The UL8 Subunit of the Heterotrimeric Herpes Simplex Virus Type 1 Helicase-Primase Is Required for the Unwinding of Single Strand DNA-binding Protein (ICP8)-coated DNA Substrates*

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The herpes simplex virus type 1 primosome consists of three subunits that are the products of the UL5, UL8, and UL52 genes. The heterotrimeric enzyme has DNA-dependent ATPase, helicase, and primase activities. Earlier studies show that a subassembly consisting of the UL5 and UL52 gene products was indistinguishable from the heterotrimeric enzyme in its helicase and primase activities. We demonstrate here that the UL8 protein is required for the helicase activity of the UL5/52 subassembly on long duplex DNA substrates (>30 nucleotides) with a single-stranded DNA loading site fully coated with the virus-encoded single strand DNA binding protein, ICP8. The Escherichia coli single strand DNA binding protein cannot substitute for ICP8, suggesting a specific physical interaction between ICP8 and the UL8 protein. Surface plasmon resonance measurements demonstrated an interaction between ICP8 and the UL5/52 subassembly and the UL8 protein. The Escherichia coli single strand DNA binding protein cannot substitute for ICP8, suggesting a specific physical interaction between ICP8 and the UL8 protein. Surface plasmon resonance measurements demonstrated an interaction between ICP8 and the UL5/52/8 heterotrimer but not with the UL5/52 subassembly or the UL8 protein alone. At a sub saturating level of ICP8, the UL5/52 subassembly does show helicase activity, suggesting that the subassembly can bind to single-stranded DNA but not to ICP8-coated DNA.

Herpes simplex virus type 1 (HSV-1)1 encodes seven proteins that are essential for the replication of its genome (1). These include an origin binding protein (2, 9), a single strand DNA binding protein (4), a DNA polymerase (5) with its associated processivity factor (6–8), and a heterotrimeric primosome. The primosome encoded by HSV-1 is composed of the products of the UL5, UL52, and UL8 genes and has DNA-dependent ATPase, DNA helicase, and DNA primase activities (9–12). Although the UL8 gene product is essential for HSV-1 DNA replication in vivo, a subassembly consisting of only the 99-kDa UL5 and 114-kDa UL52 gene products was found to be indistinguishable in its DNA-dependent ATPase, DNA helicase, and DNA primase activities from the heterotrimeric enzyme (13, 14). It was subsequently observed that the 80-kDa UL8 protein, which lacks enzymatic and DNA binding activities, can stimulate primer synthesis (15–16). It can also enhance the utilization of primers synthesized by the UL5/52 heterodimer (17). More recently, it was found that the UL8 protein can stimulate the DNA-dependent ATPase, helicase, and primase activities of the UL5/52 heterodimer in the presence of the HSV-1-encoded single strand DNA binding protein, ICP8 (18). In the present study we have found that with duplex DNA substrates >30 nucleotides in length containing a single-stranded loading site fully coated with ICP8, helicase activity shows an almost complete dependence on the UL8 subunit. However, at a narrow range of subsaturating levels of ICP8, unwinding of the helicase subunit can be promoted by the UL5/52 subassembly alone. The helicase activity of the HSV-1-encoded primosome therefore appears to be modulated by ICP8.

MATERIALS AND METHODS

Cells and Viruses—Spodoptera frugiperda (SF9 and SF21) cells were grown at 27 °C in SF-900 11 SFM medium (Life Technologies, Inc.) on a gyratory shaker rotating at 150 rpm. Stocks of Autographa californica nuclear polyhedrosis virus recombinant for the UL8, UL5, UL52, and UL29 genes were prepared by infecting 500 ml of SF9 cells grown to 1 × 106 cells/ml at 0.1 plaque forming unit/cell. The medium was supplemented with 5% fetal calf serum (Life Technologies, Inc.) just before infection. After incubation for 3 days, the cells were pelleted by centrifugation (1500 g, 10 min) at room temperature, and the virus in the supernatant fluid was either used directly or frozen at −80 °C. The final titer was approximately 1010–10 plaque forming units/ml.

Preparation of Infected Cell Extracts—Five hundred milliliters of SF21 cells were grown to a density of 2 × 106 cells/ml in medium supplemented with 1% fetal calf serum. The cells were infected with 5–10 plaque forming units/cell recombinant baculovirus, grown for 48 h, and pelleted by centrifugation (1500 × g, 10 min) at 4 °C. After washing with 500 ml of ice-cold phosphate-buffered saline (Life Technologies, Inc.), the cells were collected by centrifugation (1500 × g, 10 min) at 4 °C. The pellet was frozen in liquid nitrogen and kept at −80 °C. To lyse the cells, the frozen cell pellet was resuspended in 5 volumes of ice-cold lysis buffer containing 20 mM Hepes (pH 7.0), 1 mM dithiothreitol, 10 mM sodium bisulfite (pH 7.5), 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 0.5 mM phenylmethylsulfonyl acid, 1.0 mM EDTA (pH 8.0), and 1 mM EGTA (pH 8.0). The resuspended cells were incubated for 20 min on ice, transferred to a Dounce homogenizer, and disrupted using 15 strokes of a tight-fitting pestle. For the UL5/52 and UL5/52/8 proteins, nuclei were removed from the extract by centrifugation (500 × g, 10 min) at 4 °C. The cytosolic fraction was used directly or frozen in liquid nitrogen and stored at −80 °C. In the case of ICP8, NaCl was added to the suspension of nuclei to a final concentration of 1.2 M, and the mixture was rotated for 45 min at 4 °C. The extract was cleared by centrifugation at 55,000 rpm for 60 min using a Beckman 70.1 Ti rotor.

