A Deubiquitinating Activity Is Conserved in the Large Tegument Protein of the *Herpesviridae*

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The largest tegument protein of herpes simplex virus 1 (HSV-1), UL36, contains a novel deubiquitinating activity embedded in it. All members of the *Herpesviridae* contain a homologue of HSV-1 UL36, the N-terminal segments of which show perfect conservation of those residues implicated in catalysis. For murine cytomegalovirus and Epstein-Barr virus, chosen as representatives of the beta- and gammaherpesvirus subfamilies, respectively, we here show that the homologous modules indeed display deubiquitinating activity in vitro. The conservation of this activity throughout all subfamilies is indicative of an important, if not essential, function.

Modification of proteins by ubiquitin (Ub) plays a pivotal role in a multitude of cellular processes, including proteolysis, cell cycle control, receptor internalization, and sorting within the endo/lysosomal system (7, 14, 16). Ubiquitination is achieved by an enzymatic cascade comprising a Ub-activating enzyme (E1), several Ub-carrier proteins (E2s), and hundreds of Ub ligases (E3s). Ubiquitination can be reversed by several families of enzymes collectively designated deubiquitinating enzymes (DUBs) (1, 15).

A number of viruses have evolved strategies to manipulate the ubiquitination status of host cell proteins, both through conjugation and deconjugation (2, 4, 6, 10, 13). Recently, we reported the identification of a novel viral ubiquitin-specific protease (USP), UL36^{USP}, encoded by the herpes simplex virus 1 (HSV-1) genome (9). UL36^{USP} is a polypeptide of approximately 420 amino acids (aa) carried within the N-terminal portion of UL36, the largest tegument protein (3,164 aa) of HSV-1. This activity was detected through the use of mechanism-based, active-site-directed probes and confirmed by expression in *Escherichia coli* of a corresponding fragment that cleaves ubiquitin-based substrates. UL36^{USP} activity peaks at late stages of viral replication and appears to require proteolytic processing from full-length UL36 (9). The N-terminal UL36 fragment is well conserved in alphaherpesviruses, and a low homology to corresponding genes of the betaherpesvirus and gammaherpesvirus subfamilies was apparent in sequence alignments, but with strict conservation of the proposed catalytic residues. DUB activity may therefore be well conserved across the herpesvirus family and, if this is proven to be correct, would suggest an important function for this type of activity.

We therefore set out to investigate the possible DUB activity of two phylogenetically distant homologues of HSV-1 UL36^{USP}, each representing a different subfamily of the *Herpesviridae*. We chose UL36 homologues encoded by mouse cytomegalovirus (MCMV, M48) and Epstein-Barr virus (EBV, BPLF1) as representatives of the beta- and gammaherpesvirus subfamilies, respectively. In order to assess the degree of homology between UL36 from HSV-1 and its MCMV and EBV counterparts, a sequence alignment was generated, covering the first 336 aa (the numbering refers to HSV-1) of UL36 (Fig. 1). Overall, the homology to HSV-1 is rather low, with only 10% and 15% sequence identity for MCMV and EBV, respectively. Nevertheless, the putative catalytic triad Cys-His-Asp is strictly conserved, along with a putative oxyanion hole-forming Gln residue (Fig. 1). The conserved Cys65 is the active-site cysteine in HSV-1 UL36^{USP} (9). We therefore propose that the two homologues under investigation likewise represent cysteine protease-type DUBs. In support of this notion, a secondary structure prediction (11) shows a high degree of structural similarity despite limited sequence identity, suggesting that all members may adopt a similar tertiary structure (Fig. 1). Based on the observations that (i) the putative catalytic triad is contained within a stretch of ~200 aa and (ii) the secondary structure prediction diverges beyond position ~280, we speculate that a fragment of 280 aa or less should include the minimal domain necessary and sufficient for catalysis.

