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The DNA methyltransferase DNMT3A associates with viral proteins and impacts HSV-1 infection

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Abstract

Viral infections can alter the cellular epigenetic landscape, through modulation of either DNA methylation profiles or chromatin remodeling enzymes and histone modifications. These changes can act to promote viral replication or host defense. Herpes simplex virus type 1 (HSV-1) is a prominent human pathogen, which relies on interactions with host factors for efficient replication and spread. Nevertheless, the knowledge regarding its modulation of epigenetic factors remains limited. Here, we used fluorescently-labeled viruses in conjunction with immunoaffinity purification and mass spectrometry to study virus-virus and virus-host protein interactions during HSV-1 infection in primary human fibroblasts. We identified interactions among viral capsid and tegument proteins, detecting phosphorylation of the capsid protein VP26 at sites within its UL37-binding domain, and an acetylation within the major capsid protein VP5. Interestingly, we found a nuclear association between viral capsid proteins and the *de novo* DNA methyltransferase DNMT3A, which we confirmed by reciprocal isolations and microscopy. We show that drug-induced inhibition of DNA methyltransferase activity, as well as siRNA- and shRNA-mediated DNMT3A knockdowns trigger reductions in virus titers. Altogether, our results highlight a functional association of viral proteins with the mammalian DNA methyltransferase machinery, pointing to DNMT3A as a host factor required for effective HSV-1 infection.

Keywords

DNMT3A; HSV-1; phosphorylation; protein-protein interaction; virus-host interactions

INTRODUCTION

Viruses are intracellular parasites that have co-evolved with their hosts to become operators of host cellular systems. In order to replicate and spread, viruses depend on dynamic and temporally-regulated interactions with host proteins. Herpes simplex virus type 1 (HSV-1) is a human virus that affects ~60% of the adult US population [1]. HSV-1 infects the epithelial surface and peripheral nervous system where the disease caused by the virus most

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commonly manifests itself as skin lesions. However, HSV-1 also accounts for more serious conditions, such as causing 10–20% of viral encephalitis cases reported in the United States [2]. Herpetic keratitis is another complication and is the leading cause of infectious blindness in industrialized countries [3]. Pathogenesis by HSV-1 and related herpesviruses relies on the virus's ability to coopt the cellular protein machinery; therefore, defining virus-host protein interactions has the potential to advance our understanding of the biochemical processes occurring during infection with important applicability for the development of anti-viral therapies.

The virus life cycle begins with the attachment and entry of an infectious virion in a host cell, and ends with the release of newly assembled viral progeny capable of infecting other cells. The HSV-1 virion is made up of three distinct compartments. An icosahedral nucleocapsid houses the 152kbp double stranded genome; an envelope surrounds the capsid as a lipid bilayer containing glycoproteins, and a tegument is between the capsid and envelope. The tegument is a collection of ~26 proteins, a subset of which is known to play important roles during the early stages of the infectious cycle [4]. Upon entry into host cells, the viral genome is transported to the nucleus where it undergoes an expression cascade. Immediate early genes are expressed first, which then activate early viral genes that encode enzymes necessary for DNA replication. After genome replication, the late genes coding for structural proteins are expressed. One of these structural proteins is the capsid protein VP26, which is located at the capsid periphery [5].

VP26, also known as the small capsid protein, is encoded by the UL35 gene and is composed of 112 amino acids [6]. Homologues of VP26 are essential for viral replication in the β and γ -herpes viruses HCMV, Epstein-Barr virus (EBV), and KSHV, but not thought to be essential in α -herpesviruses HSV-1, HSV-2, and VZV [7–10]. Nonetheless, α -herpesviruses lacking the small capsid protein are not equivalent to wild type, exhibiting decreased replication in animal models [8]. While knowledge of VP26 interactions with viral and host proteins is limited, several studies have suggested the contribution of VP26 interactions in capsid assembly and maturation. Specifically, since VP26 lacks a nuclear localization signal, its nuclear import requires the presence of VP5 and the triplex capsid protein VP19C [11, 12]. The interaction of VP26 with the upper domain of VP5 has been mapped using several techniques, including deletional mapping, yeast two-hybrid assay, and three dimensional modeling [5, 13, 14]. Yeast two-hybrid assays have also shown interactions of VP26 with the essential tegument protein pUL37 [14], which was also observed for its VZV homologue [15]. The site of pUL37 interaction of VP26 was mapped to residues 78–112, which encode a region exposed to the tegument [16]. The significance of this interaction is not yet understood, but it may be important for the incorporation of the tegument into the maturing virion, since infection with a virus in which VP26 is deleted reduces pUL37's association with capsids [17]. Several interactions of VP26 with host proteins have also been reported. For example, VP26 association with dynein light chains RP3 and Tctex1 has been proposed to be important at the early stages of infection, facilitating the trafficking of incoming capsids via their binding to cytoplasmic dynein [18]. Additionally, following capsid assembly, the interaction of VP26 with cellular tetraspanin membrane protein 7 (CTMP-7) is necessary for nuclear egress [19]. Despite these data on VP26 structure and its few known interactions with viral and host proteins, the functions of

VP26 in viral replication, virion maturation and trafficking are still not well defined. Specifically, VP26 interactions with host proteins in the nucleus are poorly characterized when compared to those in the cytoplasm.

To identify VP26 interactions with virus and host proteins in the context of infection, we employed a method that we have successfully utilized to characterize spatial-temporal protein interactions of diverse viruses, including HSV-1 [20], HCMV [21–24], PRV [25, 26], Sindbis [27, 28], and West Nile virus [29]. This method utilizes full-length, replication-competent viruses that express fluorescently-tagged viral proteins, thereby allowing the assessment of both cellular localization and interactions of the tagged protein in an environment mirroring a natural infection [27]. Here, by infecting primary human fibroblasts with an HSV-1 virus expressing a GFP-tagged VP26, in conjunction with cryogenic cell lysis, immunoaffinity purification (IP), mass spectrometry (MS), and microscopy, we report the first proteomic study of VP26 protein interactions. We focused on nuclear VP26 interactions, prior to its nuclear egress, identifying virus-virus and virus-host protein interactions. Of particular interest within our interaction dataset was the association of nuclear VP26 with the host *de-novo* DNA methyltransferase DNMT3A. We validated this association by both reciprocal isolations using IP-MS and co-localization using confocal microscopy. Through a series of follow-up experiments, using drug-induced inhibition of DNA methyltransferase activity and siRNA- and shRNA-mediated knockdowns of DNMT3A, we show that the function of DNMT3A is important for viral replication. Altogether, our results indicate an important functional association of viral factors with the mammalian DNA methyltransferase machinery, suggesting that DNMT3A is recruited to modulate the DNA methylation status of the host or the virus for the benefit of viral replication.

