Degradation of host ubiquitin E3 ligase Itch by human cytomegalovirus UL42

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Human cytomegalovirus (HCMV) UL42 is classified as a CMV-specific but function-unknown gene. According to its amino acid sequence, UL42 has a C-terminal hydrophobic domain predicted to be a transmembrane domain and two PPxY (PY) motifs in its N terminus, but no N-terminal signal peptide. These features resemble those of herpes simplex virus (HSV) UL56 and varicella-zoster virus ORF0. HCMV UL42 interacts with Itch, a member of the Nedd4 family of ubiquitin E3 ligases, through its PY motifs as observed in HSV UL56. HCMV UL42 was partially colocalized with the trans–Golgi network and cytoplasmic vesicles in transfected fibroblasts. Itch was co-localized with HCMV UL42 and accumulated in a fine-speckled pattern in the cytoplasm. UL42 induced the ubiquitination and degradation of Itch in HCMV-infected fibroblasts, and was partially colocalized with p62, a ubiquitin-binding protein, and CD63, a marker of lysosome and multivesicular bodies. The electrophoretic pattern of Itch was altered by infection with HCMV and the amount of Itch was increased by the deletion of UL42. Our findings suggest that the regulatory function of the Nedd4 E3 ligase family and the structural features of HCMV UL42 are conserved characteristics in herpesviruses.

INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous pathogen within the human population which establishes a lifelong infection (Knipe & Howley, 2013). Primary HCMV infection occurs in most individuals at childhood, and is generally asymptomatic in healthy children and adults, but not in immunocompromised patients, including transplant recipients, AIDS patients or cancer patients receiving immunosuppressive therapies (Boeckh & Geballe, 2011; Halwachs-Baumann, 2007). However, the transmission of HCMV to the fetus via the placenta causes congenital HCMV infection which shows a high incidence of neurodevelopmental sequelae, including mental retardation and sensorineural deafness. Therefore, the development of a vaccine for the prevention of congenital HCMV is regarded as a major public health priority (Sung & Schleiss, 2010).

HCMV is an enveloped DNA virus belonging to the subfamily Betaherpesvirinae. The viral genome of HCMV is a dsDNA molecule of 236 kbp, which is the largest amongst the herpesviruses characterized to date, and encodes > 150 ORFs and 14 microRNAs (Chee et al., 1990; Dolan et al., 2004; Döllken et al., 2009). The functions for more than half of these genes are still unknown, and > 80 ORFs are CMV-specific and are not observed in other herpesviruses. HCMV UL42 is a function-unknown non-essential, CMV-specific gene (Dargan et al., 1997; Dunn et al., 2003; Mocarski et al., 1997). According to its amino acid sequence, UL42 has two Pro-Pro-X-Tyr (PPxY) sequences, a hydrophobic region at the C terminus and no N-terminal signal peptide. These features are shared with herpes simplex virus (HSV) UL56, which is classified as a tail-anchored membrane (TA) protein (Koshizuka et al., 2002; Ushijima et al., 2008). The PPxY sequence was originally identified as the sequence responsible for binding with the WW domain (Chen & Sudol, 1995). As the WW domain also binds with the Leu-Pro-Xxx-Tyr (LPxY) sequence, the (L/P)PXy motif is known as the PY motif (Kasanov et al., 2001). The PY motifs of HSV UL56 are able to bind with Nedd4 (neuronal precursor cell-expressed, developmentally downregulated 4), an E3 ligase family protein, which contains a WW domain (Ushijima et al., 2008, 2010).

Protein ubiquitination requires the combination of an ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). E3 ligases facilitate interactions with the substrate protein and the transfer of ubiquitin to the substrate (Pickart, 2001). Nine proteins are included in the Nedd4 family, which belongs to the HECT (homology to E6-AP C-terminus)-type E3 ligase subfamily (Reimand et al., 2012; Shearwin-Whyatt et al., 2006). They are characterized by a calcium/lipid-binding C2 domain at the N terminus, two to four WW domains and a HECT domain. Nedd4, together with the other members of its family, has been reported to be involved in various cellular processes. For example, Nedd4 regulates adaptive immunity (Yang et al., 2008), signal transduction...
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(Cao et al., 2008; Wang et al., 2007) and protein trafficking (Shearwin-Whyatt et al., 2004). Moreover, Nedd4 also plays a role in retroviral gag ubiquitination and the assembly of virus-like particles (Freed, 2002; Heidecker et al., 2004; Strack et al., 2000). Itch, a member of the Nedd4 family, regulates T-cell differentiation, Jun protein turnover and chronic activation of T-helper (Th) 2 cells (Gao et al., 2004). Furthermore, Itch is included in the A20 ubiquitin-editing complex, and negatively regulates the TNF-α and IL-1β signalling pathways (Shembade et al., 2008). As a reflection of the immunological functions of Itch, knockout mice develop a variety of disorders, including pulmonary chronic interstitial inflammation (Perry et al., 1998).

