Protein Interaction Domains of the Ubiquitin-specific Protease, USP7/HAUSP*

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Melissa N. Holowaty‡, Yi Sheng§, Tin Nguyen‡, Cheryl Arrowsmith§, and Lori Frappier¶

From the ¶Department of Medical Genetics and Microbiology and §Ontario Cancer Institute, Department of Medical Biophysics, University of Toronto, Toronto M5S 1A8, Canada

USP7 or HAUSP is a ubiquitin-specific protease in human cells that regulates the turnover of p53 and is bound by at least two viral proteins, the ICP0 protein of herpes simplex type 1 and the EBNA1 protein of Epstein-Barr virus. We have overexpressed and purified USP7 and showed that the purified protein is monomeric and is active for cleaving both a linear ubiquitin substrate and conjugated ubiquitin on EBNA1. Using partial proteolysis of USP7 coupled with matrix-assisted laser desorption ionization time-of-flight mass spectrometry, we showed that USP7 comprises four structural domains; an N-terminal domain known to bind p53, a catalytic domain, and two C-terminal domains. By passing a mixture of USP7 domains over EBNA1 and ICP0 affinity columns, we showed that the N-terminal p53 binding domain was also responsible for the EBNA1 interaction, while the ICP0 binding domain mapped to a C-terminal domain between amino acids 599–801. Tryptophan fluorescence assays showed that an EBNA1 peptide mapping to residues 395–450 was sufficient to bind the USP7 N-terminal domain and did so with a dissociation constant of 0.9–2 μM, whereas p53 peptides spanning the USP7-binding region gave dissociation constants of 9–17 μM in the same assay. In keeping with these relative affinities, gel filtration analyses of the complexes showed that the EBNA1 peptide efficiently competed with the p53 peptide for USP7 binding, suggesting that EBNA1 could affect p53 function in vivo by competing for USP7.

The modification of proteins by the addition of ubiquitin controls a wide variety of cellular processes in eukaryotes. Formation of branched polyubiquitin chains on the ε amino group of select lysines in a target protein directs the protein for degradation by the 26S proteasome, and this system of selective protein degradation plays an integral role in many cellular processes, such as cell cycle progression, signal transduction, differentiation, and chromosome segregation (reviewed in Refs. 1–5). Important biological effects can also be incurred by the addition of single ubiquitin moieties to proteins, a modification that regulates protein function without targeting the protein for proteasomal degradation (reviewed in Refs. 6 and 7).

Both poly- and monoubiquitination can be reversed by deubiquitinating enzymes (DUBs)3 that specifically cleave the isopeptide bond at the C terminus of ubiquitin. DUBs also generate the pool of free ubiquitin both by liberating ubiquitin from precursor ubiquitin fusion proteins and by recycling ubiquitin from the branched polyubiquitin chains of degraded proteins (reviewed in Refs. 8–10). The DUBs are comprised of two groups of enzymes, the UCHs (ubiquitin C-terminal hydrolases) and the USPs (ubiquitin-specific proteases; referred to as UBP in yeast). The UCHs are small, closely related proteases (20–30 kDa in size) that are generally involved in cleaving ubiquitin from small processed peptides. The USPs are more numerous, much larger in size (60–300 kDa), and are thought to have specific protein targets. USPs can be identified by conserved sequences within the active site, but sequences outside of the catalytic domain are highly divergent, likely reflecting their role in mediating interactions with different protein targets (10–12). There are 16 known UBP s in Saccharomyces cerevisiae , and 63 putative USP genes in humans (8). Single and even multiple UBP deletions in yeast generally produce minimal phenotypic abnormalities, suggestive of functional redundancies among the yeast UBP family (13). However, studies have shown that USPs can play specific roles in various biological processes in higher eukaryotes, suggesting a more specialized role as cellular regulators in multicellular organisms. Specific DUBs have been shown to regulate eye development (14, 15), cell growth in response to cytokines (16), oncogenic transformation (17–19), cell cycle regulation (11), chromatin structure (7), and transcriptional regulation (20, 21).

The human DUB called USP7 or HAUSP (herpes-associated ubiquitin-specific protease), was first identified by virtue of its interaction with the herpes simplex virus type I immediate early protein, ICP0 (also called Vmw110) (22, 23). ICP0 is an E3 ubiquitin ligase (24) that is important for induction of the lytic infectious cycle. ICP0 promiscuously activates gene expression (25) and induces the destruction of some cellular proteins, including those of kinetochores (26) and ND10 nuclear structures (27). USP7 binds ICP0 through the C-terminal portion of ICP0, which is important for activating gene expression, and the ability of USP7 to bind ICP0 correlates with the ability of ICP0 to activate gene expression, suggesting a role for USP7 in this process (28).