Enzyme Purification—The UL5/52/8 and UL5/52 proteins were purified as described previously (19), with the following modifications. Heparin-agarose was replaced with a prepacked 1-ml heparin Hi-trap column (Pharmacia Biotech Inc.), and the Superose 12 column was replaced with a prepacked Sephadex 200 (16/60) column (Pharmacia). The yields of UL5/52/8 heterotrimer and UL5/52 heterodimer were approximately 5 mg each. The purity of both complexes estimated by
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Duplex DNA Substrates with an ICP8-coated Single Strand Loading Site—We reasoned that the UL8 protein might be required to enhance the processivity of the helicase-primase, an effect that would be missed with short DNA duplexes. We therefore increased the length of the double-stranded region by DNA polymerase action followed by cleavage with the appropriate restriction enzymes. The substrates produced in this way contained duplex stretches of 33- and 74-bp in addition to the 40-bp single strand loading site (Fig. 1).

With the 74-bp duplex substrate, essentially no unwinding by the UL5/UL2 heterodimer could be detected either in the presence or absence of the UL8 protein (<1%). However, when ICP8 was added, there was a significant difference in the response of the UL5/UL2 and UL5/UL2/8 proteins. In the absence of the UL8 subunit, ICP8 had only a very small stimulatory effect; in its presence, substantial unwinding was observed (Fig. 3, A and B). Essentially the same result was obtained with the substrate containing a 34-bp duplex region (data not shown). We therefore conclude that ICP8 has a strong stimulatory effect on the helicase activity of the HSV-1 helicase-primase and that this stimulation is dependent upon the UL8 protein.

Stoichiometric Amounts of UL8 Protein Are Required for Maximum Helicase Activity—To determine the amount of UL8 protein required for optimal helicase activity, increasing amounts in the UL8 protein were added to the UL5/52 subassembly, and helicase activity was determined with the substrate containing a 74-bp DNA duplex and a 40-nucleotide single strand loading site coated with ICP8. As shown in Fig. 4, helicase activity reached a plateau when the UL8 protein was present at a ratio of about 1:1 with the UL5/UL2 subassembly. This finding is consistent with the 1:1:1 stoichiometry that is found in the helicase-primase isolated from HSV-1-infected cells (10, 12).

Stimulation of Helicase-Primase Is ICP8-specific—To determine whether stimulation of the helicase activity of the UL5/UL2/8 heterotrimer by ICP8 is specific, the heterologous E. coli SSB was substituted for ICP8. As shown in Fig. 5, there was no stimulation of helicase activity by the E. coli SSB under conditions where ICP8 produced a strong stimulation. The T4 phage SSB, gene 32 protein, was similarly ineffective (data not shown). The specific stimulation of helicase activity by ICP8 suggested that there is physical interaction between ICP8 and the helicase-primase. However, attempts to detect such an
interaction by specific retention of either the UL8 protein or the UL5/52/8 heterotrimer on an ICP8-agarose column or by co-immunoprecipitation with antibody directed against ICP8 were unsuccessful.2

Demonstration of the Interaction between ICP8 and UL5/52/8 Holoenzyme by Surface Plasmon Resonance—Surface plasmon resonance detects the change in refractive index near a surface bearing one adsorbed protein as a result of the interaction with a second protein. The change in refractive index, in resonance units, recorded as a function of time after the addition of the second protein or following removal of this protein can be used to determine the rates of association and dissociation respectively. These rates are related through simple exponentials to the fundamental rate constant \( k_a \) and \( k_d \) for the binding reaction,

\[
\frac{k_a}{k_d} = \frac{A + B}{k_d} \rightarrow AB
\]

(Eq. 1)

with \( k_a/k_d = K_{on} \), the equilibrium association constant (25).

In these experiments, ICP8 was immobilized to the dextran surface of a BIAcore sensor chip, and its interaction with the UL5/52/8 holoenzyme, UL5/52 subassembly, and the UL8 protein was measured. As shown in Fig. 6, the UL5/52 subassembly and the UL8 protein failed to interact with the ICP8 surface. In contrast, the UL5/52/8 holoenzyme did interact significantly. The apparent association rate constant \( k_a \) was \( 2.8 \times 10^5 \) s\(^{-1}\) M\(^{-1}\) (S.D., \( 8.5 \times 10^4; n = 4 \)). The apparent dissociation rate constant \( k_d \) was \( 2.5 \times 10^{-3} \) s\(^{-1}\) (S.D., \( 9.8 \times 10^{-4}; n = 4 \)). We assume that a 1:1 complex is formed between ICP8 and the UL5/UL52/UL8 heterotrimer. A plot of \( \ln(R_0/R) \) versus time after the start of dissociation, where \( R_0 \) is the response at the start of dissociation and \( R \) is the response at time \( t \) after dissociation is linear, indicating that dissociation is first order (data not shown).

