Protein Profiling with Epstein-Barr Nuclear Antigen-1 Reveals an Interaction with the Herpesvirus-associated Ubiquitin-specific Protease HAUSP/USP7*

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The Epstein-Barr nuclear antigen-1 (EBNA1) protein of Epstein-Barr virus is important for the replication, segregation, and transcriptional activation of latent Epstein-Barr virus genomes; has been implicated in host cell immortalization; and avoids proteasomal processing and cell-surface presentation. To gain insight into how EBNA1 fulfills these functions, we have profiled cellular protein interactions with EBNA1 using EBNA1 affinity chromatography and tandem affinity purification (TAP) of EBNA1 complexes from human cells (TAP-tagging). We discovered several new specific cellular protein interactions with EBNA1, including interactions with HAUSP/USP7, NAP1, template-activating factor-Ip/SET, CK2, and PRMT5, all of which play important cell regulatory roles. The ubiquitin-specific protease USP7 is a known target of herpes simplex virus, and the USP7-binding region of EBNA1 was mapped to amino acids 395–450. A mutation in EBNA1 that selectively disrupted binding to USP7 was found to cause a 4-fold increase in EBNA1 replication activity but had no effect on EBNA1 turnover and cell-surface presentation. The results suggest that USP7 can regulate the replication function of EBNA1 and that EBNA1 may influence cellular events by sequestering key regulatory proteins.

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UBIQUITIN-SPECIFIC PROTEASE HAUSP/USP7

Reveals an Interaction with the Herpesvirus-associated Epstein-Barr Nuclear Antigen-1

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The Epstein-Barr virus (EBV) is a ubiquitous human γ-herpesvirus that persists for the life of the host. As part of its latent infectious cycle, EBV immortalizes the host cell and, in doing so, predisposes the cell to malignant transformation. As a result, EBV is associated with several types of cancer. EBV genomes are maintained in latently infected replicating cells as circular DNA epispisms that replicate once per cell cycle and segregate stably during cell division (reviewed in Refs. 1 and 2). Epstein-Barr nuclear antigen-1 (EBNA1) is the only viral protein required to maintain the EBV genomes in proliferating cells, which it does by binding to recognition sites in the FR (family of repeats) and DS (dyad symmetry) elements of the latent origin of DNA replication, oriP (3, 4). EBNA1 binding to the DS element is necessary to initiate DNA replication from this element (5). EBNA1 binding to the FR element is important for the partitioning of the EBV episomes during cell division and also activates the expression of other viral latency genes (6). In addition to its functions at oriP, EBNA1 has been shown to repress its own transcription (7) and to promote the development of B-cell lymphomas in transgenic mice, suggesting a direct role for EBNA1 in cell transformation (8).

While fulfilling all of its functions, EBNA1 avoids detection by host cytotoxic T-lymphocytes. This ability to hide from the immune system is biologically important, as it enables the persistence of latently infected cells that express EBNA1 in the absence of other EBV antigens. The failure of EBNA1 to elicit a cytotoxic T-lymphocyte response is due to lack of proteasomal processing, which prevents the presentation of EBNA1 by major histocompatibility complex class I molecules on the cell surface (9). This property of EBNA1 has been attributed to the central Gly-Ala repeat, which varies in length in different EBV isolates and is not required for any of the EBNA1 functions measured in tissue culture (10, 11).

EBNA1 has no apparent enzymatic activities and is thought to fulfill its functions by mediating interactions with specific host cellular proteins. However, few of these cellular protein interactions have been identified. To date, only yeast one- and two-hybrid approaches have been used to screen for EBNA1-interacting proteins; these screens have identified importin-α (also called karyopherin-α2 or Rch1), karyopherin-α1, p32/TAP, and EBP2 as EBNA1-binding proteins (12–17). Since importin-α and karyopherin-α1 are known nuclear transport factors, their interaction with EBNA1 may be important for EBNA1 entry into the nucleus. p32/TAP (also called gC1q-R) is predominantly a mitochondrial protein, but has been found to bind to a wide variety of proteins with diverse functions (Refs. 13 and 18–20 and references therein). Thus, the significance of the interaction of EBNA1 with p32/TAP remains unclear.

The interaction of EBNA1 with EBP2 seems to be important for the segregation function of EBNA1. EBP2 is a component of the cellular mitotic chromosomes, and EBNA1 appears to attach to EBP2 to partition EBV plasmids (15, 21, 22). In addition to the EBNA1-binding proteins identified by screening, studies that have specifically tested for interactions of EBNA1 with replication protein A and the origin recognition

1 The abbreviations used are: EBV, Epstein-Barr virus; EBNA1, Epstein-Barr nuclear antigen-1; TAP, tandem affinity purification; DTT, dithiothreitol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TAP, template-activating factor; TBP, TATA box-binding protein.