The functions of the Nedd4 family of E3 ligases described above are regulated by adaptor proteins (Shearwin-Whyatt et al., 2006). For example, Ndfip2 leads Nedd4 to specific cellular compartments such as the Golgi complex and multivesicular bodies (MVBs) (Shearwin-Whyatt et al., 2004), whereas Spartin, an Itch adaptor protein, recruits Itch to lipid droplets and induces the ubiquitination of Adipophilin (Hooper et al., 2010) and PCBP2 [poly(C)-binding protein 2] regulates the Itch-dependent degradation of the MAVS (mitochondrial antiviral signalling) adaptor (You et al., 2009). Amongst the various herpesvirus-encoding proteins, Epstein–Barr virus (EBV) LMP2A has been reported to act as an Itch regulator, inducing ubiquitination and the turnover of Lyn and Syk, and subsequently blocking B-cell receptor signalling (Ikeda et al., 2003; Winberg et al., 2000).

Here, we report that HCMV UL42 was able to interact with Itch through its PY motifs. UL42 recruited Itch to the cytoplasmic vesicles, including the lysosomes, and induced the degradation of Itch in HCMV-infected cells. Furthermore, TA proteins containing a PY motif, as found in UL42, might be conserved in all human herpesviruses. Our findings suggest that the regulation of the Nedd4 family of E3 ligases is ubiquitous amongst herpesviruses.

RESULTS

Identification of PY motif-containing TA proteins in human herpesviruses

According to its amino acid sequence, HCMV UL42 has two PY motifs, a C-terminal hydrophobic domain but no N-terminal signal peptide (Fig. 1). The SOSUI algorithm (Hirokawa et al., 1998) indicated that the SOSUI algorithm (Hirokawa et al., 1998) indicated that the C-terminal hydrophobic region is a putative transmembrane domain (TMD) and the PY motifs of UL42 might be oriented toward the cytoplasm. These features resemble those of HSV UL56 and varicella-zoster virus (VZV) ORF0, even though there was no significant sequence homology. The UL56 protein of HSV-1 and -2 has three PPxY and one LPxY sequence, respectively, at its N terminus. VZV ORF0 has both a PPxY and LPxY sequence. Although no Nedd4-interacting proteins have been identified amongst human herpesvirus (HHV)-6, HHV-7 and Kaposi sarcoma-associated herpesvirus (KSHV), the U24 proteins of HHV-6 and HHV-7 have a PPxY sequence, whilst ORF16 of KSHV has an LPxY sequence. All of them have a C-terminal hydrophobic domain, but no N-terminal signal peptide. Another gammaherpesvirus, EBV, has no obvious PY motif-containing TA protein, but LMP2A, which is a much larger protein with 12 TMDs, has two PPxY sequences in its N-terminal cytoplasmic domain.

Interaction of UL42 with Itch

When haemagglutinin (HA)-tagged UL42 (HA-UL42) protein was overexpressed in fibroblasts, a marked accumulation of the expressed protein was observed in the perinuclear region of the cytoplasm, with part of the HA-UL42 dispersed in the cytoplasm in a fine-speckled pattern (Fig. 2). In the perinuclear region, HA-UL42 was colocalized with p230, a trans-Golgi network (TGN) marker. The subpopulation of HA-UL42 forming the fine-speckled pattern was colocalized with EEA1, an early endosome marker, and CD63, a lysosome/multivesicular body marker (Fig. 2, arrowheads).

UL42 possesses two PY motifs, as described above, indicating the possibility of an interaction between UL42 and Itch. In order to confirm this interaction, an immunoprecipitation assay was carried out using cell lysates from 293T cells expressing the HA-UL42wt or HA-UL42PA protein. The HA-UL42PA protein was a PY motif-disrupted mutant in which tyrosine was substituted by alanine in the PY motifs (Fig. 3a). As shown in Fig. 3(b), endogenous Itch was highly modified in the presence of HA-UL42wt (Fig. 3b, lane 2, arrowheads), but not in the presence of HA-UL42PA (Fig. 3b lane 3). Both modified (open arrowheads) and unmodified (closed arrowheads) Itch species were co-precipitated with HA-UL42wt (Fig. 3b, lanes 5

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**Fig. 1.** Schematic representation of TA proteins with PY motifs in human herpesviruses. These proteins, except for EBV LMP2A, have a C-terminal hydrophobic domain which is a putative TMD (black box), but no N-terminal signal peptide. The sequences of the PY motifs are indicated as grey (PPxY) and hatched (LPxY) boxes.
and 8), whilst UL42PA was also associated with endogenous Itch, although with a markedly reduced affinity (Fig. 3b, lanes 6 and 9). This result indicated that the PY motifs of UL42 were important for the interaction with Itch, although it is possible that other segments within the UL42 amino acid sequence could contribute to this interaction.

