Deletion of UL21 causes a delay in early stages of the herpes simplex virus type 1 replication cycle

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ABSTRACT

The HSV-1 U L21 gene encodes a 62-kDa tegument protein with homologs in the alpha, beta, and gamma herpesvirus subfamilies. In the present study we have characterized a novel U L21-null virus and its genetic repair to determine whether this protein plays a role in early stages of the HSV-1 replication cycle. Single-step growth analyses, protein synthesis time courses, and mRNA quantifications indicated the absence of U L21 results in a delay early in the HSV-1 replication cycle.
Herpes simplex virus type 1 (HSV-1) virions, like those of all herpesviruses, contain a proteinaceous layer termed the tegument localized between the nucleocapsid and viral envelope. The HSV-1 tegument is composed of ~20 different viral proteins of varying stoichiometry. Tegument proteins have been shown to play a variety of roles in infection including the regulation of viral and host gene expression and the promotion of virus assembly and egress (2, 5, 12, 13). Tegument proteins are delivered to the host cell upon infection and, thus, can play roles at early times in infection as well as at late times when they are produced.

The UL21 protein is a 535 amino acid-long component of the HSV-1 tegument (1, 17). The majority of work on UL21 has been performed with HSV-1 and pseudorabies virus (PRV), though homologs of this protein have been identified in the alpha, beta, and gamma herpesvirus subfamilies. Although the role of UL21 late in infection has been studied in both HSV-1 and PRV, the role this protein plays at early times in infection is unknown. In the present study we sought to characterize the role(s) of the UL21 protein at early times in the HSV-1 replication cycle.

To identify the function(s) of UL21 at early times in HSV-1 infections, we generated both a UL21-null virus (UL21−) that lacks the entire UL21 ORF and a repair virus (UL21R) in which the UL21 ORF was restored using the HSV-1(F) BAC pYEbac102 (18) with the En passant recombination system (19) and the PCR primers 5'-CCGTAGGGGGCCTCTGGGCCGTGTTACGTCGCCGCCCGCGAAGACCCCAATAAACGTATATAGGGATAACAGGGTAATCGATTT-3' and 5'-ACACAAGGGTGTAGTAGCGATATACGTTTATTGGGGTCTTCGCGGGCGGCGGACGT AACACGCCAGTTACAACCAATTAACC-3'. The deletion in the UL21-null virus spanned HSV-1 bps 42074-43678, a region beginning with the start codon and ending with the stop codon of the UL21 ORF. This entire sequence was restored to its original location in the UL21R virus. Following transfection of BAC DNAs into Vero cells and subsequent virus stock production, restriction fragment length polymorphism analysis of purified viral DNAs was
performed and showed the genotype was as expected (data not shown). Viral DNAs were also
used for PCR amplification of the manipulated areas. Sequencing of the PCR products showed
the genetic manipulations were made as planned (data not shown). Southern blot analysis
showed the deletion in the UL21-null genome and the repair in the UL21R genome were correct
in size (data not shown). Immunoblot analysis showed the UL21 protein was present in lysates
of Vero cells infected with the wild-type and UL21R viruses, but not the UL21-null virus (data not
shown).

To determine whether the newly-generated UL21-null virus possessed a defect in virus
replication, single-step growth analyses were performed. Vero cells infected at an MOI of 5
PFU/cell with the WT, UL21-, and UL21R viruses were collected at 0, 6, 12, 18, and 24 hours
post-infection, lysed, and intracellular virus was quantified by plaque assay (Fig. 1A). Media
overlaying the infected cell monolayers were clarified and assayed separately (Fig. 1B). The
UL21-null virus showed a 99% (two-log) reduction in both intracellular and extracellular virus
yields as early as 6 hours post-infection as compared to the wild-type and UL21-repair viruses.
This reduction was less pronounced late in infection, with intracellular and extracellular virus
yields reduced by approximately 5- and 10-fold, respectively, at 18 and 24 hpi. The above data
indicate that the absence of UL21 causes a delay in the production of infectious virus.

