Cysteine 111 Affects Coupling of Single-stranded DNA Binding to ATP Hydrolysis in the Herpes Simplex Virus Type-1 Origin-binding Protein

(Received for publication, September 30, 1999, and November 5, 1999)

Deborah A. Sampson, Mercedes E. Arana, and Paul E. Boehmer

From the Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida 33101-6129

Herpes simplex virus type-1 origin-binding protein (UL9 protein) initiates viral replication by unwinding the origins. It possesses sequence-specific DNA-binding activity, single-stranded DNA-binding activity, DNA helicase activity, and ATPase activity that is strongly stimulated by single-stranded DNA. We have examined the role of cysteines in its action as a DNA helicase. The DNA helicase and DNA-dependent ATPase activities of UL9 protein were stimulated by reducing agent and specifically inactivated by the sulfhydryl-specific reagent N-ethylmaleimide. To identify the cysteine responsible for this phenomenon, a conserved cysteine in the vicinity of the ATP-binding site (cysteine 111) was mutagenized to alanine. UL9C111A protein exhibits defects in its DNA helicase and DNA-dependent ATPase activities and was unable to support origin-specific DNA replication in vivo. A kinetic analysis indicates that these defects are due to the inability of single-stranded DNA to induce high affinity ATP binding in UL9C111A protein. The DNA-dependent ATPase activity of UL9C111A protein is resistant to N-ethylmaleimide, while its DNA helicase activity remains sensitive. Accordingly, sensitivity of UL9 protein to N-ethylmaleimide is due to at least two cysteines. Cysteine 111 is involved in coupling single-stranded DNA binding to ATP-binding and subsequent hydrolysis, while a second cysteine is involved in coupling ATP hydrolysis to DNA unwinding.

The herpes simplex virus type-1 (HSV-1) origin-binding protein (UL9 protein) is one of seven virus-encoded proteins that are required for origin-dependent DNA replication (1–6). The UL9 protein is an 851-amino acid polypeptide with a calculated mass of 94,250 Da (7). It binds cooperatively and with high affinity to 10-base pair inverted repeats that flank an A/T-rich region within the origins of replication (8–10). The sequence-specific DNA-binding activity resides in the C-terminal one-third of the UL9 protein (residues 535–851) (11–17).

The UL9 protein also catalyzes the hydrolysis of ATP, which is greatly stimulated by single-stranded DNA (ssDNA), reflecting the ability of the protein to translocate along ssDNA and to unwind DNA with a polarity of 3’ to 5’ (18–22). The ATPase and DNA helicase activities as well as a distinct ssDNA-binding activity localize to the N-terminal two-thirds of the UL9 protein (residues 1–534) (23). Mutations in the ATP-binding site or the conserved DNA helicase motifs of the UL9 protein abolish origin-dependent replication in a transient system (24, 25).

The function of the UL9 protein is to initiate replication by unwinding the DNA at the origins (26, 27). This function is probably performed in conjunction with the HSV-1 single strand DNA-binding protein (ICP8) which interacts with the C-terminal domain of the UL9 protein (28). This interaction is important for origin-dependent replication and has been shown to stimulate the DNA helicase activity of the UL9 protein by preventing its dissociation from the DNA substrate (29, 30). Presumably, the sequence-specific DNA-binding activity of the UL9 protein targets a complex of UL9 protein and ICP8 to the origins to promote efficient DNA unwinding (1, 27).