To investigate the functionality of this minimal DUB consensus domain, we cloned the relevant genomic fragments for heterologous expression and subsequent biochemical characterization. The corresponding DNA fragments, specifying aa 1 to 205 of EBV (EBV^{205}) and aa 1 to 285 of MCMV (MCMV^{285}), were PCR-cloned into pET28 according to standard procedures, using MCMV strain Smith and EBV B958 type 1 DNA as templates. Following expression in *E. coli* BL21-DE3, the fragments, equipped with an N-terminal His tag, were purified, using a Ni-NTA resin (QIAGEN), and subsequently subjected to gel filtration (S75 HiLoad; Amersham Pharmacia Biotech) in 50 mM Tris, 50 mM KCl, 50 mM NaCl, 0.5 mM EDTA, 2 mM dithiothreitol, and 10% (vol/vol) glycerol (pH 7.5) to achieve apparent homogeneity. To examine whether the purified constructs indeed display DUB activity, we used hemagglutinin (HA)-tagged Ub-vinylmethyl ester (HAUbVME), a probe that acts as a suicide inhibitor for DUBs by forming a thioether

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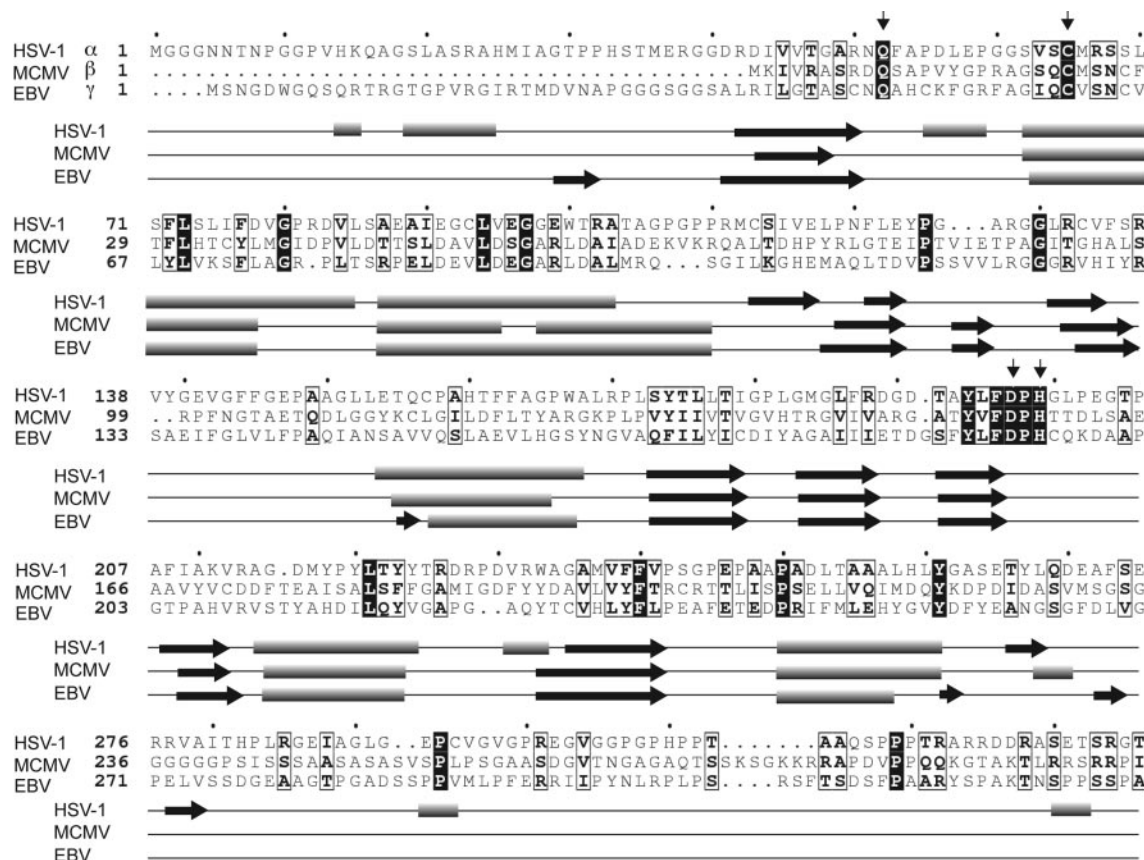
FIG. 1. Key catalytic residues and the secondary structure of a ubiquitin-specific protease are conserved in three herpesvirus subfamilies. The sequence alignment of UL36 of HSV-1 (alphaherpesvirus subfamily) and its MCMV (betaherpesvirus subfamily) and EBV (gammaherpesvirus subfamily) homologues is shown. For clarity, only the N-terminal portion of UL36 is shown. Identical residues are white on a black background, and conserved residues are boxed. Putative active-site residues are marked by vertical arrows. The predicted secondary structure is depicted in alignment with the primary sequence. Symbols: cylinder, helix; horizontal arrow, strand; line, coil.