2 or Rch1), karyopherin-α1, p32/TAP, and EBP2 as EBNA1-binding proteins (12–17). Since importin-α and karyopherin-α1 are known nuclear transport factors, their interaction with EBNA1 may be important for EBNA1 entry into the nucleus. p32/TAP (also called gC1q-R) is predominantly a mitochondrial protein, but has been found to bind to a wide variety of proteins with diverse functions (Refs. 13 and 18–20 and references therein). Thus, the significance of the interaction of EBNA1 with p32/TAP remains unclear. The interaction of EBNA1 with EBP2 seems to be important for the segregation function of EBNA1. EBP2 is a component of the cellular mitotic chromosomes, and EBNA1 appears to attach to EBP2 to partition EBV plasmids (15, 21, 22). In addition to the EBNA1-binding proteins identified by screening, studies that have specifically tested for interactions of EBNA1 with replication protein A and the origin recognition

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complex have detected these interactions, suggesting that they might be important for the replication function of EBNA1 (23, 24).

Since the present set of known protein interactions with EBNA1 is unlikely to account for all of the EBNA1 functions, we have used additional methods to screen for human proteins that specifically recognize EBNA1. Using affinity chromatography and tandem affinity purification (TAP)-tagging approaches, we have identified novel EBNA1-interacting proteins, including the deubiquitinating enzyme USP7 (also known as HAUSP).

**EXPERIMENTAL PROCEDURES**

Purification of EBNA1 and Δ325–376—EBNA1 (lacking most of the Gly-Ala repeat region) was expressed as a hexahistidine fusion from a baculovirus. EBNA1 was purified from insect cell nuclei on a metal chelating column, followed by a heparin-agarose column (25). Δ325–376 (lacking amino acids 325–376 in addition to most of the Gly-Ala repeat) was expressed as a hexahistidine fusion from pET15b in *Escherichia coli* and purified as described for EBNA1.

**EBNA1 Affinity Columns**—HeLa S3 cells (10 g; National Cell Culture Center) were lysed in 11 ml of 10 mM HEPES (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM diethiothreitol (DTT), and complete protease inhibitors (Roche Applied Science). 10 ml of 50 mM HEPES (pH 7.5), 1.5 mM MgCl₂, 1.26 mM NaCl, 0.5 mM DTT, 0.6 mM EDTA, and 75% glycerol was added, and the lysate was homogenized in a Dounce homogenizer. After 30 min on ice, the extract was clarified by centrifugation for 3 h at 64,000 × g and then dialyzed overnight against 50 mM HEPES (pH 7.5), 20% glycerol, 0.5 mM DTT, 5 mM MgCl₂, and 75% KCl. Prior to loading onto affinity columns, CaCl₂ was added to 4 mM, and lysates were incubated with RhNase A and RNase D1 (1.4 μg/ml of lysate) for 30 min at 25 °C to remove nucleic acid.

Purified EBNA1 or Δ325–376 was covalently linked to Affi-Gel 10 (Bio-Rad) by incubating 2 ml of protein/ml of Affi-Gel in buffer A (20 mM HEPES (pH 7.5), 10% glycerol, 0.1 mM EDTA, 1 mM DTT, and 1 mM NaCl) and blocked as described previously (26). Coupled resin was washed with buffer A and equilibrated in buffer A containing 0.1 mM NaCl (buffer B) prior to being poured into 40-μl microcolumns. 400 μl of HeLa lysate (at 14 mg/ml) was applied to the columns. Columns were washed with 400 μl of buffer B and 160 μl of buffer B containing 1% Triton X-100 and then subsequently eluted with buffer A and 1% SDS. For chromatography assays, the buffer A eluates from three columns were pooled, dialyzed against buffer A containing 200 mM NaCl, and reapplied to an EBNA1 affinity column. This column was washed and eluted as described above. Column eluates were analyzed by SDS-PAGE and silver staining.

**Mass Spectrometry**—Gel slices containing the protein bands were identified by reduction in DTT, alkylation in iodoacetamide, and then subjecting to in-gel trypsin hydrolysis. The peptides were purified and analyzed by MALDI-TOF mass spectrometry using a cyano-4-hydroxy-cinnamic acid matrix (Sigma) on a Voyager DE-STR instrument (Applied Biosystems) (27). Identification of the proteins using these mass fingerprinting data was carried out using the ProFound software.

**Co-immunoprecipitation from Insect Cells**—Baculoviruses expressing EBNA1AGA (with a long Gly-Ala repeat) (11), EBNA1 or EBNA1 mutants 330–641, 330–619, 452–641, and Δ41–376 have all been described previously (28, 29). The baculovirus expressing EBNA1 with a small Gly-Ala repeat and lacking amino acids 395–450 was constructed by QuikChange mutagenesis of EBNA1 in pFast-Bac (Invitrogen). Baculovirus was generated according to the manufacturer’s specifications. A baculovirus expressing USP7 with an N-terminal hexahistidine tag was similarly constructed using USP7 cDNA in pET-3a, kindly provided by Roger Everett (30). For co-immunoprecipitation assays, 10° S9 cells were infected with baculovirus expressing either EBNA1 or a Δ325–376 construct, cells were labeled with 50 μCi of [35S]methionine for 16 h as described previously (15). Cells were lysed in 1 ml of immunoprecipitation buffer (20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM MgCl₂, 1% Triton X-100, and protease inhibitors) on ice for 30 min. After centrifugation, the supernatant was precleared with protein A-Sepharose. The supernatant was then precipitated with rabbit anti-EBNA1 polyclonal antibody K67 (kindly provided by Jaap Middeldorp) and protein A-Sepharose as described previously (15). Immunoprecipitated proteins were analyzed by SDS-PAGE, followed by autoradiography of the dried gels.

