactivation from latency although it is required for activation of IE gene expression during the early stages of a primary infection of permissive cells (Tal-Singer et al. 1999; Yang et al. 2002).

Since RP4 is dramatically more compromised in IE gene activation compared to RP3 in permissive cells (Tal-Singer et al. 1999; Yang et al. 2002) it was surprising that RP3 was incapable of stress-induced reactivation whereas RP4 was capable of substantial forskolin-induced reactivation. To determine if this outcome was due to trans-activation properties specific to neuronal cells, IE gene activation was measured in ND-PC12 cells. Consistent with the relative influence of the individual sub-regions of VP16 TAD (Tal-Singer et al. 1999; Yang et al. 2002), IE gene activation was reduced 80–90% and 99%, compared to the parental virus (RP1), following infection of Vero cells with recombinants lacking the VP16C (RP3) and VP16N (RP4) sub-regions, respectively (Fig. 4b). The same hierarchical

effects were observed following infection of neuronal cells (Fig. 4a), albeit the degree of reduction was substantially more pronounced. Importantly, virus yield following infection of neuronal cells with these same recombinant viruses was consistent with their ability to efficiently or inefficiently express IE genes. One explanation for the contrasting requirements for the VP16N and VP16C sub-regions for IE gene activation and stress-induced reactivation may be the states of the viral genome that would be expected to be different during early and late stages following delivery of viral DNA to the cell nucleus. Interestingly, the VP16N sub-region (present in RP3 and absent in RP4) is critical for strong interaction with the Mediator coactivator complex component MED25 (Yang et al. 2004) and is critical for efficient VP16-mediated transcription of naked DNA templates (Ikeda et al. 2002). This may account for the greater IE gene activation and virus yield requirement following infection of permissive and neuronal cells with RP3 compared to RP4. In contrast, the VP16C sub-region binds numerous transcription factors including TBP (Stringer et al. 1990), TFIIA (Kobayashi et al. 1998), TFIIB (Roberts et al. 1993), the 32 kDa subunit of TFIID (Klemm et al. 1995), and the p62-Tfb1 subunit of TFIIF (Xiao et al. 1994; Langlois et al. 2008) and is required for VP16's interaction with the histone acetyltransferase CBP and in vitro trans-activation of chromatin templates (Ikeda et al. 2002). These features may account for the unique requirement of this specific region during reactivation of latent viral genomes that are presumed to be associated with nucleosomes in chromatin structures in QIF-PC12 cells (Su et al. 2002; Ferenczy et al. 2011) as they are during HSV-1 latency in trigeminal ganglia (Deshmane and Fraser 1989).

In conclusion, our data indicate that neurons are unique in regulating IE gene activation, and the VP16 TAD is critical for reactivation of HSV-1 from a quiescent state in neurons. Current evidence supports that VP16 migrates from the cytoplasm to the nucleus in response to stress conditions where the VP16N is critical for reactivation. The neuronal factors that regulate these processes remain to be identified.

Acknowledgments

This research was supported by a grant from the National Institute of Dental Craniofacial Research, no. DE014142 (to C.S.M.).

References

- Ace CI, McKee TA, et al. Construction and characterization of a herpes simplex virus type-1 mutant unable to transduce immediate-early gene-expression. *J Virol.* 1989; 63(5):2260–2269. [PubMed: 2539517]
- Akhova O, Bainbridge M, et al. The neuronal host cell factor-binding protein Zhangfei inhibits herpes simplex virus replication. *J Virol.* 2005; 79(23):14708–14718. [PubMed: 16282471]
- Bertke AS, Swanson SM, et al. A5-positive primary sensory neurons are nonpermissive for productive infection with herpes simplex virus 1 in vitro. *J Virol.* 2011; 85(13):6669–6677. [PubMed: 21507969]
- Block T, Barney S, et al. Long term herpes simplex virus type 1 infection of nerve growth factor-treated PC12 cells. *J Gen Virol.* 1994; 75(9):2481–2487. [PubMed: 8077952]
- Bloom DC, Giordani NV, et al. Epigenetic regulation of latent HSV-1 gene expression. *Biochim Biophys Acta.* 2010; 1799:246–256. [PubMed: 20045093]
- Bloom DC, Stevens JG. Neuron-specific restriction of a herpes simplex virus recombinant maps to the UL5 gene. *J Virol.* 1994; 68(6):3761–3772. [PubMed: 8189514]
- Cleary MA, Stern S, et al. Differential positive control by Oct-1 and Oct-2: activation of a transcriptionally silent motif through Oct-1 and VP16 corecruitment. *Genes Dev.* 1993; 7(1):72–83. [PubMed: 8422989]

