The herpes simplex virus type 1 origin-binding protein interacts specifically with the viral UL8 protein

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The products of herpes simplex virus type 1 (HSV-1) genes UL5, UL8 and UL52 form a complex in virus-infected cells that exhibits both DNA helicase and DNA primase activities. UL8 protein was purified from insect cells infected with a recombinant baculovirus and used to generate monoclonal antibodies (MAbs). MAb 0811 was shown to recognize the UL8 protein in both Western blots and immunoprecipitation assays and to co-precipitate the other two proteins in the complex from insect cells triply infected with recombinants expressing the UL5, UL8 and UL52 polypeptides. Experiments performed using extracts from doubly infected cells indicated that UL8 could interact separately with both the UL5 and UL52 proteins. Similar experiments using a recombinant virus that expressed the HSV-1 origin-binding protein (OBP), UL9, demonstrated a direct physical interaction between the helicase–primase complex and OBP which involved the UL8 subunit. The C-terminal DNA-binding domain of OBP is dispensable for this interaction, as evidenced by the ability of MAb 0811 to co-precipitate a truncated UL9 protein, containing only the N-terminal 535 amino acids, with UL8.

Introduction

The linear dsDNA genome of herpes simplex virus type 1 (HSV-1) contains three elements that function as origins of replication and encodes seven proteins that play direct and essential roles in viral DNA synthesis. Genes UL30 and UL42 encode catalytic and accessory subunits of a heterodimeric DNA polymerase and gene UL29 specifies an ssDNA-binding protein. The product of the UL9 gene functions as a sequence-specific origin-binding protein (OBP) which interacts with each of the replication origins. The remaining proteins encoded by genes UL5, UL8 and UL52, form a trimeric complex which possesses DNA helicase and DNA primase activities (for reviews see Challberg, 1991; Weller, 1991).

Experiments in which different combinations of the subunits of the helicase–primase complex were co-expressed by mixedly infecting insect cells with recombinant baculoviruses demonstrated that a sub-assembly of UL5 and UL52 exhibited enzymic activities identical to those of the complete complex (Calder & Stow, 1990; Dodson & Lehman, 1991). Although neither of these proteins alone exhibits either DNA helicase or DNA primase activity it is noteworthy that the UL5 protein contains several motifs conserved in many known helicases and that these have been shown in mutagenesis experiments to be crucial for UL5 function in viral DNA synthesis (Gorbalenya et al., 1989; Zhu & Weller, 1992). Two possible roles for UL8 in the helicase–primase complex have been suggested by experimental results; stabilizing primer–template interactions (Sherman et al., 1992) and promoting efficient uptake of the complex into the nucleus of infected cells (Calder et al., 1992).

In addition to its origin-binding activity UL9 also functions as a helicase on partially double-stranded templates (Bruckner et al., 1991) although specific unwinding of an HSV-1 origin of replication has not yet been demonstrated (Fierer & Challberg, 1992). The 851 amino acid protein appears to be organized into at least two separate functional domains. The C-terminal 317 residues contain all the information required for sequence-specific DNA binding (Weir et al., 1989; Deb & Deb, 1991) and a set of essential motifs characteristic of helicases is confined to the N-terminal half of the protein (Gorbalenya et al., 1989; Martinez et al., 1992). The N-terminal 534 amino acids are also required for the observed dimerization of the intact protein (Bruckner et al., 1991; Fierer & Challberg, 1992) and cooperative binding to the origins of replication (Hazuda et al., 1992; Elias et al., 1992).

Relatively little is understood about how the seven HSV-1 DNA replication proteins interact to coordinate viral DNA synthesis and whether cellular proteins are also involved. This has been due in part to the low levels of these proteins present in HSV-1-infected cells and the likelihood that these interactions may be weak or
transient in nature, making their detection difficult. In the absence of a cell-free replication system for HSV-1 DNA, we are attempting to approach this problem by preparing antibodies with which to study interactions between different components of the replicative machinery. In this paper we describe the generation of monoclonal antibodies (MAbs) reactive with the UL8 protein and the use of one such MAb in immunoprecipitation assays. These experiments demonstrate that UL8 can interact separately with both UL5 and UL52, and additionally with UL9 via sequences present in the N-terminal 535 amino acids of this protein.