**TAP-tagging Experiments**—The DNAs coding for EBNA1, Δ395–450, and Δ325–376 were PCR-amplified from plasmids containing these EBNA1 mutants and cloned between the XhoI and NotI sites of pM7ZI. pMZI expresses proteins with C-terminal TAP tags (31) in mammalian cells under the control of an ecsvdrome-inducible promoter. The pM7ZI and the pMZZi-LacZ construct, which encodes TAP-tagged β-galactosidase, will be described elsewhere. 293T cells at 60% confluence in 150-mm dishes were cotransfected by calcium phosphate precipitation with 8 μg of pM7ZI expressing EBNA1 or an EBNA1 mutant and 8 μg of pVgRKR (Invitrogen), which encodes the ecsvdrome receptor heterodimer. The precipitate was removed 15 h post-transfection, and expression of the EBNA1 proteins was induced by adding medium containing 3 μM Ponasterone A (Invitrogen). The cells were harvested 22–48 h later, and a whole cell extract was prepared from 4 × 10⁶ cells as described by Xiao et al. (32), except that the extract was dialyzed against 10 mM HEPES (pH 7.9), 0.1 mM potassium acetate, 0.1 mM EDTA, 0.1 mM DTT, and 10% glycerol. Eluted protein was further purified on a 40-μl column of calmodulin-Sepharose 4B. Protein complexes were eluted with 10 mM HEPES (pH 8), 100 mM NaCl, 0.1% Triton X-100, 0.5 mM EDTA, 1 mM DTT, and 10% glycerol. Eluted protein was then further purified on a 40-μl column of chymotrypsin-Sepharose 4B. Protein complexes were eluted with 10 mM HEPES (pH 8), 100 mM NaCl, 0.25% Triton X-100, 0.5 mM MgCl₂, 0.6 mM EDTA, 1 mM DTT, and 1% Triton X-100; concentrated by lyophilization; and analyzed by SDS-PAGE and silver staining.

**Protein Turnover Assays**—2 × 10⁶ 293T cells were transfected with 5 μg of pc3oriPEPNA1 or pc3oriP, which contain the EBV oriP sequence and express EBNA1 or EBNA1, respectively, was described previously (15). At 48 h post-transfection, plasmids were recovered, linearized, and digested with DpnI, and analyzed by Southern blotting. Plasmid bands were visualized by autoradiography and quantified by PhosphorImager analysis using ImageQuant software (Amersham Biosciences). For transactivation assays, C33A cells were transfected with 5 μg of pc3oriP plasmids and 2 μg of the pRTKCAT reporter construct (kindly provided by Bill Sugden). 24 h later, cell lysates were prepared, and 50 μg of each was assayed for chloramphenicol acetyltransferase activity using several reaction times as described previously (34). Reaction products at each time point were quantified by PhosphorImager analysis and used to determine the acetylation rate.

**Protein Turnover Assays—**2 × 10⁶ 293T cells were transfected with 5 μg of pc3oriPEPNA1 or pc3oriP3995–450 using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's recommendations. Cells were grown for 1 week under selection for the plasmid (400 μg/ml G418) before blocking the cells in 100 μg/ml cycloheximide (Sigma). Cells were harvested at various times after blocking by resuspending in 9 ml of 5 mM Tris-HCl (pH 7.5). Lysates were clarified by sonication and centrifugation, and 50 μg of each was separated by SDS-PAGE and Western-blotted using anti-EBNA1 antisera R4 (raised against EBNA1(452–641)). The same lysates were also probed with anti-interferon regulatory factor 1 (IRF-1) antibody C20 (Santa Cruz Biotechnology).

**Immunofluorescence Microscopy**—C33A cells expressing EBNA1 were generated by transfecting C33A cells with pc3oriPEPNA1 and growing the cells under selection for the plasmid. The cells were then grown on coverslips to 60% confluence, fixed with formaldehyde (5% v/v) in phosphate-buffered saline containing 2% FBS and permeabilized with acetone/methanol (70:30) at −20 °C. Coverslips were washed with phosphate-buffered saline and blocked for 1 h in 10% bovine serum albumin. The cells were stained with anti-EBNA1 monoclonal antibody OT1x (kindly supplied by Jaap Middeldorp) at a 1:500 dilution and rabbit anti-USP7 antibody r201 (kindly provided by Roger Everett) at a 1:200 dilution, followed by staining with Texas Red.

