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Ovarian Tumor Domain-Containing Viral Proteases Evade Ubiquitin- and ISG15-Dependent Innate Immune Responses

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SUMMARY

Ubiquitin (Ub) and interferon-stimulated gene product 15 (ISG15) reversibly conjugate to proteins and mediate important innate antiviral responses. The ovarian tumor (OTU) domain represents a superfamily of predicted proteases found in eukaryotic, bacterial, and viral proteins, some of which have Ub-deconjugating activity. We show that the OTU domain-containing proteases from nairoviruses and arteriviruses, two unrelated groups of RNA viruses, hydrolyze Ub and ISG15 from cellular target proteins. This broad activity contrasts with the target specificity of known mammalian OTU domain-containing proteins. Expression of a viral OTU domain-containing protein antagonizes the antiviral effects of ISG15 and enhances susceptibility to Sindbis virus infection in vivo. We also show that viral OTU domain-containing proteases inhibit NF-κB-dependent signaling. Thus, the deconjugating activity of viral OTU proteases represents a unique viral strategy to inhibit Ub- and ISG15-dependent antiviral pathways.

INTRODUCTION

Viruses have evolved a panoply of different mechanisms to subvert cellular processes to their own advantage. In particular, viruses must overcome the potent antiviral and inflammatory effects of innate immune cytokines such as type I interferon (IFNαβ) and tumor necrosis factor alpha (TNFα). The induction and activity of these antiviral cytokines is controlled by, among other factors, Ub and Ub-like (UBL) molecules (Kirkin and Dikic, 2007). The activation of the transcriptional regulator nuclear factor-κB (NF-κB) by TNFα was one of the first immune regulatory pathways found to be dependent on protein ubiquitination (Karin and Ben-Neriah, 2000). NF-κB is sequestered in the cytoplasm of unstimulated cells through binding to IκB. TNFα binding to its receptor induces IκB phosphorylation followed by K^{48}-linked poly-ubiquitination targeting IκB for proteasomal degradation. This releases NF-κB dimers for translocation to the nucleus, where they regulate transcription. NF-κB-induced genes include type I IFN and other cytokines, hence this Ub-controlled pathway plays a vital role in innate and adaptive immunity, as well as in inflammation (Tergaonkar, 2006). In addition to ubiquitination of IκB, K^{63}- and K^{48}-linked ubiquitination regulates other molecules involved in this signaling pathway (Chen, 2005).

ISG15 is an interferon-induced UBL molecule with important antiviral activity against Sindbis, herpes simplex, influenza A, and influenza B viruses (Lenschow et al., 2007). ISG15, like Ub, is covalently conjugated to target proteins via a C-terminal LRLRGG sequence (Haas et al., 1987; Loeb and Haas, 1992; Narasimhan et al., 1996). Although conjugation is critical for the antiviral activity of ISG15 (Lenschow et al., 2005, 2007), the mechanism by which ISG15 mediates its antiviral function is not completely understood.

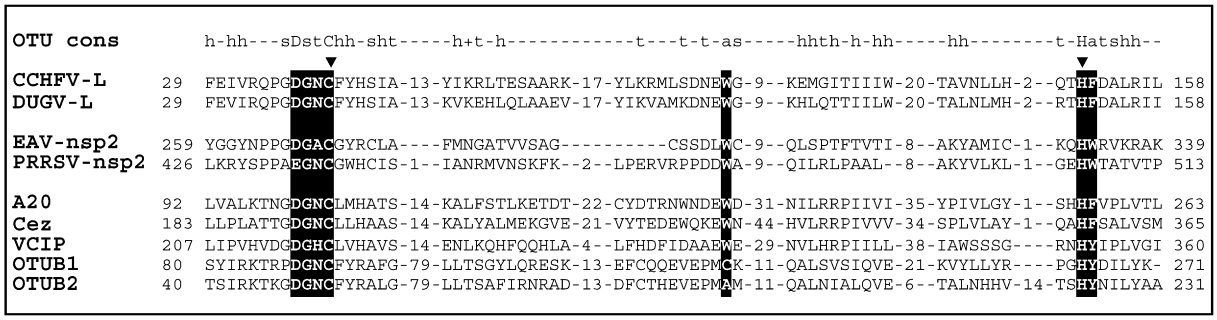

Figure 1. The OTU Domain Sequence Is Conserved across Viral and Mammalian Proteins

Multiple alignment of the OTU domains present in the proteins used in this study. In the consensus (Makarova et al., 2000), "h" indicates hydrophobic residues (A, C, F, L, I, M, V, W, Y, T, S, G), "s" indicates small residues (A, C, S, T, D, V, G, P), "+" indicates positively charged residues (R, K), "a" indicates aromatic residues (W, Y, F, H), and "t" indicates residues with high β-turn-forming propensity (A, C, S, T, D, E, N, V, G, P). Highly conserved residues are shaded in black. Numbers at the beginning and end of each sequence indicate the positions of the first and last aligned residue in the respective protein sequences; the numbers between aligned blocks indicate the numbers of residues that are not shown. CCHFV, Crimean Congo hemorrhagic fever virus; DUGV, Dugbe virus; EAV, equine arteritis virus; PRRSV, porcine respiratory and reproductive syndrome virus; VCIP, VCIP135; Cez, Cezanne; OTUB1 and OTUB2, Otubains 1 and 2, respectively.