bond with the active-site cysteine (3, 12). Reactions were performed using final concentrations of 1 μM enzyme (EBV[205] or MCMV[285]) in the absence and presence of 2 μM HAUbVME in reaction buffer (50 mM Tris, 100 mM NaCl, 1 mM dithiothreitol pH 7.5) for 30 min at 37°C. Following incubation, samples were boiled in reducing sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A shift in electrophoretic mobility, indicative of covalent modification by HAUb, was observed for both the MCMV and EBV constructs after silver staining (Fig. 2 A, upper panel). The identity of the covalent enzyme-HAUb adducts was confirmed by immunoblotting (Fig. 2 A, lower panel), using an HRP-conjugated anti-HA antibody (3F10; 1:4,000 dilution ratio; Roche) in conjunction with the Western Lightning chemiluminescence reagent kit (Perkin Elmer).

Are the proteins under investigation indeed cysteine proteases? Pretreatment of EBV[205] or MCMV[285] with 10 mM N-ethylmaleimide (NEM) for 10 min completely blocked labeling with the probe (Fig. 2 A). Replacement of the putative active-site cysteine by alanine (C61A) abrogated labeling with the probe for EBV[205] (Fig. 2 A). In the case of MCMV, we also constructed a longer variant that terminates at position 575 (MCMV[575]). Although we were unable to obtain a pure preparation owing to low

expression levels and susceptibility to proteolytic attack, a polypeptide of the expected molecular mass (62 kDa) was clearly detectable by immunoblotting, using a penta-His (QIAGEN) antibody (Fig. 2B). This variant likewise reacted with HAUbVME in NEM-sensitive fashion, and a nonconservative mutation of the putative active site (C23A) resulted in complete loss of labeling with the probe (Fig. 2B). Taken together with the established specificity of the electrophilic derivatives of Ub, we conclude that both the MCMV- and EBV-derived fragments are Ub-specific cysteine proteases.

