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Identification of a novel higher molecular weight isoform of USP7/HAUSP that interacts with the Herpes simplex virus type-1 immediate early protein ICP0

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ABSTRACT

The Herpes simplex virus type-1 (HSV-1) regulatory protein ICP0, a RING-finger E3 ubiquitin ligase, stimulates the onset of viral lytic replication and the reactivation of quiescent viral genomes from latency. Like many ubiquitin ligases ICP0 induces its own ubiquitination, a process that can lead to its proteasome-dependent degradation. ICP0 counteracts this activity by recruiting the cellular ubiquitin-specific protease USP7/HAUSP. Here we show that ICP0 can also interact with a previously unidentified isoform of USP7 (termed here USP7$_\beta$). This isoform is not a predominantly ubiquitinated, SUMO-modified, or phosphorylated species of USP7 but is constitutively expressed in a number of different cell types. Like USP7, USP7$_\beta$ binds specifically to an electrophilic ubiquitin probe, indicating that it contains an accessible catalytic core with potential ubiquitin-protease activity. The interaction formed between ICP0 and USP7$_\beta$ requires ICP0 to have an intact USP7-binding domain and results in its susceptibility to ICP0-mediated degradation during HSV-1 infection.

1. Introduction

The Herpes simplex virus type-1 (HSV-1) immediate early (IE) protein ICP0 is required for the efficient initiation of lytic replication and the reactivation of quiescent viral genomes from latency (reviewed in Everett, 2000, 2006). Virus mutants that fail to express ICP0 are impaired in their ability to replicate and are more likely to establish a quiescent state of infection where viral gene expression becomes highly restricted (reviewed in Everett et al., 2004). The capability of ICP0 to trans-activate gene expression stems from its zinc-binding RING-finger domain, a motif that confers E3 ubiquitin ligase activity to ICP0 (Boutell et al., 2002) and which is required for ICP0 to mediate the proteasome-dependent degradation of several cellular proteins during infection, including the promyelocytic leukaemia protein (PML) (reviewed in Everett, 2006; Hagglund and Roizman, 2004). The ability of ICP0 to target specific cellular proteins for ubiquitination and proteasome-dependent degradation is thought to inhibit and/or relieve cellular repression mechanisms that either instigate or maintain viral DNA genomes in a state of transcriptional quiescence (Everett et al., 2006; Preston, 2000).

Like many RING-finger ubiquitin ligases, ICP0 catalyses its own ubiquitination, a process that can lead to its proteasome-dependent degradation. ICP0 restricts this process by recruiting the cellular ubiquitin-specific protease USP7 (also known as HAUSP (Herpes-Associated-Ubiquitin-Specific-Protease), Everett et al., 1997; Meredith et al., 1995, 1994). USP7 is capable of cleaving the ubiquitin moieties from ICP0, thereby promoting its stabilisation (Canning et al., 2004). Concurrently however, ICP0 can also catalyse the ubiquitination and proteasome-dependent degradation of USP7 during the initial stages of HSV-1 infection (Boutell et al., 2005). The resulting balance between degradation and stabilisation of both ICP0 and USP7 during infection contributes to the overall expression levels of ICP0 and subsequently its ability to act as a viral trans-activator.

USP7 is encoded by a large multiply-spliced transcript containing 32 exons that spans a genomic contig of 70793 base pairs. USP7 has been shown to interact with a number of other important viral and cellular regulatory proteins including the Epstein Barr Virus nuclear antigen EBNA-1 (Holowaty et al., 2003a,b), p53 and its associated regulators Hdm2, Hdmx, and hDaxx (Cummins et al., 2004; Cummins and Vogelstein, 2004; Li et al., 2004, 2002; Meulmeester et al., 2005; Tang et al., 2006), and histone H2A (van der Knaap et al., 2005). In each instance the catalytic activity of USP7 has been shown to directly influence the ubiquitin modification status of its associated protein-partner.

Here we identify a novel higher molecular weight (MW) isoform of USP7, termed USP7$_\beta$, which is constitutively expressed in a number of different cell types and shares a significant level of amino acid homology to the major 135 kDa USP7 isoform. Electrophilic ubiquitin-binding studies demonstrate that USP7$_\beta$ contains an

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accessible catalytic core with potential ubiquitin-protease activity. Like USP7, USP7$_\beta$ can form a direct interaction with ICP0 during HSV-1 infection that results in its susceptibility to ICP0-mediated degradation in a proteasome-dependent manner. These data suggest that multiple isoforms of USP7 may contribute to the overall stabilisation and trans-activation capability of ICP0 during HSV-1 infection.

2. Materials and methods

2.1. Viruses and cells

The wild-type (wt) HSV-1 strain used was 17+. Other viruses used were *dl1403* (ICP0 null mutant; Stow and Stow, 1986), FXE (RING-finger deletion mutant; Everett, 1989), and E52X, E58X, M1, M4, D13, D14, A78, and A8X that express forms of ICP0 that carry mutations (either substitutions or deletions) within the USP7-binding region (Everett, 1989; Everett et al., 1999; Meredith et al., 1995, 1994). All viruses were grown and titrated as previously described (Boutell et al., 2005; Everett et al., 2004). U2OS, HEp-2 and Human fetal foreskin fibroblast cells (HFFF-2; European Collection of Cell Cultures) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Vero and HeLa cells were grown in GMEM containing 10% FCS. All media was supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