2 M. Zeghouf, J. Li, G. Butland, A. Borkowska, V. Canadien, D. Richards, B. Beattie, A. Emili, and J. Greenblatt, submitted for publication.

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EBNA1-USP7 Interaction

RESULTS

EBNA1-interacting Proteins Identified by Affinity Chromatography—EBNA1-binding proteins were isolated from a HeLa cell extract by retention on columns containing purified EBNA1. Elution profiles were compared for EBNA1 columns—\textit{A} and negative control columns (with no protein coupled) in four experiments. To identify these proteins, we compared the cellular proteins eluted from the EBNA1 column with those eluted from a column containing TATA box-binding protein (TBP), which has a pI similar to that of EBNA1. The elution profile from the TBP column shows that the EBNA1-binding proteins importin-\(\beta\), TAF-1\(\alpha\), p32/TAP, and pp32 were also retained on the TBP column, suggesting that they interact nonspecifically with basic proteins (Fig. 1). We also excised bands from the TBP elution profile that migrated at positions similar to those of NAP1, importin-\(\alpha\), USP7, and TAF-I\(\alpha\). USP7 was present in the TBP eluate. We conclude that USP7, NAP1, importin-\(\alpha\) and TAF-1\(\beta\) interact specifically with EBNA1. Karyopherin-\(\beta\) and CK2 bands were not observed on the TBP column; however, since they are close to the limit of detection in the EBNA1 profile, we cannot be certain that they are not present in the TBP profile.

We next tested the relative affinity of the EBNA1-binding proteins for EBNA1 by rechromatography of EBNA1 column eluates on a second EBNA1 column. Eluates from three EBNA1 columns were pooled, dialyzed to lower the salt to 100 mM, and then reapplied to another EBNA1 column. When the high salt elution profiles were compared from the first- and second-pass EBNA1 columns, bands corresponding to USP7, NAP1, importin-\(\alpha\), CK2\(\alpha\), and pp32/TAP were all observed to be enriched upon rechromatography relative to the other bands (Fig. 1B), suggesting that these proteins bind EBNA1 with higher affinity than the other proteins. Two bands corresponding to NAP1 were identified in this experiment; the lower band is a proteolytic fragment of the full-length protein.

We have previously shown that EBNA1 amino acids 325–376 are essential for both the transcriptional activation and partitioning functions of EBNA1, suggesting that these residues mediate interactions with cellular proteins involved in these processes (15, 34). To uncover cellular proteins that interact with this functionally important region of EBNA1, we constructed affinity columns using an EBNA1 mutant lacking amino acids 325–376 (\textit{\Delta}325–376) and compared the profile of cellular proteins retained on this column with that on an EBNA1 column (Fig. 2). In each of three experiments, importin-\(\alpha\) and NAP1 were not detected on the \textit{\Delta}325–376 column.
indicating that residues 325–376 are important for importin-α and NAP1 binding. No other consistent differences were observed between the EBNA1 and Δ325–376 profiles.

Mapping of the USP7-interacting Region of EBNA1—The EBNA1-USP7 interaction had not been previously identified and passed all of our criteria for a bona fide interaction, viz. reproducibility, specificity, and strength of binding. To further verify this interaction and to determine whether it is direct or mediated by another human protein, we coexpressed both proteins in insect cells using baculoviruses, metabolically labeled the cells, and performed co-immunoprecipitation experiments using anti-EBNA1 antibody (Fig. 3B). Immunoprecipitates from cell lysates expressing both EBNA1 and USP7 contained labeled bands corresponding to EBNA1 and USP7 (lanes 5 and 14). USP7 also immunoprecipitated with a version of EBNA1 containing a longer Gly-Ala repeat region (EBNA1GA) (lane 13). The USP7 band was not detected in anti-EBNA1 immunoprecipitates from cells expressing either USP7 alone or EBNA1 alone (lanes 8 and 15). Thus, the EBNA1-USP7 interaction does not require other human proteins.

We used this co-immunoprecipitation assay to determine the region of EBNA1 that interacts with USP7 (Fig. 3B). The series of EBNA1 truncation and deletion mutants in Fig. 3A were coexpressed with USP7 in insect cells. In keeping with the affinity column results, the Δ325–376 mutant retained the ability to bind USP7 (lane 2). USP7 was also found to interact with EBNA1 mutants lacking any or all of the N-terminal 395 amino acids, but not with the 452–641 fragment (lane 6), suggesting that residues between amino acids 395 and 452 of EBNA1 mediate the USP7 interaction. In keeping with this conclusion, an EBNA1 deletion mutant lacking residues 395–450 did not detectably interact with USP7 in this assay (lane 17).

