The 152-kb herpes simplex virus type 1 (HSV-1) genome encodes at least 84 proteins, 7 of which are early genes essential for viral DNA replication in vitro (49). Functionally, the proteins specified by the UL5, UL8, and UL52 genes comprise the viral DNA polymerase and polymerase accessory protein, respectively, which together replicate the UL42 genes to produce the viral DNA polymerase. The UL29 gene encodes infected cell protein 8 (ICP8), a single-stranded DNA (ssDNA) binding protein that stabilizes replicating ssDNA. The products of the UL30 and UL42 genes comprise the viral DNA polymerase and polymerase accessory protein, respectively, which together replicate the viral genome. The product of the UL9 gene is the origin binding protein (OBP), the viral DNA replication initiator protein, and the subject of this paper.

Initiator proteins are functionally similar in all life forms and are characterized by their ability to bind to specific sequences in origin DNA. This binding results in unwinding of the origin at a site that usually contains an AT-rich sequence. DNA binding and unwinding are followed by the recruitment of the DNA replication machinery. As a means of regulating the DNA replication process, the activities of initiator proteins themselves are highly regulated. For example, the activities of many viral initiator proteins including simian virus 40 large T antigen (44), polyomavirus large T antigen (59), human papilloma virus E1 (27), bovine papillomavirus E1 (65), and the NS1 protein of minute virus of mice (11) are regulated by differential phosphorylation. The activities of some initiator proteins such as the bacteriophage lambda O (60–62) and bacteriophage phiX174 (12).

HSV-1 OBP is a 95-kDa, 851-amino-acid nuclear phosphoprotein that forms homodimers and binds to specific sites within HSV-1 origin DNA via a domain in the C-terminal half of the protein (Fig. 1). The C terminus of the protein also contains a binding domain for ICP8 (5). The N-terminal half of OBP contains binding domains for at least two other viral proteins essential for viral DNA synthesis (UL8 and UL42) (5, 35, 36) as well as seven helicase domains that facilitate the unwinding of the double-stranded DNA at the AT-rich apices of origin palindromes.

Based on work from other laboratories using temperature-sensitive mutants of UL9 (4, 51), a two-stage model of HSV DNA replication has been proposed (4, 51, 63). Stage I is thought to begin early in infection, followed by a switch to stage II. Stage I replication begins when OBP binds to one or more sites in origin DNA and recruits other essential viral replication proteins to these sites through direct protein-protein interactions (Fig. 2). As viral DNA circularizes upon infection (17, 42, 57), stage I replication likely proceeds by a theta-like mechanism. In contrast to initiation at early times postinfection (stage I), it has been demonstrated that OBP is not essential for viral DNA synthesis at later times postinfection or to elongate DNA using a preprimed template (4, 19, 51–53). Rather, overexpression of OBP following stage I initiation is detrimental to viral DNA synthesis and, by extension,
viral replication (2, 4, 29, 32, 51). It has also been demonstrated that the inhibitory effect of OBP is dependent upon its ability to bind DNA (2, 30, 39, 56). These observations suggest that OBP is functionally altered and/or dissociates from origins and replication complexes following stage I initiation (32). This functional alteration/dissociation may result in a switch to stage II, origin-independent replication, which may proceed via a rolling circle and/or recombination-dependent mechanism.

Based on these observations, it has been hypothesized that OBP functions and/or levels must be regulated during infection (8, 9). Specific posttranslational modifications of OBP may be used for this purpose. First, OBP is phosphorylated during infection (22), a common mechanism by which the functions of replication initiator proteins are regulated. Second, OBP contains a putative PEST motif (Fig. 1) (32). PEST motifs target proteins for degradation via the ubiquitin/proteasome pathway. Third, OBP interacts with and is degraded in the presence of FBX2, a neuron specific F-box protein (13, 14). To date, the functional consequences of these modifications in the context of lytic infection have not been determined. Although the dissociation of OBP from replication complexes has not been demonstrated directly, available evidence suggests that the nuclear localization of OBP may change following initiation of viral DNA synthesis (13, 14), suggesting the dissociation hypothesis. If OBP is, in fact, removed from the initiation complex, the mechanism underlying its removal and the mechanism of OBP-mediated interference in stage II viral DNA replication have yet to be determined.