USP7 is also the target of another herpes virus protein, namely the EBNA1 protein of Epstein-Barr virus (EBV) (29).

3 The abbreviations used are: DUB, deubiquitinating enzyme; UCH, ubiquitin C-terminal hydrolase; USP, ubiquitin-specific processing protease; E3, ubiquitin-protein isopeptide ligase; EBV, Epstein-Barr virus; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; GST, glutathione S-transferase; ALLN, N-acetyl-Leu-Leu-norleucinal; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PBS, phosphate-buffered saline; TEM, N-ethylmaleimide; MES, 4-morpholineethanesulfonic acid.

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EBNA1 binds to the latent origin of DNA replication and governs the replication and segregation of the EBV episomes during latent infection (30, 31). EBNA1 also activates the expression of other viral latency genes and is implicated in the immortalization of host cells by EBV (32, 33). EBNA1 is also found to interact when co-expressed in insect cells, indicating that no additional human proteins are required for this interaction. This interaction was selectively disrupted by deletion of EBNA1 residues 395–450, just upstream of the DNA binding domain, and the resulting EBNA1 mutant had increased DNA replication activity, suggesting that USP7 can influence the efficiency of EBV replication from the latent origin.

Insight into the cellular function of USP7 came from a recent study by Li et al. (34), who showed that USP7 regulated the turnover of p53. USP7 was isolated on a p53 affinity column and subsequently shown to co-immunoprecipitate with p53. Overexpression of USP7 stabilized p53 and induced p53-dependent growth repression and apoptosis. These effects were likely due to deubiquitination of p53 by USP7, because the overexpression of USP7 resulted in increased levels of deubiquitinated p53, and because dominant negative effects on p53 levels and ubiquitination were observed using a catalytically inactive USP7 point mutant. A role for USP7 in apoptosis was also supported by a study by Vugmeyster et al. (35), who reported the caspase-dependent proteolysis of USP7 during apoptosis in thymocytes. The p53-USP7 interaction was shown to involve the N-terminal portion of USP7 and the C-terminal regulatory region of p53 (11). This N-terminal region of USP7 contains a TRAF domain (tumor necrosis factor-receptor associated factor), which interacts in vitro with the human TRAF proteins 1–6 and is necessary for the nuclear localization of USP7 (36).

The interaction of viral proteins with USP7 suggests that some viruses may influence cellular events by reprogramming or altering the activity of USP7. We have begun to explore this possibility by defining the domain structure of USP7 and determining which domains are bound by EBNA1 and ICP0. Here we show that EBNA1 binds the N-terminal USP7 domain with 10-fold higher affinity than p53, effectively competing with p53 for USP7, while ICP0 binds a C-terminal domain.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of USP7**—A USP7 baculovirus was constructed using full-length USP7 cDNA (kindly provided by R. Everett) in a pET-3a vector. The cDNA was excised with NdeI and HindIII, and cloned into the same sites of pET-15b (Novagen), downstream of the LexA fusion and an in vitro tandem affinity purification-tagging approach to profile cellular protein interactions with EBNA1, and identified USP7 as specifically interacting with EBNA1 in both assays (29). USP7 and EBNA1 were also found to interact when co-expressed in insect cells, indicating that no additional human proteins are required for this interaction. This interaction was selectively disrupted by deletion of EBNA1 residues 395–450, just upstream of the DNA binding domain, and the resulting EBNA1 mutant had increased DNA replication activity, suggesting that USP7 can influence the efficiency of EBV replication from the latent origin.

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by addition of 2X complete protease inhibitor (Roche Applied Science) and placing on ice until loading onto the affinity columns. Protein bands in SDS-PAGE were excised and identified by complete tryptic digestion, followed by identification of the tryptic peptides by MALDI-TOF mass spectrometry as previously described (29), using PAWS or ProFound software (Proteomics).