As endogenous Itch was not detectable by the antibody in an immunofluorescence assay, FLAG-tagged Itch (FLAG-Itch) was co-expressed with HA-UL42 and analysed by immunofluorescence assay using an anti-FLAG antibody (Fig. 3c). In the absence of UL42, FLAG-Itch was localized at the cytoplasm, with some accumulation observed in the perinuclear region of the cytoplasm (Fig. 3c). When co-expressed with HA-UL42wt, the localization of FLAG-Itch was altered to a fine-speckled pattern in the cytoplasm and colocalization with the subpopulation of HA-UL42wt.

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In order to determine the ubiquitination of FLAG-Itch, Myc-tagged ubiquitin was co-transfected and analysed in an immunoprecipitation assay (Fig. 4a-d). The amount of FLAG-tagged Itch was markedly decreased in the presence of HA-UL42wt (Fig. 4b, lane 2, upper panel). FLAG-Itch was also decreased in the presence of HA-UL42PA, but remained detectable by an anti-FLAG antibody (Fig. 4b, lane 3, upper panel). A dominant negative mutant Itch (ItchCA), in which the catalytic centre was substituted, was also decreased in the presence of HA-UL42wt, but not in that of HA-UL42PA (Fig. 4b, lanes 5 and 6, upper panel). Although FLAG-Itch of the input was undetectable in Fig. 4(b, lane 2), the ubiquitinated FLAG-Itch was detected (Fig. 4d, lane 2, under panel). This result indicated that FLAG-Itch existed under the detection level of anti-FLAG that we used. The ubiquitination of FLAG-Itch was increased in the presence of UL42wt (Fig. 4d, lane 2, lower panel). The ItchCA mutant was detectable even in the presence of HA-UL42wt (Fig. 4d, lane 5), indicating that the catalytic activity of Itch was important for the degradation of FLAG-Itch itself. It is speculated that transfected FLAG-ItchCA proteins are ubiquitinated by the endogenous Itch activated by HA-UL42wt. HA-UL42 was also observed to be ubiquitinated in transfected cells (Fig. 4c, lower panel).

**Increase of Itch by the deletion of HCMV UL42**

In order to determine the effect of UL42 on Itch turnover and activation, we constructed a series of UL42 recombinant HCMVs using a bacterial artificial chromosome.
Fig. 3. Induction of Itch modification by UL42. (a) Schematic representation of HA-UL42 constructs. The two PY motifs of UL42wt and alanine-substituted sites are indicated as grey and dotted boxes, respectively. The C-terminal hydrophobic domain is indicated as a black box. (b) Immunoprecipitation (IP) assay. HA-UL42 was expressed in 293T cells and co-precipitated with endogenous Itch. Control plasmid (−), HA-UL42wt (WT)- or HA-UL42PA (PA)-expressing 293T cell lysates were immunoprecipitated with anti-HA or anti-Itch mAbs with a buffer containing PBS/1 % Triton X-100. Whole-cell lysates (WCLs) and immunocomplexes were analysed by Western blotting (WB) with anti-HA polyclonal or anti-Itch mAbs. The Western blotting image of actin is shown as a loading control for the WCLs. The arrowheads indicate the modified (open arrowhead) or unmodified (closed arrowhead) Itch species. (c) Subcellular localization of Itch and UL42. FLAG-ItchWT and HA-UL42 plasmid-transfected cells were double stained with anti-FLAG mAb and anti-HA polyclonal antibody. Bar, 10 μm.
(BAC) mutagenesis method (Fig. 5a). As described in Dunn et al. (2003), the deletion of UL42 had no effect on HCMV growth (data not shown). A 23 kDa HA-UL42 polypeptide was detected by 1 day post-infection (p.i.) and this amount gradually increased up to 4 days p.i. in TowneBAC-HAUL42-infected cells (Fig. 5b). Infection with WT HCMV produced a shift in the Itch protein to a form with a higher molecular mass (Fig. 5b, arrowheads).

