A RING Finger Ubiquitin Ligase Is Protected from Autocatalyzed Ubiquitination and Degradation by Binding to Ubiquitin-specific Protease USP7*

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Herpes simplex virus type 1 immediate-early regulatory protein ICP0 stimulates lytic infection and reactivation from latency, processes that require the ubiquitin E3 ligase activity mediated by the RING finger domain in the N-terminal portion of the protein. ICP0 stimulates the production of polyubiquitin chains by the ubiquitin-conjugating enzymes UbcH5a and UbcH6 in vitro, and in infected and transfected cells it induces the proteasome-dependent degradation of a number of cellular proteins including PML, the major constituent protein of PML nuclear bodies. However, ICP0 binds strongly to the cellular ubiquitin-specific protease USP7, a member of a family of proteins that cleave polyubiquitin chains and/or ubiquitin precursors. The region of ICP0 that is required for its interaction with USP7 has been mapped, and mutations in this domain reduce the functionality of ICP0. These findings pose the question: why does ICP0 include domains that are associated with the potentially antagonistic functions of ubiquitin conjugation and deconjugation? Here we report that although neither protein affected the intrinsic activities of the other in vitro, USP7 protected ICP0 from autoubiquitination in vitro, and their interaction can greatly increase the stability of ICP0 in vivo. These results demonstrate that RING finger-mediated autoubiquitination of ICP0 is biologically relevant and can be regulated by interaction with USP7. This principle may extend to a number of cellular RING finger E3 ubiquitin ligase proteins that have analogous interactions with ubiquitin-specific cleavage enzymes.

Herpes simplex virus type 1 (HSV-1) is a common human pathogen that can establish a lifelong quiescent infection in sensory neurons following primary infection of epithelial cells. Environmental stimuli such as stress and sunlight trigger periodic recurrences of lytic infection, causing cold sores and genital lesions. HSV-1 expresses three broad groups of temporally regulated genes during lytic infection, termed immediate-early (IE), early, and late (for reviews, see Refs. 1 and 2). The IE protein ICP0 has an important role in the mechanisms that govern the switch between lytic and latent infection (reviewed in Refs. 3–5). Although not essential for viral replication, ICP0 increases the probability of the virus entering lytic infection, particularly after low multiplicity infection of human fibroblasts, and in its absence, viral genomes are more likely to become repressed and establish a quiescent infection (5–7). ICP0 stimulates the expression of all three classes of viral genes by as yet uncertain mechanisms that correlate with its ability to induce the degradation of a number of cellular proteins (8–12). ICP0 includes a zinc-binding RING finger domain in its N-terminal portion, and in its C-terminal third lies a nuclear localization signal and motifs required for self-multimerization and efficient localization at specific nuclear substructures known as ND10 or PML nuclear bodies. Consistent with its ability to induce the degradation of certain cellular proteins, the RING finger domain of ICP0 has been shown to possess the ubiquitin E3 ligase activity that is typical of RING finger family proteins both in vitro and in vivo (13–17).

ICP0 has been reported to interact with a number of viral and cellular proteins (18–21). Among these interacting proteins is USP7 (also known as HAUSP (herpesvirus-associated ubiquitin-specific protease), a member of a large family of cellular enzymes that cleave ubiquitin from either α- or isopeptide-linked chains (22). ICP0 function in gene expression and viral growth assays was reduced (but not eliminated) by mutation of residues within its USP7-binding motif, suggesting that the interaction is biologically relevant (23). Although it is clear that a major component of the functions of ICP0 is derived from its ubiquitin E3 ligase activity, it is paradoxical that it should include in its C-terminal portion a domain involved in the potentially antagonistic process of polyubiquitin chain cleavage.

This study aimed to test various possible consequences of the ICP0-USP7 interaction. For example, ICP0 might inhibit the peptidase activity of USP7 or control its specificity, or USP7 might regulate ICP0 E3 ligase activity. We have studied the activities of both proteins in vitro singly and in combination and the consequences of their interaction in vivo. Although we could not detect an effect of either protein on the intrinsic catalytic activity of the other in vitro, we found that ICP0 can induce its own ubiquitination in vitro and that binding to USP7 inhibits ICP0 autoubiquitination. Consistent with these observations, a derivative of ICP0 lacking the RING finger is more stable than the wild type (WT) protein during HSV-1 infection, indicating that the ubiquitin ligase activity of ICP0 contributes to its own turnover. Conversely, a USP7-binding-deficient form
of ICP0 is highly unstable during HSV-1 infection. These observations demonstrate a mechanism that regulates RING finger E3 ligase activity that may be applicable to other members of the RING finger family, particularly those that have also been reported to interact with ubiquitin-specific protease enzymes.

**MATERIALS AND METHODS**

**Plasmids**—Plasmid pT7-USP7 was constructed by cloning the NdeI-HindIII fragment of the USP7 cDNA into pET3a (22). The USP7 initiation codon is in the NdeI site, and the fragment includes 205 nucleotides of the 3' untranslated region. The NdeI-HindIII fragment was excised and cloned into pET24a, which provided a NotI site on the 3' side of the HindIII site. The NdeI-NotI fragment of the resultant plasmid (pET24a-USP7) was excised and cloned between the EcoRI-NotI sites of pFastBac-HTa (Invitrogen), downstream of an oligonucleotide flanked by EcoRI and NdeI sites and encoding a polyhistidine tag preceding by the sequence MRGS. This plasmid was named pFastBac-HTa-USP7, and subsequently the derivative pFastBac-HTa-USP7(C223S) was constructed in which the active site cysteine residue (Cys-225) had been mutated to a serine residue. The C223S mutation is as follows.