Potential mechanisms for both of these events came to light when Baradaran et al. reported the identification of a transcript lying in frame with and comprising the C-terminal half of the UL9 transcript (1). This transcript, which is expressed with delayed early (DE) kinetics, was termed UL8.5. In vitro transcription/translation assays using the UL8.5 transcript and an antibody specific for the C terminus of OBP, a protein of 53 kDa, similar in size to a protein observed in infected cell extracts, was observed (1). Based on these observations, it was assumed, though not tested, that the C-terminal protein, designated OBPC, was expressed from the UL8.5 transcript. OBPC expressed by in vitro transcription/translation assays using the UL8.5 transcript retained the ability to bind to HSV-1 origin DNA but, based on size, was assumed to lack the majority of the helicase domains as well as the N-terminal binding domains for UL42 and UL8 (2). Based on these properties, OBPC was hypothesized to be involved in mediating the switch from theta to rolling circle viral DNA replication by binding to origin sequences and blocking the binding of full-length OBP to origin sequences and, hence, formation of the initiation complex (2). If correct, this mechanism would be similar to that of gene X of bacteriophage f1 and gene A* of bacteriophage phiX174 (12, 16). This hypothesis is supported by several studies which have demonstrated that overexpression of C-terminal forms of OBP that are able to bind site I
DNA are inhibitory to viral DNA synthesis and viral replication, while C-terminal forms that are not able to bind site I DNA are noninhibitory and at times are even potentiating (2, 9, 52, 56).

Many C-terminal overlapping transcripts have been reported in the HSV-1 genome including UL8.5, UL9.5, UL12.5, UL26.5, UL49.5, US1.5, US3.5, and US8.5 (1–3, 6, 7, 18, 21, 25, 34, 43, 45, 64, 66). Each of these overlapping transcripts encodes a protein that is in frame with and comprises the C terminus of the open reading frame (ORF) encoded by the larger transcript. Because these truncated C-terminal proteins share a subset of functional domains with their larger counterparts, the possibility that they play modulatory roles with regard to the proteins they overlap is intriguing. The discovery of a second protein of ~35 kDa that is in frame with and comprises the C-terminal-most portion of OBP, designated OBPC-2 (Fig. 1), prompted us to ask whether the 53-kDa OBPC-1 (formerly designated OBPC) is indeed the product of the UL8.5 transcript.

We have shown that OBPC-1 is not the product of the UL8.5 transcript but, rather, is a cathepsin B-mediated cleavage product of OBP. Intriguingly, cleavage is dependent upon viral DNA replication. Cathepsin B-mediated cleavage of OBP may indicate a change in OBP structure and/or localization following the initiation of viral DNA replication and may serve to regulate OBP levels and/or function during infection.

MATERIALS AND METHODS

Cells and viruses. Vero cells (ATCC CCL-81) were cultured in Dulbecco’s modified Eagle medium supplemented with 5% fetal bovine serum, and all experiments in Vero cells were performed using Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum. Cells were propagated and maintained at 37°C in 5% CO2.

The wild-type virus used in these studies was HSV-1 strain KOS and was propagated as previously described (50). Strain 17 was obtained from the Medical Research Council at the University of Glasgow, Scotland, United Kingdom. Strain F was obtained from Bernard Roizmann, University of Chicago, Chicago, IL. Strains Seibert and Gayle were obtained from William Rawls, Baylor College of Medicine, Houston, TX. The h94 virus, whose genome contains a β-galactosidase expression cassette inserted at codon 534 of the OBP ORF, was isolated and kindly provided by Sandra Weller (University of Connecticut Health Sciences Center, Farmington, CT) (29).

