Tsg101 Interacts with Herpes Simplex Virus 1 VP1/2 and Is a Substrate of VP1/2 Ubiquitin-Specific Protease Domain Activity

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Ubiquitination/deubiquitination of key factors represent crucial steps in the biogenesis of multivesicular body (MVB) and sorting of transmembrane proteins. We and others previously demonstrated that MVB is involved in herpes simplex virus 1 (HSV-1) envelopment and budding. Here, we report that the HSV-1 large tegument protein, VP1/2, interacts with and regulates the ubiquitination of Tsg101, a cellular protein essential in MVB formation, thus identifying the first cellular substrate of a herpesviral deubiquitinating enzyme.

In order to optimize their replication, many viruses have evolved strategies to manipulate the cell ubiquitination/deubiquitination machinery. Interestingly, all members of the *Herpesviridae* family contain within their large tegument protein a cysteine protease module that displays deubiquitinating (DUB) activity, named herpesvirus tegument ubiquitin-specific protease (htUSP) (1). Even though htUSPs are in general dispensable for viral growth (2), they play important roles in viral replication and pathogenesis (3–6). However, information is still lacking on their cellular and/or viral substrates. In the case of herpes simplex virus 1 (HSV-1), the htUSP activity maps to the large tegument protein VP1/2, which is encoded by the UL36 gene. VP1/2 is an essential protein involved in several crucial steps of HSV replication (7–11), and its USP domain displays an autocatalytic activity (2). While VP12 hUSP activity has been shown to reside in the N-terminal 533 amino acids of the protein (1), the relevance of this autocatalytic activity and the presence of additional viral/cellular targets remain open issues. It has been proposed that VP1/2 hUSP might play a role during viral budding (1, 12). Indeed, the ubiquitination/deubiquitination machinery is part of a system involved in the formation of the multivesicular body (MVB), which is required for the exit of several enveloped viruses. Viruses hijack cellular proteins essential for MVB biogenesis by a direct binding mediated by short proline-rich motifs, named late domains (L-domains) (13). L-domains are divided into three classes: P(T/S)AP, PPxY, and YPxL, known to recruit specific cellular factors, Tsg101, Neddf4-like ubiquitin ligases, and AIP1, respectively (13). We and others have demonstrated that MVB is also involved in HSV-1 envelopment and budding and that ubiquitination of specific viral protein plays a role in this context (14–16). Thus, it is conceivable that VP1/2 could influence the ubiquitination status of specific proteins during crucial steps of viral replication. Moreover, it is well known that several cellular proteins involved in the formation of MVB undergo ubiquitination/deubiquitination cycles that are essential for their function (17). In this context, it has been reported that ubiquitination of Tsg101 plays an important role in regulating its activity (18, 19). Tsg101 is, in fact, involved in crucial cellular processes, such as the final stages of cytokinesis, in endosomal sorting, and in the degradation of membrane receptors. As an alteration of Tsg101 levels can severely affect the cell, Tsg101 expression is finely controlled posttranslationally. The Tsg101-associated ligase (Tal) has a crucial role in mediating this control (19) by ubiquitination of Tsg101-containing sorting complexes, thus impairing cargo proteins to be loaded and recycled (18). On the other hand, it is conceivable that DUB activities, not yet identified, are involved in the assembly of new sorting complexes, by balancing Tal’s action. Interestingly, VP1/2 contains two different sequences overlapping the canonical Tsg101 binding site, a PSAP and a PTAP motif, as shown in Fig. 1A.

Starting from these considerations, we cloned into the eukaryotic expression vector pcDNA3.1 (+) the full-length version of VP1/2 from the HSV-1 strain 17 (20) and a sequence corresponding to the N-terminal 767 amino acids of the viral protein, both enriched with a 5′ Flag tag (Flag-VP1/2 and Flag-VP1/21-767). The N-terminal 767 amino acids of VP1/2 were selected because they encompass the hUSP activity, the interaction sites for both VP16 and UL37, and a single canonical Tsg101 binding site (Fig. 1A). We also generated the mutant counterparts in which the cysteine of the USP active site (Cys65) was replaced by a serine residue to obtain proteins lacking the deubiquitinating activity (2) (Flag-VP1/2 C65S and Flag-VP1/21-767 C65S). As mentioned above, it has recently been shown that in transfected cells, both full-length and N-terminal variants of VP1/2 encompassing the core USP domain itself display autocatalytic activity (2). In order to analyze whether this feature was conserved in VP1/21-767 and whether it was influenced by other viral proteins, wild-type and C65S mutant forms of VP1/2 and VP1/21-767 were transfected into human kidney 293T cells. Twenty-four hours later, cells were either mock infected or infected with HSV-1 (19) at a multiplicity of infection (MOI) of 3 PFU/cell. Forty-eight hours postinfection, cell lysates were prepared and the protein complexes were immunoprecipitated with a monoclonal antibody (MAB) to the Flag epitope (21). Both VP1/2 C65S and VP1/21-767 C65S were monoubiquitinated, thus demonstrating that both VP1/2 and VP1/21-767 display autocatalytic activity in infected and transfected cells (Fig. 1B). More...
over, the proteins generated were mainly localized in the cytosol of infected cells (data not shown).