Fig. 4. UL42 expression-induced ubiquitination and degradation of Itch. 293T cells were transfected with HA-UL42-, FLAG-Itch- and Myc-ubiquitin (mycUb)-expressing plasmids. At 24 h post-transfection, cells were lysed under denaturing conditions. (a–d) Input fractions were analysed by Western blotting (WB) with anti-HA (a, c, upper panel), anti-FLAG (b, d, upper panel) or anti-Myc (a–d, lower panels) antibodies. HA-UL42 and FLAG-Itch were immunoprecipitated with anti-HA or anti-FLAG mAbs, respectively. Immunoprecipitated proteins were analysed with anti-HA polyclonal (c, upper panel), anti-FLAG monoclonal (d, upper panel), anti-Myc monoclonal (c, d, lower panel) antibodies. The positions of the immunoglobulin light chain (LC) are indicated.
**Fig. 5.** Structure of recombinant viruses and time course of protein expression in infected cells. (a) Schematic representation of the HCMV TowneBAC mutagenesis of the UL42 locus. A HA-tag was inserted at the start codon of UL42 to generate TowneBAC-HAUL42. In order to generate the UL42 deletion mutant (TowneBAC-ΔUL42), the HAUL42 sequence was deleted from TowneBAC-HAUL42. Thereafter, the HA-UL42 sequence was inserted into TowneBAC-ΔUL42 to generate a repair virus (TowneBAC-HAUL42R). (b) Expression of UL42, pp150 and Itch in recombinant HCMV-infected fibroblast cells. hTERT-BJ1 cells were mock-infected or infected with TowneBAC viruses at m.o.i. 3 and harvested at the indicated days post-infection. Cell lysates were analysed by Western blotting. m, Mock-infected cell lysate. Positions of the molecular mass markers (kDa) are indicated on the left. (c) Immunoprecipitation (IP) of Itch from HCMV-infected cell lysate. hTERT-BJ1 cells

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were infected with the recombinant viruses at m.o.i. 3. At 5 days p.i., cells were harvested with PBS/1 % Triton X-100 buffer and subjected to immunoprecipitation. Immuno-precipitated complexes were analysed by Western blotting. (d) Ubiquitination of Itch in HCMV-infected cells. hTERT-BJ1 cells were infected with the recombinant viruses at m.o.i. 3. At 3 days p.i., cells were harvested under denaturing conditions and subjected to immunoprecipitation with anti-Itch antibody. Immuno-precipitated complexes were analysed by Western blotting (WB). The modified (open arrowhead) and unmodified (closed arrowhead) Itch species are indicated on the right. WCL, whole-cell lysate; m, mock; wt, TowneBAC-wt; HA, TowneBAC-HAUL42; Δ, TowneBAC-ΔUL42; R, TowneBAC-HAUL42R; LC, IgG light chain.

However, as indicated by the closed arrowheads in Fig. 5(b), the lower-molecular-mass band of Itch increased markedly on infection with the UL42 deficient-HCMV (Fig. 5b). HA-UL42 and endogenous Itch were co-precipitated in HCMV-infected cell lysates (Fig. 5c).

In order to elucidate whether the shift in the molecular mass of Itch was due to ubiquitination or not, endogenous Itch was analysed by immunoprecipitation assay under denatured conditions. As shown in Fig. 5(d), detection with an anti-ubiquitin antibody clearly revealed the presence of high-molecular-mass ubiquitinated Itch in HCMV-infected cell lysates (Fig. 5d). The ubiquitinated Itch was detected even in the absence of UL42, indicating that the HCMV infection itself induced the ubiquitination of Itch. Although the ubiquitinated species of Itch was detected in HCMV-infected cells, the ubiquitinated UL42 was not detectable under our experimental conditions (data not shown).

**Subcellular localization of UL42 in HCMV-infected cells**

As shown in Fig. 6(a), HA-UL42 was detected at 1 day p.i. in HCMV TowneBAC-infected hTERT-BJ1 cells. The HA-UL42 was observed to accumulate in the perinuclear region of the cytoplasm, with some dispersal into the cytoplasm in a fine-speckled pattern. GFP fluorescence was used as a marker for TowneBAC infection in cells and revealed that the number of dispersed HA-UL42 foci gradually increased up to 4 days p.i. The HA-UL42 foci were partially colocalized with p62, a ubiquitin-binding protein, in TowneBAC-HAUL42-infected cells (Fig. 6b). A portion of the HA-UL42 foci in the vacuolar structure of HCMV-infected cells were surrounded by the CD63 cellular protein (Fig. 6b, arrowheads). In order to elucidate the stability of UL42, recombinant HCMV-infected cells were treated with chloroquine and MG132. HA-UL42 was increased in the presence of chloroquine, a lysosome inhibitor, but not in the presence of MG132, a proteasomal inhibitor, indicating that UL42 was degraded by the lysosome (Fig. 6c). However, the band pattern of Itch was not significantly changed even in the presence of the inhibitors.

