Protoemics of Herpes Simplex Virus Replication Compartments: Association of Cellular DNA Replication, Repair, Recombination, and Chromatin Remodeling Proteins with ICP8

Travis J Taylor and David M. Knipe*

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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In this study, we have used immunoprecipitation and mass spectrometry to identify over 50 cellular and viral proteins that are associated with the herpes simplex virus 1 (HSV-1) ICP8 single-stranded DNA-binding protein. Many of the coprecipitating cellular proteins are known members of large cellular complexes involved in (i) DNA replication or damage repair, including RPA and MSH6; (ii) nonhomologous and homologous recombination, including the catalytic subunit of the DNA-dependent protein kinase, Ku86, and Rad50; and (iii) chromatin remodeling, including BRG1, BRM, hSNF2H, BAF155, mSin3a, and histone deacetylase 2. It appears that DNA mediates the association of certain proteins with ICP8, while more direct protein-protein interactions mediate the association with other proteins. A number of these proteins accumulate in viral replication compartments in the infected cell nucleus, indicating that these proteins may have a role in viral replication. WRN, which functions in cellular recombination pathways via its helicase and exonuclease activities, is not absolutely required for viral replication, as viral yields are only very slightly, if at all, decreased in WRN-deficient human primary fibroblasts compared to control cells. In Ku70-deficient murine embryonic fibroblasts, viral yields are increased by almost 50-fold, suggesting that the cellular nonhomologous end-joining pathway inhibits HSV replication. We hypothesize that some of the proteins coprecipitating with ICP8 are involved in HSV replication and may give new insight into viral replication mechanisms.

Herpes simplex virus 1 (HSV-1) is a large, double-stranded DNA virus that replicates in the host cell nucleus. HSV encodes over 80 gene products that contribute to viral replication in either cultured cells or animal hosts (76). Due to the limited size of the HSV-1 genome, the virus cannot code for every function required for its propagation; thus, HSV-1 must rely upon factors supplied by the host cell for replication. For example, HSV exclusively uses the host cell RNA polymerase II for the transcription of viral genes (4, 16). The exact number and identity of the cellular factors required for HSV replication is unknown, but the identification of such factors is an active area of research as it may shed light on mechanisms of viral replication, the cellular process, or the factor itself. It is this concept that induced us to identify cellular proteins that associate with HSV-1 ICP8.

The HSV-1 single-stranded DNA-binding protein, ICP8, is a 128-kDa multifunctional zinc metalloprotein (31, 37) encoded by the U129 gene (61). ICP8, in concert with the other HSV DNA replication proteins, including the helicase-primase complex (U1.5, U1.8, U1.52), the origin-binding protein (U1.9), and the polymerase holoenzyme (U30/U42), is required for viral DNA synthesis (11, 12). While the seven HSV DNA replication proteins are known, it is currently unclear as to what host proteins are involved in viral DNA replication. In addition to its role in DNA synthesis, ICP8 has been shown to affect viral transcription in at least two ways: (i) by repressing transcription from input parental viral genomes (33–35) and (ii) by stimulating late gene transcription (32).

ICP8 and a number of other viral proteins, including the aforementioned viral replication proteins, the major viral transactivator ICP4, the immediate-early protein ICP27, and the major capsid protein VP5 accumulate within intranuclear structures referred to as replication compartments (9, 19, 47, 59, 71, 73). Many of the processes required for viral replication, including viral DNA synthesis (18, 71, 74), viral transcription (47, 57, 71, 74, 75), virion assembly, and DNA packaging (19, 51, 93, 96), occur within replication compartments. Because numerous viral processes take place in replication compartments, it is expected that cellular proteins that are required for viral replication may accumulate there as well. Indeed, the host cell RNA polymerase II is redistributed to replication compartments during HSV infection (57, 71, 75). Other proteins such as p53 and the cellular single-stranded DNA-binding protein replication protein A (RPA) have been observed in replication compartments (95), but their role in viral replication remains unknown.