Identification of Protein Interactions by TAP of EBNA1—We used a TAP-tagging approach to profile cellular proteins that stably interact with EBNA1 in vivo. TAP tagging, which was originally developed for use in yeast, involves expressing the protein of interest fused at the C terminus to a calmodulin-binding peptide, followed by a tobacco etch virus protease cleavage site and a protein A IgG-binding domain (31). The tagged protein is isolated from cell lysates on IgG resin, eluted by tobacco etch virus protease cleavage, further purified on calmodulin resin, and eluted with EGTA. Because the native elution conditions in this procedure enable protein complexes to remain intact throughout the purification, this is a powerful method for profiling in vivo protein interactions. TAP-tagging results also tend to be cleaner than single pull-down experiments since the two-column purification procedure leads to the isolation of only relatively stable protein complexes.

To apply the TAP-tagging approach to EBNA1, we designed a vector expressing TAP-tagged EBNA1 from an ecdysone-inducible promoter and used it to express EBNA1 in human 293 cells. TAP-tagged EBNA1 was purified from cell lysates, and copurifying proteins were separated by SDS-PAGE and identified by MALDI-TOF mass spectrometry (Fig. 4A). This method identified a subset of cellular proteins that interacted with EBNA1 in the affinity column approach, viz. USP7, importin-α, CK2 (α-, α’-, and β-subunits), and p32/TAP. In some experiments, we also identified an interaction with the protein-arginine methyltransferase PRMT5 (Fig. 4B). PRMT5 was also found to be retained on EBNA1 affinity columns, but was eluted only with SDS (data not shown).

We used the TAP-tagging approach to assess the effects of the Δ395–450 and Δ325–376 deletions on cellular protein interactions with EBNA1 (Fig. 4B). A comparison of the EBNA1 and Δ395–450 profiles showed that this deletion selectively disrupted the USP7 interaction without altering interactions with any of the other cellular proteins. This indicates that the Δ395–450 mutant is not grossly misfolded and is suitable for functional studies to assess the significance of the EBNA1-USP7 interaction. Sequence 395–450 likely corresponds to a functional entity/domain since it is bordered on one side by the DNA-binding domain (amino acids 452–607) and on the other side by the nuclear localization signal (amino acids 379–387) (41). The TAP-tagging results with Δ325–376 showed that, like EBNA1, this mutant stably interacted with USP7, CK2, and p32/TAP, which is consistent with the affinity column results. A band was also observed at the position of PRMT5, suggesting that PRMT5 interacts with Δ325–376. In the experiments in Fig. 4B, we cannot comment on interactions with importin-α since this band would be obscured by the bands from the EBNA1 deletion mutants and by the bands corresponding to partially proteolyzed EBNA1.

Effect of USP7 Interaction on EBNA1 Turnover and Presentation—EBNA1 is not efficiently processed by the 26 S proteasome and, as a result, has an extremely long half-life and is not presented on the cell surface through the major histocompatibility complex class I pathway. The interaction of EBNA1 with the USP7 ubiquitin protease suggested that the lack of proteasomal processing of EBNA1 could be due, at least in part, to the removal of conjugated ubiquitin from EBNA1 by USP7. If this hypothesis were correct, then the Δ395–450 mutant, which does not bind USP7, should remain ubiquitinated and be degraded by the proteasome. To test this possibility, we compared the turnover of EBNA1 and Δ395–450 in 293 cells expressing these proteins by following the amount of each protein remaining at various times after blocking protein synthesis with cycloheximide (Fig. 5). Little change in the levels of either of the EBNA1 proteins was observed over the course of the experiment (32 h), indicating that the Δ395–450 mutation did not significantly alter the stability of EBNA1 in these cells. Turnover of the unstable IRF-1 protein was observed, indicating that the cells were blocked by the cycloheximide treatment. Identical results were obtained when turnover experiments were conducted in C33A cells and when the Δ395–450 mutation was made within a version of EBNA1 containing the full-length Gly-Ala repeat (data not shown).

We also tested the presentation of Δ395–450 mutants (containing full-length or small Gly-Ala repeats) expressed in B-cells as determined by lysis by major histocompatibility complex class I-restricted cytotoxic T-lymphocytes. The results indicated that the Δ395–450 mutation did not significantly increase EBNA1 presentation over the levels seen with the
parental EBNA1 proteins (data not shown). Thus, the EBNA1-USP7 interaction does not appear to be a major determinant in the lack of turnover or presentation of EBNA1.