The UL9 protein contains a remarkably high content of cysteines (24 out of 851 amino acids) that are randomly distributed throughout the primary sequence (7). None of these cysteines are arranged into motifs that are involved in metal binding such as zinc fingers or RING fingers. Thus far there have been no reports on the importance of cysteines for the activities of the UL9 protein. In this study, we show that the UL9 protein contains N-ethylmaleimide (NEM)-sensitive cysteines that overlap with the ssDNA-binding site and are involved in signal transduction between ssDNA-binding and ATP hydrolysis and movement of the UL9 protein along DNA. The inability to identify a specific cysteine that is modified by NEM prompted us to use site-directed mutagenesis to identify the cysteine that is responsible for the inhibitory effect of NEM. Since the ATPase and DNA helicase activities of the UL9 protein reside in the N-terminal domain (23), the susceptible cysteine(s) must be in this region. Although this part of the UL9 protein contains 17 cysteines, only four of these are perfectly conserved in the origin-binding proteins of at least 10 different herpesviruses. Since amino acids that perform critical tasks are likely to be conserved, these cysteines may be responsible for NEM-induced inactivation of the UL9 protein. One of these cysteines (cysteine 111) is located in the vicinity of the "Walker" type A adenine nucleotide-binding site and DNA helicase motif I (Fig. 1) (31–33) and may therefore represent the cysteine responsible for NEM-induced inactivation of the UL9 protein. Our results indicate that cysteine 111 is an essential residue that is involved in coupling ssDNA-binding to ATP hydrolysis, which represents a key step in the mechanism of action of a DNA helicase.

* This work was supported by National Institutes of Health Grant AI38335. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Miami School of Medicine, P.O. Box 016129, Miami, FL 33101-6129. Tel.: 305-243-2934; Fax: 305-243-3955; E-mail: pboehmer@mmbio.med.miami.edu.

‡ The abbreviations used are: HSV, herpes simplex virus; NPV, nuclear polyhedrosis virus; DTT, dithiothreitol; EPPS, N-(2-hydroxyethyl)piperazine-N’-(3-propanesulfonic acid); m.o.i., multiplicity of infection; NEM, N-ethylmaleimide; ssDNA, single-stranded DNA.

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DNA-dependent ATPase and DNA Helicase of HSV-1 UL9 Protein

**EXPERIMENTAL PROCEDURES**

**Chemicals**—ATP (disodium salt), phosphoenolpyruvate (potassium salt), NADH, malachite green, and ammonium molybdate were obtained from Sigma. [32P]ATP (4,500 Ci/μmol) and [α-32P]dATP (3,000 Ci/μmol) were purchased from ICN. N-Ethylmaleimide was purchased from Pierce. Its concentration was determined by using an extinction coefficient of 620 M⁻¹ cm⁻¹ at 280 nm in 20 mM HEPES-NaOH, pH 7.0.

**DNA**—[13C]ATP (disodium salt, phosphoenolpyruvate (potassium salt), NADH, malachite green, and ammonium molybdate) was obtained from Sigma. [32P]ATP (4,500 Ci/μmol) and [α-32P]dATP (3,000 Ci/μmol) were purchased from ICN. N-Ethylmaleimide was purchased from Pierce. Its concentration was determined by using an extinction coefficient of 620 M⁻¹ cm⁻¹ at 280 nm in 20 mM HEPES-NaOH, pH 7.0.

**CELL CULTURES**—Sf21 cells at 27 °C in Sf900 II-serum-free medium (Life Technologies, Inc.). Recombinant NPV isolates were used to screen for expression of UL9C111A protein in S. frugiperda cells infected with a 1-mL Resource S column (Amersham Pharmacia Biotech) as described for UL9 protein on a MonoS HR 5/5 column (28), except that DTT was omitted from the buffer. The peak of UL9C111A protein, containing nearly homogenous UL9C111A protein, eluting at the same position as UL9 protein, was subjected to size exclusion chromatography on a 1-ml Resource S column (Amersham Pharmacia Biotech) at 1 mL/min. The protein was then dialyzed against 1 mL of 10 mM sodium phosphate, pH 7.2, 0.1 M NaCl, 10% glycerol, and 10 mM DTT and stored at -80 °C. The peak of UL9C111A protein, containing nearly homogenous UL9C111A protein, eluting at the same position as UL9 protein, was subjected to size exclusion chromatography on a 1-ml Resource S column (Amersham Pharmacia Biotech) at 1 mL/min. The protein was then dialyzed against 1 mL of 10 mM sodium phosphate, pH 7.2, 0.1 M NaCl, 10% glycerol, and 10 mM DTT and stored at -80 °C.