EBNA1 Affinity Columns—EBNA1(lacking most of the Gly-Ala repeat region) was expressed in insect cells with an N-terminal hexahistidine tag and purified as previously described (29). Purified EBNA1 was coupled to Affi-Gel-10 at a concentration of 1 mg of EBNA1/ml of resin as described in Holowaty et al. (29). 25-μl affinity columns were equilibrated in buffer C (20 mM Tris-HCl, pH 7.6, 0.2 mM NaCl) before addition of 64 μg of the partially proteolyzed USP7 or an equimolar mixture of individually expressed and purified USP7 fragments (400 pmol each fragment). Columns were washed with 8 column volumes of buffer C, and bound polypeptides were eluted by increasing the NaCl concentration to 1 M and subsequently by the addition of 1% SDS (to buffer C). Column fractions were analyzed by using SDS-PAGE and Coomassie Blue staining.

ICP0 Affinity Columns—Amino acids 594–775 of ICP0 of HSV-1 was expressed as a GST fusion in BL21(DE3)pLysS cells from pGEX-ES2 (kindly provided by R. Everett (22)). After a 4-h induction at room temperature with 0.1 mM IPTG, cells from 25 ml of culture were lysed by sonication in PBS plus 0.5% Triton X-100 and complete protease inhibitors (Roche Applied Science). After clarification of the lysate by centrifugation, the supernatant was incubated with 100 μl of glutathione-Sepharose beads (Amersham Biosciences) overnight at 4 °C. The beads were washed three times with PBS, twice with PBS containing 300 mM NaCl, and twice more with PBS, before being resuspended in PBS. 25-μl columns were generated from the ICP0-bound beads, and these were equilibrated in buffer C and used to assess binding of partially proteolyzed USP7 fragments as described for EBNA1 affinity columns.

Generation of EBNA1 Peptide—An EBNA1 fragment coding for amino acids 395–450 was PCR-amplified and cloned between the NdeI and BamHI sites of pET15b (Novagen), downstream of the hexahistidine tag. The EBNA1 peptide was expressed at 25 °C in BL21(DE3)pLysS cells after a 4-h induction with 0.5 mM IPTG. Cells were lysed by sonication in buffer D (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 1% Triton X-100, 5 μM imidazole, and complete protease inhibitor (Roche Applied Science)), and the lysate was clarified by centrifugation for 30 min in an SS34 Sorvall rotor at 15,000 rpm. The supernatant was incubated with Talon cobalt resin (Clontech) overnight at 4 °C, and, after extensive washing with buffer D lacking detergent, the peptide was eluted with buffer D containing 250 μM imidazole. The eluted EBNA1 peptide was dialyzed against buffer C and further purified by passing through a 50,000 molecular weight cut-off Centricon filter (Millipore). The concentration of the peptide was determined by A280.

Generation of p53 Peptide—Human p53 fragments encoding amino acids 311–393 and 355–393 were cloned between the NdeI and BamHI sites of pET15b downstream of a hexahistidine tag. p53 proteins were overexpressed in BL21 CodonPlus (Stratagene) overnight at 30 °C. Three hours after induction at 25 °C, cells were lysed by sonication in buffer D, and the tagged peptides were purified from the clarified lysate on Talon cobalt columns as described for the EBNA1 peptide. After concentration by ultracentrifugation in a Centricon with a 5000 molecular weight cut-off, the concentration of the p53(311–393) peptide was determined by A280, and the concentration of the p53(355–393) peptide (which lacks aromatic residues) was measured by comparing with protein standards on a SDS-PAGE gel.

Tryptophan Fluorescence Assay—Fluorescence measurements were performed in an Aviv ATP105 ratio spectrofluorometer. Peptide binding titrations were performed by stepwise mixing of the target peptide (monomeric p53(355–393), tetramer of p53(311–393), or EBNA1(395–450)) with the USP7(1–205) fragment, using a MicroLab 500 series automated titrator. Binding reactions contained 1 μM USP7 and in 2 ml of 20 mM Tris, pH 7.6, 200 mM NaCl. After mixing, the samples were incubated for 1 min at room temperature, and the degree of binding was assessed by measuring the total tryptophan fluorescence at 350 nm, using an excitation wavelength of 295 nm. The change in fluorescence signal was plotted as a function of target peptide concentration and fit to the following equation as described in Maxford and Davidson (37), using Kaleidograph software to determine the Kd values (38).

\[ I = \left( I_0 - \left( I_{\text{cleaved}} - I_{\text{uncleaved}} + K_d I_{\text{protein}} \right) \right) \frac{[\text{Peptide}][I_{\text{protein}}]}{[\text{Peptide}]} + K_d I_{\text{protein}} + I_0 \] (Eq. 1)

