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E6AP/UBE3A catalyzes encephalomyocarditis virus 3C protease polyubiquitylation and promotes its concentration reduction in virus-infected cells

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Abstract

The encephalomyocarditis virus (EMCV) 3C protease (3C^{pro}) is one of a small number of viral proteins whose concentration is known to be regulated by the cellular ubiquitin-proteasome system. Here we report that the ubiquitin-conjugating enzyme UbcH7/UBE2L3 and the ubiquitin-protein ligase E6AP/UBE3A are components of a previously unknown EMCV 3C^{pro}-polyubiquitylating pathway. Following the identification of UbcH7/UBE2L3 as a participant in 3C^{pro} ubiquitylation, we purified a UbcH7-dependent 3C^{pro}-ubiquitylating activity from mouse cells, which we identified as E6AP. *In vitro* reconstitution assays demonstrated that E6AP catalyzes the synthesis of 3C^{pro}-attached Lys⁴⁸-linked ubiquitin chains, known to be recognized by the 26S proteasome. We found that the 3C^{pro} accumulates to higher levels in EMCV-infected E6AP knockdown cells than in control cells, indicating a role for E6AP in *in vivo* 3C^{pro} concentration regulation. We also discovered that ARIH1 functions with UbcH7 to catalyze EMCV 3C^{pro} monoubiquitylation, but this activity not influence the *in vivo* 3C^{pro} concentration.

Keywords

3C protease; ARIH1; E6AP/UBE3A; encephalomyocarditis virus; UbcH7/UBE2L3; ubiquitylation

1. Introduction

Protein polyubiquitylation can function as a part of a selective protein concentration control mechanism in eukaryotes by marking proteins for destruction by 26S proteasome-mediated proteolysis. Polyubiquitylation occurs through repetition of a sequential set of reactions catalyzed by a ubiquitin-activating enzyme, or E1, a ubiquitin-conjugating enzyme, or E2, and a ubiquitin-protein ligase, or E3, the latter of which recognizes ubiquitylation target

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

substrates [1–4]. Many viruses have been found to make use of, and even direct, the ubiquitin-mediated proteolysis of host cell proteins to facilitate the generation of a cellular environment favorable for virus replication [5], but relatively few studies have examined the extent to which the polyubiquitylation of viral proteins contributes to their concentration and concentration ratio regulation [5]. The 3C proteases produced by EMCV and certain other picornaviruses have been shown to be targeted for polyubiquitylation and 26S proteasome-catalyzed degradation [6–9], although the function of this degradation has not been fully explicated. 3C proteases are critical for successful virus replication, and limiting their concentrations may serve to help maximize replication efficiency through minimizing 3C^{pro} cytotoxicity [10] or may represent an antiviral defense mechanism [11].

Understanding the interplay between the cellular protein ubiquitylation machinery, picornaviral 3C^{pro} concentration regulation, and virus reproduction requires the identification of the ubiquitin system participants in 3C^{pro} polyubiquitylation. We previously identified the existence of two pathways by which EMCV 3C^{pro} polyubiquitylation might occur [12]. One of these includes the RING-type E3 UBR1, which requires a ten-amino acid destruction signal sequence/structure in the 3C^{pro} for substrate recognition, and the other includes isoforms of the E2 UbcH5. The kinetic parameters that characterize UBR1-catalyzed polyubiquitylation, however, suggest that this pathway may not be a major contributor to marking the 3C^{pro} for destruction *in vivo* [13]. The E3 that functions in the UbcH5-containing pathway is probably DTX3L, a RING-type E3 recently reported to catalyze EMCV 3C^{pro} polyubiquitylation and contribute to 3C^{pro} degradation in virus-infected cells [11]. DTX3L complexed with PARP9 also functions as a component of a potent antiviral response system that promotes interferon-stimulated gene expression [11].