Next, with the aim of identifying new viral/cellular targets for VP1/2 htUSP activity, 293T cells were cotransfected with a construct expressing a hemagglutinin (HA)-tagged version of Tsg101 (21), along with constructs encoding either Flag-VP1/2, Flag-VP1/2 C65S, Flag-VP1/21–767, or Flag-VP1/21–767 C65S. Twenty-four hours later, cells were infected with HSV-1, strain 17, at an MOI of 3 PFU/cell. Forty-eight hours postinfection, cell lysates were prepared and the protein complexes were immunoprecipitated with an MAb to either the Flag epitope or the HA epitope, as previously described (21). The immunoprecipitated complexes were analyzed by Western blotting with an MAb to the HA tag or with MAb to Flag. We were able to demonstrate that, indeed, both VP1/2 and VP1/21–767 interact with Tsg101, independently from their htUSP activity (Fig. 2A). However, surprisingly enough, the interaction did not depend on the PSAP motif, at least not exclusively. Indeed, a VP1/21–767 mutant characterized by an AAAA replacing the PSAP motif (VP1/21–767AAAA) still interacted with Tsg101 (Fig. 2B).

VP1/21–767 is characterized by the presence of a PSGP motif (Fig. 1A), which partially resembles the canonical Tsg101-interacting motif and, at least in the context of the retroviral Gag, functions as a noncanonical L-domain (data not shown). However, also the disruption of this motif did not abrogate VP1/21–767/Tsg101 interaction (Fig. 2C). In conclusion, while there is a clear interaction between VP1/2 and Tsg101, the binding domain remains to be identified. Importantly, neither VP1/2 nor VP1/21–767 was able to interact with the glycoprotein B of the human cytomegalovirus (HCMV), an HA-tagged protein employed as a control (Fig. 2D). This finding further confirms the specificity of VP1/2-Tsg101 interaction.

Having demonstrated the Tsg101-VP1/2 interaction, we next addressed its functional relevance. 293T cells were transfected with constructs expressing Flag-Tsg101 (21), HA-ubiquitin (21) and the different forms of VP1/2. Cell lysates were harvested, and Western blotting was performed on Flag-immunoprecipitated proteins. Tsg101 was heavily ubiquitinated in cells expressing the htUSP-defective forms of VP1/21–767 and VP1/2 (Fig. 3A). In order to further validate this finding, the effects of VP1/21–767 and VP1/2 were also assayed on endogenous Tsg101. To this end, the experiment was repeated without overexpressing Flag-Tsg101 and by employing a Tsg101-specific antibody (4A10 mouse monoclonal antibody; GeneTex, Prodotti Gianni, Milan Italy) for the immunoprecipitation. Despite the low levels of endogenous Tsg101 in 293T cells, the results show that this protein is ubiquitinated in cells expressing the htUSP-defective form of VP1/21–767 (Fig. 3B).

To further correlate Tsg101 ubiquitination with VP1/2 htUSP activity, upon transfection with the Flag-Tsg101-expressing construct, 293T cells were infected with a VP1/2-defective HSV-1 (kindly provided by Helena Browne, University of Cambridge). As shown in Fig. 3C, in the absence of VP1/2, Tsg101 was clearly ubiquitinated in infected cells (Fig. 3C, lane 1). Tsg101 ubiquitination was even more evident when the mutant virus was comple-
VP1/2 specifically interacts with Tsg101 independently from the PSAP motif and the noncanonical L-domain PSGP. 293T cells were cotransfected with a plasmid expressing HA-Tsg101 along with constructs expressing Flag-VP1/2, Flag-VP1/2 C65S, Flag-VP1/21–767, or Flag-VP1/21–767 C65S with the wild-type PSAP motif (A), or characterized by different mutations (B), or with the PSGP domain changed into AAAA (C). In a series of transfections, HA-Tsg101 was replaced with a construct expressing the HCMV glycoprotein B (HCMV-gB) enriched with an HA tag at the C terminus (D). Twenty-four hours later, cells were infected with HSV-1, strain 17, at an MOI of 3 PFU/cell. Forty-eight hours postinfection, cell lysates were prepared and the proteins were immunoprecipitated (IP) with MAb to either the Flag epitope or the HA tag, as indicated. The immunoprecipitated complexes were analyzed by Western blotting with an MAb to HA at the C terminus (Tsg101 and gB) enriched with an HA tag at the C terminus (Tsg101 and gB). The asterisks indicate the heavy chain of the antibody. C stands for cells transfected with the pcDNA3.1 empty vector. In panel B, the table on the left lists the mutations at the level of the PSAP motif introduced into the Flag-VP1/21-767 construct. The numbers on the left of each gel represent molecular weight markers.
mented with VP1/2, C65S (Fig. 3C, lane 3). In contrast, when VP1/2 was employed for the complementation, the levels of Tsg101 ubiquitination were reduced (Fig. 3C, lane 2). Overall, these findings demonstrate that VP1/2 activity affects the levels of Tsg101 ubiquitination. It has been previously reported that in cells infected with a temperature-sensitive VP1/2 defective HSV-1, ubiquitin conjugates accumulated in excess in cytoplasmic clusters, overlapping or in close proximity to the VP1/2 clusters (12). Those authors argued that there is a defect in the stage in which mutant VP1/2 removes ubiquitin moieties from specific substrates necessary for late stages of viral assembly, such as cellular factors involved in the MVB biogenesis (14, 15). Our results strongly support this conclusion. In addition, in the absence of htUSP, even though Tsg101 was ubiquitinated, its intracellular levels were clearly not reduced (Fig. 3A and B, right). These results indicate that, in this case, Tsg101 ubiquitination is not linked to protein degradation. This finding is not unexpected if ubiquitination/deubiquitination of Tsg101 has a role in regulating the cellular protein activity more than its stability, as has previously been proposed (18). In this model, monoubiquitinated membrane receptors or structural viral proteins are sorted into cytoplasmic vesicles by a specific interaction with Tsg101 (18). Upon ubiquitination mediated by specific enzymes (i.e., Tal), Tsg101 is functionally inactivated, leaves the sorting complexes, and ends up in a