EXPERIMENTAL PROCEDURES

Cell culture, virus strains and infection

The GFP-VP26 HSV-1 KOS strain (gift from Dr. Lynn Enquist, Princeton University) has VP26 tagged at its N-terminus with a GFP tag and expressed under its native promoter at levels equivalent to wild type strains, as described [30]. As control, an HSV-1 strain expressing free GFP not coupled to a viral protein was used. Virus stocks were propagated by infection of Vero cells grown in Dulbecco's Modified Eagle's Medium (DMEM) with the addition of 10% fetal bovine serum and 1% penicillin/streptomycin (Gemini Bio-Products). Infections for propagation were performed at an MOI = 0.01 pfu/ml and harvested 72 hours post infection (hpi) by scraping into the media. Collected cells were freeze-thawed and sonicated to release viral particles from the cytoplasm. Virus concentration was determined by plaque assays on Vero cells. Confluent human foreskin fibroblasts (HFFs) with less than 12 passages and MRC5 fetal lung fibroblast cells with less than 30 passages were cultured as described for Vero cells and infected with viral stocks at an MOI = 5 pfu/ml.

Generation of DNMT3A-GFP cell line

GFP-FLAG-tagged DNMT3A was stably expressed in a human embryonic kidney (HEK) 293 cell line using the PhoenixTM retrovirus expression system (Orbigen, San Diego, CA). Full-length DNMT3A cDNA was amplified using PCR, digested with restriction enzymes and ligated to the 3' end of GFP cDNA (pGFP-N1; Clontech). Cloning of the digestion products into the pLXSN vector (Clontech) resulted in the pLXSN-FLAG-GFP-DNMT3A retroviral vector containing a LTR promoter, as previously described for tagging of other proteins [31]. The vector was transfected into Phoenix cells using FuGENE (Roche Applied Science) and these cells were then grown to a confluency of 90%. The generated retroviral particles were transduced into HEK293 cells. This process was repeated in another HEK 293 cell line for stable expression of GFP alone. Cells stably expressing tagged DNMT3A were selected using 400 µg/ml G418 (EMD, Gibbstown, NJ) and sorted with fluorescence-assisted cell sorting. Stable expression and localization of the GFP-DNMT3A fusion protein was verified with confocal microscopy and immunoblotting.

Immunoaffinity purification

HFFs were infected with GFP-VP26 HSV-1 or the control strain, and the cells were harvested 14 hpi for immunoaffinity purification using anti-GFP conjugated magnetic beads, as described [25, 32]. Five 15 cm dishes of HFFs were washed with 4 ml of cold DPBS (Invitrogen), and cells were scraped and collected in cold DPBS, then pelleted by spinning at 200× g at 4° C. The cell pellet was suspended (100 µl/g cells) in 20 mM HEPES-NaOH, pH 7.5, 1.2% polyvinylpyrrolidone (w/v), 1:100 (v/v) protease inhibitor cocktail (Sigma, and then frozen drop wise in liquid nitrogen. Cryogenic disruption was performed using a Retsch MM 301 Mixer Mill (8 steps × 2 min at 30 Hz) (Retsch, Newtown, PA). A total of 0.8 g of frozen cell powder was recovered for each sample. Optimized lysis buffer was added to the frozen cell powder where both stringent and relatively milder buffers were used. The milder buffer contained 20 mM HEPES-KOH, pH 7.4, containing 0.1 M potassium acetate, 2 mM MgCl₂, 0.1% Tween 20, 1 µM ZnCl₂, 1 µM CaCl₂, 1% Triton X-100, 250 mM NaCl, 1/100 (v/v) protease inhibitor cocktail (Sigma). The more stringent buffer contained 20 mM HEPES-KOH, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.3% sodium N-lauroyl-sarcosine, 0.1 mM MgCl₂, 1 mM DTT, and 1/100 (v/v) protease inhibitor cocktail. The lysate was homogenized with a Polytron (Kinematica) for 2 × 10 s followed by centrifugation at 8000 × g for 10 min. The resulting supernatant was incubated with anti-GFP conjugated magnetic beads (M270 Epoxy Dynabeads; Invitrogen) for 1 hour at 4°C. The beads were washed, and the proteins eluted as previously described [33].

Mass Spectrometry

Eluted samples were loaded onto a NuPAGE 4–12% Bis-Tris gel and partially resolved to about half the length of the gel. The gel was then stained with Simply Blue Coomassie stain (Invitrogen), sliced, and destained in 50 mM ammonium bicarbonate, 50% acetonitrile (ACN), followed by three rounds of dehydration and rehydration steps. The samples were incubated overnight at 37°C with 12.5 ng/µl of sequencing grade trypsin (Promega). To extract the peptides, 1% FA solution was added to the gel pieces and incubated at room

temperature for 4 hours then washed for a second extraction in 50% ACN/0.5% FA for 2 hours. Liquid samples were concentrated and analyzed by nLC-MS/MS using a Dionex Ultimate 3000 RSLC coupled to a LTQ-Orbitrap Velos ETD mass spectrometer (ThermoFisher Scientific), as described [34]. The peptides were washed onto the trap column (Magic C18 AQ, 3 μ m, 100 μ m $\times$ 2.5 cm; Michrom Bioresources, Inc.) in 0.5% TFA, 1% ACN, 98.5% water and desalted for 5 min at 5 μ l/min and then separated by reverse phase chromatography (Acclaim PepMap RSLC, 1.8 μ m, 75 μ m $\times$ 25 cm) at a flow rate of 250 nl/min using a 90-min discontinuous gradient of ACN as follows: 4% to 20% B over 50 min, 20% to 40% B over 40 min (Mobile phase A: 0.1% formic acid, 0.1% acetic acid in water, Mobile phase B: 0.1% formic acid, 0.1% acetic acid in 97% ACN). The mass spectrometer was operated in data-dependent acquisition mode with FT preview scan disabled and predictive AGC and dynamic exclusion enabled (repeat count, 1; exclusion duration, 70 s). A single acquisition cycle comprised a single full scan mass spectrum (m/z = 350–1700) in the Orbitrap (r = 30,000 at m/z = 400), followed by CID fragmentation on the top 20 most intense precursor ions (minimum signal = 1E3) in the dual pressure linear ion trap. The following instrument parameters were used: FT MS and IT MSn (tandem MS) target values of 1E6 and 5E3, respectively; FT MS and IT MSn maximum injection time of 300 and 100 ms, respectively. CID fragmentation was performed at an isolation width of 2.0 Th, normalized collision energy of 30, and an activation time of 10 ms.