A delay in virus production could result from a delay in the initiation of virus infection. To
measure the kinetics and efficiency of UL21-null virus attachment, 25 cm² flasks of Vero cells
were infected at room temperature with 100 PFU of WT, UL21-, or UL21R viruses for 5, 10, 20,
30, or 45 minutes. At each time point, infected cells were washed to remove unattached virus
and overlaid with media containing human gamma globulins to neutralize any remaining
unattached virus. Infected cells were then incubated at 37°C for ~2 days at which time viral
plaques were counted to determine the percentage of the original infecting dose that had
initiated viral infection by each time point. We found no difference in the number of viral plaques
produced by the wild-type, UL21-null, and UL21-repair viruses (data not shown).
The reduced intra- and extracellular virus yields observed early in UL21-null virus infections could be caused by a defect or delay in viral protein synthesis. We examined whether total protein synthesis was decreased at various times early in UL21- virus infections by performing $[^{35}\text{S}]$-labeling time courses. Vero cells were either mock infected or infected with the WT, UL21-, or UL21R viruses at an MOI of 10 PFU/cell by rocking slowly at 4°C for 1 hour, followed by removal of unattached virus and incubation at 37°C to synchronize virus entry. At 0, 2, 4, 6, or 8 hours post-infection, media overlaying the infected cells was replaced with media containing 200 $\mu$Ci $[^{35}\text{S}]$-methionine and -cysteine and cells were returned to 37°C for 2 hours. Following each two-hour labeling period, the infected, labeled cells were washed, collected, and boiled in SDS-PAGE sample buffer. Ten-microliter samples were assayed for incorporated radiolabel via scintillation counting (Fig. 2A). We found no difference in total protein synthesis between cells infected with the wild-type, UL21-null, or UL21-repair viruses for any of the time periods assayed. To examine the relative abundance of individual proteins synthesized following SDS-PAGE separation of the above radiolabeled cell lysates (Fig. 2B). The absence of UL21 caused a 2-4 hour delay in the induction of viral protein synthesis inasmuch as some proteins synthesized in infected cells, but not in mock infected cells, began accumulating 2-4 hours later in UL21- infections as compared to WT and UL21R infections (open circles). The absence of UL21 also corresponded with a 2-4 hour delay in the shut-off of host protein synthesis, inasmuch as proteins present in mock infected cells were synthesized in UL21 infected cells for 2-4 hours longer than in either WT or UL21R infected cells (filled squares).

We next examined how the absence of UL21 affects the accumulation of specific immediate-early, early, and late viral proteins. We analyzed the accumulation, over time, of ICP0, ICP4, ICP27, ICP8, and VP16 by performing immunoblots on lysates from Vero cells infected with the WT, UL21- or UL21R viruses at an MOI of 10 PFU/cell for 2, 4, 6, 8, or 10 hours (Fig. 3A). For each experiment, immunoblots were quantified and the signal from each
sample was normalized to the signal obtained from the WT 10 hpi sample. Normalized values from three different experiments were used to calculate relative means and standard deviations (Fig. 3B). We found the delay in protein synthesis observed in the absence of UL21 was more pronounced for the immediate-early proteins ICP0, ICP4, and ICP27 than for either the early protein ICP8 or the late protein VP16. We therefore focused on ICP0, ICP4, and ICP27 for the following experiments examining mRNA levels. The quantifications also revealed the largest difference between wild-type and UL21- ICP0, ICP4, and ICP27 protein levels occurred at 4 hours post-infection. This time point was therefore chosen to examine relative mRNA levels.

To determine whether the delay in accumulation of ICP0, ICP4 and ICP27 proteins in UL21- infections reflected a delay in accumulation of their respective mRNAs, we performed quantitative real-time RT-PCR. Total RNA was collected from cells infected at an MOI of 10 PFU/cell with the WT, UL21-, and UL21R viruses for 4 hours. The RNAs were treated with DNase to remove contaminating DNA and then used as templates to synthesize cDNAs using random primers. Equal amounts of cDNA were used in qRT-PCR reactions containing primers to the gene of interest and SYBR green for product quantification. Parallel qRT-PCR reactions contained primers to 18S rRNA as a control to normalize template input. The 18S rRNA Ct value for a given template was subtracted from the Ct obtained for each mRNA of interest (ICP0, ICP4, ICP27) from the same template to obtain the normalized Ct value (ΔCt) for each template. Fold change was then calculated using the ΔΔCt method relative to wild-type. At 4 hours post-infection ICP4 and ICP27 steady-state mRNA levels were decreased ~10-fold and ~13-fold, respectively, in UL21- infected cells as compared to WT and UL21R infected cells (Fig. 3C). ICP0 mRNA levels were also affected by the absence of UL21, although to a lesser extent with a 2-fold decrease in UL21- infected cells as compared to WT and UL21R infected cells.