In this communication, we report the identification of a third EMCV 3C^{pro} polyubiquitylation pathway. After first identifying the E2 UbcH7/UBE2L3 as an EMCV 3C^{pro} polyubiquitylation-stimulating factor, we pursued the identification of the UbcH7-dependent E3 that contributes to 3C^{pro} concentration regulation during virus infection. By using UbcH7 as part of an affinity ligand and as a 3C^{pro}-ubiquitylating activity assay component, we partially purified and identified by mass spectrometry three UbcH7-interacting E3 ubiquitin-protein ligases with the potential to participate in 3C^{pro} concentration regulation: the RBR (ring-between-ring) ligases ARIH1 and ARIH2 and the HECT (homologous to E6AP carboxyl terminus) ligase E6AP/UBE3A. *In vitro* assays revealed that in the presence of UbcH7 ARIH2 does not ubiquitylate the EMCV 3C^{pro}. ARIH1, however, catalyzes 3C^{pro} monoubiquitylation while E6AP catalyzes the synthesis of 3C^{pro}-attached Lys⁴⁸-linked ubiquitin chains, which are known to bind ubiquitin receptors in the 26S proteasome complex [3,14]. An evaluation of 3C^{pro} accumulation in EMCV-infected control and E3 knockdown cells implicates E6AP, but not ARIH1, in the regulation of 3C^{pro} concentration *in vivo*.

2. Material and Methods

Unless otherwise noted all reagents were from Sigma-Aldrich.

2.1. Plasmid constructions and expressed protein purification

All enzymes used for recombinant DNA constructions were obtained from New England BioLabs; PfuTurbo DNA polymerase (Agilent Technologies) was used for all PCR reactions.

To construct pET3d-Ub-UbcH7-GST, the UbcH7 sequence was amplified by PCR from pGEX-2T-UbcH7 [15] using the primers 5'-AAAACCATGGCGGCCAGCAGGAGGCTGATG-3' (1F) and AGGGGACATCCCGGGGTCCACAGGTCGCTTTTCCCC-3', and the GST-encoding sequence was amplified from pET42a(+) using the primers 5'-CCTGTGGACCCCGGGATGTCCCCTATACTAGGTTATGG-3' and 5'-AAAAGGATCCTTAATCCGATTTTGGAGGATGGTC-3' (2R). The products of these reactions were combined and amplified by PCR using the primers 1F and 2R to generate the sequence encoding the UbcH7-GST fusion protein, which was ligated into pET3d using the BamHI and NcoI sites to generate pET3d-UbcH7-GST. The sequence encoding ubiquitin was amplified by PCR from pGST-PKA-TEV-Ub [16] using the primers 5'-CAGATTTTCGTCAAGACTTTGACCGGTAAAACCATAACATTG-3' and 5'-GCCACACCTCTTAGCCTTAGCACAAGATGTAAGGTGG-3' and this product was ligated into pET3d-UbcH7-GST at the NcoI site following treatments with Klenow fragment, to place the ubiquitin-encoding sequence in frame with that of UbcH7-GST and generating pET3d-Ub-UbcH7-GST. Ub-UbcH7-GST was expressed from *E. coli* BL21-CodonPlus-RIL cells (Agilent Technologies) transformed with pET3d-Ub-UbcH7-GST. The expression of ubiquitin-UbcH7-GST was induced by treatment with 0.4 mM IPTG for 4 h at 28°C, and the cells were lysed by sonication at 4°C in PBS containing 1 mM PMSF, 1 mM benzamidine, and 2 mg/ml protease inhibitor cocktail. The fusion protein was recovered from the cleared lysate by glutathione-agarose column chromatography.

To construct pT7CFE1-GST-ARIH1 and pT7CFE1-GST-ARIH2, the mouse ARIH1 and ARIH2 E3 ligase-encoding sequences (NM_019927 and NM_011790, respectively) were amplified by PCR from the pTruORF plasmids containing the ARIH1- or ARIH2-encoding sequence (Origene) using the primers 5'-TTTTCATATGGTGAATGTATCCGGGAGGTCAACGAG-3' and 5'-TTTTCGCGCCGCGTCCTCAATGTACTCCACAGATCTTTTTC-3' for ARIH1 and 5'-AAAAGGATCCATGTCTGTGGACATGAACAGCCAAGGGTCAG-3' and 5'-AAAAGCGGCCGCGGTGTCGTGAAAATCCTTTAGCAGGG-3' for ARIH2. These products were ligated into pT7CFE1-NHIS-GST-CHA (Pierce/ThermoFisher Scientific) using the NdeI and NotI sites. ARIH1 and ARIH2 were synthesized in large scale *in vitro* transcription-translation reactions programed with these plasmids using the High Yield Mini IVT System (Pierce/ThermoFisher Scientific) and were purified by glutathione-agarose chromatography and treatment with HRV 3C^{Pro} (Pierce/ThermoFisher Scientific) as instructed by the supplier.