Ub and ISG15 are synthesized as inactive precursors with C-terminal extensions that undergo cleavage to reveal the motif required for conjugation. Coordinated activities of an enzymatic cascade comprising an activating enzyme (E1), a conjugating enzyme (E2) and a ligase (E3) result in the conjugation of Ub or ISG15 to the ε-amino group of a lysine residue present in the target protein. Both Ub and ISG15 conjugation can be reversed by the activity of deconjugating enzymes. Deubiquitinating (DUB) proteolytic enzymes are also involved in processing of Ub precursors. Five classes of DUBs have been described (Nijman et al., 2005); one of the most recently identified is the ovarian tumor (OTU) domain family. This family comprises a group of putative cysteine proteases, homologous to the OTU protein in *Drosophila*, and includes more than a hundred proteins from eukaryotes, bacteria, and viruses (Makarova et al., 2000). Several OTU domain-containing mammalian proteins, such as Cezanne (Evans et al., 2003), Otubain 1 and 2 (Balakirev et al., 2003), and A20 (Evans et al., 2004) have been identified as proteases that participate in substrate-specific deubiquitinating processes. For example, A20 is an important downregulator of TNFα signaling via its deubiquitination of TRAF6 (Boone et al., 2004) and its dual function of poly-Ub^{63} deconjugation followed by poly-Ub^{48} modification of RIP (Wertz et al., 2004). These activities are mediated by its N-terminal OTU domain and its C-terminal zinc finger domain, respectively. However, the substrate specificity and physiologic role of most OTU domain-containing proteins remains unknown.

As protein ubiquitination and ISGylation are both important for innate immunity, rely on terminal LRLRGG sequences, and share a common mechanism of conjugation, we tested the hypothesis that viral OTU domain-containing proteases regulate Ub- and ISG15-dependent innate immunity via deconjugating protease activity. We found that two unrelated families of RNA viruses express OTU domain-containing proteases with the capacity to decrease Ub and ISG15 conjugation. Nairoviruses are negative-sense, segmented, RNA viruses of the *Bunyaviridae* family. Their large (L) protein contains an OTU domain and an RNA polymerase domain, placing this protein in the growing category of multifunctional viral proteins. Crimean Congo hemorrhagic fever virus (CCHFV) is a human nairovirus that causes hemorrhagic fever with up to 30% mortality (Whitehouse, 2004). Arteriviruses, including equine arteritis virus (EAV) and porcine respiratory and reproductive syndrome virus (PRRSV), are the causative agents of important diseases in horses and pigs. They are positive-sense, nonsegmented RNA viruses that contain an OTU domain within their nonstructural protein 2 (nsp2), which is also involved in viral replicase polyprotein processing (Snijder et al., 1995). We found that these viral OTU domains, in contrast to known mammalian OTU proteases, display a broad deconjugating activity toward ubiquitinated and ISGylated products and consequently inhibit innate immunity pathways that are dependent on Ub and ISG15. Thus, the deconjugating activity of viral OTU domains represents a unique strategy used by nairoviruses and arteriviruses to evade the host antiviral response, probably by targeting a common biochemical structure in Ub and the UBL protein ISG15.

RESULTS

OTU Domains in Viral and Mammalian Proteins

Sequencing of the L gene of the highly pathogenic human virus CCHFV (NFS and AGS, data not shown) (Honig et al., 2004; Kinsella et al., 2004) led to the identification of an OTU domain in the N-terminal region of the viral protein (Figure 1). This domain was also present in the L proteins of the nairoviruses Dugbe virus (DUGV) and Nairobi sheep disease virus (Honig et al., 2004) but has not been found in the L proteins of any other genus in the *Bunyaviridae*