Data processing

Raw files containing MS/MS spectra for each biological sample were processed through Proteome Discoverer (version 1.3; ThermoFisher Scientific) and searched against the 2010 UniProt SwissProt sequence database containing human, herpesvirus, and contaminant sequences (21,570 sequences) in SEQUEST. Spectra were searched against peptide databases generated from both forward and reverse protein sequence entries with the following parameters: maximum of two missed cleavages, full enzyme specificity, parent and fragment ion mass tolerances of 0.5 Da and 10 ppm, S, T, Y phosphorylation (+80 Da), fixed modification of carbamidomethylcysteine (+57 Da), and variable acetyl-lysine (+42 Da) and methionine oxidation modifications (+16 Da). Peptide spectrum matches were loaded into Scaffold (ver. 3.2; Proteome Software, Inc.) with the X! Tandem re-search enabled (GPM 2010.12.1.1). Peptide and protein probabilities were calculated by the PeptideProphet and ProteinProphet algorithms. To reduce false-positive identifications to <1%, probability filters were empirically set to 99% protein confidence, 95% peptide confidence and two unique peptides per protein in at least one biological sample. Protein groups and unweighted spectrum counts were exported to Excel for further data processing.

Western Blotting

SDS-PAGE gels (10%) were transferred to PVDF membranes and blocked in buffer containing 5% milk in 50 mM Tris, 150 mM NaCl, 0.1% Tween 20 (TBST) for 1 hour at RT then incubated overnight at 4°C in primary antibodies diluted in blocking buffer. Membranes were washed in TBST then incubated in blocking buffer at RT containing horseradish peroxidase (HRP)-conjugated secondary antibodies for 45 mins. Membranes were washed as above and proteins were visualized with ECL (GE Healthcare).

Confocal microscopy

HFFs were cultured on Millicell EZ slides (Millipore) pretreated with poly-d-Lysine (Sigma, St. Louis, MO). Cells were infected with either GFP-VP26 HSV-1 or wt HSV-1 at an MOI = 0.1. After 10, 14, 18, or 24 hpi, cells were fixed with 2% paraformaldehyde, permeabilized with 2% paraformaldehyde with 0.1% Triton-X100, and washed with 10 mM glycine to halt fixation. Cells were then washed with PBS-T, and incubated with 1 µg/ml DAPI in PBS for 15 min. For antibody stained cells, samples were blocked overnight at 4° C in 10% (v/v) human serum, 2% (w/v) BSA, in PBS-T. Primary anti-DNMT3A (Cell Signaling Technology) diluted 1/100 in blocking buffer was added, incubated at room temperature for 1 hour, washed in PBS-T, followed by incubation of secondary Rbt368 antibody (Invitrogen) diluted 1/1000 in blocking buffer for 45 min. Cells were visualized by confocal microscopy on a Zeiss LSM510 (Zeiss, Dublin, CA) using 63×oil-immersion lens. GFP-tagged VP26 was visualized by direct fluorescence, DNMT3A by indirect fluorescence using an Alexa 568 antibody, and nuclei were stained with DAPI.

Knockdown of DNMT3A

siRNA knockdowns were performed in MRC5 cells. Stocks of siRNA (#1 and #2) were diluted to 10 µM with RNase free water and added to Opti-MEM (Invitrogen). The solution was combined with lipofectamine RNAiMAX (Invitrogen) and incubated at room temp for 10 mins. shRNA knockdowns were performed using custom MISSION shRNA lentiviral transduction particles (Sigma). HFFs were grown to 80% confluence in 10cm dishes; DMEM containing 8µg/ml hexadimethrine bromine was added to the media, followed by 20µl of lentivirus from four separate stocks (#sh-1-4). A control, non-mammalian shRNA lentivirus was also used. After 2 days, puromycin was added to the media (1µg/ml) to select for integration. The efficiency of DNMT3A knockdown was measured by western blot.

Drug treatment

RG108 (Sigma), dissolved in DMSO, was diluted in DMEM to concentrations ranging from 50–200 µM. HFFs were pre-treated with DMEM containing RG108 and incubated at 37 °C for 2 hours prior to infection. Control cells were treated with DMEM with equivalent concentrations of DMSO alone. Cells were infected with HSV-1 at an MOI of 5 and after viral inoculum was removed, RG108 containing DMEM was returned to the infected cells and progeny virus was harvested 24 hpi. Cell viability was assessed by both trypan blue staining and TUNEL assay. For TUNEL assay, cells were grown on chamber slides and treated with RG108 as described above. The cells were fixed in 2% paraformaldehyde, permeabilized in 2% paraformaldehyde and 0.1% Triton-X100, and washed with PBS. The cells were treated with 50 µl of enzyme solution and label solution from commercial kit (Roche) and incubated at 37 °C for 1 hour before mounting with coverslips.

Viral titers

Virus concentration was measured by plaque assay using Vero cells. Viral inoculum was serially diluted in DMEM and added to Vero cells at 80% confluence for 1 hour. Inoculum was removed and replaced with a DMEM solution containing 1% methocel (Dow) and 2% FBS then, cells were incubated at 37°C for about 3 days or until the formation of plaques.

Once plaques appeared, cells were fixed and stained in 0.5% methylene blue in 70% methanol. Plaques were counted manually and MOI was calculated as plaque forming units per milliliter (pfu/ml) based on the dilution factor.