2.2. Infections, Western blots, and antibodies

Cells were seeded into 35 mm dishes at a cell density of 4×10^5 cells per dish and infected the following day at the stated multiplicity of infection (moi). Cell monolayers were washed with PBS prior to harvesting in SDS-PAGE boiling mix containing 4 M urea and 50 mM DTT. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis. USP7 was detected using rabbit polyclonal antibodies; BL851 (Bethyl Laboratories, peptide antibody raised against sequences encoded by exon 3), Rb201 (peptide antibody raised against the C-terminal 16 amino acids; Everett et al., 1999, 1997), and R1 (antibody raised against full length USP7; Holowaty et al., 2003a,b). Other antibodies used were ICP0 mAb 11060 (Everett et al., 1993), Actin mAb (AC-40, Sigma–Aldrich), p53 mAb (Ab6) and phospho-specific p53ser15 mAb (Ab3, Oncogene), myc mAb (9E10), myc polyclonal (A-14), p53 polyclonal (FL-393), SUMO-1 polyclonal (FL-101), HA polyclonal (Y-11) and ubiquitin mAb (P4D1) antibodies (Santa Cruz). Densitometry analysis was carried out as previously described (Boutell et al., 2005).

2.3. GST-E52 pull down, immunoprecipitation and alkaline phosphatase analysis

Cell monolayers (equivalent to 3×10^6 cells) were harvested in 1.5 ml of buffer A (50 mM Tris pH 7.4, 500 mM NaCl, 1% NP40, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) supplemented with protease inhibitors (Roche Applied Sciences). Cells were lysed by bath sonication, incubated on ice for 30 min and clarified by centrifugation (13,000 rpm for 15 min at 4 °C). Glutathione-S-Transferase (GST) pull-downs were carried out using 75 μl of beads bound to either GST alone or GST linked to the C-terminal 594–775 amino acids of ICP0 (GST-E52; Meredith et al., 1995) for 2 h at 4 °C. The beads were washed (3×1 ml) in buffer A and soluble complexes eluted in buffer A + 50 mM reduced glutathione. USP7 immunoprecipitation experiments were carried out using 1 μg of USP7 polyclonal antisera as stated. Immune-complexes were precipitated using 30 μl (50% w/v) of equilibrated Protein G sepharose for 2 h at 4 °C. The beads were washed (3×1 ml) in buffer A and protein complexes eluted in 35 μl of SDS-PAGE loading buffer. Alkaline phosphatase (AP) assays were carried out on immunoprecipitated USP7/USP7$_\beta$ or p53 (from ultra violet (U.V.) irradiated HFFF-2 cells as previously described; Boutell and Everett, 2004) in the presence of 15 units AP (New England Biolabs) in the appropriate buffer. ICP0 immunoprecipitation experiments were carried out on U2OS cells infected with wt or mutant HSV-1 viruses at a moi of 5. One hour post-infection (PI) the cells were treated with MG132 (Calbiochem, final concentration 10 μM) to prevent ICP0-mediated degradation of USP7. The cells were harvested 4 h PI and ICP0 was immunoprecipitated using 1 μg of the 11060 mAb in buffer A.

2.4. Transfection and shRNAi knock down experiments

HEp-2 cells were transfected with either 1 μg of empty vector or vector expressing myc-USP7 (pCDNA3.1myc-HAUSP; Meulmeester et al., 2005) using Exgen500 transfection reagent (Fermentas). The cells were harvested 16 h post-transfection for immunoprecipitation using 1 μg of anti-myc polyclonal antibody (A-14) or Rb201 (as described above). USP7 shRNAi knock down experiments were carried out as previously described (Canning et al., 2004).

2.5. Mass spectrometry

USP7/USP7$_\beta$ were immunoprecipitated from HeLa cell extracts (equivalent to 2×10^8 cells/precipitation) in 15 ml of buffer A using 100 μg of Rb201 polyclonal anti-sera. Immune-complexes were eluted in SDS-PAGE loading buffer, resolved by SDS-PAGE, and stained with Coomassie Brilliant blue. The bands of interest were excised, digested and analysed by LC-MSMS using a QSTAR Pulsar XL (MDS Sciex, Toronto, Canada) and LC Packings nanoLC (Dionex, Amsterdam, Netherlands). Spectra were searched against the MSDB and NCBI databases using Mascot Daemon 2.1 (Matrix Science, London, UK).

2.6. Eletrophilic ubiquitin labelling of USP7 and cellular fractionation

Immunoprecipitated USP7/USP7$_\beta$ was incubated either in the presence or absence of 5 mM N-ethylmaleimide (NEM) for 10 min at room temperature (RT) prior to the addition of 0.8 μg of electrophilic ubiquitin probe (HA-UbVME, a gift from Hidde Ploegh; Borodovsky et al., 2001, 2002). Reaction mixtures were incubated for 1 h at RT and terminated by the addition of SDS-PAGE loading buffer. Cellular fractionation experiments were performed on HEp-2 cells (equivalent to 3×10^6 cells/lysis) using a commercial kit (NucBuster™, Novagen) following the manufacturers instructions.

3. Results

3.1. Identification of a novel higher molecular weight isoform of USP7

Following comparative Western blot analysis on whole cell HeLa extracts utilising a panel of USP7 polyclonal anti-sera (Fig. 1A), a number of potential higher MW species of USP7 were identified in addition to the major 135 kDa form. One predominant higher MW band (labelled (*), Fig. 1A) was clearly identified by three independently derived USP7 anti-sera. To determine the commonality of this potential USP7 higher MW isoform, Western blot analysis was performed on whole cell extracts from a number of different cell lines in conjunction with purified 6 × His tagged USP7 expressed from a cDNA clone (Canning et al., 2004) as a com-