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AAAGATACGGGcGcACTagGTACATGAC
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**Sequence I**

The changed bases are in lowercase, the two silent changes in the glycine and alanine codons create a NruI site for ease of tracking, and codon 223 is shown in bold. Plasmids pCl-USP7 and pCl-USP7(C223S) contain the wild type and C223S USP7 open reading frames inserted downstream of the human cytomegalovirus IE promoter in vector pClneo (Promega) for expression in transfected mammalian cells. Plasmid pFastbac-HTa-ICP0 (13) was modified to create pFastBac-HTa-M1 by replacing the MluI-Sall ICP0 coding region fragment with the corresponding fragment from pCM1. The N-terminal truncations eliminate the ability of ICP0 to bind to USP7 (23). Plasmid pGEXUb52 expresses ubiquitin with a natural C-terminal extension fused to GST (22). Plasmid pET28-E56 was constructed by linking the NcoI-NotI fragment of ICP0 cDNA plasmid p110-C1 (24) and the NotI-EcoRI fragment of ICP0 EcoRI linker insertion mutant p110E56 (25) in vector plasmid pET28a. Plasmid pET28-E56 expresses in bacteria a polypeptide containing ICP0 residues 1–59 linked to a C-terminal polyhistidine tag.

**Viruses and Cells—HSV-1 strain 17+ was the wild type strain used.** The mutant virus derivatives used were PXE and M1, that express forms of ICP0 lacking the RING finger (FXE) or contain amino acid substitutions in the USP7 interaction region (M1) (23, 24). All viruses were grown in baby hamster kidney (BHK) cells and titrated in T20S cells, in which ICP0 is not required for efficient replication of HSV-1 (27). U2OS cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HK2 cells were grown in Glasgow modified Eagle's medium supplemented with 10% newborn calf serum and 10% tryptophan phosphate broth. All cell growth media contained 100 units of penicillin and 100 μg streptomycin/ml.

**Baculoviruses—**Recombinant baculoviruses Ac.HTa-USP7, Ac.HTa-USP7(C223S), and Ac.HTa-M1 were generated from the plasmids described above using the Bac-to-Bac system (Invitrogen) and grown according to the supplier's guidelines. Baculovirus Ac.HTa-ICP0 has been described previously (13). For the preparation of baculovirus-infected cell extracts, Sf21 insect cells were grown in suspension in TC100 medium supplemented with 10% fetal calf serum and antibiotics as above. When the cells reached a density of 1 × 10^6 cells/ml, they were infected for 72 h using a multiplicity of infection (m.o.i.) of 2 plaque-forming units (pfu)/cell. The cells were harvested by low speed centrifugation, washed in phosphate-buffered saline (PBS), and stored at −70 °C until required.

**Expression and Purification of ICP0, ICP0-M1, and USP7**—Pellets of infected Sf21 cells harvested from 75 ml of an infected culture were resuspended in 5 ml of buffer A (100 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol, 20 mM β-mercaptoethanol, 1% Nonidet P-40) in the presence of a mixture of protease inhibitors (Roche Diagnostics). The cell suspension was gently sonicated for 30 s in a soni-bath and incubated on ice for 30 min. The extracts were diluted with an equal volume of 100 mM Tris-HCl, pH 8.0, before centrifugation at 13,000 revolutions/minute for 5 min at 4 °C. The supernatant was mixed with 200 μl of nickel-nitrotriacetic acid-agarose beads (Qiagen) for 1 h at 4 °C. The beads were washed four times with 1 ml of Buffer B (100 mM Tris-HCl, pH 8.0, 250 mM NaCl, 5% glycerol, 20 mM imidazole, pH 8.0). Proteins were eluted in three aliquots of 300 μl of Buffer C containing 250 mM imidazole, dialyzed into 100 mM Tris-HCl, pH 8.0, 250 mM NaCl, 5% glycerol, 0.1% Nonidet P-40, 2.5 mM MgCl2, and stored at −70 °C.

**Expression and Purification of GST-UB2—**Plasmid pGEX-UB2 was transformed into BL21(DE3) (LysS) bacteria, and fresh colonies were inoculated into 100 ml of yeast tryptone broth and grown to mid-log phase. Isopropyl-β-D-galactopyranoside was added to a final concentration of 0.1 mM, and then incubation was continued for 2 h. The cells were harvested by centrifugation at 3000 revolutions/minute for 15 min, resuspended in 2 ml of PBS, and lysed by sonication. Nuclei (0.54 mg) were added to 1 ml of (v/v), and the extract was clarified by centrifugation at 8000 revolutions/minute for 20 min. The soluble extract was stored at −20 °C. GST-UB252 was purified by incubating 300 μl of extract with 100 μl of glutathione-agarose beads (50% v/v in PBS) for 30 min, washing the beads three times with PBS, and eluting bound protein with 150 μl of elution buffer (50 mM reduced glutathione, 250 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 0.1% Nonidet P-40). Purified GST-UB252 protein was stored at −20 °C.

**Ubiquitin-specific Cleavage Assays—**Ub252 cleavage assays were performed by incubating GST-UB252 with USP7 in a 10-μl reaction volume in the presence of 10 nm dithiothreitol. The amount of USP7 used varied from ~200 to 600 ng, and the amount of USP7 used varied from 10 to 5 ng. The assay was carried out at 37 °C for 1 h in a buffer containing 135 mM Tris-HCl, pH 8.0, 7.5 mM NaCl, 0.06% Nonidet P-40, and 0.5% glycine and alanine codons create an NarI site for ease of tracking, and the changes were analyzed by electrophoresis on 10% SDS-polyacrylamide gels.

**Ubiquitin Ligase Assays—**In vitro ubiquitin ligase assays were performed as described by Boutell, et al. (13). Briefly, recombinant histidine-tagged ubiquitin activating enzyme (E1) and the recombinant ubiquitin-conjugating enzyme (E2) UbcH5a were expressed and purified by guest on July 24, 2018http://www.jbc.org/Downloaded from

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sham Biosciences ECL protocol prior to probing with different antibodies.