**Hydroxyapatite chromatography**—was performed as described for UL9 protein on a MonoS HR 5/5 column (28), except that DTT was omitted from the buffer. The peak of UL9C111A protein, containing nearly homogenous UL9C111A protein, eluting at the same position as UL9 protein, was subjected to size exclusion chromatography on a 1-ml Resource S column (Amersham Pharmacia Biotech) at 1 mL/min. The protein was then dialyzed against 1 mL of 10 mM sodium phosphate, pH 7.2, 0.1 M NaCl, 10% glycerol, and 10 mM DTT and stored at -80 °C.

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assays. The effect of DTT on the DNA-dependent ATPase activity of the UL9 protein was measured in reactions (25 μl) containing increasing concentrations of DTT (0–10 mM) and 20 mM EPPS-NaOH, pH 8.3, 50 mM NaCl, 4.5 mM MgCl₂, 2 mM ATP, 10 μM (nucleotide) (d)T₆₀, and 0.1 mg/ml bovine serum albumin in the absence or presence of 100 mM UL9 protein. Reactions were incubated for 30 min at 37 °C followed by the addition of 750 μl of acidic ammonium molybdate solution containing malachite green to measure the formation of inorganic phosphate (35). After 5 min of color development at room temperature, the absorbance at 650 nm was determined. Rates of ATP hydrolysis were determined using an extinction coefficient of 6,220 M⁻¹ cm⁻¹ for NADH.

The positions of UL9C111A protein and of molecular weight standards were indicated. The vertical arrows indicate the peak of elution. The horizontal arrows indicate the position of UL9C111A protein.

![Fig. 2. Purification of UL9C111A protein.][1]

UL9C111A protein was purified as described under “Experimental Procedures.” A, Coomassie Blue-stained 10% SDS, 9% polyacrylamide gel of fractions eluting from the Resource S column. B, fractions shown in A were analyzed by immunoblotting using an anti-UL9 protein rabbit serum. The positions of UL9C111A protein and of molecular weight standards are indicated. The vertical arrows indicate the peak of elution. The horizontal arrows indicate the position of UL9C111A protein.

### Table I

**Effects of dithiothreitol and N-ethylmaleimide on the UL9 protein**

Reactions were performed as described under “Experimental Procedures.” The effects of NEM were determined after modification with 1 mM NEM.

| Effect Activity | Without NEM | With neutralized NEM | With NEM |
|----------------|-------------|----------------------|----------|
| DNA helicase | 0.21 ± 0.07 | 0.24 ± 0.03 | 0.14 ± 0.04 |
| ssDNA-binding | 32.2 | 35.5 | 23.3 |
| Origin-binding | 3.56 | 3.31 | 3.17 |

* DNA helicase activity was determined after 15 min at 37 °C and is expressed as percentage of DNA unwound.
* Activity is expressed in absorbance units at 650 nm.
* Activity is expressed as pmol of ATP hydrolyzed s⁻¹.
* Activity is expressed as fmol of DNA bound.
* Activity is expressed as fmol of DNA bound at 200 mM UL9 protein.

**RESULTS**

**Effects of Dithiothreitol and N-Ethylmaleimide on the UL9 Protein**—DTT stimulated the DNA helicase and DNA-dependent ATPase activities of UL9 protein up to a maximum of 5- and 2-fold, respectively (Table I). Incubation of UL9 protein with 1 mM NEM rapidly (within 30 s) inactivated its DNA helicase activity (Fig. 3). Similarly, NEM inactivated the DNA-depend-
Incubation of UL9 protein with ssDNA ((dT)60) fully protected protein. Reactions were performed as described under “Experimental Procedures.” At the times indicated, 10-μl aliquots were removed to measure DNA helicase activity. Filled circle, UL9 protein; open triangle, UL9 protein with neutralized NEM; filled square, NEM-modified UL9 protein.

ent ATPase activity of UL9 protein but had no significant effect on its DNA-independent ATPase, ssDNA-binding, and origin binding activities (Table I). Inactivation of the DNA helicase and DNA-dependent ATPase activities required excess concentrations of NEM (IC50 ~ 200 μM NEM) (data not shown). Incubation of UL9 protein with ssDNA ((dT)60) fully protected its DNA-dependent ATPase activity from NEM inactivation (Fig. 4). In addition, both ATP (5 mM) and duplex DNA (15 μM nucleotide) also provided partial protection from NEM inactivation (data not shown). Using N-[ethyl-1,2-3H]maleimide, the stoichiometry of modification was determined as 14:1 NEM:UL9 protein, indicating that the majority (14 out of 24) of cysteines in the UL9 protein are susceptible to modification by NEM. In the presence of (dT)60, this ratio was decreased to 2:1 NEM:UL9 protein, indicating that the majority (12 out of 14) of the NEM-susceptible cysteines are protected by ssDNA.