RESULTS

A proteomic approach for studying VP26 interactions in HSV-1 infected human fibroblasts

To study VP26 protein interactions during infection in primary human fibroblasts (HFFs), we used a proteomic approach that we previously reported for studying other viral proteins tagged with GFP [22, 27, 32]. An HSV-1 KOS viral strain expressing VP26 tagged with GFP, previously shown to have comparable infectivity and titers to the untagged wild type strain [30], was used for infection of HFF cells (Figure 1A). To control for effects of GFP expression in HFFs, a HSV-1 strain expressing free EGFP was utilized. The GFP tag was used to observe the localization of VP26 at several time points after infection using confocal immunofluorescence. GFP-VP26 was localized exclusively to the nucleus at 10 hours post infection (hpi) (Figure 1B), primarily within discrete puncta. The nuclear localization of VP26 is expected during the stage of infection when the capsid structure is assembled. Indeed, this nuclear punctate localization was previously observed for both untagged and tagged VP26 [30, 35–38], and sites at the C-terminus of VP26 were shown to be important for both its ability to bind capsid and to acquire this localization [13]. Further studies would be needed to determine if this precise subnuclear localization corresponds to capsid assembly sites [30] or other functionally relevant sites during early stages of viral replication, or are artifacts from tagging [39]. While at 14 hpi VP26 remained predominantly nuclear, at 18 hpi, initial egress of VP26 from the nucleus into the cytoplasm was evident, and by 24 hpi, it appeared spread throughout the cytoplasm (Figure 1B). Previous studies of VP26 during HSV-1 infection have focused on its initial transport to the nucleus or its functions during late stages of infection (e.g., assembly). We were interested in characterizing its roles, and therefore its interactions, prior to nuclear egress. Therefore, we selected the 14 hpi time point (see Figure 1B) to perform immunoaffinity isolation and proteomic analysis of VP26 protein interactions (Figure 1C). Following infection with either the EGFP-VP26 HSV-1 or control EGFP HSV-1 virus at an MOI of 5 pfu/mL, HFF cells were harvested at 14 hpi. Cells were lysed under either mild or stringent lysis buffer conditions to capture both indirect and strong protein interactions, respectively. Immunoaffinity purifications were performed using anti-GFP antibodies conjugated to magnetic beads, and the co-isolated proteins were resolved by 1-D SDS-PAGE. GFP-VP26 was effectively isolated, as supported both by the expected Coomassie-stained band at ~37 kDa for both conditions and by western blots, showing the recovery of GFP-VP26 with little remaining GFP-VP26 in the flow through or bound to the beads after elution (Figure 1D–E, compare IP to FT and B fractions). In addition to GFP-VP26, other bands were present on Coomassie gels (Figure 1E). Several of the most prominent bands were common between the lysis conditions, though the mild lysis condition clearly showed greater intensity and number of distinct bands compared to the stringent condition. The Coomassie-stained lanes were subjected to in-gel trypsin digestion and subsequently analyzed by nLC MS/MS on an LTQ-Orbitrap Velos ETD mass spectrometer, as in [32].

VP26 associates with numerous viral proteins during infection

Proteomic analysis of EGFP-VP26 immunoisolates identified several virus-virus protein interactions. Some of these were preserved under both mild and stringent lysis conditions (Figure 2A, rectangles), suggesting strong associations with VP26. These strong interactions were exclusively within the viral capsid, as might be predicted for such a highly ordered structure. Among these was the known VP26 main binding partner VP5 [5, 40, 41], as well as the triplex capsid proteins VP23 and VP19C, which are known to interact with each other and with VP5 to form the icosahedral structure [42]. The identification of the capsid proteins VP5, VP23, and VP19C, acted as a validation, indicating that we isolated VP26 within its known functional complex. Additionally, the capsid protein UL25, which is located on the external vertices of the capsid structure [43–45], was also still maintained in the stringent conditions, albeit at significantly reduced levels when compared to the milder condition. Several VP26 interactions with other capsid proteins, as well as with tegument and envelope viral proteins were observed only under milder conditions, suggesting weaker associations (Figure 2A, hexagon shapes). Among these were the tegument protein UL17 [46], which is known to bind to UL25 and form the capsid vertex-specific complex, as well as the UL31 and UL34 proteins, the former of which also directly binds UL25 [47]. Also present in the mild condition were the members of the viral DNA packaging machinery, the tripartite terminase complex, UL15, UL28, and UL6, as well as the protease precursor UL26, all of which are known to form an interaction network [48–51]. Additionally, the known interaction of VP26 with the most proximal, inner tegument protein pUL37 was observed in our mild condition. pUL37 is known to form a complex with other tegument proteins - UL46, UL48, UL36, and with the envelope protein gD [16, 52–54], which were also present in our VP26 isolation. Finally, VP26 associated with proteins with no known associations to the aforementioned interaction networks (Figure 2A). These included the tegument proteins US10, residing in the host nuclear matrix, the DNA polymerase processivity factor UL42, and the envelope protein gI, required for cell-to-cell spread [55–57]. Altogether, this IP-MS study provided a view of the stronger and weaker VP26 interactions with other viral proteins within the nuclei of infected fibroblasts. Additionally, the association with pUL37 and other tegument proteins suggests that one of the VP26 structural roles is to provide a link between different virion compartments, i.e. the macromolecular proteinaceous capsid and tegument layers. Alternatively, these associations may be reflective of diverse intermediate virion structures.

VP26 is phosphorylated within the C-terminal UL37 binding region

Many viral proteins are known to be phosphorylated, and these modifications can regulate different aspects of the viral life cycle. The virus itself encodes two kinases, but HSV-1 proteins are known targets of host kinases as well [58, 59]. Therefore, evaluation of the post-translational modification status of VP26 may inform on other aspects of its function, including potential regulation by cellular and viral kinases, and/or phosphorylation-dependent changes in localization and protein associations. Previous biochemical data predict that VP26 contains multiple sites of threonine phosphorylation and at least one serine phosphorylation; however, only a single phosphorylation at Thr111 has been confirmed thus far [60]. Whether other predicted phosphorylation sites are present during infection has not yet been determined [61]. Our immunoaffinity enrichment of VP26 lead to high (84%)