**Immune Precipitation**—Protein A-Sepharose beads coupled with anti-ICP0 mAb 11060 were incubated with equivalent amounts of either ICP0-E56 or full-length ICP0 and mixed end-over-end for 1 h at room temperature. USP7 radiolabeled with [35S]methionine was prepared by coupled in vitro transcription/translation using a rabbit reticulocyte system (Promega). The radiolabeled extracts were precleared by incubation with protein A-Sepharose beads, and then equivalent amounts of the clarified supernatant were added to either mAb 11060-coupled beads alone (control) or to beads with bound ICP0-E56 or full-length ICP0. The mixtures were incubated for 1 h at room temperature, and then the beads were washed thoroughly before being boiled in SDS-PAGE loading buffer. The complexes were analyzed by SDS-PAGE and Western blotting for ICP0 and then autoradiography to detect USP7.

**Fluorescence-activated Cell Sorter (FACS) Assays**—U2OS cells in 35-mm dishes were infected with WT HSV-1 or mutant virus M1 at a m.o.i. of 0.1 pfu/cell and harvested for FACS analysis 6 h later. The cells were washed and suspended in cell dissociation buffer (Sigma), and then total cell numbers in the sample were determined in a cell counting chamber. The cells were fixed with 5% (v/v) formaldehyde in PBS at room temperature for 1 h, the cells were washed twice and then resuspended in 0.5 ml PBS/2% calf serum containing antibodies to detect ICP4 (58S hybridoma supernatant), or serum containing antibodies to detect USP7. The fluorescence signal was analyzed on a BD Biosciences FACsCalibur machine in comparison with mock-infected control samples prepared in parallel.

**Transfection and siRNA Treatment**—U2OS cells were transfected with an equimolar mixture of three USP7 siRNAs (designed and synthesized by Ambion) or with control anti-GFP siRNA (Qiagen). The siRNA sense sequences are as follows: USP7-1, 5′-GGCAACCUUUAGUGUCUCCAT-3′; USP7-2, 5′-GGCUAGCUCCGUUAAGATT-3′; USP7-3, 5′-GGAGGAGUUVUAAAGUGATT-3′. U2OS cells were transfected with a control extract (Mock) (a rabbit reticulolysate transcription/translation using a rabbit reticulocyte system (Promega)). The radiolabeled extracts were precleared by incubation with protein A-Sepharose beads, and then equivalent amounts of the clarified supernatant were added to either mAb 11060-coupled beads alone (control) or to beads with bound ICP0-E56 or full-length ICP0. The mixtures were incubated for 1 h at room temperature, and then the beads were washed thoroughly before being boiled in SDS-PAGE loading buffer. The complexes were analyzed by SDS-PAGE and Western blotting for ICP0 and then autoradiography to detect USP7.

**RESULTS**

**Enzymatic Activity of Purified USP7 on Model Substrates in Vitro**—Previous reports have demonstrated that USP7 has ubiquitin-specific protease activity in vitro, both on model α-peptide-linked ubiquitin substrates and on isopeptide-linked p53 ubiquitin conjugates (32–34). We expressed and purified a His-tagged version of wild type USP7 (Fig. 1A) and confirmed that it can cleave a C-terminal extension from a natural ubiquitin precursor (Fig. 1B). This cleavage activity was very efficient, and by measuring the rate of cleavage of increasing amounts of substrate in the presence of limiting amounts of enzyme, we estimated a $K_m$ value of less than 5 μM for this reaction (data not shown). In contrast, USP7 displayed comparatively little isopeptidase activity on a range of model substrates in vitro. At concentrations that were sufficient to cleave all of the ubiquitin C-terminal extension substrate (Fig. 1B), USP7 did not efficiently cleave Lys48-linked tetraubiquitin (Fig. 1C) or forms of the E2 conjugating enzyme UbcH5a, autoubiquitinated with either normal or methyl ubiquitin (the latter giving only monoubiquitinated isopeptide-linked derivative) (Fig. 1, D and E). Higher amounts of USP7 (on the order of 1 μg in similar assays instead of the 5 ng used here) have, however, been shown to cleave a similar model isopeptide-linked substrate (32), and therefore the protein does not lack the potential for this activity.

It is possible that specific isopeptide-linked ubiquitinated substrates are preferentially cleaved by USP7. Indeed, USP7 has been shown to bind to and cleave ubiquitinated forms of both p53 and Epstein-Barr virus regulatory protein EBNA-1 (32, 34); although in the case of p53, the $K_m$ value was estimated to be greater than 50 μM (39). As a control for the intrinsic activity of our preparations of USP7, we generated ubiquitinated p53 in reactions using ICP0 as the E3 ubiquitin ligase (14). The addition of wild type, but not C22S3 active site mutant, USP7 protein clearly reduced the levels of ubiquitinated p53 in this system, whether the USP7 was added during the conjugation reaction (Fig. 2, left panel) or after (Fig. 2, right panel). In the latter case, the ubiquitination reaction was stopped by the addition of 20 mM EDTA (see Fig. 5B).
functional consequences in vitro of the USP7-ICP0 interaction—USP7 binds strongly to ICP0 in vitro and in vivo, and the interaction with USP7 contributes to the ability of ICP0 to transactivate gene expression and stimulate viral lytic infection (22, 23). We wished to test whether the interaction has any functional consequences for the in vitro activities of either protein. We first asked whether binding to ICP0 affects the peptidase activity of USP7. We found that 2 ng of USP7 can cleave approximately half of 500 ng of GST-Ub52 in one h. Allowing USP7 to bind to an excess of ICP0 prior to the addition of substrate had no effect on the extent of GST-Ub52 cleavage (Fig. 3 A) despite apparently quantitative binding of the available USP7 by ICP0 (data not shown). ICP0-M1 is a derivative of ICP0 with two amino acid substitutions in the USP7-binding domain. ICP0-M1 is unable to bind to USP7 (23), but its intrinsic ubiquitin ligase activity is similar to that of the wild type protein (data not shown). Like wild type ICP0, the presence of ICP0-M1 in the peptidase assay had no effect on USP7 activity (Fig. 3A).