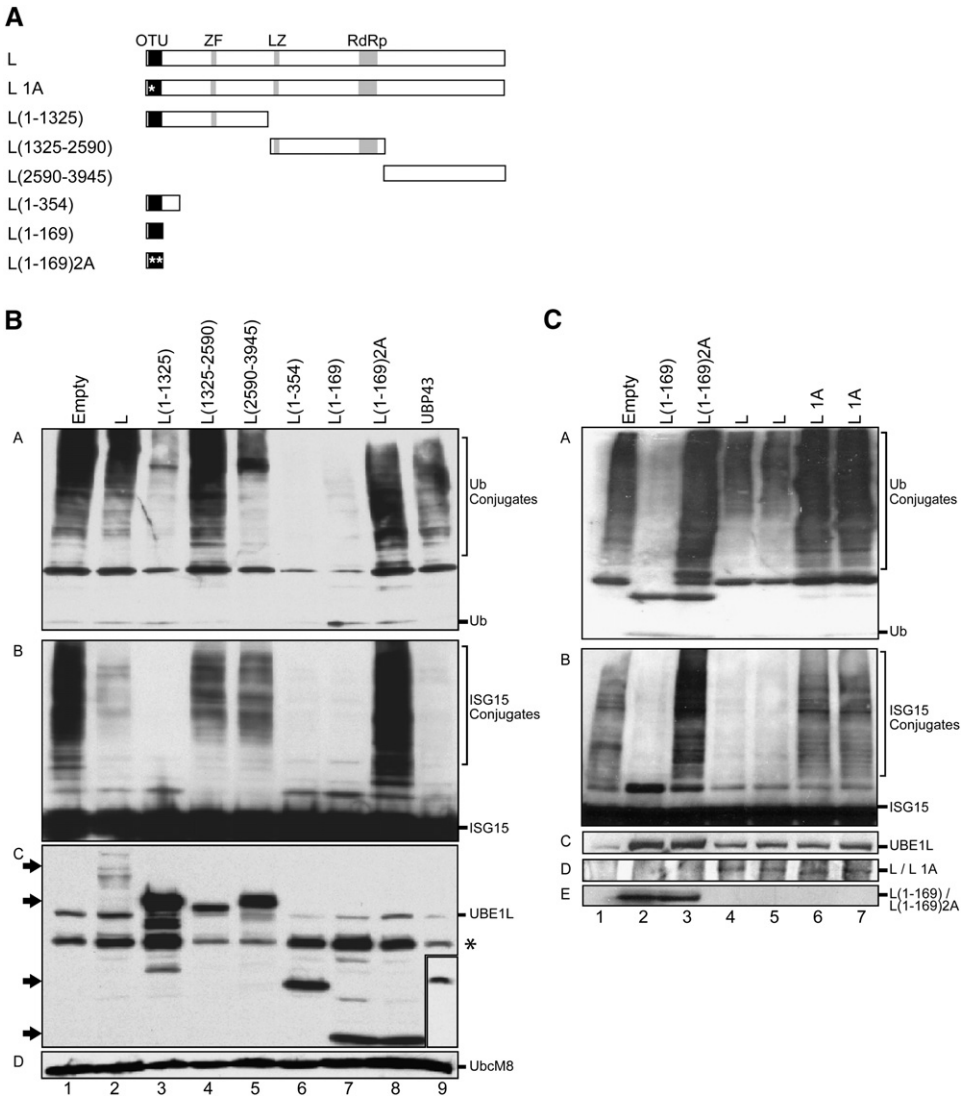

Figure 2. Levels of Ubiquitinated and ISGylated Proteins in Cells Expressing CCHFV-L and CCHFV-L Mutants

(A) Schematic representation of the CCHFV-L constructs utilized in these studies. Predicted protein domains within the protein: OTU domain; ZF, zinc finger; LZ, leucine zipper; RdRp, RNA-dependent RNA polymerase conserved motifs. White stars represent mutations in the OTU domain: 1A (C40A) or 2A (C40A and H151A). All constructs were HA tagged.

(B) 293T cells were transfected with either HA-Ub (panel A) or His-ISG15, HA-mUBE1L, and Flag-UbcM8 (panels B–D) along with HA-tagged CCHFV-L constructs, Flag-UBP43, or empty plasmid. Total protein ubiquitination was visualized by immunoblotting with anti-HA (panel A), and protein ISGylation was visualized by anti-ISG15 immunoblot (panel B). ISG15-transfected samples were also probed with anti-HA or anti-Flag (panels C and D) for detection of the CCHFV-L constructs (left arrows), mUbE1L, UBP43 (inset), and UbcM8. Asterisk indicates a nonspecific band.

(C) L and L 1A were analyzed for their effect on total ubiquitination (panel A) or ISGylation (panel B) as described in (B). Expression of HA-tagged UBE1L (panel C), L and L 1A (panel D), and L(1–169) and L(1–169)2A (panel E) is shown.

family. An alignment of the OTU domains present in nairoviruses, arteriviruses, and mammalian proteins A20, Cezanne, VCIP135, Otubain 1, and Otubain 2 revealed limited identity; however, a strong conservation of D^{37}, G^{38}, N^{39}, C^{40}, W^{71}, H^{151}, and an aromatic amino acid at position 152 (numbering based on the CCHFV-L sequence) was observed (Figure 1, black boxes). Among these amino acids, C^{40} and H^{151} (Figure 1, black arrows) were predicted to be the catalytic residues present in the putative protease active site (Balakirev et al., 2003; Makarova et al., 2000; Nanao et al., 2004; Snijder et al., 1995).

Impact of CCHFV-L Expression on Protein Ubiquitination and ISGylation

Given that host OTU domain proteins have deubiquitinating activity (Nijman et al., 2005), we tested the hypothesis that the L protein of CCHFV (CCHFV-L) has deubiquitinating and deISGylating activity (Figure 2). Transfection of

cells with plasmids expressing CCHFV-L slightly decreased the overall expression of Ub-conjugated proteins (Figure 2B, lanes 1 and 2, panel A; Figure 2C, lanes 1, 4, and 5, panel A). To test the effect of CCHFV-L expression on protein ISGylation, ISG15 conjugates were generated by transfecting plasmids expressing ISG15 and its specific E1 (UBE1L) (Yuan and Krug, 2001) and E2 (UbcM8) (Kim et al., 2004; Zhao et al., 2004) enzymes, since endogenous levels of ISGylated proteins are low in the absence of IFN stimulation. Cotransfection of CCHFV-L resulted in a clear decrease in the level of ISGylated proteins (Figure 2B, lanes 1 and 2, panel B; Figure 2C, lanes 1, 4, and 5, panel B). This decrease was also observed when CCHFV-L was untagged (data not shown). The decrease in total ISGylation was comparable to the effect of UBP43, a known ISG15 deconjugating enzyme (Figure 2B, lane 9, panel B). Expression of CCHFV-L did not affect levels of expression of UBE1L or UbcM8 (Figure 2B, lane 2, panels C and D; Figure 2C, lanes 4 and 5, panel C), consistent with CCHFV-L acting via inhibition of ubiquitination and ISGylation reactions or by directly deubiquitinating or deISGylating proteins.