Effect of USP7 Binding on the Replication and Transactivation Functions of EBNA1—We also tested the possibility that USP7 binding may affect the replication, segregation, and transactivation functions of EBNA1. Such effects could be due to the ubiquitination state of either EBNA1 or cellular proteins. To this end, we compared the activities of EBNA1 and Δ395–450 in plasmid maintenance, replication, and transcriptional activation assays. Plasmid maintenance assays were performed by transfecting C33A cells with an oriP plasmid containing the EBV origin of replication and segregation elements and expressing either EBNA1 or Δ395–450. After 2 weeks of cell growth, the plasmid levels were compared by Southern blotting. We observed that Δ395–450 maintained the plasmids at higher levels (3-fold higher on average) than EBNA1, whereas oriP plasmids that did not express EBNA1 proteins were not maintained (Fig. 6A). This effect was not due to abnormally high expression levels of Δ395–450, as this protein was expressed at levels similar to those of wild-type EBNA1 (data not shown). Since stable plasmid maintenance requires both replication and segregation functions, the results could reflect an increase in the efficiency of either of these processes by Δ395–450. To distinguish between these possibilities, we measured the replication efficiency of the same plasmids in a transient replication assay. In this assay, the plasmids were recovered from cells 3 days post-transfection, linearized, and incubated with the methylation-sensitive enzyme DpnI, which digests the unreplicated plasmids, leaving replicated plasmids intact. The amount of DpnI-resistant plasmid was then quantified by Southern blotting. As shown in Fig. 6B, oriP plasmids were replicated more efficiently in the presence of Δ395–450 compared with EBNA1; replication levels calculated from four experiments were 4-fold higher on average in the presence of Δ395–450 compared with EBNA1, which could account for the increased plasmid levels seen in the plasmid maintenance assays. The results suggest that the EBNA1-USP7 interaction decreases the efficiency with which EBNA1 activates replication from oriP, but has no obvious effect on EBNA1 segregation function.

We also tested the possibility that the USP7 interaction affects the transcriptional activation function of EBNA1 by comparing the ability of EBNA1 and Δ395–450 to activate the expression of a chloramphenicol acetyltransferase reporter gene under the control of the EBV enhancer element (FR). For these assays, C33A cells were cotransfected with the oriP plasmids expressing EBNA1 or Δ395–450 and with pFRTKCAT, and the level of chloramphenicol acetyltransferase gene expression was determined by measuring the acetylation rates for each cell lysate using equal amounts of protein. Results from four experiments indicated that Δ395–450 was slightly less active than EBNA1 in these assays, yielding 68 ± 7% of the transcriptional activation levels of EBNA1. Thus, the interaction with USP7 may modestly increase the transcriptional activity of EBNA1, but does not appear to play a major role in this process.

**Fig. 3.** Mapping of the USP7-binding region of EBNA1 by co-immunoprecipitation. A, shown is a schematic representation of EBNA1 and EBNA1 mutants and summary of their ability to bind USP7 in the co-immunoprecipitation assay. BD, binding domain; NLS, nuclear localization signal. B, shown are the results from co-immunoprecipitation assays in which EBNA1 or the indicated EBNA1 mutant was expressed in insect cells from a baculovirus with (+ USP7) and without (lanes 1, 10, 15, and 18) USP7 expression from a second baculovirus. After metabolic labeling of the insect cells, EBNA1 proteins were immunoprecipitated with anti-EBNA1 antibody, and EBNA1 and co-immunoprecipitating proteins were separated by SDS-PAGE and visualized by autoradiography. The position of the USP7 protein is marked by asterisks. Negative control experiments in which insect cells were infected with USP7 baculovirus alone (lane 8) or with no baculovirus (lanes 7 and 16) are also shown.
EBNA1-USP7 Interaction

A.

B.

Fig. 4. Isolation of TAP-tagged EBNA1 and associated cellular proteins. TAP-tagged proteins were expressed in 293T cells and sequentially purified on IgG and calmodulin columns. Purified proteins were separated by SDS-PAGE, visualized by silver staining, and identified by MALDI-TOF mass spectrometry. The positions of molecular mass markers are indicated beside each gel in kilodaltons. Bands that were not labeled were not identified by mass spectrometry. A, experiment performed with TAP-tagged EBNA1; B, experiments performed with TAP-tagged EBNA1, Δ395–450, Δ325–376, or β-galactosidase (β-gal) as a negative control. The brackets indicate the positions of the TAP-tagged EBNA1 proteins and the proteolytic fragments of these proteins. The band at 116 kDa in the β-gal lane is TAP-tagged β-galactosidase. CK II, CK2.

EBNA1

Δ395–450

α EBNA1

α IRF-1

0 1 5 9 25 32

0 1 5 9 25 32

Fig. 5. Comparison of turnover rates of EBNA1 and Δ395–450. 293T cells expressing either EBNA1 or Δ395–450 were blocked with cycloheximide, and cells were collected and lysed 0, 1, 5, 9, 25, and 32 h post-transfection as indicated. Equal amounts of protein from each cell lysate were analyzed by Western blotting using anti-EBNA1 and anti-IRF-1 antibodies.

Cellular Localization and Abundance of USP7 and EBNA1—To better understand the extent of interaction between EBNA1 and USP7 in human cells, we used antibodies raised against USP7 and EBNA1 to determine the localization of these proteins both in an EBV-positive Burkitt’s lymphoma cell line (Raji) and in the C33A epithelial cells expressing EBNA1 that were used for the functional assays described above (Fig. 7). In both cases, EBNA1 was found throughout the nucleus, and USP7 was found throughout most of the nucleus, with the exception of the nucleolus. Thus, there is a considerable overlap between EBNA1 and USP7 in interphase cells. One of the C33A cells captured in Fig. 7 was in mitosis, and immunofluorescence staining of this cell showed that USP7 and EBNA1 have very different staining patterns in mitosis; EBNA1 was localized to the mitotic chromosomes, whereas USP7 was found outside of the chromosomes. The localization of EBNA1 and USP7 is therefore consistent with a possible interaction between the two proteins in interphase, but not in mitosis.