**Association of UL42 with membranous structures in infected cells**

According to the results of the intracellular localization and the amino acid sequence of UL42, it was suggested that UL42 was associated with membranous structures. In order to determine whether UL42 was associated with these membranous structures, the UL42 protein was fractionated with detergents from HCMV-infected cell lysates. Almost all UL42 proteins were soluble in the 1 % Triton X-100-containing buffer, but not in the absence of detergent (Fig. 7, lanes 2–5). HCMV-encoded glycoprotein B (gB), which possesses a TMD, was also soluble in the 1 % Triton X-100-containing buffer. A viral tegument protein, pp150, was pelleted in the 1 % Triton X-100-containing buffer, but soluble in the 1 % sarcosyl-containing buffer (Fig. 7, lanes 4–7). This result indicates that some tegument proteins might associate with the 1 % Triton X-100-insoluble structures such as virions. Almost all of the EEA1 protein, a cellular peripheral membrane protein (Mu et al., 1995), was collected in the soluble fraction even in the absence of detergent (Fig. 7, lanes 2 and 3). As a marker of cellular cytoplasmic protein, more than half of actin protein was collected in the soluble fractions, although significant amounts of the protein were detected in the pelleted fractions of the buffer without detergent and 1 % Triton X-100-containing buffer (Fig. 7, lanes 2–5). The properties of UL42 closely resembled those of gB, indicating that UL42 associated with the membranous structures as an integral membrane protein. As UL42 has a hydrophobic region at its C-terminus, as shown in Fig. 1, and it is speculated that this domain functions as a TMD.

**DISCUSSION**

In this study, we found that HCMV UL42 interacts with Itch through its PY motif. As the PY motif is a target of the WW domain of Nedd4 family proteins (Kasanov et al., 2001), it is also possible that UL42 is able to interact with other Nedd4 family proteins. To the best of our knowledge, this is the first report to demonstrate that a HCMV protein binds to the Nedd4 family of E3 ligases. Taken together with the data for transfected cells, we demonstrated that UL42 was able to induce the relocalization of the subcellular distribution of Itch. In addition, UL42 induced the ubiquitination of Itch both in transfected and infected cells. UL42 has a putative C-terminal TMD and PY motifs that are speculated to be oriented toward the cytoplasm. The binding of UL42–Itch occurred at the cytoplasm as Itch is a cytoplasmic protein, thus the N-terminal side of UL42 was oriented toward the cytoplasm.
Fig. 6. (a) Intracellular distribution of UL42 in recombinant virus-infected cells. hTERT-BJ1 cells were infected with TowneBAC–HAUL42 or TowneBAC–ΔUL42 at m.o.i. 3. Cells were fixed with 4 % paraformaldehyde at the indicated days (d) post-infection. Samples were double stained with anti-HA polyclonal antibody (upper panels). The intrinsic fluorescence and EGFP (+GFP) fluorescence were merged (lower panels). Bar, 10 μm. (b) TowneBAC–HAUL42-infected cells were fixed at 3 days p.i. and double stained with anti-HA and anti-p62 or anti-CD63 antibodies as indicated. The images of EGFP, Alexa Fluor 546 and Alexa Fluor 647 are shown as blue, green and red, respectively. Bar, 10 μm. (c) Lysosomal degradation of UL42. Mock-infected or recombinant HCMV-infected hTERT-BJ1 cells (m.o.i. 3) were treated with a proteasome inhibitor, MG132 (MG, 10 μM), or a lysosome inhibitor, chloroquine (CQ, 100 μM), from 80 to 96 h p.i. IE, Intermediate-early.
The electrophoretic pattern of Itch was changed by HCMV infection even in the absence of UL42. The higher-molecular-mass species of Itch was modified by ubiquitin (Fig. 5d). These results indicate that Itch was activated by HCMV infection based on that fact that the activation of Itch induces auto-ubiquitination, as seen in other HECT domain ubiquitin E3 ligases (Gao et al., 2004). The ubiquitinated Itch was increased in UL42-deficient virus-infected cells, indicating that the total amount of Itch was increased.

It is unclear whether UL42 is ubiquitinated in HCMV-infected cells as the expression level of UL42 in HCMV-infected fibroblasts was lower than that in transfected 293T cells. Furthermore, the transfected Myc-tagged ubiquitin might be more readily detectable than the endogenous ubiquitin. Itch was activated by c-Jun N-terminal kinase (JNK) and the subsequent phosphorylation of Itch induced a conformational change after activation (Gao et al., 2004; Garnier et al., 1996). This is consistent with the activation of JNK by HCMV infection (Chaumorcel et al., 2012).