We hypothesized that host proteins that coprecipitate with viral proteins in replication compartments might be cellular proteins that play a role in viral replication. We thought that ICP8 was a good candidate for this analysis because it is highly expressed in infected cells and it is believed to interact with multiple cellular or viral complexes to mediate its various func-

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115. Phone: (617) 432-1934. Fax: (617) 432-0223. E-mail: david_knipe@hms.harvard.edu.
tions during infection. Here, we report the identification of numerous cellular proteins that coprecipitate with ICP8, which suggests that they may have a functional role in HSV replication.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) and human epidermoid (HEp-2) cells obtained from the American Type Culture Collection (Manassas, Va.) were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM: Media Tech Inc., Herndon, Va.) supplemented with 5% fetal bovine serum (Gibco, Carlsbad, Calif.)-5% bovine calf serum (HyClone, Logan, Utah), streptomycin (100 µg/ml), and penicillin (100 U/ml) DMEM—10% fetal calf serum (FCS)]. V529 cells (17) were grown in DMEM—10% FCS supplemented with G418 (400 µg/ml). Normal and Ku70-deficient murine embryonic fibroblasts (MEFs) (36), kindly provided by David Sinclair, Harvard Medical School, Boston, Massachusetts, were grown and maintained in DMEM—10% FCS. Normal (number AG14591) and WRN-deficient (number AG00780H) primary human fibroblasts were obtained by passage number AG14591 and WRN-deited ewes were grown and maintained in modified Eagle’s medium (MEM) with Earle’s balanced salt solution supplemented with 15% heat-inactivated fetal bovine serum, a 2x concentration of MEM essential and nonessential amino acids (Gibco), and vitamins (Gibco).

The HSV-1 wild-type (wt) KOS strain was propagated and assayed on Vero cells. The HSV-2-derived d55 and d529 mutant strains were propagated on V529 cells as previously described (17).

Antibodies. The anti-HSV ICP8 39S mouse monoclonal (81) and 3-83 rabbit polyclonal (47) antibodies were described previously. The anti-RPA p70-9 mouse cells as previously described (17).

10% FCS supplemented serum [FCS]). V529 cells (17) were grown in DMEM—10% FCS supplemented with G418 (400 µg/ml). Normal and Ku70-deficient murine embryonic fibroblasts (MEFs) (36), kindly provided by David Sinclair, Harvard Medical School, Boston, Massachusetts, were grown and maintained in DMEM—10% FCS. Normal (number AG14591) and WRN-deficient (number AG00780H) primary human fibroblasts were obtained by passage number AG14591 and WRN-deleted ewes were grown and maintained in modified Eagle’s medium (MEM) with Earle’s balanced salt solution supplemented with 15% heat-inactivated fetal bovine serum, a 2x concentration of MEM essential and nonessential amino acids (Gibco), and vitamins (Gibco).

The HSV-1 wild-type (wt) KOS strain was propagated and assayed on Vero cells. The HSV-2-derived d55 and d529 mutant strains were propagated on V529 cells as previously described (17).

RESULTS

Coprecipitation of cellular and viral proteins with ICP8. To identify potential ICP8-associated cellular and viral proteins, we immunoprecipitated ICP8 and associated molecules from wt HSV-infected or mock-infected whole-cell HEp-2 lysates prepared at 6.5 h postinfection using the conformation-specific 39S monoclonal antibody, which predominantly recognizes ICP8 at prereplicative sites or within HSV replication compartments (89). At this time of infection, the bulk of ICP8 is in replication compartments (18, 72), and the majority of ICP8 is associated with replication complexes or progeny viral DNA (48). The IPs were separated by SDS-PAGE, and the resolved proteins were visualized by Coomassie blue staining (Fig. 1A). Protein bands present only in IPs from virus-infected cells were excised, digested with trypsin, and analyzed by tandem MS.

We identified, in total, over 50 cellular and viral proteins that coprecipitated with ICP8 with 39S antibody (Table 1). Table 1 lists the majority of the coprecipitating proteins categorized by general function with the corresponding number of peptides detected by MS from a representative experiment. Some proteins, such as cytoplasmic constituents or cytoskeletal elements, were not included in this analysis, but the entire list of coprecipitating proteins may be found at http://knipelab.med.harvard.edu/. Certain proteins identified by one peptide were included in Table 1 because of their relationship or interaction with other coprecipitating proteins on the list.
To confirm the identity of these proteins and the authenticity of their association with ICP8, we performed several lines of experiments. First, we confirmed the presence of representative members of the larger protein complexes in ICP8 IPs by Western blotting with specific antibodies (Fig. 1B). Second, we determined if DNA plays a role in the coprecipitation (Fig. 1B). Third, we used immunofluorescence to determine if the coprecipitating proteins colocalize with ICP8 in viral replication compartments (Fig. 2). Finally, to determine if a cellular protein plays a role in viral replication, we have initiated studies to analyze HSV-1 replication in cells deficient for the cellular protein (Tables 2, 3, and 4).