sequence coverage (Supplementary figure 1), thus allowing us to experimentally evaluate its phosphorylation. We identified three phosphorylation sites in the VP26 C-terminal region (Figure 2B–C): the previously identified Thr111 phosphorylation, as well as two unreported sites at Ser107 and Thr108. All three VP26 phosphorylation sites are located within the region that binds the tegument protein pUL37 [16]. Interestingly, the region immediately surrounding the Ser107 corresponds to a binding motif for 14-3-3 chaperone proteins (R-X-X-pS-X-P), whose functions depend on binding to phosphorylated residues [62]. This putative 14-3-3 binding motif has not been previously described for VP26. Consistent with this result, we also identified multiple 14-3-3 subunits (zeta/delta, theta, epsilon, beta/alpha, eta, and gamma) in our isolations of VP26. While the interaction of VP26 with 14-3-3 has yet to be confirmed, it is possible that host 14-3-3 is needed for viral signaling or transport upon infection. In agreement with a role for 14-3-3 proteins during HSV-1 infection, our lab has recently reported an interaction between the 14-3-3 chaperones and the HSV-1 tegument protein pUL46, the latter of which was also found to be phosphorylated within a 14-3-3 binding motif [20].

In addition to the phosphorylation sites found on VP26, we identified one previously undiscovered lysine acetylation on the major capsid protein VP5. While this constitutes the first report of a lysine acetylation on an HSV-1 protein, such modifications have been well documented for the human immunodeficiency virus (HIV) tat and integrase proteins and human papillomavirus (HPV) E2 protein [63, 64]. VP5 is acetylated at Lys1218, which is located in the middle region of the protein and is not part of any characterized secondary structural element [65]. Additionally, we identified several previously described phosphorylations on the tegument protein pUL47, the transcriptional regulator pUS1, and ICP22 [60]. Phosphorylation of pUL47 was found at Ser20 in the 15–120 loop region known to be a target of the viral kinase pUS3, while two phosphorylations were found on ICP22 at Ser22 and Ser167, where ICP22 is known to be phosphorylated *in vitro* by the viral kinases pUS3 and pUL13 [66, 67].

VP26 associates and co-localizes with DNMT3A during infection with HSV-1

As mentioned previously, several host proteins co-isolated with GFP-VP26 at 14 hpi (Supplementary Table 1). Of particular interest was the association with the *de-novo* DNA methyltransferase, DNA (cytosine-5)-methyltransferase 3A (DNMT3A) (Figure 3A), a protein known to localize primarily in the nucleus [68]. DNMT3A was only identified under stringent isolation conditions, suggesting that harsher detergents were necessary to access VP26 complexes that included DNMT3A. This association is of interest as recent reports have highlighted that viral infections modulate host epigenetic factors, impacting both viral and host gene expression and creating an environment conducive to viral replication. These virus-induced changes in the cellular epigenetic landscape can occur through modulation of DNA methylation profiles or through alteration of chromatin remodeling enzymes and histone modifications [69–71]. DNMT3A is an important cellular epigenetic factor, targeting unmethylated CpG sites and being generally connected to transcriptional repression [72, 73]. Therefore, we selected to focus on this association.

We designed several validation experiments employing reciprocal isolations in alternately tagged systems to confirm the VP26-DNMT3A interaction we observed in our proteomic experiments. We generated an HEK293 cell line stably expressing DNMT3A with a dual tag, FLAG and GFP, similar to our recent studies on histone deacetylases [31]. This dual tagging gave us the flexibility to investigate this association following infection with both GFP-tagged and wild type HSV-1. First, we infected these cells with GFP-VP26 HSV1 and isolated DNMT3A via the FLAG tag (Figure 3B, left). Using western analysis, we observed that GFP-VP26 co-isolated with DNMT3A, but not with the IgG control (Figure 3B, right). Second, we infected the DNMT3A-GFP-FLAG expressing cells with wild type HSV-1. Using mass spectrometry, we confirmed that untagged VP26 was co-isolated with DNMT3A-GFP (Figure 3C, right). In conjunction with our proteomic analyses showing the association of VP26 with endogenous DNMT3A, these experiments confirm that this association is not an artifact of tagging VP26 or DNMT3A. Finally, we examined the co-localization of endogenous DNMT3A with VP26 by confocal immunofluorescence imaging in primary human fibroblasts infected with GFP-VP26 HSV-1. The imaging of GFP-VP26 was performed at two time points of infection (6 and 14 hpi) using direct fluorescence to avoid cross-talk of antibodies. Indeed, we observed the co-localization of endogenous DNMT3A with GFP-VP26 at both 6 and 14 hpi in nuclear puncta (Figure 4). Overall, using multiple strategies of proteomics, reciprocal isolations, and co-localization imaging, we demonstrate a previously unrecognized association between the HSV-1 viral capsid protein VP26 and the host DNMT3A in the nucleus of HSV-1 infected cells.

Inhibition of DNA methyltransferase activity suppresses HSV-1 infection

In other viruses, such as Hepatitis B virus and Kaposi's sarcoma-associated herpesvirus, viral proteins interact with DNMT3A to enhance viral replication [74–76]. However, it is also possible that DNMT3A could be acting as part of an antiviral host protection mechanism. To further understand the role of DNMT3A during HSV-1 infection, we examined whether the enzymatic DNA methyltransferase activity of DNMT3A was required for HSV-1 infection. Towards this goal, we used the small molecule RG108, which was shown to have low cell toxicity (when compared to other DNA methyltransferase inhibitors), to trigger concentration-dependent de-methylation of genomic DNA in cells, and to directly inhibit DNA methyltransferase activity *in vitro* [77, 78]. To confirm that the previously reported RG108 concentration, which lacked cytotoxicity (~100 μ M) [78], would also be suitable for treatment in primary human fibroblasts (HFF cells), we assessed cell viability and morphology of HFFs after treatment with two concentrations of RG108, 50 and 200 μ M. After RG108 treatment for 24 hours, no apparent morphological changes were observed by light microscopy at either concentration (Figure 5A). Also, total cell death (data not shown) and apoptosis was not significantly different than the DMSO-treated control as measured by TUNEL assays (Figure 5A). Therefore, we proceeded with our assessment of HSV-1 propagation. After pre-treatment of fibroblasts with increasing concentrations of RG108 and subsequent infection with wild type HSV-1, viral titers were measured. A dose-dependent decrease in virus titers was observed, achieving a ~10-fold reduction in virus titer at the highest RG108 concentration (200 μ M) (Figure 5B). Overall, these results suggest that methyltransferase activity is critical for HSV-1 virus propagation.