We next tested the ability of the enzymes to cleave Ub C-terminal 7-amido-4-methylcoumarin (Ub-AMC; Boston Biochem) (5). Ub-AMC hydrolysis assays were performed in reaction buffer supplemented with bovine serum albumin (50 μg/ml) by incubating the enzymes (100 pM) with a 1,000-fold excess of Ub-AMC (100 nM). EBV[205], MCMV[285], and MCMV[575] efficiently hydrolyzed Ub-AMC, as had been shown for UL36[USP] (9). In accordance with HAUbVME labeling experiments, Ub-AMC hydrolysis was sensitive to NEM and was not observed for the active-site mutants, corroborating the identity of the proposed catalytic cysteine residue (Fig. 3 and data not shown). While a minimal DUB domain extending little beyond the active-site residues is active, we sought to determine
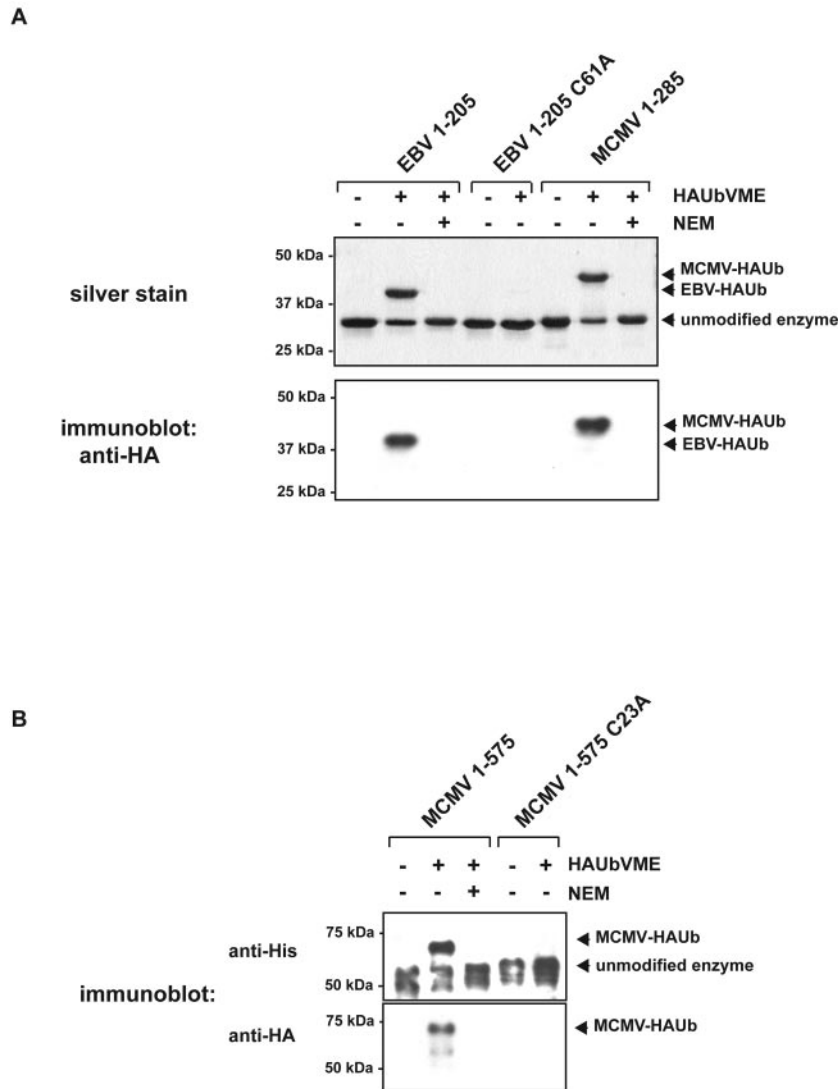
A

B

FIG. 2. MCMV and EBV encode cysteine proteases that are targeted by a ubiquitin-derived probe. (A) Labeling of MCMV and EBV protease/ tegument protein domains by HAUbVME. EBV[205], EBV[205] C61A, and MCMV[285] were incubated with a twofold molar excess of HAUbVME in the absence or presence of NEM and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Silver stain was used for the upper panel. Anti-HA antibody directed against the probe was used for the immunoblot in the lower panel. Positions of the unmodified enzymes and the covalent HAUb adducts are indicated by arrowheads on the right. (B) Labeling of MCMV[575] and its active-site mutant as for panel A, visualized by immunoblots using anti-His (upper panel) and anti-HA (lower panel) antibodies.

whether the shorter constructs are also sufficient to confer substrate specificity. To address this issue, we tested the potential of several inhibitors to block DUB activity in Ub-AMC hydrolysis assays. Enzymes were preincubated for 1 h with a 100-fold molar excess of electrophilic derivatives of Ub, SUMO, Nedd8, and ISG15, all carrying a VME moiety as the electrophile at their C terminus (8). While UbVME completely inhibited hydrolytic activity, none of the Ub-related inhibitors impaired DUB activity (Fig. 3). We conclude that a domain consisting of little more than 200 aa, as exemplified by EBV[205], mediates both catalytic activity and specificity towards Ub.