Construction of the pUL9n24 plasmid. In order to construct a plasmid that would express the UL8.5 transcript and its protein product(s), OBP, a DNA fragment containing base pairs 20140 to 24136 of the HSV genome and containing the UL8.5 and UL9 genes in their entirety (see Fig. 4) was ampliﬁed by PCR using the following primers: 5′-CACAACATGTAGCAGCTTGTTGTATAGTG-3′ and 5′-CATACAAAAATACACCGGGCGTGGAAGTAC-3′. The PCR product was cloned into the pcR-Blunt II-Topo vector using a Zero Blunt Topo PCR Cloning Kit (Invitrogen, Carlsbad, CA) following the manufacturer’s protocols. The resulting plasmid (pUL9DE) was used to clone the DraIII-EcoRV fragment of the UL9 ORF into the pALTER (Promega, Madison, WI) backbone. This plasmid, pAΔE, was used for site-directed mutagenesis of the UL9 ORF. The pUL9n24 nonsense mutation was introduced into the wild-type sequence 5′-CATTGGGACCAGCGTAGCTACGTGAACGTCGAG-3′ (pAΔE) by PCR using the following mutagenic primer: 5′-CATTTGGGACCAGCGTAGCTACGTGAACGTCGAG-3′ (pAΔE) for 30 min, and the supernatant was dialyzed in 20 mM HEPES, pH 7.9, 20% glycerol, 1 mM KCl, and 0.2 mM EDTA containing protease inhibitors and digested at 4°C for 30 min. Cell debris was removed by centrifugation at 13,000 g for 30 min, and the supernatant was dialyzed in 20 mM HEPES, pH 7.9, 20% glycerol, 1 mM KCl, and 0.2 mM EDTA containing protease inhibitors and DTT. Nuclear extracts were aliquoted, snap frozen in liquid nitrogen, and stored at −80°C.

Whole-cell extracts. Cells (3.1 × 106) were seeded in 100-mm dishes and infected 12 h later at a multiplicity of infection (MOI) of 10 PFU/cell with the virus indicated in the ﬁgure legend. At 8 hpi, cells were washed twice with cold PBS and harvested by scraping into 5 mL of cold PBS. Cells were pelleted and resuspended in 100 μL of NET buffer (50 mM Tris, pH 7.8, 100 mM NaCl, 1 mM EDTA) supplemented with leupeptin, aprotinin, pepstatin, and phenylmethylsulfonyl fluoride, and DTT. Cells were snap frozen in liquid nitrogen and quickly thawed at 37°C. The supernatant was then sonicated twice for 30 s, cellular debris was pelleted at 13,000 g, and the supernatant was retained.

Western blot analysis. Laemmli buffer (2.5 μL of 4×) (23) was added to 10 μL of each whole-cell extract or 10 μg of each nuclear extract. Samples were boiled for 5 min, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8% gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) using a Mini Trans-Blot cell (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in Tris-buffered saline ([TBS] 0.1 M, pH 7.4, 150 mM NaCl, containing 0.05% Tween-20) for 1 h at room temperature. The membranes were washed for 30 min, and the membranes were exposed on CL-X Posure film (Pierce, Rockford, IL).

RESULTS

Two proteins in frame with and comprising the C terminus of OBP are expressed in HSV-1-infected cells. OBPC was first identified by Baradaran et al. during mapping studies of the UL8, UL9, and UL10 regions of the HSV-1 genome (1). To determine whether OBPC was present in extracts of cells infected with other strains of HSV-1, Western blot analysis of nuclear extracts of Vero cells infected with each of three lab strains (KOS, F, and 17), two clinical isolates (Siebert and Choriquine (Sigma, St. Louis, MO) (200 μM) was used to inhibit viral DNA replication. Calu74 methyl ester (Calu74Me) (Sigma, St. Louis, MO) (1 μM) was used to speciﬁcally inhibit cathepsin B, and calpain inhibitor peptide (American Peptide, Sunnyvale, CA) (1 μM) was used to speciﬁcally inhibit calpains I and II. To inhibit cathepsin B as well as calpains I and II, MDL 28170 (Sigma, St. Louis, MO) (100 μM) was used. Clasto-lactacystin β-lactone (Boston Biochem, Cambridge, MA) (5 μM) was used as a speciﬁc inhibitor of the proteosome. GM132 (Z-Leu-Leu-Leu-CHO, 20 μM) inhibits the proteosome, cathepsin B, and calpains I and II (Boston Biochem, Cambridge, MA). Chloroquine (Sigma, St. Louis, MO) (200 μM) was used as an inhibitor of lysosomal proteases.