ICP0 can generate high molecular weight, unanchored polyubiquitin chains in vitro in the presence of human E1 and either of the E2 enzymes UbcH5a and UbcH6 (13). These ubiquitin moieties are linked through lysine 48, because ICP0 cannot conjugate ubiquitin in which lysine is substituted by arginine (data not shown). To assess whether USP7 can cleave ICP0-generated polyubiquitin, we carried out an ICP0 ubiquitin conjugation reaction, inactivated the enzymes by heating to prevent further conjugation, and then added USP7. Consistent with its lack of activity on other model isopeptide-linked substrates, USP7 showed little ability to cleave ICP0-generated polyubiquitin chains (Fig. 3B). Similarly, combining USP7 with ICP0 prior to conjugation did not significantly inhibit the ubiquitin ligase activity of ICP0 (Fig. 3C). These results showed that neither USP7 nor ICP0 affect the intrinsic catalytic activities of each other in vitro.

USP7 Protects ICP0 from Autoubiquitination in Vitro—Many RING finger ubiquitin ligases can efficiently autoubiquitinate in vitro (for reviews, see Refs. 35 and 36). We tested whether full-length ICP0 could induce its own ubiquitination in vitro and whether binding to USP7 could inhibit this process. We found that ICP0 could indeed produce autoubiquitinated forms in ubiquitination assays in vitro using either methyl ubiquitin (Fig. 4A) or normal ubiquitin (Figs. 4B and 5A) in the reaction mixture. Methyl ubiquitin can be conjugated to a target protein but cannot itself serve as a target for ubiquitination. The multiple ICP0 bands formed in the presence of methyl ubiquitin indicate therefore that ICP0 has several lysine residues that are targets for ubiquitination. Interestingly, ICP0 possesses only eight lysine residues, and the presence of a ladder of at least six ubiquitinated forms with methyl ubiquitin indicates that most or all of these can be ubiquitinated.

The autoubiquitination activity of ICP0 required its RING finger domain (data not shown) and specifically utilized E2 conjugation enzymes UbcH5a or UbcH6, but not UbcH7, UbcH10, or cdc34 (UbcH3) (Fig. 4B). When ICP0 was allowed to bind to USP7 before adding the remaining components of the ubiquitination reaction, ubiquitinated forms of ICP0 were not produced (Fig. 4A). As before, the addition of USP7 did not inhibit the production of polyubiquitin chains by ICP0 in this assay (see Fig. 5D). Therefore, rather than interfering with each other’s intrinsic catalytic activity, it appears that ICP0 may recruit USP7 to protect itself from its own E3 ligase activity.

In ICP0 autoubiquitination reactions using normal ubiquitin, a smear of very high molecular weight ubiquitinated forms of ICP0 was produced (Fig. 5A, set of tracks to the right). This indicates that, in addition to autoubiquitination at multiple lysine residues, ICP0 activity can extend the ubiquitin chains by the sequential addition of multiple ubiquitin moieties. Inclusion of USP7 in the reaction mixture restored ICP0 to a ubiquitin-free form (Fig. 5A, set of tracks to the right). In this experiment, the initial ubiquitination reaction was stopped...
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Fig. 4. A, ICP0 ubiquitinates itself at multiple lysine residues in vitro, and USP7 efficiently removes the ubiquitin adducts. Purified ICP0 (50 ng) was mixed with increasing amounts of purified USP7 or the C223S active site mutant of USP7 (10 or 50 ng), as indicated, and then a mixture containing E1, UbcH5a and methyl ubiquitin in E3 ligase buffer was added. The reactions were stopped after 3 h, and then the products were analyzed by SDS-PAGE and Western blotting for ICP0. The arrow indicates the position of unmodified ICP0. B, ICP0 utilizes UbcH5a and UbcH6 for autoubiquitination but not UbcH7, UbcH10, or cdc34. Purified ICP0 was incubated with a mixture containing E1, ubiquitin, and each of the E2 conjugation enzymes, as indicated. After 5 min, the reactions were stopped and analyzed by SDS-PAGE and Western blotting to detect ICP0.

Prior to USP7 treatment by the addition of EDTA to 20 mM, EDTA inhibits the ubiquitin conjugation reaction because the E1 enzyme reaction is dependent on ATP and divalent cations, as shown in Fig. 5B. The minor level of residual ubiquitination conjugation in Fig. 5B occurred because the EDTA was added after the initial incubation of E1, E2, and ubiquitin, enabling the formation of a limited amount of ubiquitin-charged E2 enzyme and hence high molecular weight polyubiquitin chains (Fig. 5B, left) and ICP0 with a very limited number of ubiquitin adducts (Fig. 5B, right). This residual activity is negligible compared with that in samples not treated with EDTA.

To test whether binding of USP7 to ICP0 was important for the ability of the former (USP7) to deubiquitinate the latter (ICP0), plasmid pET28-E56 was constructed, which expresses the N-terminal 509 residues of ICP0 linked to a C-terminal polyhistidine tag. The expressed protein ICP0-E56 lacks the USP7-binding motif of ICP0 and was found to have an efficient autoubiquitination activity in vitro, producing high molecular weight ubiquitin adducts that must presumably be linked to the three lysine residues present in the ICP0 moiety (Fig. 5A, set of tracks to the left). ICP0-E56 does not bind to USP7 (Fig. 5C). Full-length ICP0 and ICP0-E56 (20 ng of each) were allowed to autoubiquitinate in vitro for 10 min, and then the ubiquitination reaction was stopped by the addition of 20 mM EDTA. The ubiquitinated substrates were then incubated for 5 min with 2.5 or 5 ng of USP7, and the products were analyzed by gel electrophoresis and Western blotting for ICP0. As discussed below, USP7 deubiquitinated full-length ICP0 more efficiently than ICP0-E56.