Subsaturating Levels of ICP8 Can Stimulate the Helicase Activity of the UL5/52 Subassembly—The experiments described this far were all performed at excess ICP8 to ensure that free ICP8 would be available to bind the single-stranded DNA generated during the helicase-promoted unwinding of the substrate (26, 27). Under these conditions, the UL5/52 heterodimer was inactive. There was, however, a narrow range of subsaturating ICP8 concentrations at which unwinding of the substrate could be observed in the absence of the UL8 protein (Fig. 7, A and C). As the level of ICP8 was increased, the stimulatory effect was suppressed. In contrast, increasing amounts of ICP8, in the presence of the UL8 protein, produced no such inhibition (Fig. 7, B and C). In a control experiment using *E. coli* SSB, no stimulation of helicase activity was observed at any of the concentrations tested. It therefore appears that low concentrations of ICP8 can have a specific stimulatory effect on the helicase activity of the helicase-primase even in the absence of the UL8 protein.

DISCUSSION

The primosome encoded by HSV-1 is a heterotrimer consisting of the products of the UL5, UL8, and UL52 genes (9–12). Helicase activity is associated with the UL5 subunit (17, 28), and primase activity is associated with the UL52 subunit (29). The specific function of the UL8 subunit has remained obscure. In the studies reported here, we have found that the UL8 subunit is not needed for helicase action on short, 20-nucleotide duplex DNA substrates in the absence of ICP8 but is required for longer, 30–74-bp duplex DNA substrates with a single-stranded loading site fully coated with the HSV-1 single strand DNA binding protein, ICP8. A plausible inference from these findings is that the UL8 subunit of the primosome interacts with ICP8-coated single-stranded DNA to facilitate binding of the UL5/52 heterodimer. The reaction is specific for ICP8, and a heterologous single strand DNA-binding protein (*E. coli* SSB) was ineffective, suggesting a physical interaction of the primosome with ICP8.

By using surface plasmon resonance we did, in fact, observe that the UL5/52/8 holoenzyme, but neither the UL5/52 subassembly nor the UL8 subunit alone, was able to bind to ICP8. The estimated \( k_a \) of \( 2.5 \times 10^{-3} \) s\(^{-1}\) confirmed that the interaction is specific and occurs with a relatively high affinity. Attempts to measure the interaction of the UL5/52/8 holoenzyme, the UL5/52 subassembly, or the UL8 protein with ICP8 bound to single-stranded DNA were complicated by the rapid association and dissociation of ICP8 with and from the single-stranded DNA.3

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2 M. Falkenberg, P. Elias, and I. R. Lehman, unpublished data.

3 D. Bushnell, M. Falkenberg, P. Elias, and I. R. Lehman, unpublished data.
What is the mechanism by which the UL8 protein promotes the binding of the helicase-primase to an ICP8-coated substrate? One possibility is that it permits the displacement of ICP8 from DNA, perhaps by stabilizing a conformation of ICP8 that prevents it from binding to single-stranded DNA. Alternatively, ICP8-coated single-stranded DNA itself may serve as the loading site for the helicase-primase. Since the UL8 protein alone does not bind ICP8, interaction of the UL8 protein with the UL5/52 subassembly either enhances its binding to ICP8, or alternatively, facilitates interaction of the UL5/52 subassembly with ICP8.

The effects of ICP8 on the HSV-1 primosome are reminiscent of the effects of the T4 bacteriophage single strand DNA-binding protein, the gene 32 protein, on the T4 primosome, the product of T4 genes 41 and 61. The gene 32 protein stimulates the helicase activity of the primosome but confines the synthesis of RNA primers to those sites that are used to initiate synthesis of an Okazaki fragment (30). An additional T4-encoded protein, the product of gene 59, is required to assemble the gene 41 protein (the helicase) onto gene 32-coated single-stranded DNA. This reaction is mediated by a specific interaction between the gene 59 and gene 32 proteins (30). Thus, the HSV-1-encoded UL8 protein may be the functional analogue of the T4 gene 59 protein.

A surprising finding was that over a narrow range of sub-saturating ICP8 concentrations, the UL5/52 subassembly can unwind long DNA duplexes even in the absence of the UL8 subunit. Possibly the UL5/52 heterodimer can bind to a single-stranded DNA loading site free of ICP8 and promote helicase activity. The appropriate concentration of ICP8 would then be needed to bind the single strands produced by helicase action, preventing them from reannealing. At levels of ICP8 sufficient to fully coat the single-stranded DNA, the UL5/52 subassembly is unable to bind the ICP8-coated single strands, and helicase activity is inhibited.