The above experimental findings show the absence of UL21 leads to a delay in the accumulation of ICP0, ICP4 and ICP27 mRNAs, their corresponding proteins, and infectious
intra- and extracellular virus. Ul21 could promote timely progression through the HSV-1 replication cycle by promoting efficient packaging of tegument proteins into virions. For example, if the absence of Ul21 leads to decreased virion levels of tegument proteins with known regulatory roles, such as VP16, there could result a delay in IE transcription during subsequent infection with these virions. To test this hypothesis, we purified virions from WT, Ul21−, and Ul21R infected Vero cells as described previously (4) and examined their relative protein compositions. Figure 4A shows WT, Ul21−, and Ul21R purified virions separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Figure 4B shows a panel of immunoblots performed on the purified virions. VP5 was used as a loading control. As expected, the Ul21 protein (65 kDa) was absent in Ul21− virions. Of note, VP16, vhs, ICP0 and ICP4 were present in approximately equimolar amounts in WT, Ul21−, and Ul21R virions. As shown previously (9), levels of the Ul16 protein (41 kDa) were reduced in Ul21− virions. These data show the delay in the HSV-1 replication cycle observed in the absence of Ul21 is not caused by reduced virion packaging of the regulatory proteins VP16, vhs, ICP0 or ICP4.

By specifically studying the role of Ul21 in early stages of HSV-1 replication, we have made the novel finding that deletion of Ul21 causes a delay early in the virus replication cycle. Single-step growth analyses showed the deletion of Ul21 results in a delay in the production of infectious virions. This was previously demonstrated in a study by Baines et al. in which a HSV-1 mutant virus missing the first 484 codons of Ul21 was generated (1). When compared to the wild-type and Ul21 repair viruses, the Ul21 mutant produced virus yields that were reduced by 1.5 logs on Vero cells at 8 hours post-infection. However, by 20 and 28 hours post-infection, the difference between wild-type and Ul21-null virus yields was decreased to approximately 0.5 logs. In the current study, the delay in the virus replication cycle was also observed in [35S]-labeling protein synthesis time courses (Fig. 2B). By following the accumulation of individual proteins over time via immunoblots (Figs. 3A, 3B), we found the Ul21− associated delay in viral protein synthesis was more pronounced for the immediate-early proteins ICP0, ICP4, and
ICP27 than for either the early protein ICP8 or the late protein VP16. Similarly, steady-state ICP4, ICP27 and, to a lesser extent, ICP0 mRNA levels were decreased at 4 hours post-infection in Ul21-null virus infected cells as compared to wild-type and Ul21-repair virus infected cells (Fig. 3C). Interestingly, virus-induced shut-off of several host proteins was also delayed in Ul21-null virus infected cells as compared to wild-type or Ul21-repair virus infected cells (Fig. 2B, filled squares). The effect of Ul21 on host protein shut-off is likely an indirect consequence of its role in promoting the synthesis of ICP27 since several studies have shown that ICP27 inhibits host cell pre-mRNA splicing, resulting in the shut-off of host protein synthesis (6, 7, 14, 15).

There are a number of possible roles Ul21 may play in promoting timely progression through early stages of the HSV-1 replication cycle. For example, Ul21 may play a role in ensuring timely transport of the DNA-filled capsid to the nucleus and/or in nuclear genome delivery. Alternatively, Ul21 delivered to infected cells may act directly or indirectly to aid in guiding the cellular transcription machinery toward the synthesis of viral mRNAs and away from the synthesis of cellular mRNAs. Finally, the delay in HSV-1 replication we observed in the absence of Ul21 may be an indirect consequence of reduced Ul16 quantities present in Ul21- virions (Fig. 4B) (9). Sequence analysis of Ul16 homologs from several herpesviruses identified a possible zinc-binding domain in the C-terminus (20) and studies with the Ul16 protein from HSV-2 showed this protein may possess DNA binding activity (11). It is not known whether Ul16 is involved in transcriptional regulation but, if so, Ul21 could act indirectly to promote timely viral protein synthesis by promoting the incorporation of Ul16 into virions. In any case, future studies that further define the role of Ul21 in promoting timely progression through early stages of the HSV-1 replication cycle will be of interest.