Purification of UL9C111A Protein—A UL9 protein variant, designated UL9C111A protein, in which cysteine 111 is substituted with alanine, was expressed and purified to near homogeneity from A. californica NPV-infected Sf21 cells. The purity of the final protein preparation, eluting from a Resource S column, is shown in Fig. 2A. The identity of purified UL9C111A protein was confirmed by immunoblot analysis with an anti-UL9 protein rabbit serum (Fig. 2B).

Characterization of the ATPase and DNA Helicase Activities of UL9C111A Protein—The rate of ATP hydrolysis by UL9C111A protein was compared with that of UL9 protein both in the absence and presence of a ssDNA cofactor, (dT)60. Fig. 5A shows that the rate of DNA-independent ATP hydrolysis by UL9C111A protein did not significantly differ from that of UL9 protein. In contrast, UL9C111A protein exhibited ~50% of the rate of DNA-dependent ATP hydrolysis observed with UL9 protein (Fig. 5B). The rate of DNA unwinding exhibited by UL9C111A protein was also reduced to ~50% of that of UL9 protein (Fig. 6A). Likewise, the specific activity of DNA unwinding of UL9C111A protein was approximately half that of UL9 protein (Fig. 6B).

Mutagenesis of Cysteine 111 to Alanine Does Not Affect the Stability of the UL9 Protein or Its Origin Binding Activity—To confirm that UL9C111A protein is not structurally altered, we compared its thermal stability to that of UL9 protein. The thermal inactivation curves of UL9 and UL9C111A proteins, both in the absence and presence of (dT)60, were the same (data not shown). Similarly, substitution of cysteine 111 with alanine had no effect on the origin binding activity of the UL9 protein (data not shown).

Effect of N-Ethylmaleimide on the ATPase and DNA Helicase Activities of UL9C111A Protein—We predicted that cysteine 111 is responsible, at least in part, for the sensitivity of the UL9 protein DNA-dependent ATPase and DNA helicase activities to NEM. Consistent with our previous data (Table I, Fig. 3), the DNA-dependent ATPase and DNA helicase activities of the UL9 protein were inhibited by NEM modification (Fig. 7). In agreement with our prediction, NEM modification had no effect on the DNA-independent ATPase activity of UL9C111A protein and did not inhibit its DNA-dependent ATPase activity (Fig. 7, A and B). Interestingly, NEM-modified UL9C111A protein actually exhibited an increased rate of DNA-dependent ATP hydrolysis (Fig. 7B). In contrast to the ATPase activities of UL9C111A protein, its DNA helicase activity, like that of UL9 protein, was inhibited by NEM modification (Fig. 7C).

Kinetic Characterization of UL9C111A Protein—To determine the basis of the defect in UL9C111A protein, a steady-state kinetic analysis of the DNA-dependent ATPase activity of UL9 and UL9C111A proteins was performed. Determination of kcat and Km ATP for the DNA-independent ATPase activity of
UL9 and UL9C111A proteins was also attempted. However, rates of ATP hydrolysis failed to saturate with increasing ATP concentrations, exhibiting a linear relationship up to 10 mM ATP, making it impossible to determine $k_{\text{cat}}$ and $K_m^{\text{ATP}}$ (data not shown). In contrast, the addition of increasing ATP concentrations to reactions containing UL9 or UL9C111A proteins and (dT)$_60$ cofactor resulted in typical Michaelis-Menten behavior (data not shown). The kinetic parameters for the DNA-dependent ATPase activity of UL9 and UL9C111A proteins are shown in Table II. The values for $K_m^{\text{ATP}}$ and $K_m^{\text{ssDNA}}$ are in agreement with those previously reported for UL9 protein (22). Consistent with the data shown in Fig. 5, $k_{\text{cat}}$ of UL9C111A protein is 2-fold lower than that of UL9 protein. Interestingly, the defect in UL9C111A protein does not appear to be in its interaction with ssDNA cofactor, since it actually possess a lower $K_m^{\text{ssDNA}}$ than UL9 protein. Consequently, the $k_{\text{cat}}/K_m$ ssDNA ratios of UL9 and UL9C111A proteins are not significantly different. However, UL9C111A protein exhibits a 3-fold higher $K_m^{\text{ATP}}$ for ATP than UL9 protein. Accordingly, the $k_{\text{cat}}/K_m$ ATP ratio of UL9C111A protein is almost 7-fold lower than that of UL9 protein.