The OTU Domain of CCHFV-L Decreases the Levels of Ubiquitinated and ISGylated Proteins

To determine the region of the L protein responsible for decreasing ubiquitinated and ISGylated proteins, plasmids expressing three portions of the L protein were constructed (Figure 2A). Expression of the OTU domain-containing N-terminal portion, L(1–1325), resulted in the greatest decrease of Ub and ISG15 conjugates (Figure 2B, lane 3, panels A and B). To further map this region, truncation mutants of the L protein expressing only the first 354, L(1–354), or 169 amino acids, L(1–169), were tested. The results indicated that the region responsible for the decreased levels of Ub and ISG15 conjugates mapped to the OTU domain, L-(1–169) (Figure 2B, lanes 6 and 7, panels A and B; Figure 2C, lane 2, panels A and B).

The Predicted Protease Active Site of the CCHFV-L OTU Domain Is Required for Reducing Ub and ISG15 Conjugates

To test whether the amino acids C^{40} and H^{151} (Figure 1, black arrows) were critical for the observed reduction in ubiquitinated and ISGylated proteins by the CCHFV-L OTU domain, we expressed a full-length L protein with a C^{40} to A^{40} mutation (L 1A) and a mutant L(1–169) protein in which both amino acids were replaced by alanines [L(1–169)2A] (Figure 2A). Expression of L 1A and L(1–169)2A proteins did not decrease levels of ubiquitinated or ISGylated proteins (Figure 2B, lane 8, panels A and B; Figure 2C, lanes 3, 6, and 7, panels A and B), strongly suggesting that the OTU domain contains a cysteine protease activity that mediates the decrease in ubiquitinated and ISGylated proteins.

The OTU Domain of CCHFV-L Is a Deconjugating Enzyme with Specificity for Poly-Ub Conjugates and ISG15

To determine whether the CCHFV-L OTU domain directly deconjugates Ub and ISG15 from target proteins, we expressed and purified L(1–169) and a catalytic C^{40} to A^{40} mutant, L(1–169)1A, for in vitro deconjugation assays (Figure 3A). Recombinant L(1–169) cleaved both K^{48}- and K^{63}-linked poly-Ub chains into monomers (Figure 3B, lanes 2 to 5), similarly to isopeptidase T, a known DUB enzyme (Figure 3B, lane 10). This activity was markedly decreased by mutating the amino acid C^{40} (Figure 3B, lanes 6 to 9), indicating that this residue is required for optimal protease activity. The small amount of deconjugation observed with L(1–169)1A is not unexpected, as mutation of Cezanne's catalytic cysteine yielded similar data where most, but not all, catalytic activity was impaired (Evans et al., 2003). This result shows that the OTU domain of CCHFV-L has bona fide DUB activity in the absence of other cellular proteins.

We next determined whether L(1–169) can deconjugate ISGylated proteins. For this, we generated cell lysates from IFNβ-treated *Ubp43*$^{-/-}$ murine embryonic fibroblasts (MEFs) that are rich in ISG15 conjugates (Malakhov et al., 2003). Incubation of these lysates with recombinant L(1–169) protein, but not L(1–169)1A, appreciably decreased ISGylated proteins (Figure 3C, top panel) in a L(1–169) concentration-dependent manner. This result suggests that the CCHFV OTU domain has C^{40}-dependent ISG15 deconjugating activity but does not exclude the possibility that the protease activity of the viral OTU domain was activating another deISGylating enzyme present in cell lysates. To address this possibility, we enriched 6xHisISG15 conjugates using affinity chromatography. Incubation of ISG15 conjugates with increasing amounts of L(1–169) resulted in ISG15 deconjugation (Figure 3C, lanes 2 to 5, bottom panel). No deconjugation was detected with the mutant L(1–169)1A protein (Figure 3C, lanes 6 to 9, bottom panel). In addition, L(1–169) processed a pro-ISG15 protein into its mature form (Figure 3E, lane 2, panel A). These data suggest that the CCHFV-L OTU domain directly deconjugates ISGylated proteins through its predicted cysteine protease activity.

To gain further insights on the specificity of the OTU domain, we assessed the ability of L(1–169) and L(1–169)1A to hydrolyze poly-SUMO-2 (Figure 3D, top panel) and poly-SUMO-3 chains (Figure 3D, bottom panel), pro-SUMO-1 (Figure 3E, panel C), and pro-Nedd8 (Figure 3E, panel B). While the catalytic domain of SENP2 ($SENP2_{CD}$), a SUMO-specific protease, could hydrolyze SUMO chains to monomers (Figure 3D, lane 10) and process pro-SUMO-1 (Figure 3E, lane 5, panel C), neither L(1–169) nor L(1–169)1A was able to cleave SUMO chains or a pro-SUMO-1 precursor. However, similar to its ability to process pro-ISG15 (Figure 3E, lane 2, panel A), L(1–169) hydrolyzed pro-Nedd8 into a mature form (Figure 3E, lane 2, panel B). In summary, the OTU domain of CCHFV-L hydrolyzes Ub and ISG15, but not SUMO-2 or SUMO-3,