RESULTS

Assessment of Protein Interactions by Gel Filtration—The USP7 N-terminal fragment spanning amino acids 62–205, the p53(355–393) fragment, and the EBNA1(395–450) fragment were analyzed individually and in combination by size exclusion chromatography. To this end, 0.88 μmol of each protein (individually and in combination) in a 2-ml volume was dialyzed overnight at 4 °C against 20 mM Tris, pH 7.5, 200 mM NaCl, 2 mM DTT, 1 mM PMsF, and 1 mM benzamidine, then applied to a HiLoad 26/60 Superdex-75 prep grade column (Amersham Biosciences). 6-ml fractions were collected from this column, and 20-μl aliquots of each fraction containing protein were analyzed by using SDS-PAGE and Coomassie Blue staining. Analysis of molecular mass standards (Amersham Biosciences) on this column showed that markers of molecular weights 67, 43, 25, and 14 peaked at fractions 28, 31, 35, and 37, respectively.

Purification and Biochemical Characterization of USP7—Full-length USP7 was expressed as a hexahistidine fusion in insect cells from a baculovirus. Whole cell lysates showed that full-length USP7 (130 kDa) was expressed at high levels in a soluble form (Fig. 1A). The tagged USP7 was purified on a metal chelating column yielding ~9 mg of purified USP7 from 1.3 × 10^9 insect cells (Fig. 1A). To determine if the purified recombinant USP7 was catalytically active, we tested its ability to cleave a linear ubiquitin fusion protein, GST-Ub52, which was previously shown to be cleaved by USP7 when the two proteins were expressed in E. coli (23). Purified USP7 was combined with purified GST-Ub52 at an enzyme:substrate molar ratio of 1:1000, and the cleavage of the ubiquitin moiety within GST-Ub52 was monitored over time by SDS-PAGE, followed by SYPRO-orange protein staining. As shown in Fig. 1B, USP7 efficiently cleaved GST-Ub52 under these conditions, cleaving 50% of the substrate in ~2 min.

![Fig. 1. Cleavage of linear and conjugated ubiquitin by purified USP7.](image-url)
Because in vivo USP7 is thought to cleave conjugated (as opposed to linear) ubiquitin chains, we also tested the ability of the purified USP7 to cleave conjugated ubiquitin chains. These were generated on the Epstein-Barr virus EBNA1 protein, which is known to bind USP7 and therefore likely to be a target of the deubiquitination activity. Purified EBNA1 was ubiquitinated by incubation with ubiquitin in a rabbit reticulocyte lysate, which contains ubiquitin-conjugating enzymes, and the resulting EBNA1 species were detected by Western blotting using an EBNA1 antibody. As shown in Fig. 1C (lanes 2–5), EBNA1 was quickly converted to a ladder of higher molecular weight conjugates, typical of a heterogeneously ubiquitinated protein. After incubation with purified USP7, the high molecular weight EBNA1 conjugates were reduced to the position of EBNA1 alone (lanes 6 and 7). Western blot of the lysate lacking EBNA1 showed that the EBNA1 antibody did not cross-react with proteins in the lysate (lane 1). We have also purified the EBNA1 conjugates after the ubiquitination reaction (by virtue of a histidine tag on EBNA1) and shown that the conjugates are recognized by anti-ubiquitin antibodies (data not shown). The results indicate that EBNA1 can be efficiently ubiquitinated in vitro and can be deubiquitinated by the purified USP7.

Because the linear ubiquitin cleavage assay could be easily quantified, we used it to define the sensitivity of the catalytic activity of purified USP7 to various salt concentrations, divalent cations, pH, and protease inhibitors. Time courses of GST-Ub52 cleavage were performed multiple times, and average values are shown in Fig. 2. USP7 cleaved GST-Ub52 at a similar rate in NaCl concentrations up to 120 mM, but this cleavage rate sharply decreased when the NaCl concentration was increased to 195 mM and higher (Fig. 2A and Table I). However, USP7 remains partially active even at 520 mM NaCl, as 80% of the substrate was cleaved in this condition upon overnight incubation with a 1:1000 molar ratio of USP7:GST-Ub52 (data not shown). USP7 was also found to be quite tolerant of high pH, because it exhibited complete GST-Ub52 cleavage activity at pHs between 7.0 and 9.5 (Fig. 2C). A marked decrease in activity was seen at pH 6.2, however. As expected based on studies with other USPs, USP7 catalytic activity was effectively inactivated by the thiol-blocking reagent N-ethylmaleimide (NEM) at 1 mM, but was not affected by the proteasomal inhibitor, N-acetyl-Leu-Leu-norleucinal (ALLN), or the serine protease inhibitor, PMSF, at the same concentration (Fig. 2D). The inhibition by NEM was reversible, because USP7 activity was restored upon quenching of the NEM with DTT (data not shown).