DNMT3A downregulation triggers a decrease in virus titers

Although RG108 is a potent inhibitor of DNA methyltransferase activity ($IC_{50} = \sim 115$ nM), it has selectivity towards several human DNMTs [77]. To test if DNMT3A specifically has a functional role in HSV-1 propagation, we used several knockdown strategies targeting endogenous DNMT3A. First, we transiently knocked down the expression of DNMT3A in infected fibroblasts with siRNA. MRC5 human fibroblast cells were selected for these experiments, as these cells are amenable for nucleic acid transfections. Using two different siRNA constructs, expression of DNMT3A was reduced to undetectable levels, while the other *de novo* methyltransferase, DNMT3B, was unaffected (Figure 6A). This knockdown did not significantly affect cell proliferation (Figure 6B). Under these conditions of DNMT3A knockdown, the production of infectious HSV-1 was measured by viral titers at two time points of infection—15 hpi and 25 hpi, the latter of which represents the end of one viral life cycle. We observed a decrease in viral titer at both time points in DNMT3A knockdown cells relative to control siRNA (Figure 6C), with 25 hpi, showing the greatest decrease in infectious virus released (~ 7 -fold) relative to control.

To further validate these observations, we constructed DNMT3A shRNA knockdown cell lines using lentiviral transduction of primary human fibroblasts (HFFs). Non-clonal populations of DNMT3A knockdown cells were generated using four different lentiviral particles (Figure 6D, sh-1-4), as well as a control cell line using shRNA against non-mammalian sequences. Notably, cell lines derived from all targeting shRNAs, except shRNA-2, had significant knockdown of DNMT3A, as showed by western blot analysis (Figure 6D). Given the lack of DNMT3A knockdown in sh-2 cell line, it was employed as an additional negative control. First, we screened these shRNA knockdown cell lines for their ability to produce infectious HSV-1 virus. Consistent with our results from siRNA-mediated knockdown of DNMT3A, shRNA-mediated knockdown resulted in a substantial reduction of viral titers in all knockdown cell lines except sh-2 (Figure 6E), consistent with the levels of DNMT3A expression (Figure 6D). On average, shRNA-mediated knockdown of DNMT3A reduced viral titers by $\sim 70\%$. Next, shRNA cell lines sh-1 and sh-4 were selected to test if DNMT3A knockdown induces defects in viral production over multiple rounds of replication. In this experiment, cells were infected at a lower multiplicity of infection ($MOI = 0.25$ pfu/ml) and viral titers were measured at 36 hpi, selected to reflect secondary HSV-1 infections. This is in contrast to the initial screening conditions above in which $> 99\%$ cells were simultaneously infected. In agreement with our observations using siRNA, a significant effect on viral titer was observed (Figure 6F), further supporting the requirement for DNMT3A expression in virus propagation. Moreover, a decreased viral titer at the 36 hpi suggests that additional rounds of viral replication likely cannot compensate for DNMT3A deficiency. Therefore, these results point to the specific requirement of DNMT3A for viral propagation, which does not seem to be compensated by the presence of the other *de novo* methyltransferase, DNMT3B. Taken together with the association of DNMT3A with viral capsid proteins in the nucleus and the requirement for DNA methyltransferase activity, these siRNA and shRNA knockdown experiments specifically support DNMT3A as a host factor required for efficient viral replication of herpes simplex virus-1. Based on these data, we hypothesize that DNMT3A is recruited to viral capsids during early stages of infection and could function in viral genome replication and/or packaging.

DISCUSSION

In this study, we have successfully isolated the HSV-1 viral capsid protein, VP26, and examined its potential functions and regulation by systematically studying its protein interactions and post-translational modifications. This work is the first proteomic study examining VP26, which provides additional insights into its yet-unknown functions. Examining interactions among viral proteins during infection can provide knowledge critical for understanding virion assembly and intracellular trafficking of viral complexes, in which multiple interactions must take place in an environment that is regulated both temporally and spatially.

Computational network analysis of VP26 protein associations at 14 hpi illustrated that viral protein interactions have varying strengths of association. The proteins that form the capsid are predicted to bind in a highly ordered and strict conformation; therefore, it was not surprising that they are co-isolated with VP26 even under stringent lysis conditions (high detergent and salt concentrations). Interestingly, the capsid protein pUL25 was also recovered under both mild and stringent conditions, although at significantly reduced levels under stringent conditions. It is known that pUL25 exists on the capsid surface [45] and directly binds the inner tegument protein pUL36 [43]. Our protein interaction results add additional support to the idea that pUL25 is involved in the tethering of the tegument to the capsid. Another interesting result from our IP-MS analysis of VP26 interactions was the identification of pUL17, a viral protein with nuclear functions in capsid maturation and trafficking. For example, pUL17 is part of a subcomplex referred to as the C-capsid-specific component, which also contains pUL25 and pUL36, and may function to stabilize genome-containing viral capsids [79]. Previous studies have shown that pUL17 and pUL14 can influence the localization of VP26 [80], but no evidence has indicated that these proteins associate with VP26 or to other HSV-1 proteins. Given that these proteins are either absent or substantially reduced in the stringent isolation condition, it is likely that their association with VP26 is indirect.

Our proteomic analysis also found US10 and gI, which do not participate in known complexes with any of the other proteins identified in our isolation of VP26. While it is unlikely that an envelope protein, such as gI, would bind VP26 directly in the context of intact virions, one possibility is that an intermediate viral complex exists during virion maturation that contains VP26 and gI, for example, potentially in the gE/gI complex that accumulates in the endosomes and TGN [57]. Though, given gI is also a receptor for human Fc IgG, it is also possible that gI may associate non-specifically to VP26 complexes. In contrast, pUS10 is more likely to interact directly with VP26. pUS10 is a tegument protein that resides in the host nuclear matrix and can associate with capsids, yet its function remains unknown [55]. Due to this localization, one could hypothesize that pUS10 is involved in nuclear reorganization or capsid egress from the nucleus upon interaction with VP26 or other capsid proteins. Overall, the results obtained by IP-MS studies are information-rich, generating different hypotheses related to the functional significance of viral protein interactions. Therefore, future studies on VP26 interactions may benefit from examining these associations in different subcellular fractions and different time points

during infection to determine how VP26 interaction networks may be spatially and temporally regulated.