In summary, we have demonstrated that a DUB activity previously identified in HSV-1 is conserved across all subfamilies of the *Herpesviridae*. Unexpectedly, a comparatively small structurally conserved module of ~200 aa is sufficient to confer

both hydrolytic activity and Ub specificity. Sequence elements adjacent to the 200-aa core domain may be required to confer additional specificity for ubiquitinated substrates in vivo or to mediate other interactions, for example, those required for subcellular localization. It will be necessary to raise antibodies against the N-terminal domain of these tegument proteins to establish their occurrence in virus-infected cells, to explore precursor-product relationships, and to relate these parameters to the deubiquitinating activity associated with them. Having demonstrated the enzymatic activity embedded within the tegument proteins of the alpha-, beta-, and gammaherpesviruses, we postulate an important and conserved function for this activity, the identification of which will require the creation of suitable mutants in the context of the intact genomes of the corresponding viruses.
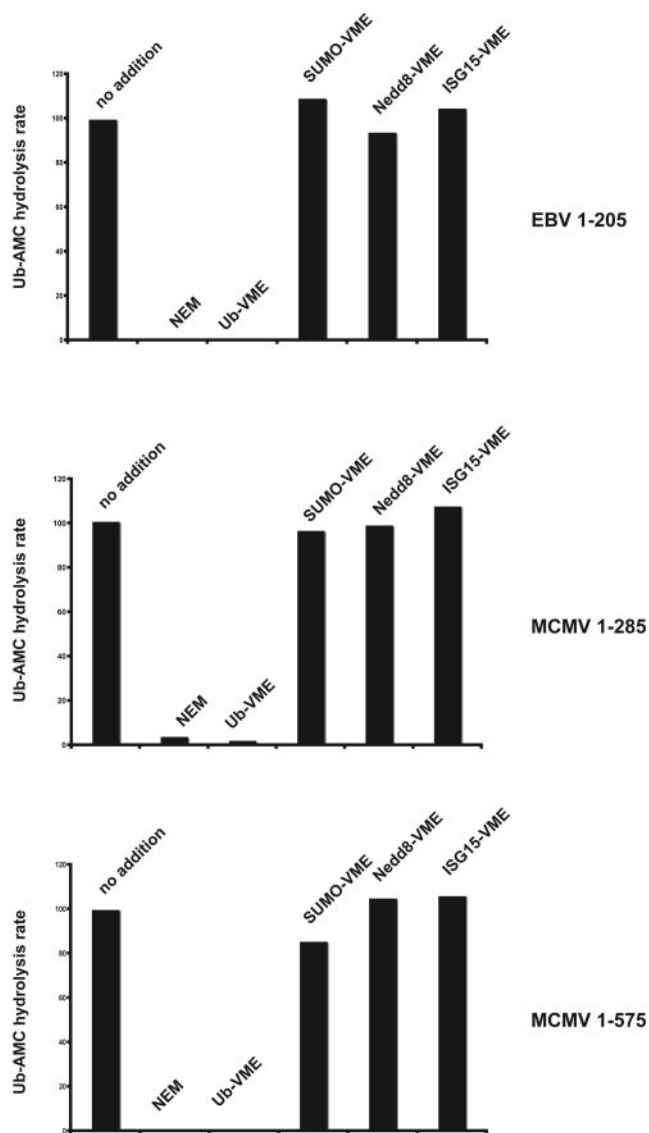
FIG. 3. UL36 homologues of EBV and MCMV contain a protease module highly specific for ubiquitin. EBV and MCMV protease/tegument protein domains used for Ub-AMC hydrolysis are indicated on the right. Enzymes were either untreated or preincubated with a 100-fold molar excess of specific inhibitors (indicated on top) for 1 h at RT prior to the addition of Ub-AMC. The rate of Ub-AMC hydrolysis by the respective untreated construct was set to 100%.

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