- Cohrs J, Randall J, et al. Analysis of individual human trigeminal ganglia for latent herpes simplex virus type 1 and varicella-zoster virus nucleic acids using real-time PCR. *J Virol.* 2000; 74(24): 11464–11471. [PubMed: 11090142]
- Cousens DJ, Greaves R, et al. The C-terminal 79 amino acids of the herpes simplex virus regulatory protein, Vmw65, efficiently activate transcription in yeast and mammalian cells in chimeric DNA-binding proteins. *EMBO J.* 1989; 8(8):2337–2342. [PubMed: 2676518]
- Danaher RJ, Jacob RJ, et al. Heat stress activates production of herpes simplex virus type 1 from quiescently infected neurally differentiated PC12 cells. *J NeuroVirol.* 1999a; 5(4):374–383. [PubMed: 10463859]
- Danaher RJ, Jacob RJ, et al. Establishment of a quiescent herpes simplex virus type 1 infection in neurally-differentiated PC12 cells. *J NeuroVirol.* 1999b; 5(3):258–267. [PubMed: 10414516]
- Danaher RJ, Jacob RJ, et al. Histone deacetylase inhibitors induce reactivation of herpes simplex virus type 1 in a latency-associated transcript-independent manner in neuronal cells. *J NeuroVirol.* 2005; 11(3):306–317. [PubMed: 16036811]
- Danaher RJ, Savells-Arb AD, et al. Herpesvirus quiescence in neuronal cells: Virus activation induced by pituitary adenylate cyclase-activating polypeptide (PACAP) involves the protein kinase A pathway. *J NeuroVirol.* 2001; 7(2):163–168. [PubMed: 11517389]
- Decman V, Kinchington PR, et al. Gamma interferon can block herpes simplex virus type 1 reactivation from latency, even in the presence of late gene expression. *J Virol.* 2005; 79(16): 10339–10347. [PubMed: 16051826]
- Deshmane SL, Fraser NW. During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. *J Virol.* 1989; 63(2):943–947. [PubMed: 2536115]
- Eom CY, Heo WD, et al. The neural F-box protein NFB42 mediates the nuclear export of the herpes simplex virus type 1 replication initiator protein (UL9 protein) after viral infection. *Proc Natl Acad Sci USA.* 2004; 101(12):4036–4040. [PubMed: 15010529]
- Ferenczy MW, Ranayhossaini DJ, et al. Activities of ICP0 involved in the reversal of silencing of quiescent herpes simplex virus 1. *J Virol.* 2011; 85(10):4993–5002. [PubMed: 21411540]
- Garrido JJ, Carnicero E, et al. Differential effects on the survival of neuronal and non-neuronal cells after infection by herpes simplex virus type 1 mutants. *J NeuroVirol.* 1999; 5(3):280–288. [PubMed: 10414518]
- Goodrich JA, Hoey T, et al. Drosophila TAF(II)40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. *Cell.* 1993; 75(5):519–530. [PubMed: 8221891]
- Hafezi W, Lorentzen EU, et al. Entry of herpes simplex virus type 1 (HSV-1) into the distal axons of trigeminal neurons favors the onset of nonproductive, silent infection. *PLoS Pathog.* 2012; 8(5):e1002679. [PubMed: 22589716]
- Ikedo K, Stuehler T, et al. The H1 and H2 regions of the activation domain of herpes simplex virion protein 16 stimulate transcription through distinct molecular mechanisms. *Genes Cells.* 2002; 7(1): 49–58. [PubMed: 11856373]
- Kemp LM, Latchman DS. Regulated transcription of herpes simplex virus immediate-early genes in neuroblastoma cells. *Virology.* 1989; 171(2):607–610. [PubMed: 2548331]
- Kim JY, Mandarino A, et al. Transient reversal of episome silencing precedes VP16-dependent transcription during reactivation of latent HSV-1 in neurons. *PLoS Pathog.* 2012; 8(2):e1002540. [PubMed: 22383875]
- Klemm RD, Goodrich JA, et al. Molecular cloning and expression of the 32-kDa subunit of human TFIID reveals interactions with VP16 and TFIIB that mediate transcriptional activation. *Proc Natl Acad Sci USA.* 1995; 92(13):5788–5792. [PubMed: 7597030]
- Knez J, Bilan PT, et al. A single amino acid substitution in herpes simplex virus type 1 VP16 inhibits binding to the virion host shutoff protein and is incompatible with virus growth. *J Virol.* 2003; 77(5):2892–2902. [PubMed: 12584313]
- Kobayashi N, Horn PJ, et al. DA-complex assembly activity required for VP16C transcriptional activation. *Mol Cell Biol.* 1998; 18(7):4023–4031. [PubMed: 9632787]
- Kristie TM, Vogel JL, et al. Nuclear localization of the C1 factor (host cell factor) in sensory neurons correlates with reactivation of herpes simplex virus from latency. *Proc Natl Acad Sci USA.* 1999; 96(4):1229–1233. [PubMed: 9990006]