Methods

Viruses and cells. The recombinant baculoviruses AcUL9, AcUL9CT, AcUL5, AcUL8 and AcUL52 have been described previously (Calder & Stow, 1990; Stow, 1992). AcUL9NT is essentially identical to AcUL9 but contains only nucleotides 23542 to 21656 of HSV-1 DNA, specifying the N-terminal 535 amino acids of UL9, followed by a synthetic XbaI linker containing termination codons in all three reading frames. Spodoptera frugiperda (Sf) cells (strain IPLB-SF-21; Kitts et al., 1990) were maintained in TC-100 medium supplemented with 5% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Virus stocks were prepared and titrated as previously described (Brown & Faulkner, 1977; Matsuura et al., 1987).

HSV-1 (strain 17 syn+) was propagated in baby hamster kidney (BHK-21, C13) cells as previously described (Brown et al., 1973).

Antibodies. Monoclonal antibodies (MAbs) were generated by immunizing female BALB/c mice with UL8 protein purified as described by Purry et al. (1993). Ten μg protein per mouse was emulsified in Freund’s complete adjuvant (for the initial immunization) or incomplete adjuvant (for subsequent immunizations). Three days after boosting with 35 μg protein in 10% glycerol, the spleen cells from a selected seropositive mouse were harvested and fused with Sp2/0Ag 14 Balb/c mouse myeloma cells using 33% polyethylene glycol. Colonies were selected in Dulbecco’s modified Eagle’s medium supplemented with 100 μM-hypoxanthine, 0.42 μM-aminopterin and 63 μM-thymidine (HAT) and the supernatants were screened for reactivity with the UL8 protein by ELISA assay (McLean et al., 1991). Cells producing positive supernatants were grown in medium without HAT and used to generate ascitic fluid in mice. MAb 0811 used throughout this paper corresponds to the ascitic fluid derived using one such cell line. MAb 13672, which reacts with UL9 protein, was similarly
generated using UL9 protein purified from SF cells infected with AcUL9 (A. P. Abbotts, A. Cross & N. D. Stow, unpublished results).

Preparation of infected cell extracts. Approximately 8 x 10^9 SF cells were seeded per well (4 cm^2 surface area) in 12-well plates 1 day prior to infection. Cells were either mock infected or infected with 5 p.f.u./cell of each of the indicated viruses and incubated at 28 °C in 2 ml medium. At 24 h post-infection (p.i.) the medium was replaced with 500 µl TC-100 salt solution (Calder & Stow, 1990) containing 2 ml medium. At 24 h p.i. the cells were scraped into the medium, pelleted in microcentrifuge tubes, washed three times with unsupplemented TC-100 medium and resuspended in 400 µl ice-cold buffer E (100 mM-Tris-HCl pH 8.0, 100 mM-NaCl, 2 mM-EDTA, 2 mM-EGTA, 1% NP40, 0.5% sodium deoxycholate, 0.5 mM-PMSF) and incubated on ice for 15 min. The extracts were clarified at 100000g for 15 min at 4°C and the supernatants were either used immediately or stored at -70 °C until required.

Extracts of SF cells for Western blotting were prepared 48 h after infection of similar monolayers with 20 p.f.u./cell AcUL8 or wild-type (wt) AcNPV. Cells were harvested by scraping into the medium, washed three times with unsupplemented TC-100 medium and resuspended in 600 µl sample buffer (50 mM-Tris-HCl pH 6.7, 2 % SDS, 700 mM-2-mercaptoethanol, 10% glycerol and 0.05% bromophenol blue) and heated at 100 °C for 2 min. Of this extract 100 µl was used per experiment. BHK cell extracts for use in Western blots were prepared as previously described (McLean et al., 1990).

Immunoprecipitation of proteins. Labelled extracts (200 µl) in 1.5 ml microfuge tubes were mixed with 1.0 µl MAb 0811 and mixed end-over-end for 5 h at 4 °C. Seventy-five µl of a 50 % (v/v) preparation of Protein A-Sepharose beads (Sigma) in buffer E was then added and mixing continued for a further 60 min. To test for association of proteins in vitro extracts were first pre-incubated together on ice for 1 h before addition of the MAb. The beads were pelleted and washed twice in buffer E containing 2 mg/ml BSA, once in buffer EN (buffer E containing 500 mM-NaCl) and four times in buffer E alone. Finally, to reduce non-specific background, they were transferred to a fresh 1.5 ml tube, resuspended in 100 µl of sample buffer and heated at 100 °C for 2 min. The beads were pelleted and the supernatants were analysed by SDS-PAGE (Marsden et al., 1978) using 8.5% polyacrylamide gels unless otherwise stated. Gels were fixed, treated with En3Hance (Du Pont), dried under vacuum and exposed to autoradiographic film at -70 °C.