EMCV 3C^{Pro} was expressed in *E. coli* BL21(DE3)pLysS cells (Promega) and purified as previously described [6,13].

2.2. 3C^{pro} ubiquitylation assays

In vitro ubiquitylation assays were performed under E3-limiting conditions, as previously described [6,12], with the following modifications. The assay mixtures included 5 μ M of purified EMCV 3C^{pro}, 50 nM human E1 ubiquitin-activating enzyme (Uba1/UBE1, BostonBiochem), 500 nM UbcH7/UBE2L3 (BostonBiochem), 100 μ M ubiquitin (wild type, methylated, K48R, or K48 only (K480); all from BostonBiochem), and the indicated cell fractions, E3 ubiquitin-protein ligase purification fractions, or purified E3s (ARIH1, ARIH2, or E6AP isoform 2 from BostonBiochem). The mixtures were incubated at 37°C for the indicated time and 3 μ l aliquots were removed for analysis by Western blotting as previously described, using rabbit anti-3C^{pro} antibodies [6,12] or rabbit anti-ubiquitin antibodies (EMD Millipore). The blots were developed using secondary antibody-conjugated alkaline phosphatase and BCIP/NBT phosphatase substrate (KPL Inc.).

2.3. Purification and identification of the UbcH7/UBE2L3 ubiquitin-conjugating enzyme and the E3 ubiquitin-protein ligases

ATP-depleted lysates from rabbit reticulocytes prepared from whole blood (Pel-Freez Biologicals) and mouse NIH 3T3 cells (American Type Culture Collection) cultured in Dulbecco's modified Eagle's medium with 4500 mg/L glucose, 2 mM L-glutamine, and 10% (v/v) newborn calf serum (Gibco/ThermoFisher Scientific) were prepared as previously described [12,17] and fractionated into anion-exchange binding (fraction II) and non-binding (fraction I) proteins [17]. The ubiquitylation-stimulating factor was purified from reticulocyte lysate fraction I using three column chromatography steps: a 6 ml column of SP Sepharose (GE Healthcare Life Sciences) equilibrated in 25 mM Tris-HCl (pH 7.6), 1 mM DTT, and 0.1 mM PMSF and eluted with a 60 ml gradient of 50 to 350 mM NaCl; a 1.5 $\times$ 100 cm column of Sephacryl S-100 HR (GE Healthcare Life Sciences) equilibrated in 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, and 1 mM DTT and eluted in the same buffer; and a 1 ml column of Bio-Gel HTP hydroxyapatite (BioRad) equilibrated in 25 mM Tris-HCl (pH 7.5) and 1 mM DTT and eluted with a 10 ml gradient of 50 to 350 mM NaH₂PO₄-Na₂HPO₄. At each step column eluent fractions were assayed for their ability to stimulate NIH 3T3 lysate fraction II-catalyzed EMCV 3C^{pro} ubiquitylation. 20 μ g of the final preparation were subjected to electrophoresis by 5 to 13% SDS-PAGE and the major protein bands, visualized by staining with Imperial Protein Stain (Pierce/ThermoFisher Scientific), were submitted for identification by liquid chromatography-tandem mass spectroscopy at the Proteomics and Lipidomics Analysis Core Facility at the Maine Medical Center Research Institute (Scarborough, Maine).