Affinity isolation of VP26 complexes also enabled detection of post-translational modifications, with phosphorylation being the most prominently detected modification. It is tempting to hypothesize that VP26 phosphorylation provides a means of regulating its binding and localization. All of the modified sites were located in the pUL37 binding region and may influence the specific timing of this interaction. Additionally, since the modification at S107 is part of a putative 14-3-3-binding motif, it is possible that this site regulates signaling or transport during infection. Phosphorylation could trigger events such as nuclear import or the initiation of tegumentation. We also reported the first acetylation on an HSV-1 protein, VP5. In general, the number of known acetylated viral proteins is limited, with acetylated proteins including HIV-1 integrase and tat, the E2 protein of human papilloma virus, and the coat protein in Poliovirus virions [63, 64, 81–83]. HSV-1 VP5 acetylation at K1218 is located in a region of the protein that is not part of any secondary structural elements. It is possible that modification within this region could cause a structural change to regulate binding to other proteins. VP5 can exist as a monomer, or in higher order complexes (hexons and pentons), which serve as the major structural elements of the icosahedral capsid. Interestingly, these VP5-containing structures exhibit different protein binding profiles [12, 40, 42], and thus, these post-translational modifications may contribute to capsid assembly. Overall, the significance of these novel PTMs on viral proteins remain an open question. For example, could these sites serve a structural function to facilitate protein-protein interactions or regulate protein conformation? Our identification of these PTMs opens the door for such future studies.

In this investigation, we sought to gain further insights into the functions of the HSV-1 capsid protein VP26 by identifying its associations with host factors during infection. While several host proteins were identified, we chose to focus on its association with DNMT3A, as it is known that viruses from diverse families utilize methyltransferases for efficient replication, and specifically that HBV and KSHV co-opt DNMT3A [76, 84]. However, HSV-1 has not been previously shown to target DNMT3A. Using immunoaffinity purifications, mass spectrometry, microscopy, and reciprocal IPs, we established an association between VP26 and DNMT3A in human fibroblasts. These results demonstrate that VP26 and DNMT3A associate in the nucleus during HSV-1 infection. However, immunoaffinity enrichment approaches cannot distinguish between direct and indirect interactions. It is possible that another viral or host protein that is in complex with VP26 mediates the association with DNMT3A; for example, the larger major capsid protein VP5 or a viral DNA-binding protein, such as ICP8. Nevertheless, this study presents the first evidence that a DNA methyltransferase has a functional role during HSV-1 infection.

We hypothesize several possible mechanisms for the involvement of DNMT3A in HSV-1 infection. While we can't rule out an anti-viral role for DNMT3A, perhaps in silencing of HSV-1 genes to slow viral growth, our knockdown and methyltransferase inhibitor studies suggest that the predominant role of DNMT3A is to promote virus propagation. In this role, HSV-1 may coopt DNMT3A to silence host genes, and thereby establish a host environment permissive for HSV-1 replication. This could include the silencing of genes involved in

apoptosis, cell cycle control, or immune recognition. Additionally, given that herpesvirus gene expression patterns are temporally regulated, DNMT3A could silence specific classes of viral genes to regulate the cascade of gene expression during infection.

DNA methyltransferase inhibitors, such as RG108, have been studied for their potential in anti-cancer therapies and have been shown to be effective in changing methylation patterns in abnormal cells [78]. In our study, since pre-treatment of cells with RG108 inhibited viral titers, it may be possible to target these methyltransferases in antiviral therapeutics against HSV-1. While our results show pre-treatment is an effective approach, this is often unrealistic for therapeutic intervention, particularly for viruses with short life cycles. Therefore, additional studies will need to define the treatment windows during which methyltransferase inhibition functions to inhibit virus replication, as well as the mechanism by which this inhibition of replication occurs. It is also noteworthy that DNA methyltransferases are thought to have distinct cell-type and tissue-type expression levels, which can lead to context-dependent functions. In human fibroblasts, we did not observe a significant impact for DNMT3A knockdown on cell proliferation. However, the levels of DNMT3A have been previously associated with cell proliferation in the context of development and tumorigenesis (e.g., [85–87]). For example, DNMT3A was reported to have low levels of expression in normal liver cells, but increased levels in hepatocellular carcinoma cells (HCC) [85]. Knockdown of DNMT3A decreased cell proliferation and colony formation in these HCC cell lines. Other studies focused on DNMT3A functions in embryogenesis, assessing the impact of its levels on stem cell growth and differentiation. Interestingly, while DNMT3A was reported to associate, functionally and physically, with DNMT3B [88], its levels seemed to specifically affect stem cell proliferation and neural cell differentiation [86]. Nevertheless, DNMT3A and DNMT3B have been observed to have coordinated functions [88–90], which are likely tissue and context specific. It remains to be determined whether HSV-1 infection triggers changes in the levels of DNMT3A and DNMT3B, as suggested for DNMT3A by our microscopy results, and whether there are selected functional redundancies between these enzymes, as well as with DNMT1. For example, KSHV, another herpesvirus, was reported to upregulate the expression of DNMT1, thereby enhancing cell proliferation [91]. Further studies will be needed to characterize the cell-type specific functions of DNA methyltransferases in the context of viral infection, as well as the temporal nature of these functions. For example, analyses of the levels of other late proteins would help identify the exact point(s) of regulation through which DNMT3A plays its roles during HSV-1 infection. The lack of disruption in the levels of VP26 would suggest that the viral DNA replication is not altered, since this would affect L genes. However, other points of inhibition could also account for the observed growth defects, including altered transport of viral proteins, disruption of tegumentation and/or envelopment, or block of virion egress. It is also possible that released viral progeny are less infectious due to altered structure or virion composition. Based on our immunofluorescence experiments the association of DNMT3A occurs at least at 6 hpi, at which point the capsids have not yet egressed into the cytoplasm. If the DNMT3A-dependent promotion of virus replication requires modification of host methylation to promote virus genome replication, this would occur prior to 14 hpi. This mechanism of action may or may not be a VP26 dependent mechanism. In fact, it is possible that DNMT3A may serve multiple roles, an

enzymatic role early during infection to coordinate host and/or viral gene expression, as well as a structural role later in infection to support genome packaging and/or capsid maturation. This latter mechanism is feasible for VP26 given the proteomic identification of pUL17.