**Effect of the UL9C111A Mutation on Origin-dependent DNA Replication**—The ability of UL9C111A protein to support origin-dependent DNA replication in vivo was determined by detecting DpnI-resistant oriS-containing pGEM822 in Southern blots. Fig. 8 shows a representative result of such an experiment. While replicated, DpnI-resistant pGEM822 was detectable in cells infected with *A. californica* NPV encoding wild-type UL9, no detectable DNA replication was observed in cells infected with *A. californica* NPV encoding UL9C111A or in mock-infected cells. The defect in origin-dependent DNA replication was not due to the lack of expression of UL9C111A protein, since cells used for determining DNA replication activity exhibited comparable expression of UL9 and UL9C111A proteins (data not shown).

**DISCUSSION**

In this paper, we have examined the importance of cysteine residues for the functions of the HSV-1 UL9 protein and described the properties of a mutant UL9 protein that bears a cysteine to alanine substitution at position 111.

The data show that the DNA helicase and DNA-dependent ATPase activities of the UL9 protein were stimulated by dithiothreitol, indicating the requirement for reduced cysteines (sulfhydryl groups). In addition, both activities were inactivated by modification with NEM. However, NEM modification of the UL9 protein did not affect its DNA-independent ATPase
and ssDNA- and origin-binding activities. These findings indicate that NEM modification does not indiscriminately inactivate the activities of the UL9 protein and therefore does not result in any gross structural alterations. Preincubation of the UL9 protein with ssDNA protected it from NEM inactivation presumably by masking crucial cysteine(s). Lower levels of protection were also provided by preincubation of UL9 protein with ATP and duplex DNA. These data indicate that the susceptible cysteine(s) is primarily associated with the ssDNA-binding site although they do not participate in ssDNA-binding directly, since NEM-modified UL9 protein retains ssDNA-binding activity. Because NEM modified the majority of cysteines in the UL9 protein and given that the majority of these were also protected from NEM modification by ssDNA, it was impossible to utilize this approach to identify the crucial cysteine(s). However, the cysteine(s) must be located in the N-terminal two-thirds of the UL9 protein, since this domain retains ssDNA-binding, ATPase, and DNA helicase activities (23). Consequently, we employed site-directed mutagenesis to identify the pertinent residue. Cysteine 111 is one of four cysteines that are conserved in the N-terminal domain of herpesvirus origin-binding proteins. The proximity of cysteine 111 to the “Walker” type A adenine nucleotide-binding site and DNA helicase motif I (31–33) suggested to us that it may be responsible for NEM-induced inactivation of the DNA-dependent ATPase and DNA helicase activities of UL9 protein.

Our results show that cysteine 111 is an essential residue, since the mutant UL9C111A protein failed to support origin-dependent DNA replication in vivo. Substitution of cysteine 111 with alanine did not affect the DNA-independent ATPase activity of UL9 protein, suggesting that this residue is not directly involved in ATP binding or hydrolysis. This finding is consistent with the observation that NEM modification did not affect the DNA-independent ATPase activity of UL9 protein. Furthermore, the fact that UL9C111A protein retains an unaltered level of DNA-independent ATPase activity indicates that substitution of cysteine 111 with alanine did not lead to any gross structural alterations that may have impaired its catalytic activity. This conclusion is substantiated by the finding that UL9C111A protein retains wild-type levels of origin binding activity and also exhibits similar thermal stability to UL9 protein.