As expected, we identified several coprecipitating viral DNA replication proteins, including U15, U18, and U42 (Table 1), which are known to associate with ICP8 in the viral DNA replication complex (6, 7, 9, 39, 59). In agreement with previous reports (84, 86, 99), we also identified U12, ICP27, and ICP4 as ICP8-coprecipitating proteins.

We also identified numerous cellular proteins (Table 1) that are predominantly involved in cellular DNA replication and damage repair, including RPA and MSH6; homologous and nonhomologous recombination, including Rad50, DNA-PKcs, Mre11, and Ku86; chromatin remodeling, including BRG1, BRM, hSNF2H, BAF155, mSin3a, and HDAC2; and mRNA splicing, including SAP130, SAP155, and NMP200. Some of the other coprecipitating proteins have been identified as possibly playing a role during HSV replication, including HAUSP, a ubiquitin-specific protease that has been shown to associate with the HSV-1 immediate-early ICP0 protein (26); the mRNA splicing factors SAP130 and SAP155, which interact with SAP145 which in turn binds HSV-1 ICP27 (8); and Ku70, which has been demonstrated to bind the downstream activating sequence present in some HSV late gene promoter sequences (70).

We isolated multiple members of individual complexes, arguing that the coprecipitation of these complexes was specific.
TABLE 1. Proteins that coimmunoprecipitate or colocalize with ICP8 categorized by function

<table>
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<tr>
<th>Protein Type</th>
<th>Protein Name</th>
<th>No. of peptides by MS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Confirmed by IP-Western blot&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Replication compartment localization&lt;sup&gt;c&lt;/sup&gt;</th>
<th>DNA-independent ICP8 association&lt;sup&gt;d&lt;/sup&gt;</th>
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<td>Rad50</td>
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<td>U3</td>
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<sup>a</sup> Number of peptides identified by MS after trypsin digestion of an excised polyacrylamide gel band.

<sup>b</sup> Coimmunoprecipitation was confirmed by IP-Western blot.

<sup>c</sup> Immunofluorescence was used to determine if the indicated protein localized to viral replication compartments during infection. References are given for previously published observations.

<sup>d</sup> DNA-independent ICP8 association was determined by IP in the presence of EtBr. ++, increased coimmunoprecipitation in the presence of EtBr; +, similar or slightly decreased coimmunoprecipitation in the presence of EtBr; +/-, more than 50% decreased coimmunoprecipitation in the presence of EtBr; - -, no coimmunoprecipitation in the presence of EtBr.

<sup>e</sup> Protein that was predicted to associate with ICP8 or localize to replication compartments based upon its interaction with another protein shown in Table 1.
For example, we identified 4 of the 5 known protein partners in the cellular nonhomologous end-joining (NHEJ) complex (10), including DNA-PKcs, Ku86, Ku70, and XRCC4. Based on the coprecipitation of DNA repair and recombination complexes, we hypothesized that BRCA1, WRN, BLM, and MSH2, each known to interact with other proteins shown in Table 2 (5, 14, 28, 29, 45, 98, 100), may also coprecipitate or colocalize with ICP8 in infected cells and were included in this study as described in more detail below.

Role of DNA in association of the proteins with ICP8. Many of the cellular complexes identified have DNA-binding components, and ICP8 is also bound to viral DNA in infected cells (48, 53), so we attempted to determine if the associations were mediated by DNA-protein or protein-protein interactions by performing the immunoprecipitations in the presence of EtBr at concentrations known to disrupt DNA-protein but not protein-protein interactions (50). The presence of EtBr did not significantly disrupt the coprecipitation of the host proteins.
Ku86, Rad50, RPA, and WRN, all of which are involved in DNA repair or recombination, or the viral proteins ICP27 and U15 (Fig. 1B). As observed previously for ICP8 and ICP27 (99), disrupting DNA binding increased the coprecipitation of ICP8 with ICP27, U15, and WRN (Fig. 1B). This may be due to the release of ICP8 or one of the proteins from DNA, allowing them to associate more readily. In contrast, coprecipitation of BAF155, hSNF2H, mSin3a, and HDAC2, which are all involved in chromatin remodeling, was reduced by greater than 50%, and ICP4 and BRG1 coprecipitation was abolished in the presence of EtBr (Fig. 1B).