As Itch is self-ubiquitinated via an intermolecular mechanism (Scialpi et al., 2008), transfected FLAG-ItchCA might be ubiquitinated by endogenous Itch and then degraded (Fig. 4b, lane 5, upper panel). The association of UL42 with endogenous Itch might induce the ubiquitination of FLAG-ItchCA. As shown in Fig. 4(c), UL42PA mutant was more ubiquitinated than UL42wt. This result indicates that some cellular ubiquitin ligases might ubiquitinate UL42PA mutant; however, the ubiquitination by Itch was important for the degradation of UL42 protein. The degradation pattern of protein is changed by the linkage of ubiquitin, such as at Lys48 or Lys63. Lys48-linked polyubiquitin tagging is mostly used to target protein for degradation by the proteasome, but Lys63 was not (Tenno et al., 2004). Although we did not determine whether UL42 was ubiquitinated by Lys48 or Lys63, the difference in protein amounts might indicate a difference in polyubiquitin linkage.

The unmodified species of Itch was increased in UL42-deleted virus-infected cells (Fig. 5b). Together with the results for transfected cells shown in Fig. 4, this result indicates that UL42 induced the ubiquitination of Itch. The deletion of UL42 might cause an increase in the level of Itch protein. The Itch would then ubiquitinate itself and the level of ubiquitinated Itch would increase in the UL42-deficient virus-infected cells as shown in Fig. 5(d). HSV-2 UL56, a homologue of HCMV UL42, induces the degradation of Itch through the lysosomes (Ushijima et al., 2010). As with the UL56 of HSV-2, UL42 might induce the lysosomal degradation of Itch as well as its ubiquitination, although Itch was found to be relatively stable, as reported previously (Scialpi et al., 2008). As the level of UL42 was readily decreased by treatment with cycloheximide, despite Itch being highly stable (data not shown), we were unable to elucidate the half-life of Itch in the presence of UL42.

As shown in Fig. 3(c), Itch was accumulated in a fine-speckled pattern in the presence of UL42 in transfected fibroblasts. This result suggests that UL42 acts to alter the localization of Itch. It is, therefore, possible that Itch is able to approach new substrate(s) and ubiquitinate them through its interaction with UL42. Although the interaction with Nedd4 family proteins has not been clarified, the U24 of roseolovirus, which is a homologue of HCMV UL42, is known to be involved in the downregulation of CD3ε from the cell surface in transfected Jurkat cells through its PY motifs (Sullivan & Coscoy, 2008, 2010). Alternatively, HCMV might possess some substrates that are protected from ubiquitination by Itch. Itch and other Nedd4 E3 ligases are able to ubiquitinate PY motif-containing proteins (Shearwin-Whyatt et al., 2006). Amongst the PY motif-containing HCMV proteins, UL55, which encodes gB, has a PY motif in its C-terminal cytoplasmic domain. It is not yet clear whether Itch ubiquiticates gB or not and the stability of gB might have an important role in HCMV infection. It is speculated, therefore, that UL42 is able to protect them from unexpected ubiquitination by modifying Itch function.

In transfected cells, UL42 induced marked ubiquitination and degradation of Itch (Fig. 4). However, the effect of UL42 depletion in HCMV-infected cells was not so clear, although it did result in a significant increase in unmodified Itch (Fig. 5). This result indicates that UL42 induced the ubiquitination of Itch in HCMV-infected cells. These differences between infection and transfection imply that other viral proteins containing PY motifs might affect the stability of Itch. US29 and US30 are HCMV-encoded...
function-unknown proteins which possess a PY motif at their respective C-terminal domains. Interestingly, the deletion of US29 reduces the replication of HCMV in epithelial cells and the deletion of US30 enhances the replication of HCMV in fibroblasts (Dunn et al., 2003). It is possible that these PY motif-containing viral proteins coordinate to modify Itch function in order to modulate effective viral replication. If the cellular adaptor proteins such as Nedd4-1 affect the stability of Itch, the degree of Itch degradation by UL42 might be dependent on cell type. Although UL42 is dispensable for the replication of HCMV in fibroblasts, the effects of UL42 in other cell types remain to be clarified. As HCMV laboratory strains such as Towne lose endothelial cell tropism, it might be difficult to elucidate the precise function of UL42.

UL42 was colocalized with TGN in transfected fibroblasts (Fig. 2). In infected fibroblasts, UL42 was partially colocalized with the ubiquitin-binding protein p62 and lysosome/MVB marker CD63, although intracellular organelles were disrupted by HCMV infection (Fig. 6b). The ubiquitin-binding protein p62 is incorporated into autophagosomes as an LC3-binding protein (Komatsu & Ichimura, 2010). Furthermore, the degradation of UL42 is inhibited by lysosome and/or autophagosome inhibitors (Fig. 6c). These results indicate that UL42 was transported from the TGN to the lysosomal/autophagosomal membrane.