- Lam Q, Smibert CA, et al. Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion host shutoff function. *EMBO J.* 1996; 15(10):2575–2581. [PubMed: 8665865]
- Langlois C, Mas C, et al. NMR structure of the complex between the Tfb1 subunit of TFIID and the activation domain of VP16: Structural similarities between VP16 and p53. *J Am Chem Soc.* 2008; 130(32):10596–10604. [PubMed: 18630911]
- Lilley CE, Carson CT, et al. DNA repair proteins affect the lifecycle of herpes simplex virus 1. *Proc Natl Acad Sci USA.* 2005; 102(16):5844–5849. [PubMed: 15824307]
- Liu T, Khanna KM, et al. Gamma interferon can prevent herpes simplex virus type 1 reactivation from latency in sensory neurons. *J Virol.* 2001; 75(22):11178–11184. [PubMed: 11602757]
- Liu T, Khanna KM, et al. CD8(+) T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *J Exp Med.* 2000; 191(9):1459–1466. [PubMed: 10790421]
- Liu T, Tang QH, et al. Inflammatory infiltration of the trigeminal ganglion after herpes simplex virus type 1 corneal infection. *J Virol.* 1996; 70(1):264–271. [PubMed: 8523535]
- Maillet S, Naas T, et al. Herpes simplex virus type 1 latently infected neurons differentially express latency-associated and ICP0 transcripts. *J Virol.* 2006; 80(18):9310–9321. [PubMed: 16940542]
- McFarlane M, Daksis JJ, et al. Hexamethylene bisacetamide stimulates herpes simplex virus immediate early gene expression in the absence of trans-induction by Vmw65. *J Gen Virol.* 1992; 73(2):285–292. [PubMed: 1371540]
- Miller CS, Danaher RJ, et al. ICP0 is not required for efficient stress-induced reactivation of herpes simplex virus type 1 from cultured quiescently infected neuronal cells. *J Virol.* 2006; 80(7):3360–3368. [PubMed: 16537603]
- Nichol PF, Chang JY, et al. Herpes simplex virus gene expression in neurons: Viral DNA synthesis is a critical regulatory event in the branch point between the lytic and latent pathways. *J Virol.* 1996; 70(8):5476–5486. [PubMed: 8764059]
- Pesola JA, Zhu J, et al. Herpes simplex virus 1 immediate-early and early gene expression during reactivation from latency under conditions that prevent infectious virus production. *J Virol.* 2005; 79(23):14516–14525. [PubMed: 16282451]
- Poon APW, Roizman B. The phenotype in vitro and in infected cells of herpes simplex virus 1 alpha trans-inducing factor (VP16) carrying temperature-sensitive mutations introduced by substitution of cysteines. *J Virol.* 1995; 69(12):7658–7667. [PubMed: 7494274]
- Regier JL, Shen F, et al. Pattern of aromatic and hydrophobic amino acids critical for one of two subdomains of the VP16 transcriptional activator. *Proc Natl Acad Sci USA.* 1993; 90(3):883–887. [PubMed: 8381535]
- Roberts SGE, Ha I, et al. Interaction between an acidic activator and transcription factor TFIIB is required for transcriptional activation. *Nature.* 1993; 363(6431):741–744. [PubMed: 8515819]
- Roizman, B.; Knipe, DM. Herpes simplex viruses and their replication. In: Knipe, DM.; Howley, PM.; Griffin, DE.; Martin, MA.; Lamb, RA.; Roizman, B.; Straus, SE., editors. *Fields Virology*. Lippincott Williams & Wilkins; Philadelphia: 2001.
- Sainz B, Halford WP. Alpha/beta interferon and gamma interferon synergize to inhibit the replication of herpes simplex virus type 1. *J Virol.* 2002; 76(22):11541–11550. [PubMed: 12388715]
- Sawtell NM, Triezenberg SJ, et al. VP16 serine 375 is a critical determinant of herpes simplex virus exit from latency in vivo. *J NeuroVirol.* 2011; 17(6):546–551. [PubMed: 22144074]
- Schang LM, Bantly A, et al. Explant-induced reactivation of herpes simplex virus occurs in neurons expressing nuclear cdk2 and cdk4. *J Virol.* 2002; 76(15):7724–7735. [PubMed: 12097586]
- Schmelter J, Knez J, et al. Identification and characterization of a small modular domain in the herpes simplex virus host shutoff protein sufficient for interaction with VP16. *J Virol.* 1996; 70(4):2124–2131. [PubMed: 8642633]
- Seipel K, Georgiev O, et al. Different activation domains stimulate transcription from remote (enhancer) and proximal (promoter) positions. *EMBO J.* 1992; 11(13):4961–4968. [PubMed: 1464321]
- Smibert CA, Popova B, et al. Herpes simplex virus VP16 forms a complex with the virion host shutoff protein vhs. *J Virol.* 1994; 68(4):2339–2346. [PubMed: 8139019]