Inhibitors. Acyclovir (Sigma, St. Louis, MO) (200 μM) was used to inhibit viral DNA replication. Calu74 methyl ester (Calu74Me) (Sigma, St. Louis, MO) (1 μM) was used to speciﬁcally inhibit cathepsin B, and calpain inhibitor peptide (American Peptide, Sunnyvale, CA) (1 μM) was used to speciﬁcally inhibit calpains I and II. To inhibit cathepsin B as well as calpains I and II, MDL 28170 (Sigma, St. Louis, MO) (100 μM) was used. Clasto-lactacystin β-lactone (Boston Biochem, Cambridge, MA) (5 μM) was used as a speciﬁc inhibitor of the proteosome. GM132 (Z-Leu-Leu-Leu-CHO, 20 μM) inhibits the proteosome, cathepsin B, and calpains I and II (Boston Biochem, Cambridge, MA). Chloroquine (Sigma, St. Louis, MO) (200 μM) was used as an inhibitor of lysosomal proteases.

Transfections. Cells (~5 × 106) were seeded in 35-mm dishes. Twenty-four hours later, cells were transfected with 3 μg of the indicated plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were infected 18 h post-transfection as indicated in the ﬁgure legends.

Nuclear extracts. Nuclear extracts were prepared as previously described from 1 × 106 cells infected with 10 PFU/cell of the indicated virus or mock-infected and harvested at 8 h postinfection (hpi) unless otherwise noted (20). Briefly, cells were harvested by scraping into medium. Cells were pelleted by low-speed centrifugation (5 min at 300 × g), washed twice with phosphate-buffered saline (PBS), and resuspended in resuspension buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 5 mM MgCl2) supplemented with 0.5 mM dithiothreitol (DTT) (Sigma, St. Louis, MO) and 1 μg/mL of the following protease inhibitors: leupeptin (Sigma, St. Louis, MO), aprotinin (Roche, Indianapolis, IN), pepstatin (Sigma, St. Louis, MO), and phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO). Cells were then pelleted and resuspended in resuspension buffer containing 0.5% NP-40 and protease inhibitors. Cells were again pelleted and were resuspended in 20 mM HEPES, pH 7.9, 25% glycerol, 0.2 M KCl, and 0.2 mM EDTA containing protease inhibitors and DTT. Nuclear extracts were aliquoted, snap frozen in liquid nitrogen, and stored at −80°C.

Western blot analysis. Western blot analysis was performed as described in Materials and Methods.
forms have been designated OBPC-1 (viral strains tested but not in mock-infected cells. The two presence of two C-terminal forms of OBP in nuclear extracts of all purified antibody directed against the 20 C-terminal amino Gayle), or mock-infected cells was performed using an affinity-purified antibody directed against the 20 C-terminal amino acids of OBP (anti-OBPCT). This analysis revealed the presence of two C-terminal forms of OBP in nuclear extracts of all viral strains tested but not in mock-infected cells. The two forms have been designated OBPC-1 (~53 kDa) and OBPC-2 (~35 kDa) (Fig. 3). This unexpected finding represents the first evidence that a second N-terminally truncated form of OBP (OBPC-2) of uniform size is expressed in cells infected with multiple strains of HSV-1.

**OBPC-1 is a cleavage product of OBP.** Although the UL8.5 transcript was shown by Baradaran et al. (1) to specify a protein product of the approximate size of OBPC-1 in in vitro transcription/translation assays, expression of OBPC-1 from the UL8.5 gene has never been directly demonstrated in cell culture. Therefore, the possibility remained that OBPC-1 is a specific cleavage or degradation product of OBP. To test this possibility, a plasmid with nonsense mutations 24 and 29 amino acids downstream of the OBP translational initiation codon (pUL9n24) was constructed (Fig. 4A and B) (see Materials and Methods). These mutations were designed to eliminate OBP translation while preserving transcription and translation of the UL8.5 gene. Because the UL8.5 transcript is expressed with DE kinetics, cells were transfected with the wild-type or mutant plasmid and then infected 24 h later with the OBPC null virus, hr94, in order to induce maximum expression of the UL8.5 transcript. Cell extracts were harvested at 18 hpi.