We also compared the amount of USP7 and EBNA1 in Raji cells by Western blot analyses of Raji whole cell extracts in comparison with known amounts of purified EBNA1 and USP7. We found that USP7 was very abundant in Raji cells, with ~130,000 ± 68,000 copies/cell calculated from four separate experiments. USP7 was also abundant in the C33A cells in which the functional assays were performed, with a copy number calculated at 50,000 copies/cell. USP7 was severalfold more abundant than EBNA1 in the Raji cells, which our experiments indicated was present at 18,000 copies/cell and which has previously been reported to be present at 37,000 copies/cell (42). The relative abundance of EBNA1 and USP7 is consistent with the possibility that a significant portion of EBNA1 could be bound by USP7 in EBV-infected cells.

DISCUSSION

We have used in vitro affinity chromatography and in vivo TAP-tagging approaches to uncover several cellular protein interactions with EBNA1 and to gain insight into the EBNA1 regions that mediate these interactions. Interactions were observed with the nuclear import factors importin-α, importin-β (also called karyopherin-β), karyopherin-β2, and karyopherin-β3. The interaction with importin-α, which had been previously detected in yeast two-hybrid assays (12, 14), was shown to involve sequence 325–376 of EBNA1. However, the Δ325–376 mutant, which is impaired in importin-α binding, retains the ability to enter the nucleus and has wild-type DNA replication activity (15, 21), suggesting that the importin-α interaction is not solely responsible for nuclear entry or that a weak interaction with importin-α is sufficient for nuclear transport. Direct interactions with the importin/karyopherin-β proteins may also contribute to nuclear entry. Alternatively, these proteins may serve as chaperones for EBNA1 since importin-β and karyopherin-β3 have both been recently shown to protect exposed basic domains in other proteins from aggregation (43).

Both affinity column and TAP-tagging approaches detected EBNA1 interactions with the p32/TAP protein, which was previously found to bind EBNA1 in yeast two-hybrid assays (13). However, the fact that p32/TAP bound to the TBP column and has been reported to bind numerous basic proteins with no apparent functional connection (18–20) indicates that p32/TAP interacts nonspecifically with basic proteins. This promiscuity...
makes it unlikely that p32/TAP plays a role in any specific EBNA1 function, but a general role in protein folding or localization remains a possibility. It has been suggested that the interaction with p32/TAP is important for transcriptional activation by EBNA1 (44), but our finding that the EBNA1-p32/TAP interaction was not disrupted by the EBNA1 325–376 deletion, which completely abrogated transcriptional activation, does not support this conclusion.

We also identified previously unknown interactions of EBNA1 with NAP1 and the related protein TAF-I, both of which have been shown to stimulate transcription and replication of adenovirus chromatin and to affect nucleosome positioning through histone interactions (36, 37, 45). Although these interactions were detected by EBNA1 affinity chromatography, they were not detected in TAP-tagging experiments and were not enriched upon multiple rounds of EBNA1 affinity chromatography, suggesting that the interactions are relatively weak or transient. The EBNA1 interaction with NAP1, but not with TAF-I, was found to be disrupted by the 325–376 deletion, which also abrogated the transcriptional activity of EBNA1. This suggests that NAP1 might mediate transcriptional activation by EBNA1 through chromatin remodeling. EBNA1 interactions with TAF-Iα or TAF-Iβ did not correlate with transcriptional activity, but could be important for other EBNA1 functions such as activation of DNA replication and cell immortalization. TAF-Iα and TAF-Iβ are used in multiple ways to affect cellular gene expression and cell cycle progression. TAF-Iα and TAF-Iβ have been shown to be components of the INHAT complex, which inhibits histone acetylation (40); and TAF-Iβ, also known as SET, has been shown to be associated with myeloid leukemia (46) and to interact with p21Cip1 to potentially regulate cell cycle progression (38). Although our data suggest that the EBNA1 interaction with TAF-Iα may occur through nonspecific charge interactions, it is interesting that the interaction with TAF-Iβ, which can function independently of TAF-Iα, appears to be specific. It is also worth noting that the pp32 protein, which was found to interact with EBNA1 through nonspecific charge interactions, is functionally similar to TAF-I in that it also interacts with histones and is a component of the INHAT complex (40).

The remaining three new EBNA1-interacting proteins identified in this study (CK2, PRMT5, and USP7) are involved in post-translational modifications. Thus, interactions with these proteins could reflect the fact that EBNA1 is modified by these proteins and/or that EBNA1 affects the modification of cellular proteins by binding these proteins. The EBNA1 interaction with CK2 was consistently observed by both the affinity chromatography and TAP-tagging methods. CK2 is a serine/threonine kinase that has many cellular targets and is implicated in several cellular pathways, including cell cycle progression, malignant transformation, and regulation of apoptosis (39). Thus, the interaction of EBNA1 with CK2 may affect any of these pathways. The EBNA1-CK2 interaction may also reflect the regulation of EBNA1 function by CK2 phosphorylation, as EBNA1 is known to be phosphorylated at serine residues (25) and contains three putative CK2 sites.