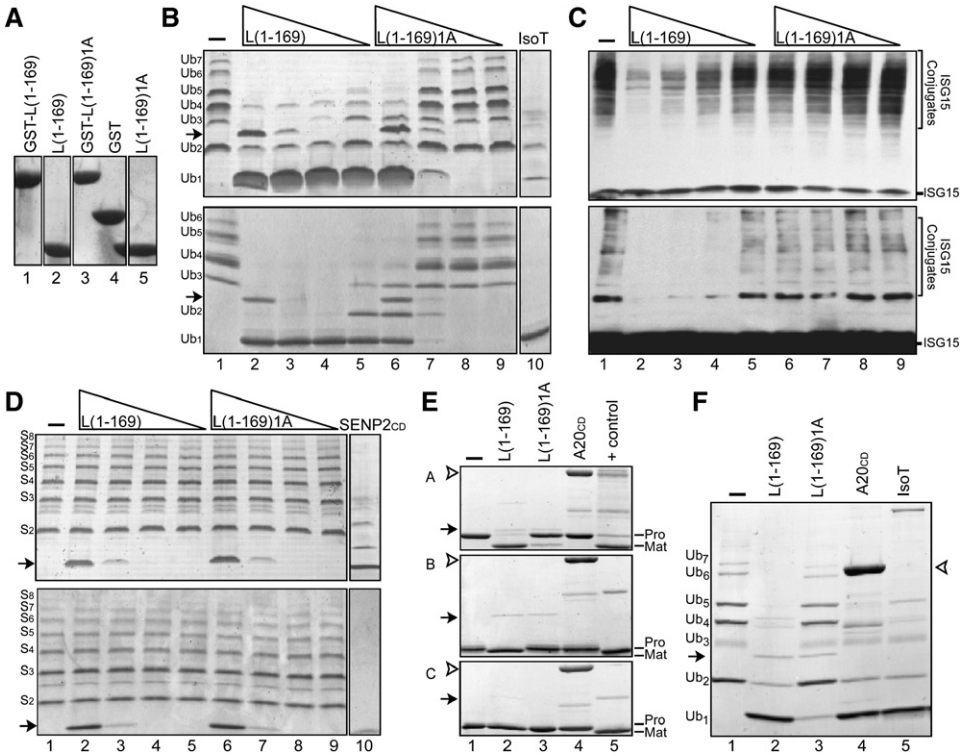

Figure 3. In Vitro Ub and ISG15 Deconjugation Activities of the CCHFV-L OTU Domain

(A) Coomassie-stained gel of GST-L(1–169), L(1–169), GST-L(1–169)1A, and L(1–169)1A recombinant proteins. L(1–169) and L(1–169)1A proteins were used for the in vitro experiments.

(B) K^{48} (top panel)- or K^{63} (bottom panel)-linked poly-Ub chains were incubated with reaction buffer (lane 1) or 10-fold dilutions of L(1–169) or L(1–169)1A recombinant proteins, subjected to SDS-PAGE and visualized by Coomassie staining. Isopeptidase T (IsoT) was used as a positive control. Black arrows indicate L(1–169) and L(1–169)1A proteins.

(C) Lysates of *Ubp43*$^{-/-}$ MEFs (top panel) or ISG15 conjugates purified from ISG15, HA-mUBE1L, and Flag-UbcM8 transfected 293T cells (bottom panel) were incubated with reaction buffer (lane 1) or 10-fold dilutions of L(1–169) or L(1–169)1A protein. ISG15 conjugates were visualized by anti-ISG15 immunoblot.

(D) SUMO-2 (top panel) or SUMO-3 (bottom panel) chains were incubated with reaction buffer (lane 1) or 10-fold dilutions of L(1–169) or L(1–169)1A and visualized by Coomassie staining. His_6-$SENP2_{CD}$ was used as a positive control. Black arrows indicate L(1–169) and L(1–169)1A proteins. $S_{2–8}$ indicates number of SUMO-2 or SUMO-3 molecules.

(E and F) ProISG15 (panel A), proNedd8 (panel B), proSUMO-1 (panel C) or K^{48}-linked Ub (F) chains were incubated with reaction buffer, L(1–169), L(1–169)1A, or A20 catalytic domain ($A20_{CD}$) and visualized by Coomassie staining. Positive controls indicate incubation with UBP43 (panel A), NEDP1 (panel B), $SENP2_{CD}$ (panel C), or IsoT (F). Black arrows indicate L(1–169) and L(1–169)1A proteins, and white arrowhead indicates $A20_{CD}$. "Pro" indicates the pro-UBL molecule form, and "Mat" indicates the mature protein.

from conjugates in vitro, suggesting that viral OTU domains have the unique ability to recognize Ub and specific UBL molecules.

Additional Viral OTU Domains Mediate Deubiquitination and DeISGylation

In addition to CCHFV, viral OTU domains are found in the L proteins of other nairoviruses and in the nsp2 proteins of arteriviruses such as EAV and PRRSV (Figure 1). The arterivirus nsp2 cysteine protease cleaves the nsp2/nsp3 site within the large viral polyprotein during replicase maturation. In the case of EAV, this process is known to be mediated by the 166 N-terminal residues of nsp2, which contains the OTU domain (Snijder et al., 1995). In our study, a slightly larger N-terminal EAV-nsp2 domain (175 amino acids; nsp2N) was used in addition to the full-length protein. The OTU domain of the L protein of DUGV, a nairovirus related to CCHFV, as well as EAV-nsp2, EAV-nsp2N, and PRRSV-nsp2, decreased Ub and ISG15 conjugates when expressed in 293T cells (Figure 4A, lanes 3–6), indicating that deconjugation may be an immune evasion strategy shared by diverse viral families.