When Vero cells were transfected with pUL9n24 and infected with hr94, Western blot analysis using anti-OBPCT demonstrated that neither OBP nor OBPC-1 was detected whereas OBPC-2 was detected. As expected, infection with the wild-type virus, KOS, as well as transfection with the wild-type plasmid expressed all three proteins while the vector-only and UL9 null virus (hr94) controls did not express OBP, OBPC-1, or OBPC-2 (Fig. 4C). These observations demonstrate that OBPC-1 expression is dependent upon expression of OBP, whereas expression of OBPC-2 is independent of OBP expression. This finding suggests either that OBP facilitates the expression of OBPC-1 or that OBPC-1 is a cleavage product of OBP. In contrast, OBPC-2 expression is independent of OBP and therefore not a degradation product of OBP. Rather, OBPC-2 is possibly the product of the UL8.5 transcript.

**Cathepsin B mediates the cleavage of OBP to produce OBPC-1.** To test the hypothesis that OBPC-1 is a cleavage or degradation product of OBP, cells were infected with KOS in the presence or absence of MG132, an inhibitor of the proteasome, cathepsin B, and calpains I and II; and cell lysates were examined for the presence of OBPC-1 by Western blot analysis. Addition of MG132 at 1 hpi inhibited the expression of OBPC-1 but not OBP or OBPC-2 (Fig. 5A). Because MG132 is able to inhibit the proteasome as well as cathepsin B and calpains I and II, a more specific proteasome inhibitor was tested for the ability to affect OBPC-1 expression to determine if OBP was degraded or specifically cleaved by a protease. Addition of clasto-lactasystin β-lactone, a more specific inhibitor of the proteasome, did not affect expression of OBPC-1 (Fig. 5A). Together, these observations indicate that OBPC-1 is not produced by the proteasome but, rather, by either cathepsin B or calpains I or II. This observation was confirmed by the addition of MDL 28170, which inhibits calpain I and II and cathepsin B but not the proteasome. The addition of MDL 28170 inhibited production of OBPC-1 but not OBP or OBPC-2 (Fig. 5A), confirming that OBP is cleaved by either calpain or cathepsin B. Addition of chloroquine, an inhibitor of lysosomal enzymes, inhibited production of OBPC-1 (Fig. 5A). Although OBPC-2 levels appear reduced in the presence of chloroquine in the experiment shown in Fig. 5A, this was not seen consistently from experiment to experiment and is likely an artifact of this particular experiment. Cathepsin B is a lysosomal enzyme, whereas calpains are not associated with lysosomes. Therefore, these data indicate that cathepsin B, not calpains I or II, cleaves OBP to produce OBPC-1. This conclusion is supported by the following experiments. Addition of calpain inhibitor peptide, which specifically inhibits calpains I and II, did not block cleavage of OBP (Fig. 5A). However, addition of Ca074Me, a highly specific cell-permeable cathepsin B inhibitor, did prevent OBP cleavage, demonstrating that OBP is cleaved in a cathepsin B-dependent manner to produce OBPC-1 (Fig. 5A). The addition of protease inhibitors did not affect the expression of OBP or OBPC-2.

Importantly, expression of an N-terminal fragment (OBPN) (~42 kDa) of nearly the same size as OBPC-1 can also be detected following cathepsin B-mediated cleavage of OBP, and its expression is inhibited by Ca074Me (Fig. 5B). The sizes of OBPC-1 (~53 kDa) and OBPN (~42 kDa) suggest that these proteins are the product of a single cleavage of OBP mediated by cathepsin B. Levels of OBPN are reduced in the nucleus compared to OBPC-1 (data not shown); however, OBPN is abundant in whole-cell extracts, suggesting that it is abundant in the cytoplasm. These findings indicate that both products of OBP cleavage by cathepsin B, OBPC-1 and OBPN, are stably expressed in infected cells.