E3 UbcH7-dependent ubiquitin-protein ligases were purified from NIH 3T3 cell lysate fraction II, first by further fractionation by (NH₄)₂SO₄ precipitation into 0–20% and 21–40% saturation fractions. The majority of the UbcH7-dependent 3C^{pro}-ubiquitylating activity partitioned into the 21–40% fraction. The remainder of the purification scheme consisted of three column chromatography steps: a 4.5 ml column of Q Sepharose (GE Healthcare Life Sciences) equilibrated in 25 mM Tris-HCl (pH 7.2), 20 mM KCl, and 1 mM DTT and eluted with a 70 ml gradient of 0 to 0.65 M NaCl; a 1.5 $\times$ 42 cm column of Sephacryl S-300 HR (GE Healthcare Life Sciences) equilibrated in 50 mM Tris-HCl (pH 7.6), 20 mM NaCl, and 1 mM DTT and eluted with the same buffer; and a 0.5 ml column of

ubiquitin-UbcH7-GST fusion protein bound to glutathione-agarose equilibrated in 50 mM Tris-HCl (pH 7.5), 20 mM NaCl, and 1 mM DTT and eluted with 2.5 ml 50 mM Tris-HCl (pH 7.5), 1 M NaCl, and 1 mM DTT. Ligase activity was followed as the purification progressed based upon the reconstitution of ubiquitin-3C^{Pro} conjugate synthesis by the addition of purification fractions to mixtures containing E1, UbcH7, and methylated or wild type ubiquitin. 25 µg of the final preparation were subjected to electrophoresis by 4 to 15% SDS-PAGE, and protein bands > approximately 15 kDa, visualized by staining with Imperial Protein Stain, were submitted for identification analysis by liquid chromatography-tandem mass spectroscopy at the Proteomics and Lipidomics Analysis Core Facility at the Maine Medical Center Research Institute.

2.4. Evaluation of EMCV 3C^{Pro} levels in virus-infected E3 ligase knockdown cell lines

Stably transfected E6AP and ARIH1 NIH 3T3 knockdown cell lines were isolated following the transfection of cells with individual plasmids from libraries consisting of pLKO.1-puro with genes encoding constitutively expressed shRNAs (Sigma-Aldrich Mission *siRNA*) designed for either mouse E6AP/UBE3A transcript variant 2 (NM_11668.2) or mouse ARIH1 transcript (NM_019927), as well as with pLKO.1-puro (to generate empty vector control cells). The transfections were carried out with cells cultured as described above and using Lipofectamine LTX Plus reagent (Invitrogen/ThermoFisher Scientific). Cells selected by culturing in the presence of 1.2 µg/ml puromycin (Gibco/ThermoFisher Scientific) were screened for E6AP or ARIH1 expression attenuation by Western blotting of 60 µg lysate protein prepared using RIPA buffer [18] containing HALT protease inhibitor cocktail with EDTA (Pierce/ThermoFisher Scientific). These blots were developed using rabbit anti-E6AP or goat anti-ARIH1 (both from Abcam) antibodies and secondary antibody-conjugated alkaline phosphatase as described above. Colonies derived from individual cells isolated from the individual plasmid-transfected populations that exhibited the largest attenuation of E6AP or ARIH1 expression relative to the pLKO.1-puro control cells were expanded and rescreened to identify cell lines with at least 70% reduction in E6AP (cell line NIH 3T3/pLKO-UBE3A.983-4) or ARIH1 (cell line NIH 3T3/pLKO-ARIH1.1033-1) expression.

Control, E6AP knockdown, and ARIH1 knockdown cells cultured in 12-well plates were infected with EMCV (American Type Culture Collection) at a multiplicity of infection of three as previously described [19]. At the indicated times, the cells were washed 2× with PBS and lysed by the addition of 200 µl RIPA buffer containing HALT protease inhibitor cocktail with EDTA. 25 µg of lysate protein were subjected to Western blotting, using rabbit anti-EMCV 3C^{Pro} antibodies ([6]) and anti-mouse β-actin antibodies (Santa Cruz Biotechnology), and the blots were developed using secondary antibody-conjugated alkaline phosphatase and enhanced chemiluminescence (Amersham/GE Healthcare ECF phosphatase substrate) and imaged with a Fuji FLA-5000 Multifunctional Imaging System.