Western blot analysis. Western blots were performed essentially as described by Towbin et al. (1979) with previously described modifications (Frame et al., 1986). Blotted proteins were incubated with a 1:2000 dilution of MAb 0811 and bound antibody was detected using the Promega Proteoblot system in conjunction with an alkaline phosphatase-conjugated secondary antibody in accordance with the manufacturer's instructions.

Results

Isolation of an MAb reactive with the UL8 protein

Following the fusion of spleen cells from a seropositive mouse immunized with purified UL8 protein, cell lines secreting antibody that reacted with the native protein were identified in an ELISA assay. Several cell lines were used to develop ascitic fluids which were aliquoted and stored at -20 °C. The ascitic fluid (MAb 0811) from one such line was used throughout the experiments described in this paper.

Fig. 1(a) shows that in a Western blot MAb 0811 recognizes a protein of the Mr expected for UL8 that is present in SF cells infected with recombinant AcUL8 (lane 4) but not in mock-infected SF cells (lane 1) or cells infected with the parental wt AcNPV (lane 3). Small amounts of a protein of similar Mr were present in HSV-1-infected, but not mock-infected BHK cells (lanes 5 and 2, respectively). Minor bands detected in SF cells infected with AcUL8 and absent in all the control tracks are thought to represent proteolytic breakdown products of the overexpressed UL8 protein.

MAb 0811 was also tested in immunoprecipitation assays using ^35S-labelled extracts from SF cells infected with AcUL8 or wt AcNPV (Fig. 1b). The antibody efficiently precipitated a protein which co-migrated with the UL8 protein overexpressed in AcUL8-infected cells (lanes 5 and 2, respectively). No protein of similar Mr was detected following reaction of an extract from wt AcNPV-infected cells with MAb 0811 or the AcUL8-infected cell extract with normal mouse serum (lanes 3 and 4, respectively). The minor bands present in lane 5 are again likely to represent UL8 breakdown products and have been shown to react with MAb 0811 following Western blotting of immunoprecipitated proteins (data...
Fig. 3. (a) Interaction of the helicase-primase complex with the viral OBP. Lanes 1 to 4 show the labelled extracts used in precipitation experiments. These are from cells infected with recombinant viruses expressing UL5, UL8, UL52 and UL9 (lane 1), UL5, UL52 and UL9 (lane 2), UL8 and UL9 (lane 3) or UL9 alone (lane 4). The corresponding immunoprecipitations using MAb 0811 are shown in lanes 5 to 8, respectively. Proteins were separated on 8.5% polyacrylamide SDS-PAGE gels, the positions of the UL5, UL8, UL9 and UL52 proteins are indicated. (b) Formation of a complex between UL8 and UL9 in vitro. Extracts were prepared from SF cells infected with AcUL8 (lane 1), AcUL8 and AcUL9 (lane 2), or AcUL8, AcUL9, AcUL5 and AcUL52 (lane 6) and subjected to immunoprecipitation with MAb 0811. Alternatively, extracts from infected cells were mixed and complexes allowed to form in vitro before precipitation with MAb 0811 as follows: UL8 and UL9 extracts (lane 3), UL8 and UL9 and UL5 plus UL52 extracts (lane 4), UL8 and UL5 plus UL52 extracts (lane 5).

not shown). The amount of UL8 protein precipitated from AcUL8-infected cells was in fact sufficient to be clearly detected using Coomassie blue staining in the absence of radiolabel (data not shown). The above experiments indicate that MAb 0811 is capable of efficiently recognizing both native and denatured forms of the UL8 protein.

Co-precipitation of UL5 and UL52 with UL8

MAb 0811 was next tested for its ability to precipitate complexes containing UL8 and other components of the helicase-primase complex. SF cells were triply infected with AcUL5, AcUL8 and AcUL52 or doubly infected in the three possible combinations (i.e. AcUL5 and AcUL8, AcUL5 and AcUL52, or AcUL8 and AcUL52) and labelled extracts were prepared as before. Fig. 2 demonstrates that MAb 0811 precipitates UL5, UL52 and UL8 from the triply infected insect cells (lane 5). Precipitation of the UL5 and UL52 proteins is due to the presence of the UL8 protein since neither was detected when an extract from cells infected with only AcUL5 and AcUL52 was reacted with the antibody (lane 6). Interestingly, precipitation of extracts from cells doubly infected with AcUL8 and either AcUL5 (lane 7) or AcUL52 (lane 8) revealed that the UL5 and UL52 proteins could each interact separately with UL8. A possible explanation for the apparent under-representation of UL5 compared with UL52 in lane 5 is that both the trimeric and the UL8/UL52 complex are present in the precipitates from triply infected cells. This could be due to a weakening of the interaction of UL5 with the
other components of the trimeric complex upon the binding of MAb 0811.