When the effect of divalent cations on USP7 catalytic activity was examined, the presence of 5 mM MnCl₂, MgCl₂, or CaCl₂ was found to decrease the rate of cleavage of GST-Ub52—3-fold (Fig. 2B and Table I). In keeping with the inhibitory effect of divalent cations on USP7, we also observed a modest stimulation of the rate of substrate cleavage by USP7 in the presence of 1 mM EDTA (Fig. 2B and Table I). The observed effects of metals and metal-chelating agents on substrate cleavage by USP7 could be due to effects on the structure of either USP7 itself or the GST-Ub52 substrate. We considered the possibility that the divalent cations might be activating a trace protease contaminant in the USP7 preparation, which could then degrade a proportion of the USP7 during the 10-min preincubation step, prior to the addition of the GST-Ub52 substrate. This possibility was ruled out by showing that the preincubation of USP7 with 5 mM CaCl₂ did not irreversibly inhibit USP7 activity; wild type activity was restored when 10 mM EDTA was added after preincubation with the divalent cation (data not shown).

Some USPs have been reported to be monomeric, whereas others appear to be dimers (39–41), but the native aggregation state of USP7 has not been determined (41). We investigated the native state of USP7 by analytical centrifugation, analyzing three different concentrations of USP7 under conditions in which the enzyme is active. The data indicated that USP7 was...
TABLE I

| NaCl concentration (mM) | Divalent cation | Relative rate of activity |
|------------------------|-----------------|--------------------------|
| 35                     | None            | 100                      |
| 120                    | None            | 75                       |
| 195                    | None            | 18                       |
| 270                    | None            | 5.8                      |
| 520                    | None            | 0.4                      |
| 35 MnCl₂ (5 mM)        |                 | 29                       |
| 35 MgCl₂ (5 mM)        |                 | 33                       |
| 35 CaCl₂ (5 mM)        |                 | 29                       |
| 35 EDTA (1 mM)         |                 | 167                      |

Effect of salt and divalent cations on GST-Ub52 cleavage by USP7

Slopes from the linear portion of the plots in Fig. 2 (A and B) were determined and expressed as a percentage of the slope obtained with 35 mM NaCl in the absence of added divalent cations or EDTA (all reactions also contained 20 mM Tris-HCl, pH 7.5, and 10 mM DTT).

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Homogeneous with an apparent molecular mass of 128,815 Da, which approximates the calculated molecular mass of 130,436 Da for hexahistidine-tagged USP7. Therefore full-length USP7 is monomeric under the conditions tested.

Mapping of the Protein Interaction Domains of USP7—USP7 has been shown to bind p53, the ICP0 protein of herpes simplex, and the EBNA1 protein of Epstein-Barr virus. Although the p53-binding region of USP7 has been mapped to the N terminus between amino acids 53 and 208 (11), the regions responsible for binding ICP0 and EBNA1 have not been determined. To map the protein interaction domains of USP7, we first used partial proteolysis to define the structural domains of USP7. To this end, purified USP7 was incubated with either trypsin or chymotrypsin for increasing amount of time until distinct proteolytic products were observed by SDS-PAGE, which were relatively resistant to further digestion. Such a pattern of bands became evident after a 2-h incubation with 1:100 mass ratio of trypsin and a 1:50 mass ratio of chymotrypsin, and examples are shown in the “load” lanes in Figs. 3A, 3B, and 5. These experiments were performed numerous times, and the protease-resistant fragments generated were identified by excising the bands from SDS-PAGE, completely digesting the excised polypeptides with trypsin and identifying the tryptic fragments by MALDI-TOF mass spectrometry. A summary of the protease-resistant USP7 fragments obtained from these experiments is shown in Fig. 4, in relation to the position of the previously mapped catalytic domain (amino acids 208–560) (11). This method identified two structural domains C-terminal to the catalytic domain, mapping approximately to amino acids 600–870 and amino acids 885–1061. It also showed that the region N-terminal to the catalytic domain consisted of a single structural domain beginning at amino acid 67. The C-terminal edge of this domain was not well defined by protease digestion but likely extends to close to the catalytic domain.