These results suggested that a number of proteins, including Ku86, Rad50, RPA, WRN, ICP27, and U15, associate with ICP8, either directly or indirectly, via protein-protein interactions. Other proteins, including BAF155, hSNF2H, mSin3a, HDAC2, BRG1, and ICP4, may require DNA to at least partially mediate or stabilize the association with ICP8.

Accumulation of cellular proteins in HSV replication compartments. If the ICP8-coprecipitating proteins physically associated with ICP8, it was expected that they would colocalize with ICP8 within the nucleus of an infected cell. To test this hypothesis, we used immunofluorescence to examine the distribution of several representative proteins in HSV-infected HEp-2 cells. Proteins involved in NHEJ (DNA-PKcs and Ku86), homologous recombination and DNA repair (BLM, BRCA1, MSH2, Rad50, and WRN), and chromatin remodeling (BAF155, BRG1, BRM, HDAC2, hSNF2H, and mSin3a) were redistributed from their normal nuclear distribution (Fig. 2, left panels) to viral replication compartments after infection (Fig. 2, middle panels) as shown by their co-staining with ICP8 or localization within replication compartments (Fig. 2, right panels). Similar staining patterns were observed in single-stained cells (data not shown). One of the most dramatic alterations involved the redistribution of WRN from its normal nucleolar pattern (Fig. 2, left WRN panel) (90) to viral replication compartments (Fig. 2, middle WRN panel). The redistribution of WRN from the nucleolus normally takes place in the presence of EtBr (Fig. 1B).

Growth of HSV-1 in WRN-deficient human fibroblasts. WRN is a mammalian RecQ helicase family member (41) and is thought to function in cellular DNA replication, repair, and recombination pathways via its helicase and exonuclease domains (68, 80). To determine if WRN was required for HSV replication, we examined viral growth and recombination in WRN-deficient primary human fibroblasts. The viral yield was decreased by three- to fivefold in WRN-deficient cells compared to control human fibroblast cells (Table 2). This result

<table>
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<th>Virus</th>
<th>Titer (10^6 PFU/ml)</th>
<th>RF (%)</th>
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</table>

* Normal or WRN-deficient fibroblasts were either singly or doubly infected with dl5 or dl29 at an MOI of 20. At 24 h postinfection, the virus was harvested and titered on both Vero and V529 cells.

* Recombination frequency (RF) was determined by dividing the titer on the noncomplementing cell line (Vero) by the titer on the complementing cell line (V529).
suggested that WRN might promote viral replication slightly but that it was not essential for HSV replication.

We next determined if HSV recombination was altered in the WRN-deficient cells by infecting normal or WRN-deficient fibroblasts singly or doubly with HSV-2 dl5 and dl29 strains (17), which contain mutations in the U1.5 and U1.29 genes, respectively, and measured the number of wt plaques that formed on Vero cells. We observed a recombination frequency of \( \sim 25\% \) in normal fibroblast cells. In contrast, we observed a lower average recombination frequency of \( \sim 18\% \) in WRN-deficient cells (Table 3). This decrease suggested that HSV recombination was not as efficient or did not occur as readily in WRN-deficient cells. In agreement with the above results with HSV-1 infection, we also observed an approximate threefold decrease in total HSV-2 yields in WRN-deficient cells compared to control human fibroblast cells.

**Growth of HSV-1 in Ku70-deficient MEFs.** Ku70 and Ku86 form the heterodimeric DNA-binding portion of the DNA-PK complex. Once the Ku70/Ku86 heterodimer is bound to DNA, the other components of the DNA-PK complex are recruited to the double-strand break to orchestrate NHEJ. In the absence of the Ku70 subunit in mammalian cells, the NHEJ pathway is disrupted. To determine the role of Ku70 during viral replication, we examined HSV growth in Ku70-deficient MEFs.

Viral yields were increased by 30- to 50-fold in Ku70-deficient MEFs compared with control MEFs (Table 4). The increased yield was observed over a range of input virus from an MOI of 1 to 50 (Table 4). These results suggested that HSV growth did not depend upon Ku70 but rather was inhibited by Ku70. This result implied that the NHEJ pathway inhibited HSV replication in some manner.