**HSV-1 OBP interacts with the helicase-primase complex through the UL8 subunit**

Early events following the interaction of HSV-1 OBP with the viral origins of replication are likely to involve an opening of the origin region by a helicase activity and the synthesis of RNA primers to allow the initiation of DNA synthesis. We therefore used MAb 0811 to test the possibility of a direct physical association between the UL9 protein (OBP) and the helicase-primase complex. Labelled extracts were prepared from Sf cells infected with various combinations of recombinant baculoviruses and precipitated with MAb 0811 (Fig. 3a). Lane 5 shows that in cells expressing the UL5, UL8, UL9 and UL52 proteins, UL9 was co-precipitated with the UL5, UL8 and UL52 proteins suggesting that OBP can specifically interact with at least one component of the helicase-primase complex. In the absence of both the UL5 and UL52 proteins, UL9 also co-precipitated with UL8 (lane 7) implicating the UL8 protein in this interaction. The UL9 polypeptide, like UL5 and UL52, is not precipitated in the absence of UL8 in the extract (lanes 6 and 8), thus ruling out non-specific interaction between UL9 and either the antibody or the Protein A beads.

The results shown in Fig. 3(a) demonstrate that UL9 can associate with UL8 when co-expressed in vivo in co-infected Sf cells. In order to determine whether similar complexes could form in vitro, extracts were prepared from Sf cells individually infected with either AcUL8 or AcUL9 or co-infected with AcUL5 and AcUL52 (UL52 could not be expressed alone for these experiments because of its low solubility). Before immunoprecipitation, various combinations of these extracts were mixed on ice. The results of the immunoprecipitation assay are shown in Fig. 3(b). UL9 co-precipitated with UL8 both when the proteins were co-expressed in vivo (lane 2) or mixed in vitro before the addition of antibody (lane 3). Similarly UL8 was able to associate in vitro with co-expressed UL5 and UL52 (lanes 4 and 5).

**The C-terminal DNA-binding domain of OBP is dispensable for the interaction with UL8**

The UL9 protein appears to be divided into at least two functional domains; the N-terminal 534 amino acids containing a set of characteristic helicase motifs and the C-terminal 317 amino acids being sufficient for sequence-specific DNA-binding activity. Recombinant baculoviruses expressing these two portions of the protein separately were constructed and used to characterize further the interaction between OBP and the UL8 protein. Sf cells were doubly infected with either AcUL8 and AcUL9CT (expressing the C-terminal region) or AcUL9NT (N-terminal region). Labelled extracts were prepared and incubated with MAb 0811. It precipitated the isolated N-terminal domain, but not the C-terminal region, together with the UL8 protein (Fig. 4, lanes 5 and 6, respectively) demonstrating that the C-terminal 317 amino acids of OBP are dispensable for the protein-protein interaction.
Further evidence in support of a specific interaction between the UL8 and UL9 proteins was provided by the ability of MAb 13672, which recognizes an epitope mapped to within the N-terminal 535 amino acids of UL9 (A. P. Abbotts, G. W. McLean and N. D. Stow, unpublished results), to co-precipitate UL8 from cells doubly infected with AcUL8 and AcUL9NT (Fig. 4, lane 8). No UL8 was detected when extracts from SF cells infected with AcUL8 alone were reacted with MAb 13672 (Fig. 4, lane 9).

Discussion

The results presented in this paper concern the interactions between the UL8 protein and three other polypeptides involved in HSV-1 DNA synthesis. These were investigated using a MAb (MAb 0811) raised against UL8 protein purified from insect cells infected with a recombinant baculovirus. In addition to reacting with the insect cell-expressed UL8 protein, MAb 0811 also recognized UL8 synthesized during the course of an infection of BHK cells with wt HSV-1. This MAb was used in immunoprecipitation experiments with extracts from insect cells mixedly infected with recombinant baculoviruses capable of expressing various HSV-1 DNA replication proteins. The interactions detected using this approach are likely to have relevance to those which occur in HSV-1 infected cells, since insect cells infected with recombinant baculoviruses expressing all seven replication proteins have previously been shown to be capable of supporting HSV-1 origin-dependent DNA synthesis (Stow, 1992).