Previous studies have demonstrated that the OTU domain-containing mammalian proteins Otubain 1, Otubain 2, Cezanne, VCIP135, and A20 cleave poly-Ub chains in vitro (Balakirev et al., 2003; Evans et al., 2003, 2004; Wang et al., 2004). In contrast, only overexpression of Otubain 1 and Cezanne moderately decreased cellular global Ub conjugate levels, while expression of A20 or Otubain 2 had no effect on total levels of ubiquitinated proteins (Balakirev et al., 2003; Evans et al., 2003, 2004). To date, there have been no published studies investigating the effects of these proteins on ISG15 conjugates. We therefore tested Otubain 1, Otubain 2, Cezanne, VCIP135, and A20 for their

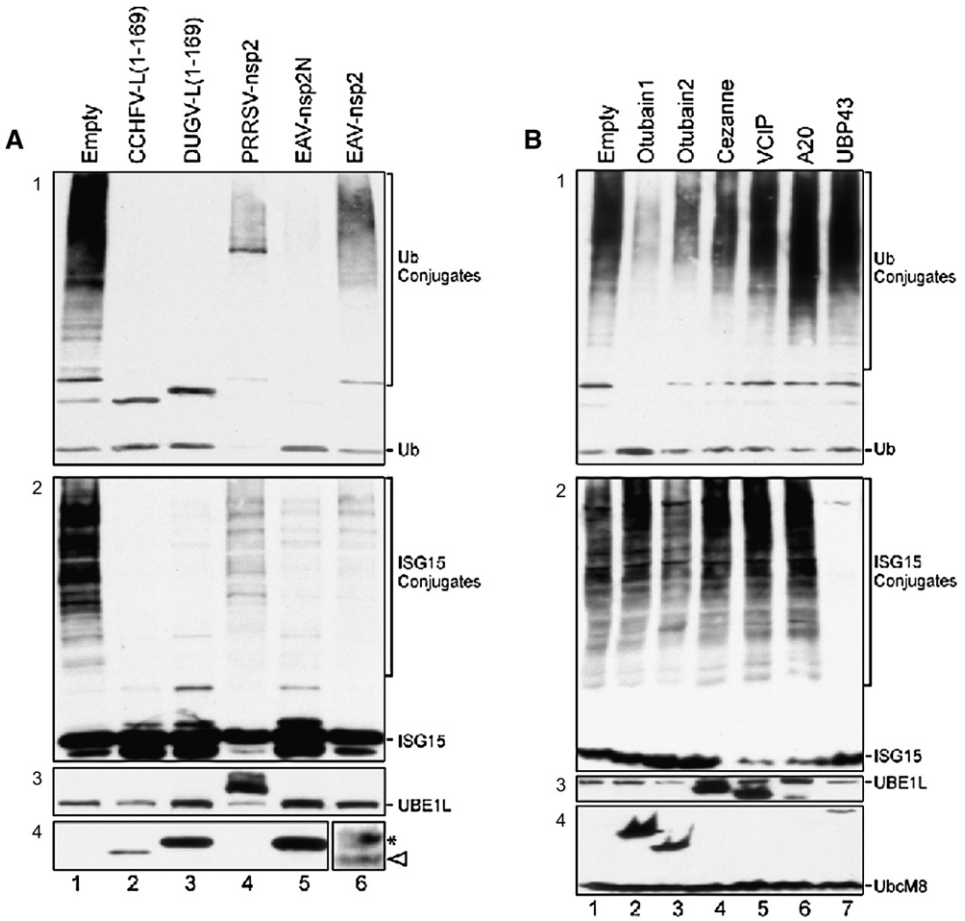

Figure 4. Ub and ISG15 Deconjugation Activity of OTU Domain-Containing Polypeptides of Viral and Mammalian Origin

(A) CCHFV-L(1–169), DUGV-L(1–169), PPRSV-nsp2, EAV-nsp2N, or EAV-nsp2 were cotransfected into 293T cells with either HA-Ub (panel 1) or ISG15, HA-mUBE1L, Flag-UbcM8, and Herc5 plasmids (panels 2 and 3). Samples were immunoblotted for HA (panel 1) or ISG15 (panel 2). ISG15-transfected samples were also probed with anti-HA (panel 3) or anti-Flag plus anti-HA (panel 4) to show expression of HA-mUBE1L, PPRSV-nsp2, CCHFV-L(1–169), DUGV-L(1–169), EAV-nsp2N, or EAV-nsp2 (inset, open triangle). Asterisk indicates a nonspecific band.