To determine which USP7 domain interacted with EBNA1, purified EBNA1 was coupled to Affi-Gel-10 to generate EBNA1 affinity columns, and partially proteolyzed USP7 was applied to the column in buffer containing 200 mM NaCl. After washing the column, the EBNA1-bound USP7 fragments were eluted, first with high salt and then with SDS. Examples of experiments performed with USP7 partially digested with trypsin and with chymotrypsin are shown in Fig. 3 (A and B, respectively). Identical experiments were performed using Affi-Gel-10 columns lacking EBNA1 as a negative control. Experiments with the USP7 tryptic fragments consistently showed that the fragments spanning the N-terminal domain of USP7 were preferentially retained on the EBNA1 column, whereas fragments from the C-terminal region flowed through the column (fragments 599–871 and 900–1050 in Fig. 3A). Preferential retention of USP7 fragments was not observed on the control column. Similar results were obtained using USP7 chymotryptic fragments; in this case fragments containing the N-terminal domain were retained on the EBNA1 column (fragments 67–322 and 67–254 in Fig. 3B), whereas fragments containing C-terminal and catalytic sequences flowed through the column (fragments 263–745 and 785–1061 in Fig. 3B). Fig. 4 shows all of the USP7 fragments that were retained on the EBNA1 columns from three experiments. The results show that EBNA1 binding occurs through USP7 sequences located in the N-terminal portion of the protein.

Although all of the USP7 proteolysis fragments that bound EBNA1 contained the USP7 N-terminal domain, most of the fragments also contained all or part of the catalytic domain. To clarify whether EBNA1 binding occurred through the USP7 N-terminal domain or involved sequences from the catalytic domain, we expressed and purified USP7 fragments containing only the N-terminal domain (1–205 and 56–205), only the catalytic domain (202–580) or both the N-terminal and catalytic domains (1–580). These fragments were combined in equimolar amounts, along with purified full-length USP7 (1–1102), and applied to an EBNA1 affinity column (Fig. 3C). All of the USP7 fragments containing the N-terminal domain were retained on the EBNA column and eluted in high salt, whereas the catalytic domain flowed through the column. None of the USP7 fragments were retained on negative control columns lacking protein (control) or containing the C-terminal portion of EBNA1 (amino acids 452–641), which contains the DNA binding domain but lacks the USP7 binding region. The results indicate that EBNA1 binds to USP7 through the N-terminal domain located between amino acids 56–205 of USP7, which is the same domain that has been shown to bind p53 (11).

We also used partially proteolyzed USP7 to determine which USP7 domain binds the ICP0 protein of herpes simplex. To this end, a fragment of ICP0 (amino acids 594–775) that was previously shown to be sufficient to bind USP7 was expressed as a GST fusion and bound to glutathione-Sepharose (22). As shown in Fig. 5A, when partially proteolyzed USP7 was passed through the ICP0 column, two USP7 fragments, 323–801 and 524–801, were retained on the column and eluted in high salt; fragments containing the N-terminal and catalytic USP7 domains and the second C-terminal domain (amino acids 885–1084) flowed through the column. USP7 fragment were not found to be retained on the negative control column containing GST alone. The analysis of the interaction of USP7 fragments with ICP0 affinity columns was conducted three times, and the results are summarized in Fig. 5B. The results indicate that ICP0 interacts with a different USP7 domain than that bound by EBNA1 and p53 and that this domain is located between the catalytic domain and amino acid 801.

Comparison of the USP7 Binding Affinities of EBNA1 and p53—We have previously shown that USP7 binds to EBNA1 but not to an EBNA1 deletion mutant lacking amino acids 395–450, suggesting that the USP7 binding site is between amino acids 395–450. To determine whether this fragment of EBNA1 was sufficient to bind USP7 and to obtain quantitative data on the USP7-EBNA1 interaction, we used a tryptophan fluorescence assay. USP7 fragment 1–205, containing the N-terminal domain shown to bind to both EBNA1 and p53, encodes 6 tryptophan residues. An EBNA1 peptide containing amino acids 395–450 (and lacking tryptophan residues) was expressed as a hexahistidine fusion, purified, and titrated with the USP7 (1–205) polypeptide. Initial emissions scans, using an excitation wavelength of 295 nm, showed that the greatest change in fluorescence intensity occurred at 350 nm, and therefore emissions at this wavelength were monitored as a measure of binding. Changes in the intensity of the fluorescence signal

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were monitored at each peptide concentration and plotted. A representative experiment is shown in Fig. 6A. Dissociation constants calculated from two separate experiments were 0.88 and 2.07 nM, indicating that EBNA1 residues 395–450 are sufficient to bind USP7 with high affinity.