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Figure 1. Single-step growth analyses of the WT, UL21−, and UL21R viruses. Vero cell monolayers were infected with the WT, UL21−, and UL21R viruses at an MOI of 5 PFU/cell for 1 h to allow virus adsorption. The cells were then washed extensively with citrate buffer to neutralize and remove unbound virus. The cells were overlaid with medium and held at 37°C. At the indicated times post-infection, the infected cells (A) and the overlaying medium (B) were analyzed separately by plaque assay to determine intracellular and extracellular virus yields, respectively. Data points are arithmetic means of three independent experiments, error bars represent one standard deviation.

Figure 2. Analysis of global protein synthesis and accumulation at early times in WT, UL21− and UL21R infections. Vero cells synchronously infected with the WT, UL21− and UL21R viruses were incubated in the presence of 35S-methionine and –cysteine from 0-2, 2-4, 4-6, 6-8, or 8-10 hours post-infection. (A) Following cell washing and lysis, labeled proteins were detected by scintillation counting. Reported values are arithmetic means of three independent experiments, error bars represent one standard deviation. (B) Labeled proteins were also detected by autoradiography following SDS-PAGE separation. The experiment was performed three times with similar results. Example cellular proteins that undergo delayed induction in UL21− infected cells are indicated by open circles, example viral proteins that undergo delayed shutoff in UL21− infected cells are indicated by filled boxes.

Figure 3. Analysis of individual protein and mRNA levels. (A, B) Immunoblot analysis of steady-state ICP0, ICP4, ICP27, ICP8, VP16 and β-actin protein levels at early times in WT, UL21− and UL21R infections. Lysates of Vero cells infected with the WT, UL21− and UL21R viruses for 2, 4, 6, 8, or 10 h were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies specific to ICP0 (Abcam), ICP4 (Rumbaugh-Goodwin Institute for Cancer Research), ICP27 (Virusys), ICP8 (16), VP16 (Santa Cruz Biotechnology)
and β-actin (Santa Cruz Biotechnology). The experiment was performed three times. (A) Autoradiographic visualization of immunoblots. Similar results were obtained from the three experiments. (B) Quantification of immunoblots. For each experiment, bands were normalized to the signal obtained from the WT 10hpi band. Normalized values from each experiment were used to calculate relative mean values. Error bars represent one standard deviation. (C) Relative quantitative real-time RT-PCR analysis of ICP0, ICP4, and ICP27 mRNA levels in Vero cells infected with the WT, U1,21- and U1,21R viruses for 4 hours. Primer pair sequences were as follows: ICP0 5’-CCTCTCCGCATCACCACAGAAGCC-3’ and 5’-CAGGTCTCGGTGCAGGAAACAC-3’; ICP4 5’-CGGCCGTCGCAGCCGTATC-3’ and 5’-CCGCCCCTCCTCCGTCTCC-3’; ICP27 5’-GGTGTCCGGGGTCGGAGAGAAGATG-3’ and 5’-GCCGGTGCCTGTCTTAGGATTTCG-3’. 18S rRNA 5’-CCAGTAAGTGCGGGTCATAAGC-3’ and 5’-GCCTCACTAAACCACATCCAA TCGG-3’. Reported values are arithmetic means of two experiments, each performed in triplicate. Error bars represent one standard deviation.

Figure 4. Immunoblot analysis of purified WT, U1,21- and U1,21R virions. Purified virions were separated by SDS-PAGE and either stained with Coomassie (A) or transferred to a nitrocellulose membrane for immunoblot analysis (B) using antibodies against VP5 (3), ICP4 (Rumbaugh-Goodwin Institute for Cancer Research), ICP0 (Abcam), U1,21 (1), vhs (8), VP16 (Santa Cruz Biotechnology), and U1,16 (10). The experiment was performed twice with similar results.
A Intracellular single-step growth

![Intracellular single-step growth graph]

B Extracellular single-step growth

![Extracellular single-step growth graph]