PRMT5 (also known as JBP1) is a protein-arginine methyltransferase and is the human homolog of the fission yeast Skr1 (47). Increasing evidence points to the importance of arginine methylation in regulating a variety of protein functions (48). Methylation most often occurs at RGG sequences, a motif that occurs multiple times in EBNA1 in the functionally important region 325–376 as well as in region 33–56 (49). Thus, the EBNA1-PRMT5 interaction detected in TAP-tagging experiments could indicate that EBNA1 is methylated by PRMT5 and/or that EBNA1 influences cellular processes regulated by PRMT5. To date, PRMT5 has been implicated in signal transduction, the assembly of small nuclear ribonucleoprotein complexes, and cell proliferation (47, 50, 51).

The interaction of EBNA1 with USP7 was identified by both affinity chromatography and TAP-tagging approaches and further verified by co-immunoprecipitation of the coexpressed proteins in insect cells. This nuclear ubiquitin-specific protease was first identified by virtue of its interaction with the ICP0 protein of herpes simplex virus type 1, which is required for efficient initiation of the herpes simplex virus type 1 lytic infectious cycle (30). ICP0 is a ubiquitin-protein isopeptide ligase that promiscuously activates gene expression and induces the destruction of specific cellular proteins (52). Although the significance of the USP7-ICP0 interaction is not fully understood, the ability of ICP0 to bind USP7 correlates with its ability to activate gene expression, suggesting a role for USP7 in this process (53). We have now shown that a protein (EBNA1) from another herpesvirus (Epstein-Barr virus) also binds USP7, suggesting that USP7 may be a common target of herpesviruses.

The interaction of EBNA1 with USP7 suggested that EBNA1 might be deubiquitinated by USP7 and that this removal of conjugated ubiquitin from EBNA1 might be part of the mechanism by which EBNA1 avoids proteasomal degradation. This possibility was supported by the finding that EBNA1 that had been ubiquitinated in vitro was deubiquitinated by purified USP7 (data not shown). However, an EBNA1 mutant defective in USP7 binding exhibited the long half-life and lack of major histocompatibility complex class I presentation typical of wild-type EBNA1, indicating that USP7 binding is not responsible for preventing the turnover and presentation of EBNA1. Several studies have shown that the Gly-Ala repeat of EBNA1 is important in preventing the turnover and presentation of EBNA1, although the mechanism by which this sequence inhibits proteasomal targeting is not clear (9–11). Our results are consistent with the findings that the Gly-Ala repeat is the dominant sequence preventing proteasomal degradation of EBNA1.

We also explored the possibility that disruption of USP7 binding to EBNA1 by the Δ935–450 mutation affects the functions of EBNA1. Δ935–450 was found to replicate oriP-containing plasmids 4-fold more efficiently than wild-type EBNA1, resulting in higher maintenance levels of these plasmids. The results are consistent with a role for USP7 in regulating EBNA1 replication function, and we envisage two models of how USP7 might influence this process. The first model assumes that the effects are due to deubiquitination of EBNA1 by
USP7; and therefore, the degree of EBNA1 ubiquitination affects its ability to function in replication. In this case, ubiquitination would stimulate EBNA1 replication activity, presumably by affecting specific protein interactions. This could be due to monoubiquitination rather than polyubiquitination since monoubiquitination is emerging as a post-translational modification that can regulate protein function without affecting turnover (54, 55). The second model assumes that the effects are due to deubiquitination of cellular proteins by USP7. In this model, EBNA1 would bring USP7 to oriP, where it could deubiquitinate one or more cellular proteins that affect the activation of replication. This could mean either that ubiquitination inactivates (or targets for proteasomal destruction) a negative regulator of DNA replication.

Although our studies have initially focused on how the EBNA1-USP7 interaction affects EBNA1 functions, it is also likely that sequestering of USP7 by EBNA1 affects cellular functions that are normally regulated by USP7. USP7 was recently shown to bind and deubiquitinate p53, resulting in p53 stabilization and p53-dependent growth arrest and apoptosis (56). Conversely, a catalytically inactive USP7 point mutant caused increased p53 ubiquitination and destabilization. If EBNA1 efficiently sequesters or inactivates USP7, then we would expect EBNA1 to destabilize p53, thereby promoting cell cycle progression and inhibiting apoptosis. Such effects may be important for host cell immortalization by EBV as well as in the development of EBV-associated tumors. The possibility that EBNA1 directly contributes to these processes is supported by the fact that some EBV tumors express only EBNA1 and by the ability of EBNA1 to induce B-cell neoplasia in transgenic mice (8). It will be interesting to determine how the interaction of EBNA1 with USP7 and the other cellular partners identified here contributes to EBV-induced immortalization and tumorigenesis.

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