FIG 3 Tsg101 represents a target for VP1/2 htUSP activity. 293T cells were cotransfected with vectors expressing Flag-VP1/2, Flag-VP1/2, or their respective Flag-tagged C65S mutants, along with a construct encoding HA-ubiquitin. In a series of transfections, a construct expressing Flag-Tsg101 was also added (Tsg101). Twenty-four hours later, cells were infected with either HSV-1 (v-wt) (A and B), strain 17, or with a VP1/2 defective virus (v-AUL36) (C) at an MOI of 3 PFU/cell. Forty-eight hours postinfection, Tsg101 was immunoprecipitated (IP) from the cell lysates with MAb to Flag or to Tsg101, and Western blotting for HA (Tsg101-Ub), Flag (Flag-Tsg101), or endogenous Tsg101 (Tsg101) was performed. The asterisk indicates the heavy chain of the antibody. The respective cell lysates were analyzed by Western blotting with MAb to Flag (VP1/2), VP1/2, and Flag-Tsg101), to Tsg101 (Tsg101), and to tubulin (Tub).
detergent-soluble membrane subdomain. Cargo sorting will start again once DUBs, by removing ubiquitin, reexpress Tsg101 (18). A similar mechanism has been shown in the case of the Epsin-dependent sorting machinery (22). Thus, ubiquitination/deubiquitination cycles would represent the switch that recycles sorting machineries and enables reloading of cargo. Our data indicate a role for VP1/2 at this level in HSV-1-infected cells. It has to be mentioned that in the case of HSV-1, viral production seems to be independent from Tsg101 (16). On the other hand, a recombinant virus expressing a C65A mutant VP1/2 also exhibited little difference in single-step growth curves (2). However, despite the absence of a phenotype for these replication parameters, the htUSP activity of VP1/2 on Tsg101 might be relevant under specific conditions, for instance, in certain cell types or in vivo.

In conclusion, we provide here evidence for a direct involvement of VP1/2 DUB activity not only on VP1/2 itself, but also on a cellular protein which is known to play several crucial roles both in cellular processes and during the life cycle of different enveloped viruses (13). Tsg101 represents the first described cellular target for a herpesviral deubiquitinating enzyme. In addition, Tsg101 ubiquitination in HSV-1-infected cells was not previously described.

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REFERENCES

RETRACTION

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Volume 87, no. 1, 692–696, 2013. Pages 692–696: The authors regretfully retract this article at the request of the Journal of Virology. All the authors take responsibility for mistakes made in the final assembly of the figures, including unacceptable digital manipulation of data in panels of Fig. 1, 2, and 3, as follows:

1. In Fig. 1B lower left panel, bands in lanes 1 and 2 were duplicated in lanes 3 and 4 and in Fig. 1B, lower right panel, the band in lane 2 was spliced into the image.
2. In Fig. 2C, middle panel, the band in lane 4 was spliced into the gel image.
3. In Fig. 2D, middle panel, the band in lane 4 was spliced into the gel image.
4. In Fig. 3B, the “tubulin” blot in panel B was copied and pasted from the last three lanes of the “tubulin” blot of panel A and rotated 180 degrees.
5. In Fig. 3C, in the area above the bands in the right panel of the left grouping, a gray rectangle was pasted over the image.

The editor in chief of the Journal of Virology has reviewed our raw data and has concluded that our original findings are supported, and, more importantly, that the major conclusion of the paper still stands, i.e., that VP1/2 interacts with Tsg101 and modulates the level of Tsg101 ubiquitination. Since the integrity of the data as presented was compromised, however, the authors retract this publication. We apologize to the editors and the readers of the journal for any inconvenience caused by our mistakes. We intend to resubmit the paper with corrected figures for reconsideration by the Journal of Virology.