To determine how the affinity of USP7 for EBNA1 compares to that for p53, we repeated the tryptophan fluorescence binding assay with the USP7-(1–205) fragment and a p53 peptide (amino acids 355–393) that contains the sequences previously shown to be sufficient to bind USP7 (11) and that lacks tryptophan residues. As shown in the titration in Fig. 6B, this p53 peptide bound USP7 with significantly lower affinity than the EBNA1 peptide, giving dissociation constants of 12.0 and 16.6 nM in two separate experiments. Because the p53-(355–393) peptide is monomeric, but full-length p53 is a tetramer, we tested the possibility that the affinity of USP7 for p53 would be higher if the USP7-binding sequences of p53 were tetramerized. To this end, we produced a larger p53 peptide spanning amino acids 311–393 that contained the tetramerization domain in addition to USP7 binding region (but still lacked tryptophan residues). When the p53-(311–393) peptide was titrated with USP7, the dissociation constants were found to be similar to those of p53-(355–393), indicating that the presence of the tetramerization domain did not significantly affect the affinity of USP7 for p53.

Fig. 3. Retention of USP7 domains on EBNA1 affinity columns. A, purified USP7 was partially digested with trypsin (load), then applied to either an EBNA1 affinity column or a column without coupled protein (control), and the flowthrough was collected (FT). After washing, the protein bound to the column was eluted with high salt (eluate) followed by SDS, and all fractions were analyzed by SDS-PAGE. The identity of the USP7 fragments were determined by MADLI-TOF mass spectrometry and are shown on the left according to their amino acids numbers. The band marked by the asterisk in the SDS lane is EBNA1. B, purified USP7 was partially digested with chymotrypsin (load) and analyzed on an EBNA1 affinity column or a control column as in A. The bands marked by the asterisk in the SDS lane are EBNA1 (top) and an EBNA1 proteolysis product (bottom). C, equimolar amounts of purified USP7-(1–1102) and purified USP7 domains containing amino acids 1–580, 202–580, 1–205, and 56–205 were mixed and applied to affinity columns containing either EBNA1, the EBNA1 DNA binding domain (amino acids 452–641; EBNA452–641) or no coupled protein (control). The flowthrough (FT) was collected and, after washing, bound protein was eluted with high salt. SDS-PAGE analysis of all fractions is shown.

Fig. 4. Summary of USP7 protease-resistant domains. Purified full-length USP7 was partially digested with trypsin or chymotrypsin then analyzed by SDS-PAGE and MADLI-TOF mass spectrometry. The identity of each USP7 fragment generated from three experiments is shown according to amino acid numbers and in relationship to the position of the USP7 catalytic domain marked in black in the scheme at the top. The USP7 fragments that were found to bind EBNA1 affinity columns are marked by the solid lines, and those that did not are marked by the broken lines.
with USP7-(1–205) in the tryptophan fluorescence assay, the observed binding was very similar to that with p53-(355–393) (Fig. 6B), with a calculated dissociation constant of 8.5 μM. Therefore, the data indicate that EBNA1 binds USP7 with ~10-fold higher affinity than p53.

**EBNA1 Efficiently Competes with p53 for USP7 Binding—**

The fact that the USP7 N-terminal domain binds with higher affinity to EBNA1 than p53, suggests that EBNA1 may compete with p53 for USP7 binding. To investigate this possibility, we used size-exclusion chromatography to compare the complexes formed between equimolar amounts of the USP7 N-terminal domain (amino acids 62–205) and the p53-(355–393) peptide in the presence and absence of the EBNA1-(395–450) peptide. The migration of these proteins on a gel filtration column was then compared with that of each protein individually to assess complex formation (Fig. 7). Although most of the p53 peptide was found to bind to USP7 in the absence of EBNA1 peptide (Fig. 7D), p53 binding to USP7 was disrupted when equal molar quantities of the EBNA1 peptide was added, as evidenced by the displacement of most of the p53 peptide to the position of unbound p53 (Fig. 7F). Displacement of all detectable p53 peptide from USP7 was observed when the amount of EBNA1 peptide added was increased to a 3-fold molar excess (Fig. 7G). In contrast, the complex formed between the EBNA1 peptide and USP7 (Fig. 7E) was not disrupted by addition of equal molar amounts of the p53 peptide (Fig. 7F). Therefore, the results show that EBNA1 can effectively compete with p53 for USP7 binding.