- Smiley JR, Duncan J. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 produces a phenotype similar to that of the in1814 linker insertion mutation. *J Virol.* 1997; 71(8):6191–6193. [PubMed: 9223515]
- Steiner I, Spivack JG, et al. A herpes simplex virus type 1 mutant containing a nontransducing Vmw65 protein establishes latent infection in vivo in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia. *J Virol.* 1990; 64(4):1630–1638. [PubMed: 2157048]
- Stringer KF, Ingles CJ, et al. Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. *Nature.* 1990; 345(6278):783–786. [PubMed: 2193231]
- Su YH, Moxley MJ, et al. Stability and circularization of herpes simplex virus type 1 genomes in quiescently infected PC12 cultures. *J Gen Virol.* 2002; 83(Pt 12):2943–2950. [PubMed: 12466470]
- Sullivan SM, Horn PJ, et al. Mutational analysis of a transcriptional activation region of the VP16 protein of herpes simplex virus. *Nucleic Acids Res.* 1998; 26(19):4487–4496. [PubMed: 9742254]
- Suzuki N, Peter W, et al. Mouse Oct-1 contains a composite homeodomain of human Oct-1 and Oct-2. *Nucleic Acids Res.* 1993; 21(2):245–252. [PubMed: 8441632]
- Tal-Singer R, Pichyangkura R, et al. The transcriptional activation domain of VP16 is required for efficient infection and establishment of latency by HSV-1 in the murine peripheral and central nervous systems. *Virology.* 1999; 259(1):20–33. [PubMed: 10364486]
- Terry-Allison T, Smith CA, et al. Relaxed repression of herpes simplex virus type 1 genomes in murine trigeminal neurons. *J Virol.* 2007; 81(22):12394–12405. [PubMed: 17855552]
- Thompson RL, Preston CM, et al. De novo synthesis of VP16 coordinates the exit from HSV latency in vivo. *PLoS Pathog.* 2009; 5(3):e1000352. [PubMed: 19325890]
- Trizzenberg SJ, Kingsbury RC, et al. Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes Dev.* 1988; 2(6):718–729. [PubMed: 2843425]
- Valyi-Nagy T, Deshmane S, et al. Induction of cellular transcription factors in trigeminal ganglia of mice by corneal scarification, herpes simplex virus type 1 infection, and explantation of trigeminal ganglia. *J Virol.* 1991; 65(8):4142–4152. [PubMed: 1649322]
- Walker S, Greaves R, et al. Transcriptional activation by the acidic domain of Vmw65 requires the integrity of the domain and involves additional determinants distinct from those necessary for TFIIB binding. *Mol Cell Biol.* 1993; 13(9):5233–5244. [PubMed: 8395001]
- Weinheimer SP, Boyd BA, et al. Deletion of the VP16 open reading frame of herpes simplex virus type 1. *J Virol.* 1992; 66(1):258–269. [PubMed: 1309245]
- Weir JP. Infection of human NT2 cells and differentiated NT-neurons with herpes simplex virus and replication-incompetent herpes simplex virus vectors. *J NeuroVirol.* 2001; 7(1):43–51. [PubMed: 11519481]
- Wheatley SC, Kemp LM, et al. Cell lines derived from dorsal root ganglion neurons are nonpermissive for HSV and express only the latency-associated transcript following infection. *Exp Cell Res.* 1990; 190(2):243–246. [PubMed: 2170153]
- Wysocka J, Herr W. The herpes simplex virus VP16-induced complex: the makings of a regulatory switch. *Trends Biochem Sci.* 2003; 28(6):294–304. [PubMed: 12826401]
- Xiao H, Pearson A, et al. Binding of basal transcription factor TFIID to the acidic activation domains of VP16 and p53. *Mol Cell Biol.* 1994; 14(10):7013–7024. [PubMed: 7935417]
- Yang FJ, DeBeaumont R, et al. The activator-recruited cofactor/Mediator coactivator subunit ARC92 is a functionally important target of the VP16 transcriptional activator. *Proc Natl Acad Sci USA.* 2004; 101(8):2339–2344. [PubMed: 14983011]
- Yang WC, Devi-Rao GV, et al. General and specific alterations in programming of global viral gene expression during infection by VP16 activation-deficient mutants of herpes simplex virus type 1. *J Virol.* 2002; 76(24):12758–12774. [PubMed: 12438601]