The results of the experiments in Fig. 5 illustrated several features of the effect of USP7 on ICP0. First, full-length ICP0 was almost completely deubiquitinated by USP7 even when the latter (USP7) was at a relative molar concentration of only approximately an eighth of the former (ICP0). Therefore, USP7 did not bind to and remain on a single molecule of ICP0 and protect that molecule from autoubiquitination, but rather it acted sequentially on several different ICP0 molecules; the two proteins interacted dynamically. Second, in that 2.5 ng of USP7 was able to almost completely deubiquitinate 20 ng of ICP0 in 5 min, the activity of USP7 on that substrate (ICP0) was similar to that on GST-Ub52 (where 2 ng of USP7 cleaved 50% of 500 ng of the substrate in 1 h; Fig. 3). Third, USP7 was less efficient at protecting ICP0-E56 than full-length ICP0 from autoubiquitination, implying that the USP7-binding site on ICP0 contributes to USP7 activity on this substrate. Fourth, despite the relative differences of its activity on the two substrates, USP7 was able to cleave some ubiquitin from ICP0-E56 (as shown by the loss of intensity of some of the ubiquitinated bands and an increase in the free form of ICP0-E56; Fig. 5A, left), implying that the strong USP7-binding site on ICP0 is not absolutely essential for this activity, at least in vitro. Finally, USP7 was preferentially active on the monoubiquitinated form of ICP0-E56 (illustrated by the loss of the first modified species in the reactions containing USP7; Fig. 5A, left), implying that there is some selectivity in USP7 activity imparted by the length of the polyubiquitin chain, the ICP0 lysine residue that is ubiquitinated, or both.

At first sight, there is an apparent possible discrepancy between the failure of USP7 to cleave ICP0-generated polyubiquitin chains (Fig. 3) and its efficient deubiquitination of polyubiquitinated ICP0 (Fig. 5A, right). To investigate this further, the fate of ICP0, polyubiquitin, and free ubiquitin were followed in ICP0 autoubiquitination reactions, which were stopped by the addition of EDTA and then incubated with USP7. Consistent with previous results (Fig. 3B), USP7 did not reduce the levels of bulk unanchored polyubiquitin chains in this assay, and despite the almost quantitative deubiquitination of ICP0, the amount of free ubiquitin in the mixture was not increased (Fig. 5D). Thus, consistent with Fig. 2C, USP7 did not cleave isopeptide-linked ubiquitin chains efficiently, and the release of unmodified ICP0 without a concomitant increase in free ubiquitin must mean that USP7 preferentially cleaves at the junction of the ICP0-polyubiquitin chain rather than reducing the length of the chain sequentially from its distal end. This property of USP7 is likely to be highly significant for its activity in vivo, because it is unlikely to be active on polyubiquitin chains in general.

The Stability of ICP0 Is Regulated by Its E3 Ligase Activity and by Binding to USP7 in Vivo—Although USP7 did not efficiently cleave a variety of nonspecific ubiquitinated substrates in vitro (Fig. 2), the use of large amounts of USP7 may enable it to act on ubiquitinated substrates to which it does not bind, albeit with considerably reduced efficiency compared with its effect on ICP0 (Fig. 5A and further data not shown). Accordingly, in vitro reactions using purified USP7 may identify substrates that might not be targeted by USP7 in vivo. Therefore, we investigated whether USP7 had a role in ICP0 metabolism during HSV-1 infection that was dependent on the interaction between the two proteins. U2OS cells were infected with HSV-1 viruses expressing wild-type ICP0 or a derivative lacking the ability to bind to USP7 (ICP0-M1). Because the rate of transcription and protein ex-
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pression of viral genes is very high in infected cells, any instability of ICP0 may be masked if the rate of de novo protein synthesis exceeds the rate of degradation. Therefore, cells were treated with cycloheximide 4 h after infection, and parallel samples were harvested at various times thereafter. Wild type ICP0 was not turned over rapidly in U2OS cells, because the amount of ICP0 was not greatly affected after treatment with cycloheximide (Fig. 6A). However, the USP7-binding-defective version ICP0-M1 was very unstable (Fig. 6A). Treatment of cells with MG132 at the same time as cycloheximide prevented the loss of ICP0-M1, indicating that proteasomal degradation is responsible (Fig. 6A). The observation that binding to USP7 stabilizes ICP0 suggests that ICP0 is subject to ubiquitination that is antagonized by USP7.

We were unable to determine whether the ubiquitin ligase activity of ICP0 itself is required for its degradation in U2OS cells, because the wild type protein and the RING finger mutant FXE were both stable over the time course of the experiment (data not shown). However, in BHK cells, wild type ICP0 was turned over more rapidly than in U2OS cells. After 4 h of cycloheximide treatment, WT ICP0 was reduced to ~30% of the levels at the time of treatment (Fig. 6B). In contrast, the ICP0-FXE protein, from which the RING finger has been deleted, was stable in BHK cells (Fig. 6B). This result demonstrates that the ubiquitin ligase activity of ICP0 is required for its turnover and strongly suggests that autoubiquitination is responsible. Consistent with the observations in U2OS cells, ICP0-M1 was degraded more rapidly than the wild type protein in BHK cells (Fig. 6B).