3. Results

3.1. Identification of the EMCV 3C^{Pro} ubiquitylation-stimulating factor as Ubch7/UBE2L3

We observed that fraction II preparations (anion-exchange matrix-binding proteins, ref. [17]) prepared from cultured mouse cell S100 lysate are much less capable of catalyzing 3C^{Pro}

ubiquitylation than is whole S100 lysate, but the addition of fraction I proteins (anion-exchange matrix flow through) to fraction II restores 3C^{pro} ubiquitylation to levels comparable to those observed in whole lysate (Fig. 1A). Fraction I prepared from rabbit reticulocyte lysate also stimulates NIH 3T3 fraction II-catalyzed 3C^{pro} ubiquitylation (Fig. 1A, lane 10), indicating that both cell types produce one or more 3C^{pro} ubiquitylation-stimulating factors that partition into fraction I. For the experiments described in Fig. 1A we used ubiquitin in which all of the primary amines have been blocked by methylation to allow for the detection of the initial attachment of ubiquitin to substrates with great sensitivity because the monoubiquitylated 3C^{pro} substrate is not distributed into polyubiquitylated products and is not subject to proteasome-dependent degradation [6,20]. The stimulating activity in fraction I also supports enhanced 3C^{pro} polyubiquitylation in a concentration-dependent manner, as is indicated by assays using wild type ubiquitin and fraction I protein added at various concentrations to fraction II at fixed concentration (Fig. 1B).

To identify the 3C^{pro} ubiquitylation-stimulating factor(s), we began with fraction I prepared from rabbit reticulocytes and applied the purification scheme described in Material and Methods. The protein composition of the final preparation enriched for the stimulating activity is shown in Fig. 1C. Three dominant protein bands were visible, one of which was obviously hemoglobin. The other two, an approximately 30 kDa protein and an approximately 18 kDa protein that was observed to track with the stimulating activity during the purification, were submitted for identification analysis by mass spectroscopy. The analysis of the approximately 30 kDa protein did not generate polypeptide fragment sequence data sufficient for identification, but the second protein was identified as the 17.8 kDa ubiquitin-conjugating enzyme UbcH7/UBE2L3. Confirmation that UbcH7 is a 3C^{pro} ubiquitylation stimulatory factor was provided by the observation that purified UbcH7 enhances both fraction II-catalyzed mono-(Fig. 1D) and polyubiquitylation (Fig. 1E) of the EMCV 3C^{pro}, although the extent to which the 3C^{pro} is incorporated into polyubiquitylated products in this system appears to be limited. The discovery that UbcH7 is involved in EMCV 3C^{pro} polyubiquitylation provided evidence for a previously unknown 3C^{pro}-polyubiquitylating pathway with the potential to mark the 3C^{pro} for destruction.

3.2. Identification and characterization of the UbcH7-dependent 3C^{pro}-targeting ubiquitin-protein ligases

To identify the E3, or E3s, that function with UbcH7 to catalyze EMCV 3C^{pro} ubiquitylation, we began with fraction II prepared from NIH 3T3 cells and followed the purification scheme described in Material and Methods. For the final UbcH7-dependent E3 purification step we used a ubiquitin-UbcH7-GST fusion protein affinity matrix, the rationale being that the ubiquitin-UbcH7 fusion protein mimics the E2 charged with ubiquitin and is therefore more likely than UbcH7 alone to associate with UbcH7-interacting E3s [21]. The ubiquitin-UbcH7 affinity column eluent was highly enriched in UbcH7-dependent 3C^{pro} ubiquitylating activity, although many proteins were detected in the preparation (Fig. 2A). The analysis by mass spectroscopy of bands representing proteins >15 kDa resulted in the identification of three ubiquitin-protein ligases. These were the 57.7 kDa RBR ligase ARIH2, the 64.0 kDa RBR ligase ARIH1, and the 100 kDa HECT ubiquitin-protein ligase E6AP/UBE3A. All three of these enzymes have been shown to use UbcH7 as

their cognate E2 [22–26]. There are three catalytically active isoforms of E6AP [26] but our analysis could not distinguish these; mouse isoform 2 is the canonical, full-length protein.