(B) Otubain 1, Otubain 2, Cezanne, VCIP135, A20, or UBP43 were analyzed for their effect on total ubiquitination (panel 1) or ISGylation (panels 2–4) as described in (A). Expression of HA-tagged Cezanne and VCIP135 (panel 3) and Flag-tagged Otubain 1 and 2 and UBP43 (panel 4) is shown.

ability to decrease overall protein ubiquitination and ISGylation in transfected cells (Figure 4B). Expression of Otubain 1 resulted in a significant decrease in Ub conjugate levels, while Otubain 2 and Cezanne had a lesser effect (Figure 4B, lanes 2–4, panel 1). Consistent with their specificity for particular ubiquitinated substrates, expression of VCIP135 and A20 did not result in a decrease in overall ubiquitination. None of the OTU-containing mammalian proteins tested decreased global levels of ISG15 conjugates (Figure 4B, lanes 2–6, panel 2). Similar results were obtained when truncation mutants expressing the OTU domains of Otubain 1, Otubain 2, Cezanne, and A20 were tested (data not shown). In addition, the OTU-containing catalytic domain of A20 ($A20_{CD}$) was unable to process either ISG15 or Nedd8 (Figure 3E, lane 4, panels A and B), even though it cleaved K^{48}-linked Ub chains (Figure 3F, lane 4). By contrast, overexpression of the mammalian deISGylating enzyme UBP43 decreased overall levels of ISG15 conjugates but not Ub conjugates (Figure 4B, lane 7). Thus, viral OTU proteases appear to be unique in their ability to target both ISG15 and Ub conjugates.

Transgenic Mice Expressing CCHFV-L(1–1325) Have Increased Susceptibility to Sindbis Virus Infection

To assess the effect of expressing an OTU domain during viral infection, we generated transgenic mice expressing the CCHFV-L(1–1325) OTU domain-containing protein, which exhibits DUB and deISGylating activities (Figure 2B, lane 3). We obtained germline transgene transmission in three lines designated 1836, 1854, and 2929, and we evaluated L(1–1325) expression in both MEFs and brain lysates from these transgenic lines. MEF cells and brain tissue from 1836 transgenic mice contained detectable L(1–1325) protein, while protein expression from the 1854 and 2929 lines was either undetectable or very low (Figures 5A and 5B). We next evaluated the

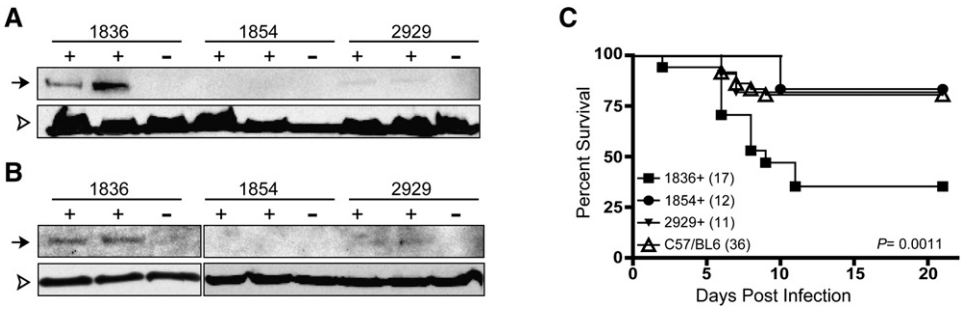

Figure 5. Expression of L(1–1325) Transgene Correlates with Increased Susceptibility to Sindbis Virus Infection

(A and B) Expression of L(1–1325) transgene and actin in MEFs (A) and brain lysates (B). + indicates a transgene-positive mouse, and − indicates a C57/BL6 mouse. The arrows indicate L(1–1325) protein and open triangles denote actin.

(C) Survival of L(1–1325) transgenic mice following infection with Sindbis virus AR86. Transgene-negative littermates from 1836, 1854, and 2929 served as C57/BL6 controls. Numbers of mice in each group are indicated in parenthesis. Comparison by statistical analysis was made between 1836+ and C57/BL6 ($p = 0.0011$).

sensitivity of L(1–1325) transgenic mice to infection with the virulent Sindbis virus strain AR86, an alphavirus that causes lethal encephalitis in young mice and is sensitive to ISG15-mediated antiviral effects (Lenschow et al., 2005). Susceptibility to Sindbis virus infection tracked with expression of the L(1–1325) protein (Figure 5C). Thirty-five percent of mice from the 1836 transgenic line survived infection compared to ≥80% survival in C57/BL6 littermate controls or transgenic mice expressing low or undetectable levels of transgene-encoded protein. The decreased survival of line 1836 transgenic mice following AR86 infection suggests that CCHFV-L OTU enhances susceptibility to viral disease in vivo.

The OTU Domain of CCHFV Overcomes ISG15-Mediated Protection from Sindbis Virus-Induced Lethality

The increased pathogenicity of Sindbis virus observed in L(1–1325)-expressing mice suggested that the CCHFV-L OTU domain might counteract the antiviral activities of ISG15 in vivo. It was previously shown that expression of ISG15 from the chimeric Sindbis virus dsTE12Q protects adult *IFNαβR*$^{-/-}$ mice from Sindbis virus-induced lethality (Lenschow et al., 2005). To determine whether expression of the CCHFV-L OTU domain would antagonize this protective effect of ISG15, we engineered four recombinant chimeric Sindbis viruses (Figure 6A). Two viruses expressed ISG15 followed by an IRES element to drive translation of either L(1–169) (169GG) or enzymatically inactive L(1–169)2A (MTGG). We also generated control viruses that expressed either L(1–169) (169) or L(1–169)2A (MT) in the absence of ISG15.

The recombinant viruses expressed the OTU domains and ISG15 as expected (Figures S1A and S1B in the Supplemental Data available with this article online) as well as similar levels of Sindbis virus proteins in infected cells (Figure S1C). All four viruses grew with similar kinetics to similar final titers under single-step growth conditions in BHK-21 cells (Figure S2). This is expected, as to date ISG15 has only demonstrated antiviral activity in vivo.