Fig. 1. Schematic representation of the (a) HSV-1 genome and (b) VP16 polypeptide indicating the region involved in assembly of the VP16-induced complex (VIC) with Oct-1 and HCF-1 and the trans-activation domain (TAD). Upper diagram adapted from (Knez et al. 2003). Lower panels adapted from (Tal-Singer et al. 1999). RP3: Truncated at codon 456. RP4: Lacks amino acids 413–452. RP5: Truncated at codon 412.

Fig. 2.

Effect of the VP16 TAD and HMBA on HSV-1 IE gene expression. Cells were plated in 6-well dishes and inoculated with recombinant viruses (MOI = 1) containing wt and deleted VP16 TAD, RP1 and RP5, respectively, as described in Material and Methods. Infected cultures in (c) were grown in the presence and absence of 3 μ M HMBA. Cells were harvested 4 h pi and cDNA was synthesized from equivalent amounts of RNA from duplicate cultures per group and quantified by qPCR in triplicate. (a) Mean basal IE gene expression of RP5 infected cells. (b) Mean VP16 TAD-dependent IE gene expression levels of RP1 infected cultures relative to RP5 infected cells for each cell line. (c) Mean HMBA-induced IE gene expression levels of RP5 infected cultures relative to cells infected with RP5 in the absence of HMBA.

Fig. 3.

The N-terminal VP16 TAD sub-region is required for forskolin-induced HSV-1 reactivation from QIF-PC12 cells. QIF-PC12 cells were established with (a) RP1, (b) RP3, (c) RP4 and (d) RP5 at MOI of 1 in duplicate experiments ($n=72$ for each strain/experiment) and maintained in 12-well plates for 10 days in the presence of ACV. Cultures were either sham-treated (triangle), forskolin-treated (circle) or HS-treated (square) seven days post-ACV removal. Cultures were screened for infectious virus on day 0 and daily beginning 2 days post treatment, by direct plaque assay in U2OS cells supplemented with HMBA. Data represents average ($\pm$ SD) of two independent experiments.

Fig. 4.

Relative IE gene expression levels during infection of Vero and ND-PC12 cells with RP1, RP3 and RP4. (a) Vero and (b) ND-PC12 cells were inoculated with RP1, RP5 or recombinant viruses lacking the VP16C and VP16N VP16 TAD sub-regions, RP3 and RP4, respectively (MOI = 1). Virus infections, RNA purification and cDNA synthesis and quantification were performed as described in Fig. 2. Average ($\pm$ SD) VP16 TAD sub-region dependent IE gene expression levels of RP1, RP3 and RP4 infected cultures are expressed relative to RP5 infected cells (α 0, $n=1$; α 4 and α 27, $n=2$).

Fig. 5. Growth of HSV-1 recombinants in ND-PC12 cells. PC12 cultures were neuronally differentiated for 7 days and infected at MOIs of (a) 1 and (b) 10. At the indicated times, triplicate cultures were harvested, frozen and thawed three times, and viral yields were measured in duplicate by plaque assay on U2OS cells supplemented with HMBA.