UL9C111A protein retains DNA-dependent ATPase activity albeit with a 2-fold lower $k_{cat}$ than UL9 protein. The ability of ssDNA to stimulate ATP hydrolysis in UL9C111A protein indicates that it has retained its ability to interact with ssDNA. This conclusion is supported by the finding that UL9C111A protein exhibits a $K_a$ for ssDNA that is actually slightly lower than that of UL9 protein. Consequently, the defect in UL9C111A protein is not in its interaction with ssDNA. Substitution of cysteine 111 with alanine also resulted in a 2-fold decrease in the specific activity of DNA unwinding.

The observation that rates of ATP hydrolysis did not saturate with increasing ATP concentrations unless ssDNA cofactor was present suggests that ssDNA binding increases the affinity of UL9 protein for ATP, possibly by inducing a conformational change. We propose that the 3-fold increase in $K_a$ for ATP of UL9C111A protein is due to the failure of ssDNA-binding to induce high affinity ATP binding, indicating that cysteine 111 is involved in coupling ssDNA-binding to ATP binding and subsequent hydrolysis. Moreover, the decrease in $k_{cat}$ and increase in $K_a$ for ATP of UL9C111A protein, as manifested by a 7-fold lower $k_{cat}/K_a$ ATP ratio, are responsible for the inability of UL9C111A protein to support origin-dependent DNA replication in vivo. Thus, at a $K_a$ of almost 2 mM ATP and at a reduced $k_{cat}$, mutant UL9C111A protein is incapable of performing its essential role in viral origin-dependent DNA replication, presumably due to a defect at the level of DNA unwinding.

We targeted cysteine 111 in the hope of identifying the residue responsible for NEM-induced inactivation of UL9 protein. Consistent with our prediction, the DNA-dependent ATPase activity of UL9C111A protein is NEM-resistant. In fact, NEM-modified UL9C111A protein exhibited elevated DNA-dependent ATPase activity. This phenomenon may be the consequence of some structural alteration analogous to that seen with UL9DM27 protein, which lacks the C-terminal 27 amino acids of UL9 protein (29). Interestingly, while the DNA-dependent ATPase activity of UL9C111A protein is NEM-resistant, its DNA helicase activity, like that of UL9 protein, is NEM-sensitive. Accordingly, sensitivity of the UL9 protein DNA-dependent ATPase and DNA helicase activities to NEM is due to modification of at least two functionally important cysteines. The first, cysteine 111, is involved in coupling ssDNA binding to high affinity ATP binding. The second, presumably one of the three remaining conserved cysteines in the N-terminal domain of UL9 protein, is involved in coupling ATP hydrolysis to DNA unwinding.

In summary, given that NEM-modified UL9 protein lacks DNA helicase and DNA-dependent ATPase activities but retains DNA-independent ATPase and ssDNA-binding activities, we propose that cysteines are not required for catalysis or substrate binding but rather for signal transduction between ssDNA binding and ATP hydrolysis and for movement of the UL9 protein along DNA. It is possible that the critical cysteines are part of a channel that contains the ATP- and ssDNA-binding sites and other elements involved in translocation of UL9 protein along DNA. The presence of a “groove” that involves the ATP- and ssDNA-binding sites has been inferred from the crystal structures of the RecA protein as well as the PerA and Rep DNA helicases (40–42). Presumably, the function of such a groove is to transduce effects in response to ATP binding and/or hydrolysis that would enable the enzyme to translocate along DNA. We have identified cysteine 111 as an
essential residue that is involved in coupling ssDNA binding to ATP binding and hydrolysis. In addition, cysteine 111 is one of at least two cysteines that are responsible for the sensitivity of the UL9 protein DNA-dependent ATPase and DNA helicase activities to NEM. Cysteines involved in metal binding in the E. coli PriA protein and HSV-1 UL52 DNA helicase-prime subunit have previously been implicated in DNA-dependent ATP hydrolysis and DNA unwinding (43, 44). Our results are the first to show that a cysteine not involved in metal binding participates in the mechanism of DNA unwinding, specifically coupling ssDNA-binding to ATP-binding and hydrolysis.

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