In summary, these studies point to an important role for DNA methylation during the progression of HSV-1 infection, opening new avenues of research for further investigation of the involved molecular mechanisms and providing possible new targets for therapeutic antiviral intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DMEM	Dulbecco's Modified Eagle's Medium
DNMT3A	DNA (cytosine-5)-methyltransferase 3A
HEK293	human embryonic kidney cells
HFF	primary human foreskin fibroblast
HSV-1	herpes simplex virus type 1
IP	immunoaffinity purification
UL	unique long
US	unique short
WT	wild type

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SIGNIFICANCE

Herpesviruses, including herpes simplex virus-1 (HSV-1), are ubiquitous human pathogens. As there is no known cure for herpesvirus infection, estimates of HSV-1 prevalence in the general population are at least 60%. HSV-1 remains latent in healthy individuals and often does not produce overt symptoms. However, when the immune system is compromised due to stress, other viral and/or bacterial infections, or medical therapies that directly inhibit the immune system, HSV-1 can become a serious health risk. While HSV-1 re-activation from latency is treated with topical drugs, such as acyclovir, to inhibit virus replication, resistance to the treatment often develops. Therefore, a greater understanding of the cellular processes that either support or hinder infection is needed. In the current study, we identified a de novo DNA methyltransferase, DNMT3A, associating with viral structural proteins during HSV-1 infection. Importantly, this association has a functional consequence, since reduction in the levels of DNMT3A also reduced virus propagation. DNMT3A is one of three mammalian DNA methyltransferases in the cell, which methylate cytosine residues to ultimately influence gene expression. This is the first example of DNA methyltransferases impacting HSV-1, which is consistent with a potential broad role of DNA methyltransferases during other virus infections.

Figure 1. Isolation of affinity-tagged viral protein 26 (VP26) during HSV-1 infection of human fibroblasts

(A) Schematic of viral genome locus where VP26 was tagged with GFP. (B) Localization of GFP-VP26 at four different hours post infection (hpi) with HSV-1 in primary human foreskin fibroblasts (HFF), as assessed by fluorescence microscopy. Direct fluorescence was used to visualize GFP-VP26 (green), and nuclei were stained by DAPI (blue). 63×oil-immersion lens; Scale bars, 10 μ m. (C) Proteomic workflow for analysis of GFP-VP26 protein interactions. HFF cells were infected with GFP-VP26 or EGFP control expressing HSV-1 strain. Infected cells were collected at 14 hpi, and the proteins co-isolated with GFP-VP26 were analyzed by mass spectrometry as described in Materials and Methods. (D) The efficiency of GFP-VP26 isolation was evaluated by anti-GFP Western analysis of the immunoisolated sample (IP) compared to the flow-through (FT) and bead (B) fractions. (E) SDS-PAGE and Coomassie staining of GFP-VP26 protein associations, isolated under stringent (left) or mild (right) lysis conditions. The most intensely stained bands are labeled with select proteins identified by nLC MS/MS.

Figure 2. VP26-viral protein interactions and post-translational modifications
(A) Interaction network of HSV-1 proteins associated with VP26 under both mild and stringent conditions (rectangular nodes) or only under mild condition (hexagon nodes). Connections indicate proteins associations previously described and line color indicates method used for identifying the interaction. Unconnected nodes signify there are no previously described interactions within the node set. Color denotes structural compartment of the virion for each protein; grey denotes non-structural viral proteins. (B) Schematic of VP26 functional domains and sites of phosphorylation within the C-terminal UL37 binding domain. Ser at position 107 conforms to a 14-3-3 binding motif (underlined residues). (C) Summary of post-translational modifications identified on viral proteins by CID-MS/MS after isolation of VP26 (see Supplemental Figure S2).

Figure 3. Association of host DNMT3A with HSV-1 VP26

(A) Immunoaffinity isolations were performed with HFFs infected with HSV-1 expressing GFP-VP26 (see Fig 1C). One of the associated host proteins identified was host DNMT3A; representative CID-MS/MS spectrum shown (right). (B and C) Reciprocal immunoaffinity isolation of (B) FLAG-DNMT3A from GFP-VP26 HSV-1 infected HFF cells and (C) GFP-DNMT3A from wild type HSV-1 infected HFF cells confirmed its association with VP26 by Western blot and CID-MS/MS, respectively.

Figure 4. VP26 and DNMT3A co-localize in the nucleus of HSV-1 infected HFF cells

Confocal immunofluorescence microscopy of co-localized mGFP-VP26 (green, visualized by direct fluorescence) and DNMT3A (red, visualized by anti-DNMT3A antibodies) in the nuclei (DAPI, blue) of infected cells at 6 and 14 hpi. At each time point, a neighboring uninfected cell, which lacks GFP-VP26 expression, is shown for comparison. Scale bars, 10 μ m.

Figure 5. Inhibition of DNA methyltransferase activity suppresses HSV-1 titers

(A) Assessment of cell (HFFs) viability and morphology after treatment with RG108 using TUNEL assay and light microscopy. (B) Workflow for determining the impact of R108 treatment on virus titers. Virus titers were measured with increasing concentrations of RG108.

Figure 6. Reduced expression of endogenous DNMT3A decreases HSV-1 propagation

(A) Transient knockdown of DNMT3A, but not DNMT3B, expression in MRC5 fibroblasts using two different targeting siRNA was confirmed by Western blot analysis. (B) siRNA-mediated knockdown of DNMT3A did not significantly affect MRC5 cell proliferation. (C) siRNA-mediated knockdown of DNMT3A in MRC5 cells prior to infection with wild type HSV-1 triggered reduced viral titers at both 15 hpi and 25 hpi (N = 3). (D and E) Stable HFF cell lines expressing one of four targeting shRNAs (sh-1 – sh-4) and a control shRNA (Ctrl) were screened for (D) DNMT3A expression and (E) propagation of HSV-1 infection. All targeting shRNAs, except sh-2, resulted in DNMT3A knockdown and corresponding reduced viral titers. (F) Biological replicates of shRNA-mediated reduction of viral propagation after multiple infection cycles at 36 hpi.