In the samples that had not been treated with cycloheximide in Fig. 6, A and B, ICP0-M1 accumulated to levels that were not greatly reduced from those of the WT protein. However, these experiments were carried out at a high m.o.i. (10 pfu/cell), which ensures rapid IE viral gene expression. Under these conditions, de novo synthesis of ICP0 may be so high that the difference in stability between ICP0 and ICP0-M1 does not substantially affect the abundance of the mutant protein. Therefore, we considered whether, at lower m.o.i. values, the rate of ICP0 synthesis might be sufficiently reduced to reveal the effect of the lack of USP7 binding on ICP0 accumulation during infection in the absence of cycloheximide. U2OS cells were infected with HSV-1 viruses expressing WT ICP0 and ICP0-M1 at various multiplicities, and then the levels of expression of ICP0 were analyzed 4 h after infection (Fig. 6C). The lowest multiplicity tested at which ICP0 was detectable at this time point in the WT HSV-1 infected cells was 0.2 pfu/cell. Cells infected with the M1 virus at this multiplicity expressed ICP0-M1 at very high molecular weight third lane are probably autoubiquiti- nated forms of ICP0 in the "right-most 3 lanes are probably autoubiquiti- nated forms of ubiquitin ligase-active truncation products containing the RING finger motif. Consistent with the ICP0-E56 results, these forms are not sensitive to USP7. B, inhibition of ubiquitin conjugation by EDTA. A ubiquitination mix containing E1, UbH5a, ubiquitin, and ATP was prepared, aliquoted, and incubated at 32 °C for 5 min. Mix- tures of purified ICP0 and EDTA at the stated final concentrations were prepared, incubated for 5 min, and then mixed with the conjugation mixture aliquots. After a further 15 min at 32 °C, the reactions were stopped by the addition of SDS-PAGE loading buffer and then analyzed by gel electrophoresis and Western blot detection of conjugated ubiqui- nitin (left panel). The filter was stripped and reprobed for ICP0 (right panel). The lower-most band is unmodified ICP0. C, ICP0E56 does not bind to USP7. Immune precipitations were conducted as described under "Materials and Methods." The immune complexes were analyzed by SDS-PAGE and Western blotting for ICP0 (upper panel) and then autoradiography to detect USP7 (lower panel). The left-most lane shows the total amount of precleared input USP7 that was added to the immunoprecipitations. D, USP7 deubiquitinates ICP0 without reducing overall levels of polyubiquitin or increasing levels of free ubiquitin. A mixture containing E1, UbH5a, ubiquitin (0.5 μg), and ATP was incu- bated alone or with ICP0 at 32 °C for 10 min, as indicated, and then EDTA was added to 20 mM. USP7 was added to one of the samples, and then all were harvested in SDS-PAGE loading buffer after a further 5 min of incubation. The products were analyzed by Western blotting for polyubiquitin, free ubiquitin, and ICP0, as indicated. The autoubiquiti- nated forms of ICP0 in the third lane are of very high molecular weight and have not resolved well or transferred to the filter efficiently in this experiment.

![Deubiquitination of autoubiquitinated ICP0 by USP7 is increased by interaction between the two proteins.](http://www.jbc.org/Downloaded from)
infected with WT HSV-1 at a m.o.i. of 10 pfu/cell, and 4 h later cycloheximide was added to 50 μg/ml. Parallel samples were harvested at the indicated times after cycloheximide addition, and levels of ICP0 were detected by SDS-PAGE and Western blotting. The samples in the right-most lanes were treated with MG132 in addition to cycloheximide. B, the RING finger of ICP0 reduces the stability of the protein. BHK cells were infected with WT HSV-1 or mutant FXE (m.o.i. 10 pfu/cell), and 4 h later cycloheximide was added to 50 μg/ml. Parallel samples were harvested at the indicated times after the addition of cycloheximide, and levels of ICP0 were detected by SDS-PAGE and Western blotting. C, the ICP0-M1 mutant protein accumulated to lower levels than the WT protein in U2OS cells infected at a low m.o.i. U2OS cells were infected at the indicated m.o.i. with WT and mutant M1 HSV-1, and the samples were harvested 4 h later. Total cell proteins were separated by SDS-PAGE, and the levels of ICP4 and ICP0 were detected by Western blotting and sequential antibody probing of the same membrane.

Fig. 6. A, the USP7-binding-defective mutant protein ICP0-M1 is unstable in infected U2OS cells. Cells were infected with WT HSV-1 or mutant virus M1 at a m.o.i. of 10 pfu/cell, and 4 h later cycloheximide was added to 50 μg/ml. Parallel samples were harvested at the indicated times after cycloheximide addition, and levels of ICP0 were detected by SDS-PAGE and Western blotting. The samples in the right-most lanes were treated with MG132 in addition to cycloheximide. B, the RING finger of ICP0 reduces the stability of the protein. BHK cells were infected with WT HSV-1 or mutant FXE (m.o.i. 10 pfu/cell), and 4 h later cycloheximide was added to 50 μg/ml. Parallel samples were harvested at the indicated times after the addition of cycloheximide, and levels of ICP0 were detected by SDS-PAGE and Western blotting. C, the ICP0-M1 mutant protein accumulated to lower levels than the WT protein in U2OS cells infected at a low m.o.i. U2OS cells were infected at the indicated m.o.i. with WT and mutant M1 HSV-1, and the samples were harvested 4 h later. Total cell proteins were separated by SDS-PAGE, and the levels of ICP4 and ICP0 were detected by Western blotting and sequential antibody probing of the same membrane.

infected of U2OS cells by the M1 virus, because levels of the major viral transcriptional regulatory protein ICP4 (which is expressed with the same IE kinetics as ICP0) in the WT and M1 virus infections were very similar, especially at the higher multiplicities (Fig. 6C). These results are consistent with the suggestion that when sufficient viral genomes are present in the cell, synthesis of ICP0 may be rapid enough to mask the difference in stability between WT ICP0 and ICP0-M1 and hence explain why the effect of USP7 binding on ICP0 stability had not previously been noted.

Another highly illustrative method to compare the expression of ICP0 by WT and M1 mutant HSV-1 viruses that could be performed, even at very low multiplicity, is to detect cells expressing viral proteins by FACS. U2OS cells were infected with either WT or M1 HSV-1 at a m.o.i. of 0.1 pfu/cell, and then replicate plates for each infection were harvested at 6 h postinfection and analyzed for either ICP4 or ICP0 expression. FACS analysis always gives a higher number of positive cells than expected from the input on the basis of pfu (5); therefore, at this multiplicity (0.1 pfu/cell), ~50% of the cells in the WT-infected sample were expressing ICP4, and an equivalent proportion were expressing ICP0 (Fig. 7). In contrast, although 32% of cells in the M1 infected culture were expressing ICP4, the proportion of cells expressing ICP0 above background levels was reduced to only 9%, and of these, 93% were below an arbitrary threshold at 10^5 on the fluorescence intensity scale. In the WT virus infection, 71% of the cells that were positive were expressing ICP0 at levels above this threshold (Fig. 7). This method therefore reveals a highly significant defect in the accumulation of USP7-binding-deficient ICP0 during HSV-1 infection of U2OS cells at low multiplicity.