**DISCUSSION**

We hypothesized that host proteins involved in HSV replication may be identified as proteins that coinmunoprecipitate with viral proteins in nuclear replication compartments, and we therefore used the HSV ICP8 DNA-binding protein as the prototype viral protein for IP studies. We first identified protein bands in ICP8 IPs by using MS, IP and Western blotting with specific antibodies confirmed the association of the host protein with ICP8. We expected that proteins associating with ICP8 would colocalize in viral replication compartments, so we next demonstrated that representative ICP8-coprecipitating proteins accumulated within viral replication compartments. We also initiated studies to analyze the functional role of these host proteins during HSV replication by examining viral growth and recombination in mutant cell lines. We hope to extend these studies by depleting specific host factors by RNA interference techniques.

In this study, we report the identification of numerous cellular proteins that coprecipitated with HSV ICP8. The majority of the proteins were components of cellular complexes that coordinate DNA recombination and repair or chromatin remodeling (for reviews, see references 2, 21, 27, 41, 46, 63, and 83). We examine the potential roles of the coprecipitating proteins in HSV replication in greater detail below.

**Interactions with cellular DNA repair and recombination complexes.** Double-strand DNA breaks (DSB) and other DNA damage are experimentally induced by irradiation, UV exposure, and chemical treatment; however, this damage may also arise naturally as a consequence of DNA replication. Many of the repair and recombination proteins reported to redistribute to DSB after irradiation or chemical treatment colocalize with cellular DNA replication sites during S phase. For example, the Rad50/Mre11/NBS complex localizes to cellular replication structures during S phase as shown by costaining with RPA or BrdU (13, 60, 62). It is believed that the presence of stalled replication forks or stretches of single-stranded DNA are responsible for recruiting these factors. In the absence of such repair functions, genomic integrity degrades. As evidence for the critical role of these proteins in genomic integrity, many of the proteins involved in DNA repair and recombination are mutated or deleted in human genetic disorders that are associated with an increased risk of cancer (58, 87).

Two major pathways in mammalian cells repair DSB: (i) NHEJ and (ii) homologous recombination repair (HRR). The NHEJ and HRR pathways are believed to compete within the cell as they both act on DSB (3, 38, 44). HSV replication and recombination are coupled in infected cells (24, 25, 94), but the significance of this process remains unclear. It is known that the HSV genome undergoes an inversion of the L and S segments (20, 40) and that recombination frequencies are high in HSV-infected cells (24, 25, 79, 82), indicating that recombination may have a role in HSV replication. Members of both NHEJ and HRR complexes coprecipitated with ICP8, suggesting that these factors may also have a role in repairing DSB that occur during HSV DNA synthesis or have an as-yet-undefined function required for viral DNA replication. It has been recently proposed that HSV DNA replication occurs initially on a linear molecule (43); thus, recombination may play a role in HSV DNA replication as observed for bacteriophage T4 DNA replication (49).

We found that HSV replication and recombination levels were decreased modestly in WRN-deficient cells, WRN-deficient cells have a decreased ability to resolve homologous recombination products (77), which may account for the lower levels of homologous recombination that we observed in our assays. The presence of other RecQ helicases in the cell, such as BLM, raises the possibility that other cellular proteins may functionally substitute for WRN, providing a possible explanation for why we did not see a significant decrease in viral replication in WRN-deficient cells. We are currently investigating whether HSV replication is inhibited in other cell lines that have reduced homologous recombination activity.

In contrast to the WRN-deficient cells, we found that HSV replication is significantly increased in Ku70-deficient cells, which suggested that NHEJ may inhibit HSV replication. In support of this, it has been shown that HSV growth is increased in cells lacking another NHEJ component, DNA-PKcs (69). Although it has been suggested that HSV-1 infection causes a degradation of DNA-PKcs (54, 69), the extent of this effect is cell type and/or virus strain specific (69). We were surprised to see DNA-PKcs as one of the major proteins coprecipitating with ICP8, but further studies have shown that DNA-PKcs decreases by less than 50% in HEp-2 and other cells infected with HSV-1 KOS (T. J Taylor, G. Melroe, and D. M. Knipe,
unpublished data). Therefore, DNA-PKcs could exert an effect during infection under our conditions. Indeed, we found that DNA-PKcs accumulated in viral replication compartments in HEp-2 cells infected with HSV-1 KOS. The accumulation of proteins involved in NHEJ within replication compartments may not reflect a functional role in HSV replication but rather the non-specific binding of these proteins to DSBs within replication compartments.