We tested the ability of each of these three ubiquitin-protein ligases to catalyze EMCV 3C^{pro} polyubiquitylation in mixtures containing the reconstituted E1-UbcH7-E3 pathways, with purified mouse ARIH1 and ARIH2 obtained from large-scale *in vitro* transcription/translation reactions and commercially sourced human E6AP isoform 2 (the sequence of which is 96% homologous with that of mouse isoform 2). For these experiments, we probed for ubiquitylated 3C^{pro} synthesis using both anti-3C^{pro} antibodies and anti-ubiquitin antibodies, the latter of which bind preferentially to polyubiquitin chains. We found that in the presence of UbcH7 ARIH1 catalyzes 3C^{pro} monoubiquitylation but is incapable of subsequent ubiquitin additions to build a polyubiquitin chain (Fig. 2B) and that ARIH2 does exhibit any 3C^{pro}-ubiquitylating activity (Fig. 2C). These results suggest that ARIH1 and ARIH2 are unlikely to flag the 3C^{pro} for recognition by the 26S proteasome. We found, however, that E6AP is capable of catalyzing EMCV 3C^{pro} polyubiquitylation (Fig. 2D), and this result indicates that the E1-UbcH7-E6AP pathway is a candidate for tagging the 3C^{pro} for degradation *in vivo*. Support for this candidacy is provided by our results showing that 3C^{pro} polyubiquitylation *via* the E1-UbcH7-E6AP pathway occurs primarily through Lys⁴⁸ isopeptide bonds (Fig. 3A and B), the linkage typically found in ubiquitin chains that function to introduce polyubiquitylated proteins to the 26S proteasome complex [3].

3.3. Effects of E6AP and ARIH1 expression attenuation on 3C^{pro} accumulation in EMCV-infected cells

To address the question of whether either the E1-UbcH7-E6AP or E1-UbcH7-ARIH1 pathways can contribute to EMCV 3C^{pro} concentration regulation *in vivo* we examined the effect of E3 expression attenuation on 3C^{pro} levels in EMCV-infected cells. We generated stably transfected E6AP and ARIH1 NIH 3T3 knockdown cell lines (Fig. 4A) and evaluated the late infection 3C^{pro} levels in these cells. EMCV infected NIH 3T3 cells typically die between 6 and 7 h post-infection, by which time new protein synthesis has become almost exclusively viral [27]. In the infected E6AP knockdown cells, the 3C^{pro} accumulated to higher late infection levels than in the control cells (Fig. 4B), which is a result consistent with the participation of E6AP in the degradation of the protein. It is important to note that because polyubiquitylated 3C^{pro} is rapidly degraded by the proteasome *in vivo*, polyubiquitylated 3C^{pro} is difficult to detect in the absence of proteasome inhibitors; but these inhibitors also prevent EMCV infection establishment [10]. These facts account for the absence of obviously visible high molecular mass polyubiquitylated 3C^{pro} in this assay. The 3C^{pro} levels in both the infected ARIH1 knockdown and control cells were similar (Fig. 4C), which suggests that this E3 does not contribute to 3C^{pro} concentration control.

4. Discussion

The discovery of the E1-UbcH7-E6AP EMCV 3C^{pro}-polyubiquitylating pathway and the evidence that E6AP has a role in determining the 3C^{pro} concentration in EMCV-infected cells will facilitate further investigations into the extent to which ubiquitin-mediated 3C^{pro} concentration regulation influences virus replication success. It also raises questions about

how this pathway contributes to flagging the 3C^{pro} for destruction. Two other 3C^{pro}-polyubiquitylating pathways are known to exist: the E1-UbcH5-DTX3L pathway, the action of which has also been shown to contribute to 3C^{pro} degradation *in vivo* [11], and the E1-UBE2-UBR1 pathway, which functions with kinetics that are not robust [12,13]. One important issue is whether these pathways operate individually or in combination to tag the 3C^{pro} for concentration reduction and to what extent. We observed that significant monoubiquitin-3C^{pro} synthesis accompanies E6AP-catalyzed 3C^{pro} polyubiquitylation *in vitro* (Fig. 2D and 3A); this may be a consequence of incomplete E6AP oligomerization, which was recently shown to be necessary for efficient E6AP catalyzed polyubiquitylation [23]. Previous studies of the *in vivo* polyubiquitylation of the cloned, inducibly expressed EMCV 3C^{pro} in NIH 3T3 cells, however, did not reveal that monoubiquitylated 3C^{pro} accumulates in cells [10]. This suggests that *in vivo* the E6AP-containing pathway functions, perhaps cooperatively, with at least one other pathway to catalyze EMCV 3C^{pro} polyubiquitylation, most likely the E1-UbcH5-DTX3L pathway. The detailed kinetic analyses of 3C^{pro} polyubiquitylation by the purified reconstituted pathways, individually and in combination, will clarify the EMCV 3C^{pro} polyubiquitylation mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