Although it was well established that UL8 is a component of the trimeric helicase–primase complex and that the other two components, UL5 and UL52, together form a stable, enzymatically active sub-assembly, a previous report had failed to detect any interaction between UL8 and either UL5 or UL52 alone (Dodson & Lehman, 1991). Using MAb 0811 we have detected both UL5/UL8 and UL8/UL52 complexes in immunoprecipitation assays. Initially complexes were washed in extraction buffer alone, however, in order to test complex stability more rigorously, a high-salt wash was included. The complexes observed are stable even in the presence of the high salt levels (500 mM-NaCl) in the washing buffer. The reason why neither of these complexes was detected when extracts from mixedly infected SF cells were subjected to heparin-agarose chromatography (Dodson & Lehman, 1991) is unclear but may indicate a disruption of the interactions under the conditions used for chromatographic separation. Whether the presence of UL8 confers helicase or primase activities on either the UL5 or UL52 proteins, which do not exhibit these activities in isolation, remains to be determined. Combined with the previous observation of a stable UL5/UL52 complex (Calder & Stow, 1990; Dodson & Lehman, 1991) our data suggest that within the helicase–primase complex each component interacts directly with the other two.

Complex interactions between components of the replicative machinery have been implicated at replication origins and replication forks in all systems that have been studied in detail, but frequently direct physical evidence for these interactions has not been obtained (for a review see Kornberg & Baker, 1992). Although the assembly of the HSV-1 UL5, UL8 and UL52 proteins to form the viral helicase–primase enzyme has been well characterized, little is known concerning the interactions of this complex with other viral or cellular proteins involved in genome replication. The results presented in this paper indicate that an interaction can occur between helicase–primase and HSV-1 OBP. This interaction involves the UL8 subunit and does not require binding of UL9 to the origin or the UL9 DNA-binding domain or the presence of any of the other HSV-1 replication proteins. The interaction between UL8 and UL9 suggests that in addition to possible roles in primer utilization (Sherman et al., 1992) and nuclear uptake of the helicase–primase complex (Calder et al., 1992), UL8 may also be involved in positioning the complex at the replication origins, where its primase activity is likely to be essential for the initiation of DNA synthesis. Although UL9 has an intrinsic DNA helicase activity (Bruckner et al., 1989) it has not yet been shown to be capable of unwinding a fully duplex viral origin, even in the presence of UL29 protein, a single-stranded DNA-binding protein that stimulates UL9 helicase activity (Fierer & Challberg, 1992; Boehmer et al., 1993). Thus, in addition to its probable role at the replication fork, the DNA helicase activity of the UL5/UL8/UL52 complex may also be necessary for the initial opening of the origins of replication. The UL8/UL9 interaction possibly also serves to modulate the enzymic activities of UL9 and/or the helicase–primase complex.

The interaction between UL8 and UL9 resembles interactions that occur in other systems. For example in Escherichia coli the dnaA OBP is thought to be responsible for loading the dnaB helicase from the dnaB/dnaC complex at oriC, an event which represents the first stage in the assembly of the initiation complex (Baker et al., 1986, 1987; Funnell et al., 1987).

The sequences responsible for interaction with UL8 were shown to lie outside the origin-binding domain of UL9 within the N-terminal two-thirds of the protein. This region of UL9 has already been shown to contain sequences essential for helicase activity, homodimerization and cooperative binding to the origins (Martinez et al., 1992; Elias et al., 1992; Hazuda et al.,
1992). Although the sequences responsible for interaction with UL8 have not been further defined, it is interesting to note that a leucine zipper motif is present within this region, between amino acids 150 and 171. The possibility that separate domains of the UL9 protein are involved in DNA binding and the recruitment of proteins to the origin has a close parallel in bacteriophage λ DNA replication. Here the N terminus of the gpO protein binds to the origin whilst the C-terminus interacts with the gpP/dnaB complex directing the host replication machinery to the origin (Dodson et al., 1985, 1989; Wickner & Zahn, 1986).

The data we have presented demonstrate the utility of recombinant baculoviruses and MAbs in identifying novel interactions between HSV-1 DNA replication proteins. It is likely that the interaction between UL8 and UL9 is much weaker than those within either the UL5/UL8/UL52 complex or UL9 dimers and therefore has not been detected previously. Further application of this type of approach will hopefully assist in uncovering other interactions and in unravelling the mechanisms of HSV-1 DNA replication.

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