The increased growth of HSV in cell lines deficient for NHEJ suggests that this pathway inhibits HSV replication. The disruption of the NHEJ pathway may increase HSV replication in at least two ways. First, it has been suggested that HSV may utilize a recombination-dependent replication mechanism similar to that used by bacteriophage T4 to replicate linear viral DNA later during infection (66). If so, then the Ku70/Ku86 DNA-binding heterodimer may block access of viral or cellular recombination proteins to DNA by competitively binding DSB. In the absence of Ku70, the competition for DSB would decrease, thus allowing greater access of other recombination factors. The loss of DNA-PKcs activity would also disrupt the NHEJ pathway because DNA-PKcs regulates the function of proteins involved in DNA repair by phosphorylation (3, 44); however, the effect on HSV replication might not be as pronounced in DNA-PKcs-deficient cells compared to Ku70-deficient cells in that Ku70 acts upstream of DNA-PKcs in the NHEJ pathway and could still bind DSB to some degree in DNA-PKcs-deficient cells. If HSV uses a recombination-dependent replication mechanism for replication, then it might be expected that viral replication occurs more readily in the absence of the competing NHEJ pathway. Second, it has recently been suggested that HSV genome circularization may actually repress the onset of HSV infection (43). If the NHEJ pathway is responsible for genome circularization, then the absence of this cellular pathway may promote the initiation of the viral life cycle. Further studies are needed to define the mechanisms of the inhibitory effect of NHEJ during HSV replication.

At least two proteins involved in DNA repair and recombination, NBS and BLM, accumulate in or near intranuclear ND10 sites (64, 91). As it is believed that HSV DNA targets to ND10 sites and replication compartments form adjacent to the ND10 sites (42, 88), it is possible that ICP8 or other viral proteins associate with one or more of these proteins for proper intranuclear localization early during infection. It will be of interest to determine if a recently identified region of ICP8 required for proper intranuclear localization (85) mediates the interaction with one or more of the ICP8-coprecipitating proteins.

**Interactions with cellular chromatin remodeling complexes.**

Two main groups of chromatin remodeling complexes exist in mammalian cells: (i) those that require ATP hydrolysis to alter histone-DNA contacts, such as the mating type switch/sucrose nonfermenting (SWI/SNF) and imitation switch (ISWI) complexes, and (ii) those that covalently modify histone proteins by a variety of posttranslational modifications (phosphorylation, acetylation, methylation), such as histone acetyltransferases and histone deacetylases complexes. We identified members of both types of complexes as ICP8-coprecipitating proteins.

The SWI/SNF and ISWI complexes have related core subunits but differ in the makeup of accessory proteins that are believed to regulate function or specificity (83). The SWI/SNF and ISWI complexes have similar but nonoverlapping functions that differ by substrate specificity and mechanism of action (1, 52, 83), again possibly regulated by various binding partners. Several ATPase subunits associated with SWI/SNF or ISWI complexes, such as BRM, BRG1, hSNF2H, and hSNF2L, coprecipitated with ICP8, suggesting a role for these proteins in viral replication. The role of chromatin remodeling proteins in HSV productive infection is not immediately obvious because it has been reported that HSV genomic DNA is kept in a relatively nucleosome-free form during lytic replication (55, 56, 72). It is possible that SWI/SNF complexes may be involved in maintaining this nucleosome-free state. In contrast to lytic infection, it is thought that latent HSV genomes are nucleosome associated (22), suggesting that SWI/SNF complexes may be involved in generating nucleosome-free genomes during HSV reactivation.

There is evidence that interactions with transcription factors may target SWI/SNF complexes to specific promoters to regulate transcription (1, 15, 52, 65, 83, 97). It is possible that the virus uses a similar form of targeted regulation by recruiting SWI/SNF complexes to weak viral promoters early in infection or during reactivation to enhance transcription.

**Other ICP8-coprecipitating proteins.**

Many other proteins that do not fall into the above categories also coprecipitated with ICP8. The most numerous of these were proteins involved in mRNA splicing or transcription factors. HSV has been shown to alter host cell mRNA maturation through the immediate-early protein ICP27. As we also identified ICP27 as a coprecipitating protein, it is possible that the mRNA splicing proteins were interacting directly with ICP27 and not ICP8. The transcription factors may have been associated with viral DNA or with RNA polymerase II, which has been shown to associate with ICP8 (99). We postulate that these interactions may play a role in virus early and/or, more likely, late gene transcription.