As previously reported, Itch was localized at the TGN and dispersed in the cytoplasm (Angers et al., 2004). As shown in Fig. 2, Itch was accumulated in the cytoplasmic vesicles in the presence of UL42, such as the endosome or lysosome, in transfected cells. Although endogenous Itch was not detectable in the immunofluorescence assay, our results indicate that Itch might colocalize with UL42 in infected cells and accumulate in the cytoplasmic vesicles. The perinuclear localization of UL42 indicated that UL42 accumulated at the assembly compartment of HCMV. Due to the location of herpesvirus envelopment, many viral tegument and envelope proteins are accumulated in the TGN-derived membrane (Mettenleiter, 2002). Endosome- or MVB-derived membranes are also candidates for HCMV final envelopment because some endosome markers such as EEA1 and CD63 are detected in HCMV virus particles (Cepeda et al., 2010). The surface of these membranous structures could be the ubiquitination site of some viral and host proteins by Itch and other Nedd4 family proteins with the assistance of UL42. Although the extracellular virus titre was shown to be slightly decreased by the deletion of HSV-2 UL56 (Ushijima et al., 2009), the deletion of UL42 did not affect the extracellular virus titre of HCMV (data not shown). In the case of HSV-2 infection, Itch was shown to completely disappear in infected cells, but not in HCMV-infected fibroblasts cells (Ushijima et al., 2010). Therefore, the extracellular titre might be not affected in UL42-deleted HCMV. However, if the UL42–Itch interaction is involved in viral envelopment, it might be important in specific cell types.

In terms of structural homology, due to the presence of a few PY motifs and a C-terminal TMD without an N-terminal signal sequence, HCMV UL42 resembles both HSV UL56 and VZV ORF0. HSV UL56 associates with Nedd4 and Itch through its PY motif (Ushijima et al., 2008, 2010). VZV ORF0, which is the most mutated gene in the VZV vaccine strain, shares amino acid sequence homology around its PY motifs with HSV UL56 (Koshizuka et al., 2010). A screening of the amino acid(s) sequences of other human herpesviruses has shown that the U24 of HHV-6A/B and HHV-7 and ORF16 of KSHV also contain a protein possessing the same features as HCMV UL42. Although KSHV ORF16, referred to as vBcl-2, possesses Bcl-2 homology (BH) domains and anti-apoptotic activity (Cheng et al., 1997; Sarid et al., 1997), none of HCMV UL42, HSV UL56, VZV ORF0 or HHV-6 U24 has a BH domain. HCMV UL43, the next gene to UL42, has some homology with HHV-6 U25 (Mocarski et al., 1997), indicating that the UL42–UL43 locus of HCMV is conserved in betaherpesviruses. EBV has no obvious UL42 homologue, but LMP2A is reported to be a Nedd4 E3 ligase family-binding membrane protein (Ikeda et al., 2000, 2003). As LMP2A has 12 TMDs, it is possible that an undefined UL42 homologous TA protein is encoded in the EBV genome. These findings indicate that the HCMV UL42 gene is conserved amongst herpesviruses and that the regulation of Nedd4 family E3 ligases is important in herpesviruses.

**METHODS**

**Cells and viruses.** Human fibroblasts and 293T cells (RIKEN Cell Bank) were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10 % FCS. Immortalized human fibroblasts (hTERT-BJ1 cells) were cultured with DMEM supplemented with 10 % newborn calf serum (NCS). HCMV strain Towne and recombinant viruses were propagated in hTERT-BJ1 cells with DMEM/2 %NCS.