Ablation of USP7 by siRNA Decreases the Stability of ICP0—To confirm that the activity of USP7 was responsible for the relative stability of WT HSV-1 compared with ICP0-M1 during HSV-1 infection, levels of USP7 were reduced by transfection of a mixture of three siRNAs targeted against the USP7 mRNA. This procedure could reduce USP7 levels to as little as 10% of those in untreated cells or cells transfected with a control anti-EGFP siRNA (Fig. 8A). U2OS cells that had been treated with siRNA were infected with WT HSV-1, and 4 h later, replicate plates were either harvested or treated with cycloheximide. After a further 4 h, the cycloheximide-treated plates were harvested and the samples analyzed by Western blotting for actin, ICP0, ICP4, and USP7 (Fig. 8B). ICP0 levels were unaffected by the anti-GFP siRNA, and in the control and anti-EGFP siRNA-treated samples, ICP0 was completely stable. However, the anti-USP7-treated cells contained only 46% of the ICP0 present in the controls, and 35% of this was lost during the cycloheximide treatment. In this experiment, the siRNA treatment reduced the level of USP7 to 26% of that in the control. None of the siRNA treatments affected the amounts of either ICP4 or actin, showing that the reduction in ICP0 levels was not due to detrimental affects on the cells or the efficiency of HSV-1 infection. These data are entirely consistent with the results of Fig. 6C, in that a decrease in the levels of USP7 has a similar effect on the accumulation of ICP0 to that caused by loss of the ability of ICP0 to bind to USP7. That the reduction in ICP0 levels did not precisely mirror that of USP7 can be explained by the ability of even low levels of USP7 to deconjugate ubiquitin from ICP0 efficiently (Fig. 5). The ICP0 that did accumulate in the anti-USP7 siRNA-treated
and Western blotting for ICP0, ICP4, USP7, and actin. Harvested after a further 4 h. The samples were analyzed by SDS-PAGE vested 4 h later, whereas the other was treated with cycloheximide and infected with WT HSV-1. One sample from each duplicate was harvested and analyzed by SDSPAGE and Western blotting. B, U2OS cells were transfected in duplicate with siRNAs, as indicated. Three days later the cells were infected with WT HSV-1. One sample from each duplicate was harvested 4 h later, whereas the other was treated with cycloheximide and harvested after a further 4 h. The samples were analyzed by SDS-PAGE and Western blotting for ICP0, ICP4, USP7, and actin.

FIG. 8. Ablation of USP7 by siRNA reduces the accumulation of WT ICP0 during HSV-1 infection. A, reduction in USP7 levels by treatment with siRNA. U2OS cells were mock-transfected or transfected with control anti-EGFP or a mixture of anti-USP7 siRNAs, as indicated. Three days later the cells were harvested and analyzed by SDS-PAGE and Western blotting. B, U2OS cells were transfected in duplicate with siRNAs, as indicated. Three days later the cells were infected with WT HSV-1. One sample from each duplicate was harvested 4 h later, whereas the other was treated with cycloheximide and harvested after a further 4 h. The samples were analyzed by SDS-PAGE and Western blotting for ICP0, ICP4, USP7, and actin.

samples was less stable than that in the control infections but not as unstable as ICP0-M1 in the cycloheximide experiment of Fig. 6A. However, this is as expected, because the siRNA treatment would not affect all the cells in the culture equivalently. Cells that were not transfected with the siRNAs would express normal levels of USP7 that would stabilize ICP0 in the usual manner, whereas cells with reduced levels of USP7 would be defective in the accumulation and stabilization of ICP0 in a similar manner to the reduction caused by the M1 mutation.

DISCUSSION

The polyubiquitin chains that act as signals for proteasomal degradation are linked by isopeptide bonds between an internal lysine residue on one ubiquitin moiety and the C-terminal glycine residue of the next. Many deubiquitinating enzymes can deconjugate polyubiquitin chains by cleaving these isopeptide bonds (37). Some deubiquitinating enzymes, such as isopeptidase T, appear to be involved in maintaining a pool of free ubiquitin in the cell by disassembling polyubiquitin chains following proteolysis. High level expression of such enzymes causes a reduction in the overall levels of ubiquitinated proteins in the cell, and they can process polyubiquitin chains in vitro (37). Another potential role for deubiquitinating enzymes is to act on certain proteins selectively, providing a “proofreading” mechanism to prevent their inappropriate degradation (34, 37, 38). Since the human genome encodes at least 90 potential deubiquitinating enzymes (37), it is likely that many of them target specific subsets of ubiquitinated proteins.

The studies reported here have investigated the functional consequences (for both proteins) of the interaction between the HSV-1-encoded ubiquitin ligase ICP0 and the cellular ubiquitin-specific protease USP7. In agreement with previous reports, (33, 34) we found that the peptidase activity of USP7 far exceeds its isopeptidase activity on model substrates. This suggests that USP7 does not play a role in maintaining a pool of free ubiquitin in the cell by deconjugating polyubiquitin chains after protein degradation. It is more likely to be a proofreading enzyme that regulates the stability of specific target proteins by antagonizing their polyubiquitination. Indeed, it has been shown that USP7 specifically deubiquitinates p53 in vitro and in vivo (34) and has a similar effect on the Epstein-Barr virus regulatory protein EBNA-1 (32). In that USP7 binds to both p53 and EBNA-1, it may be that its binding partners are protected from ubiquitination by USP7 activity. Although USP7 has also been found to bind to members of the TRAF protein family (39) and ataxin-1 (40), whether it fulfills a similar function on these partners remains to be determined.