3C protease	3C ^{pro}
EMCV	encephalomyocarditis virus
HRV	human rhinovirus
HECT	homologous to E6AP carboxyl terminus
RBR	ring between ring
RING	really interesting new gene
Ub	ubiquitin

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UBE3A/E6AP with UBE2L3 catalyzes EMCV 3C protease polyubiquitylation.

ARIH1 with UBE2L3 catalyzes EMCV 3C protease monoubiquitylation.

UBE3A/E6AP-catalyzed 3C protease polyubiquitylation occurs through Lys⁴⁸ linkages.

UBE3A/E6AP is implicated in EMCV 3C protease concentration regulation *in vivo*.

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Fig. 1.

The ubiquitin-conjugating enzyme UbcH7/UBE2L3 participates in EMCV 3C^{pro} ubiquitylation. (A) Western blot analysis of ubiquitylation assay mixtures that included methylated ubiquitin and 5 mg/ml NIH 3T3 lysate S100 or 4 mg/ml NIH 3T3 lysate fraction II, FII, to which either 4 mg/ml NIH 3T3 lysate fraction I, FI, or 2 mg/ml rabbit reticulocyte lysate fraction I was added. (B) Western blot analysis of 3C^{pro} ubiquitylation assay mixtures that included ubiquitin, 4 mg/ml NIH 3T3 lysate fraction II, and the indicated concentration of added NIH 3T3 lysate fraction I. (C) SDS-PAGE analysis of the final hydroxyapatite column eluent containing the EMCV 3C^{pro} ubiquitylation-stimulating activity. The bands identified as UbcH7 or hemoglobin (Hb) are indicated. (D) and (E) Western blot analyses of ubiquitylation assay mixtures that included 4 mg/ml NIH 3T3 lysate fraction II, UbcH7 as indicated, and methylated ubiquitin (D) or ubiquitin (E). All of the blots were developed using anti-EMCV 3C^{pro} antibodies.

Fig. 2.

Identification and activity evaluation of Ubch7-associating ubiquitin-protein ligases. (A) SDS-PAGE analysis of the final ubiquitin-Ubch7 affinity column eluent enriched for Ubch7-dependent ubiquitin-protein ligase activity. The gel regions found by mass spectroscopy to contain E6AP, ARIH1, and ARIH2 are indicated. (B), (C), and (D) Western blot analyses, developed using either anti-EMCV 3C^{pro} or anti-ubiquitin antibodies, of ubiquitylation assay mixtures that included ubiquitin, Ubch7, and ~5 μ M ARIH1 (B), ~5 μ M ARIH2 (C), or 2.5 μ M E6AP (D).

Fig. 3. The E1-UbcH7-E6AP pathway catalyzes 3C^{pro} Lys⁴⁸-linked polyubiquitylation. (A) and (B) Western blot analyses, developed using either anti-EMCV 3C^{pro} (A) or anti-ubiquitin (B) antibodies, of ubiquitylation assay mixtures that included wild type, K48R, or K480 (all lysine residues replaced with arginine except for Lys⁴⁸) ubiquitin, UbcH7, and 2.5 μ M E6AP.

Fig. 4.

Attenuated E6AP expression results in elevated late-infection 3C^{pro} levels in EMCV-infected mouse cells. (A) Western blot analyses, developed with either anti-E6AP or anti-ARIH1 antibodies, of lysates prepared from NIH 3T3 cells stably transfected with empty, E6AP/UBE3A shRNA-expressing, or ARIH1 shRNA-expressing vector. Cell lysates prepared from two separate cultures of each cell line were analyzed. (B) and (C) Western blot analyses, developed using anti-EMCV 3C^{pro} and anti- β -actin antibodies, of lysates prepared from mock-infected, EMCV-infected control, C, and E6AP (B) or ARIH1 (C) knockdown, KD, cells at the indicated post-infection times.