**BAC mutagenesis.** As described in Fig. 5(a), HA-tagged UL42 (TowneBAC-HAUL42), UL42-deleted (TowneBAC-ΔUL42) and UL42-repaired (TowneBAC-HAUL42R) recombinant viruses were constructed using two-step Red-mediated mutagenesis (Tischer et al., 2006). In order to generate recombinant HCMV, the TowneBAC genome was engineered in Esherichia coli GS1783 (Jarosinski et al., 2007). Initially, the DNA fragment from pEP-KanS was amplified with the HAUL42 + Kfs (tcggtacccttcacacagcttaccccgctgcctgtgca gacagcgcttacccgggtgatttccgctgctg) and HAUL42 + Krv (gtggggggcgctgcctgagcggc ggcgtggatttccgctgctg) primer pair, and inserted into the TowneBAC genome to generate the TowneBAC-HAUL42-Km genome. Thereafter, the kanamycin resistance (Km') sequence was removed to generate the TowneBAC-HAUL42 genome. Another DNA fragment from pEP-KanS, which was amplified with the dUL42 + Kfs (tcggtacccttcacacagcttaccccgctgcctgtgca gacagcgcttacccgggtgatttccgctgctg) and dUL42 + Krv (tcggtacccttcacacagcttaccccgctgcctgtgca gacagcgcttacccgggtgatttccgctgctg) primer pair, was inserted into the TowneBAC-HAUL42 genome to generate the TowneBAC-HAUL42-Km genome. In order to construct the TowneBAC-HAUL42R genome, the HA-UL42 fragment was amplified from the TowneBAC-HAUL42-Km genome with primers UL42up (gtcccagagccggctgcctgctg) and UL42dn (gtccagagccggctgcctgctg)
Plasmids. The HA-tagged UL42 ORF was amplified from the TowneBAC-HAUL42 genome by PCR with primers HAFw (ggggaggtacactgattcatcagtag t; MluI site underlined) and UL42rv (gggggaattcagttgccaggtcactcctgcc; XhoI site underlined), and then cloned into a pCAGGS vector (Niwa et al., 1991) to generate pCAGGS-HAUL42wt. The tyrosine residues of two PY motifs were substituted by alanines to generate pCAGGS-HAUL42PA using an inverse PCR system (TOYOBO) with primers UL42PA1up (ggcgctggg ggcgcctgcttctga), UL42PA1dn (gagccgcaacctgtctgcgccg), UL42PA2up (gagcgggctgtgctaggctcttg) and UL42PA2dn (cgacccctgatctcgcttgattc). The UL82 ORF was amplified from the HCMV Towne genome by PCR and cloned into a pEF-BOS plasmid (Mizushima & Nagata, 1990) to generate pEF-UL82 with primers UL82fwNheI (ggggaattcagttgctca; NheI site underlined) and UL82rvXhoI (gagccgcaacctgtctgcgccg; XhoI site underlined). In order to generate anti-pp150, the UL32 ORF was amplified with the primer pair UL32fwBglII (gggggaattcagttgctca; BglII site underlined) and UL32rvHindIII (gggggaattcagttgctca; HindIII site underlined), and then digested with BamHI and Smal. The 5’ region of UL32 (nt 1–1541) was cloned into pGEX6P1 (GE Healthcare Bioscience) to construct pGEX-UL32. The FLAG-tagged Itch WT (ItchWT) and dominant-negative form (ItchCA) expression plasmids were kindly provided from Dr Annie Angers (University of Montreal, Montreal, Canada). The Myc-tagged ubiquitin expressing plasmid pC neo-mycUb was kindly provided by Dr Masahiro Fujimuro (Kyoto Pharmaceutical University, Kyoto, Japan). All plasmids were sequenced.

Antibodies. The GST-UL32 (pp150, aa 1–504) fusion protein was purified with glutathione Sepharose beads (GE Healthcare Bioscience) and used as an antigen to generate rabbit anti-pp150 antibody. The anti-HCMV intermediate-early protein (MAB810; Millipore), anti-FLAG (M2), anti-actin (Sigma), anti-Itch, anti-p230, anti-EEA1 and HA monoclonal and polyclonal, anti-Myc monoclonal (MBL), anti-ubiquitin (CST) antibodies were purchased from Life Technologies. 564-, 594- or 647-conjugated antibodies were purchased from Life Technologies. TechnoGenetics.

Immunoprecipitation. In order to detect protein–protein interactions, transfected 293T cells or recombinant HCMV-infected hTERT-BJ1 cells were lysed with PBS containing 1 % Triton X-100 and a protease inhibitor cocktail (Sigma). In order to detect protein ubiquitination, cells were lysed with a buffer containing 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, 5 mM EDTA and a protease inhibitor cocktail with PBS. Cell lysates were clarified by centrifugation at 100 000 g for 1 h at 4 °C and supernatants were immunoprecipitated with specific antibodies binding with Protein G Sepharose 4B (GE Healthcare).

Cell fractionation. Cells were fractionated as described previously (Koshizuka et al., 2002). In brief, cells were suspended in reticulocyte standard buffer (RSB; 10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl2) containing protease inhibitor cocktail. Cells were homogenized with 20 strokes in a Dounce homogenizer on ice and then an equal volume of either RSB, RSB containing 2 % Triton X-100, RSB containing 2 % sarcosyl (sodium lauroylsarcosinate; Sigma) or RSB containing 0.2 % SDS was added to aliquots of cell lysates. After a brief sonication, cell lysates were incubated for 30 min on ice and then centrifuged at 100 000 g for 1 h at 4 °C.

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