**OBPC cleavage is dependent upon viral DNA replication.** In order to determine whether OBP cleavage is dependent upon viral DNA replication, acyclovir was added to cells at 1 hpi, and the expression of OBP, OBPC-1, and OBPC-2 was monitored by Western blot analysis (Fig. 6). Acyclovir inhibits viral DNA replication by inhibiting elongation of the replicating strand. In
the absence of acyclovir, i.e., during DNA synthesis, OBP, OBPC-1, and OBPC-2 were detected in KOS-infected nuclear extracts but not in mock- or hr94-infected nuclear extracts. The addition of acyclovir at 1 hpi inhibited the production of OBPC-1 (Fig. 6) but not OBP, indicating that OBP cleavage is dependent upon either DNA replication or some event following DNA replication. The addition of acyclovir also reduced levels of OBPC-2 (Fig. 6), suggesting that OBPC-2 is expressed with DE kinetics, similar to the UL8.5 transcript (1).

**DISCUSSION**

The identification of two proteins that are in frame with and comprise different lengths of the C terminus of OBP led us to investigate the origins of these two proteins. While we have demonstrated that OBPC-1 is a product of cathepsin B-mediated cleavage of OBP, OBPC-2 may well be a product of the UL8.5 transcript as (i) it is expressed, like the UL8.5 transcript, with DE kinetics, and (ii) its expression is not dependent upon OBP expression. It should be noted that previous evidence suggesting that OBPC-1 is the product of the UL8.5 transcript relied solely on in vitro transcription/translation assays (1). When the UL8.5 transcript was used in these assays, a protein product of 53 kDa was observed. This product is similar but not identical in size to the protein detected by Baradaran et al. (1) in infected cell extracts and is the predicted size of a protein transcribed from the first in-frame translational start codon in the UL8.5 transcript. Therefore, the conclusion that OBPC-1 is a product of the UL8.5 transcript was reasonable, albeit incor-
Evidence presented in this paper indicates that in the context of viral infection, the first in-frame translational start codon of the UL8.5 transcript is not utilized, as the molecular mass of OBPC-2 (35 kDa) is much smaller than would be predicted (64 kDa) if the first start codon of the UL8.5 transcript were used. In fact, the small size of the protein suggests that it was not detected by Baradaran et al. because the protein ran off the gels used for Western blotting. It is also possible that the antibody used by Baradaran et al. and obtained from Olivo et al. (37, 38), which is directed against the last 10 amino acids of OBP, does not detect OBPC-2, and only upon generation of a new antibody specific for the C-terminal 20 amino acids of OBP has OBPC-2 been detectable. These data suggest that it was not detected by Baradaran et al. because the protein ran off the gels used for Western blotting. It is also possible that the antibody used by Baradaran et al. and obtained from Olivo et al. (37, 38), which is directed against the last 10 amino acids of OBP, does not detect OBPC-2, and only upon generation of a new antibody specific for the C-terminal 20 amino acids of OBP has OBPC-2 been detectable. These data suggest that it was not detected by Baradaran et al. because the protein ran off the gels used for Western blotting. It is also possible that the antibody used by Baradaran et al. and obtained from Olivo et al. (37, 38), which is directed against the last 10 amino acids of OBP, does not detect OBPC-2, and only upon generation of a new antibody specific for the C-terminal 20 amino acids of OBP has OBPC-2 been detectable. These data suggest that in systems with complex transcriptional and translational strategies such as HSV-1, in vitro transcription/translation data should be supported by evidence from infected cells. We have recently completed a more detailed investigation of the source of OBPC-2 with the following results: we have demonstrated that OBPC-2 is a product of the UL8.5 transcript, and we have mapped its translational start site (unpublished observations).

As noted above, the activities of DNA replication initiator proteins can be regulated in many ways. One mode of regulation is through the activities of a protein that is able to compete with or inhibit the activities of the initiator protein. As OBPC-1 shares the DNA binding domain of OBP but lacks the domains necessary to unwind DNA or to recruit other viral factors essential for the initiation of viral DNA replication, OBPC-1 was considered a prime candidate for an inhibitor of OBP function. Indeed, overexpression of a protein similar in size to OBPC-1 (~50 kDa), which presumably contains amino acids 365 to 851 of OBP, is inhibitory to origin-dependent DNA replication (2). Based on these considerations, we hypothesized that OBPC-1 may play a role in the negative regulation of OBP activity by inhibiting its binding to origin sequences and thereby facilitating the switch from origin-dependent DNA replication to origin-independent DNA replication (2). It has recently been demonstrated, however, that C-terminal forms of OBP with various amounts of N-terminal sequence behave differently during infection, with some acting as inhibitors of viral replication and others actually increasing viral replication (9). Indeed, C-terminal fragments of OBP of similar size to OBPC-1 were not able to bind origin sequences in this study (9). Therefore, it is unclear if OBPC-1 is able to bind to origins. We are in the process of determining if OBPC-1 is able to bind origin sequences to begin to understand what role OBPC-1 may play during viral infection.