The in vitro assays of the USP7 peptidase and ICP0 ubiquitin E3 ligase activities enabled investigation of whether the interaction between the two proteins influences their intrinsic enzymatic activities. Given that USP7 exhibited at best low cleavage activity on isopeptide-linked polyubiquitin chains, it was perhaps not surprising that it had little effect on the ability of ICP0 to conjugate unanchored polyubiquitin chains in vitro. The simple scenario that binding of ICP0 might intrinsically inhibit USP7 activity also proved not to be the case, at least with the model substrate used in our standard in vitro assay. However, we found that ICP0 can ubiquitinate itself very efficiently at multiple lysine residues in vitro and that binding to USP7 counteracts this autoubiquitination. In HSV-1-infected U2OS cells treated with cycloheximide, the USP7-binding-defective variant of ICP0 (ICP0-M1) was degraded much more rapidly than the WT protein. Infection at low multiplicity in the absence of cycloheximide yielded lower cellular levels of ICP0-M1 compared with the WT protein and resulted in a considerable decrease in the proportion of infected cells expressing detectable ICP0-M1. Furthermore, reduction of USP7 levels by siRNA treatment caused a reduction in the accumulation of ICP0 during infection of U2OS cells in a similar manner to the reduction caused by the M1 mutation. These results are very strongly supportive of the hypothesis that ICP0 recruits USP7 to protect itself from ubiquitin-mediated proteolysis. They also indicate that the phenotypes of ICP0 mutants with lesions in the USP7-binding domain are likely to be due to failure to bind to USP7 rather than to the loss of any other potential activities of this region.

There are now very many examples of RING finger ubiquitin E3 ligase proteins (and particularly their isolated RING finger domains) that have autoubiquitination activity in vitro (for reviews, see Refs. 35 and 36). Some members of this family of proteins have also been found to ubiquitinate themselves in a RING finger-dependent manner in vivo. Although in some cases (such as the brca1/BARD1 heterodimer (41)), the physiological significance of this activity is not clear, in several others, RING-mediated autoubiquitination has been shown to reduce the stability of the protein in question (42–49). Here we show that ICP0 provides another example of the principle that E3 ligase autoubiquitination in vitro affects the stability of the E3 ligase itself. Furthermore, the autoubiquitination activity of some members of the TRAF protein family that are involved in tumor necrosis factor receptor signaling has been shown to occur in response to stimulation by ligand or substrate binding (46–48) and to regulate the anti-apoptotic effects of IAP proteins after induction of apoptosis (49). If substrate binding also stimulates the autoubiquitination activity of ICP0, this could lead to the decreased stability of ICP0 when associated with its substrates, unless USP7 was also present. Such a model could explain why USP7-binding-deficient ICP0 variants appear to degrade ND10 proteins PML and Sp100 with reduced efficiency (30), because such mutant forms of ICP0 may be turned over more rapidly than the WT protein when in complex with substrates undergoing ubiquitination. This model is also consistent with the observation that HSV-1 viruses expressing such mutant ICP0 proteins exhibit reduced replication in certain
circumstances (23, 50). However, these issues are complicated by cell type differentials in the phenotype of ICP0 mutant HSV-1 viruses and by the ability of ICP0 to induce ubiquitination and degradation of USP7 itself during virus infection.\(^2\) A full analysis of the biological consequences of the biochemical interactions between USP7 and ICP0 during virus infection is outside the scope of this paper, but it is likely that the interaction is most significant when ICP0 levels and rates of synthesis are low, such as during low multiplicity of infection and the initial stages of reactivation from latency.

As well as protecting ICP0 from autoubiquitination, it is possible that recruitment of USP7 by ICP0 could protect certain proteins from ubiquitination by ICP0. Indeed, brca1 has been reported to interact with the ubiquitin protease BAP1 (52), and several members of the TRAF protein family, can cleave ubiquitin conjugates from both TRAF2 and TRAF6 and thereby regulate TRAF-mediated signaling. For example, CYLD, a member of the UBP family, can cleave ubiquitin conjugates from both TRAF2, TRAF3, and TRAF6 in vitro (51). Therefore, despite the ability of USP7 to protect p53 from ICP0-mediated ubiquitination in vitro, there is no evidence that USP7 plays a significant role in p53 stability in the HSV-1 infection situations that we have studied.

The demonstration that autoubiquitination activity can be regulated by interaction with a specific USP enzyme provides an explanation of why a ubiquitin E3 ligase protein interacts with an enzyme with an opposing activity. This example suggests a mechanism that may be more generally relevant to other ubiquitin E3 ligases of the RING finger or other classes. Indeed, brca1 has been reported to interact with the ubiquitin protease BAP1 (52), and several members of the TRAF protein family are themselves RING finger E3 ligases that interact with USP7 (39). Because autoubiquitination appears to play an important role in the regulation of TRAF2, TRAF3, and TRAF6 activity (46–48), the potential of these proteins to interact with USP7 could in principle provide another means of regulating TRAF-mediated signaling. For example, CYLD, a member of the UBP family, can cleave ubiquitin conjugates from both TRAF2 and TRAF6 and thereby regulate NFκB signaling (53–55). Thus, in addition to their roles in the cleavage of ubiquitin precursors, recycling of ubiquitin monomers, and regulation of the ubiquitination and stability of specific protein substrates, members of the diverse ubiquitin-specific protease family may also be involved in regulation of the ubiquitination mechanism itself by protecting ubiquitin E3 ligases from self-induced degradation. Recent reports demonstrating that USP7 can protect mdm2 from ubiquitination and degradation (56–58) are fully in support of this model.

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A RING Finger Ubiquitin Ligase Is Protected from Autocatalyzed Ubiquitination and Degradation by Binding to Ubiquitin-specific Protease USP7
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