**DISCUSSION**

It is becoming increasingly clear that deubiquitinating enzymes play important roles in a variety of cellular processes, yet to date few have been purified and characterized. We have purified USP7 and shown it to be active for cleavage of linear and conjugated ubiquitin, as well as for interactions with known protein targets. The efficiency with which USP7 cleaves linear ubiquitin in vitro (50% cleaved in 2 min with a 1:100 ratio of enzyme to substrate) compares very favorably to that reported for other purified USPs. For example, incubation of human USP5 (formerly known as isopeptidase T) with a head to tail dimer of ubiquitin at a 1:120 molar ratio resulted in only 50% cleavage of the substrate in 20 min (39). Similarly, ~50% of Ub-CEP80, Ub-DHFR, or Ub-PESTc substrates were cleaved by chick UCH-8 (thought to be a UBP despite its name) in 2 h at an enzyme:substrate ratio of 1:100 (40). Purified S. cerevi-
mine how many potentially functional domains exist outside of the catalytic domain and the approximate boundaries of the domains. The results indicate that there is one structural domain N-terminal to the catalytic domain beginning at amino acid 67, which agrees well with reports of a p53 binding domain between amino acids 53–205 (11). We have shown that, in addition to binding p53, this domain mediates interactions with EBNA1. The region C-terminal to the catalytic domain can be divided into two protease-resistant domains, mapping approximately to amino acids 622–801 and amino acids 885–1061. The first of these domains binds ICP0, whereas a function has yet to be identified for the second domain. The fact that EBNA1 and ICP0 interact with different domains of USP7 argues against the possibility that these interactions occur due to a nonspecifically sticky USP7 domain, and indicates that EBV and HSV have developed different mechanisms for targeting the same cellular protein.

Although USP7 shares little sequence homology with other USPs outside of the catalytic domain, homologues of human USP7 can be readily identified in other species by BLAST analysis. The highest sequence conservation is seen in the multicellular organisms *Mus musculus* (gi 25053918) with 99% identity, *Anopheles gambiae* (gi 31220351) with 51% identity, and Drosophila melanogaster (gi 24641480) with 47% sequence identity, but USP7 is also conserved in yeast with 34% and 30% identity in *S. pombe* (SpUbp21) and *S. cerevisiae* (ScUbp15), respectively. The conserved sequence of USP7 suggests that the function of USP7 has been evolutionarily conserved. Although it is the catalytic domain that is most highly conserved across species, there is significant sequence conservation throughout most of the N- and C-terminal domains, suggesting the functional importance of these domains. Interestingly, although residues corresponding to the N-terminal protease-resistant domain are conserved in mouse (97%, identical), mosquito (48% identical), and fruit fly (44% identical), residues corresponding to the first 58 amino acids of human USP7 are either absent (mouse) or highly divergent, suggesting that they are not critical for function. It is likely that the conserved N-terminal domain can mediate interactions with several different proteins, because, in addition to binding p53 and EBNA1, this domain has been shown to bind TRAF proteins in vitro and to be important for getting USP7 into the nucleus (36).

The fact that EBNA1 and p53 interact with the same USP7 domain, raised the possibility that EBNA1 may interfere with p53 binding to USP7. Because EBNA1 appears to bind this domain 10 times more avidly than p53, it would be expected to efficiently compete with p53 for USP7 binding if the two proteins share the same or overlapping binding sites on USP7. Gel filtration analyses of USP7-p53 and USP7-EBNA1 complexes showed that EBNA1 indeed inhibited the ability of p53 to bind USP7. EBNA1 binding to USP7, however, was not affected by p53 under the same conditions, likely due to the higher affinity of USP7 for EBNA1. The p53-USP7 interaction has been shown to stabilize p53, thereby inhibiting cell cycle progression and inducing apoptosis (34). Therefore by disrupting this interaction, EBNA1 would be expected to promote cell cycle progression and prevent apoptosis, which could be important for the host cell immortalization typical of EBV. Epstein-Barr virus efficiently immortalizes cells as part of its latent infectious cycle, and this process involves a few different EBV latency proteins (reviewed in Ref. 43). Whether or not EBNA1 plays a direct role in this process has not been clear but is suggested by the findings that transgenic mice expressing EBNA1 have a tendency to develop B-cell lymphomas (32). Cellular transformation by viruses (e.g. adenovirus, SV40, and papillomavi-
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