In the course of attempts to express OBPC-1 in the absence of OBP, we have demonstrated that OBPC-1 is a cathepsin B-mediated cleavage product of OBP. As OBP is a nuclear protein while cathepsin B is most commonly a lysosomal protein, it is difficult to imagine how cathepsin B directly cleaves OBP. However, cathepsin B and cathepsin B activity have been identified in the nuclei of multiple cell types (40, 41, 48, 54). Overexpression of OBP is detrimental to viral DNA synthesis and viral plaque formation (2, 29, 32, 52, 55). Therefore, this cleavage event may play a role in regulating the levels of OBP in the nucleus during viral infection, possibly by down-regulating the initiator function of OBP and thus down-regulating viral DNA replication. Consequently, it will be of interest to study the replication kinetics of a virus that does not express OBPC-1.

To characterize an OBPC-1 null virus in cell culture and in
mice, identification of the cathepsin B cleavage site of OBP will be necessary. Unfortunately, cathepsin B cleavage sites are very poorly defined. To date, only six known sites have been mapped (26), and attempts to identify a consensus sequence for cleavage have been unsuccessful. This may be because cleavage likely occurs at a site distinct from the cathepsin B binding site and may be more dependent on the secondary structure of the protein than on a specific amino acid sequence (26). Indeed, while the cathepsin B cleavage site of sphingosine kinase I has been identified, mutagenesis of the P1, P2, and P1′ amino acids did not eliminate cathepsin B cleavage of the protein (58). These complications will make identification of the cathepsin B cleavage site in OBP and elimination of cathepsin B cleavage of OBP very difficult, even using protein-sequencing techniques. An available cathepsin B cleavage site prediction program (26) predicted a cathepsin B cleavage site at amino acids 479 to 484 of OBP. However, mutagenesis of this region did not eliminate OBP cleavage (data not shown). Furthermore, the N- and C-terminal products of cathepsin B cleavage of OBP tend to communoprecipitate (data not shown). Due to the similarities in sizes of these N- and C-terminal fragments, it has been difficult to separate them by gel electrophoresis. This has complicated attempts to analyze the start site of OBPC-1 by mass spectrometry and N-terminal sequencing.

The aim of this study was to begin to study the two C-terminal forms of OBP, OBPC-1 and OBPC-2, separately; however, the possibility remains that these two proteins share similar roles during viral replication. It will be necessary to eliminate the expression of both proteins to further test this hypothesis. These studies are the subject of a forthcoming manuscript.

What, then, may be the function of the cleavage of OBP to yield OBPC-1 and OBPN? As noted above, in order for stage II origin-independent DNA replication to begin, OBP’s initiator function must be eliminated, or OBP must be removed from viral origins and the initiator complex degraded or otherwise inactivated in order to block the initiation of origin-dependent viral DNA replication (stage I) (31, 33). Intriguingly, cathepsin B cleavage of OBP is dependent upon viral DNA synthesis. We hypothesize, therefore, that the process of viral DNA replication itself, or a late function of the virus, results in a change in the conformation or modification of OBP (possibly by phosphorylation or ubiquitination). This change may result in the release of OBP from the origins, rendering it available for cleavage by cathepsin B. Alternatively, cleavage of OBP may occur while OBP is still bound to the origin. This cleavage event may have two distinct effects on the regulation of OBP activity by (i) rendering the initiator protein inactive and (ii) producing a protein that might bind to origins, inhibiting new rounds of OBP-dependent viral DNA replication. Further study of the regulation of this cleavage event should shed light on the regulation of OBP function during viral DNA replication.

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REFERENCES