**Role of DNA in mediating ICP8 coprecipitation.**

The majority of the ICP8-coprecipitating proteins tested associated with ICP8 in a DNA-independent manner, suggesting that they physically associate with ICP8 directly or indirectly through other binding partners. The coprecipitation of proteins involved in chromatin remodeling required DNA for optimal association. In agreement with a recent study (84), we coprecipitated ICP4 with ICP8; however, this did not appear to be an authentic direct protein-protein interaction as it was dependent upon the presence of DNA.

As we precipitated ICP8 using the conformation-specific 39S monoclonal antibody that preferentially recognized ICP8 within replication compartments (89), we believe that viral DNA, not contaminating cellular DNA, mediated these interactions because ICP8 is bound to viral DNA in replication compartments (48). It is possible that ICP8 recruited these factors to replication compartments or viral DNA and was not required for their retention once assembled onto viral DNA.

**Functional role of cellular proteins in HSV replication compartments.**

The presence of representative members of the coprecipitating proteins in replication compartments argued for a potential role of these proteins in HSV replication. We observed that the distribution and number of foci within replication compartments differed from protein to protein. For
example, there were relatively few MSH2, BRM, or hSNF2H foci within the boundaries of replication compartments compared to BRG1, mSin3a, or BAF155 (Fig. 2, middle panels). This finding suggested that these cellular complexes targeted to distinct subcompartments or structures within replication compartments. This sort of subcompartimentalization has been described for viral proteins in that ICP4, ICP8, and VP5 have distinct distribution patterns within replication compartment boundaries (19). The different distributions of cellular proteins may reflect functional domains within replication compartments in which similar or related cellular machinery are recruited to aid HSV replication or sequestered to prevent possible antiviral activities.

It remains to be established that the ICP8-coprecipitating proteins have a role in viral replication. We demonstrated that the WRN and Ku70 proteins were not absolutely required for HSV growth. Further investigation is needed to examine the necessity of the other coprecipitating proteins during HSV replication. To do so, cells deficient in one or more proteins listed in Table 1 would be invaluable. This may prove to be feasible, as the proteins may be absolutely required for viability, making isolation of such cell lines difficult, or as is the case for WRN, redundant protein functions may complement the deficiency, thus masking any phenotype. However, with the increasing number of knockout mice, small-interfering RNA technology, and repositories that supply cells from humans with certain genetic disorders, it will rapidly become more feasible to investigate the role of the ICP8-coprecipitating proteins in HSV replication.

We believe that the large number of coprecipitating proteins reflects the multiple roles of ICP8 and replication compartments in viral DNA replication, viral gene expression, and capsid assembly. Not all of the coprecipitating proteins can interact with ICP8 directly, so we believe that some of the interactions are dependent upon intermediate binding partners. It remains to be determined which ICP8-coprecipitating proteins physically interact with ICP8 directly. Many of the proteins identified associate with numerous other proteins including other ICP8-coprecipitating proteins. It is possible that the association of ICP8 with one or two proteins may lead to the recruitment of numerous other proteins or complexes with a variety of functions. For example, the BRCA1-associated genome surveillance complex (BASC) is a 2-MDa "super complex" that is believed to contain over 40 proteins that are involved in genome integrity surveillance (92). The many components of BASC can detect and repair DNA damage as well as transduce signals to cell cycle control proteins to halt cell growth (30, 92). The association of ICP8 with BRCA1 or another member of this complex could recruit the numerous other BASC-associated proteins to viral replication compartments.

We hypothesize that HSV recruits some of these cellular proteins to replication compartments to participate directly in HSV replication. Alternatively, they may be targeted to damaged viral DNA that arises during replication. This demonstrates the complexity of determining the function of these proteins in the context of HSV infection. As many of the ICP8-coprecipitating proteins function in the major recombination repair pathways, it will be of interest to determine if these proteins have a necessary role in HSV replication or are recruited to sites of DNA damage or stalled replication forks and are not required for HSV replication. Further studies are needed to determine the specific role of the proteins identified in viral infection.

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REFERENCES