We assessed the ability of the L(1–169) protein expressed from within the Sindbis virus genome to deISGylate and deubiquitinate proteins by infecting BHK-21 cells (Figure 6B). Infection with 169GG or 169, but not MTGG or MT, reduced the amount of Ub conjugates detected in cells (Figure 6B, right panel), indicating that the viral OTU domain functions as a DUB enzyme when expressed from a Sindbis virus. Following transfection with ISG15 and its E1, E2, and E3 enzymes, ISGylated proteins can be detected in BHK-21 cells (Figure 6B, lane 1, middle panel). Infection with 169GG or 169 greatly reduced ISG15 conjugates, confirming that OTU expression results in deconjugation of ISGylated proteins. When cells were transfected with the E1, E2, and E3 enzymes but not ISG15, ISG15 conjugates were observed only following MTGG infection (Figure 6B, left panel). This shows that ISG15 expressed from dsTE12Q is capable of ISGylating proteins in the presence of the relevant conjugating enzymes but that this is only seen in the presence of a catalytically inactive form of the coexpressed L(1–169) protein (Figure 6B, lane 3, left panel).

We then determined whether OTU expression could counter ISG15's in vivo antiviral effect. In order to exclude effects due to IFNαβ-stimulated genes other than ISG15, we infected *IFNαβR*$^{-/-}$ mice (Figure 6C). Seventy percent of mice infected with a virus expressing ISG15 and the mutant OTU domain (MTGG) survived, consistent with previous observations that expression of ISG15 protects mice from lethality following Sindbis virus infection (Lenschow et al., 2005). In contrast, only 20% of mice infected with a virus expressing ISG15 and a functional OTU domain (169GG) survived infection ($p = 0.0015$). These data also correlate with our in vitro data demonstrating that L(1–169), but not L(1–169)2A, can deISGylate proteins following infection (Figure 6B). Mice infected with 169 or MT died with similar kinetics, demonstrating that the expression of L(1–169) did not increase the virulence of dsTE12Q in the absence of the IFN-mediated antiviral response. The slight increase in survival between 169GG and 169 ($p < 0.0001$) or MT ($p = 0.0032$) suggests that

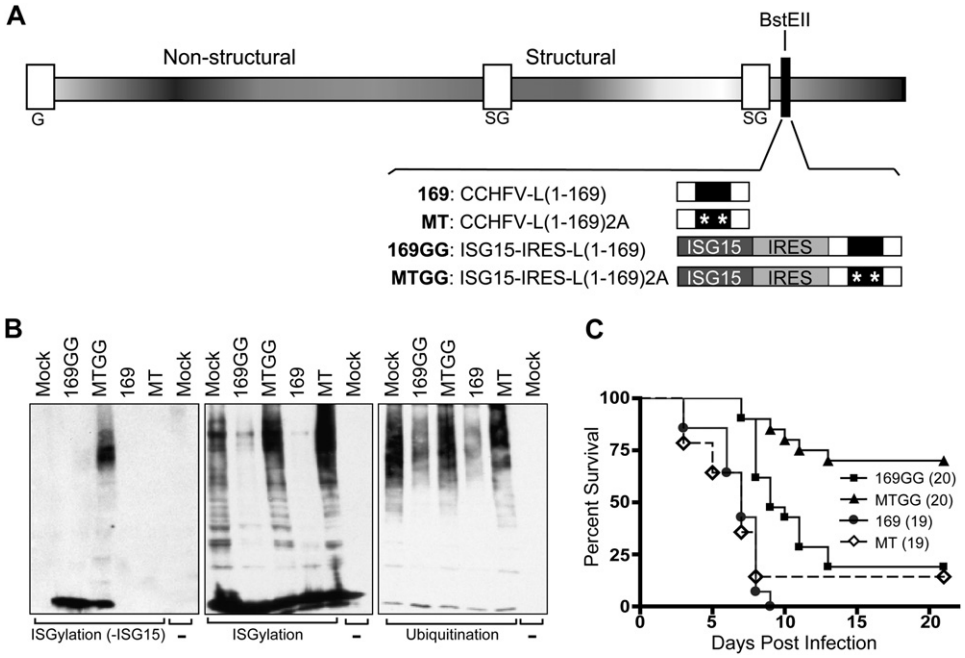

Figure 6. Sindbis Viruses Expressing the CCHFV-L OTU Domain Deconjugate Ub and ISG15 and Inhibit ISG15-Mediated Antiviral Effects in Mice

(A) Schematic diagram representing the CCHFV OTU domain-expressing Sindbis viruses utilized in these studies. G, genomic promoter; SG, subgenomic promoter.

(B) BHK-21 cells were transfected with UBE1L, UbcM8, and Herc5 (left panel), UBE1L, UbcM8, Herc5, and ISG15 (middle panel), or HA-Ub (right panel) and subsequently infected with recombinant Sindbis viruses as indicated. Cells lysates were immunoblotted with anti-ISG15 (left and middle panels) or anti-HA (right panel) antibodies. – indicates untransfected cells.

(C) *IFNαβR*$^{-/-}$ mice were infected with recombinant Sindbis viruses as indicated and monitored for survival. Data are pooled from four independent experiments, and numbers of mice in each group are indicated in parenthesis. Differences in survival were analyzed by the log-rank test: 169GG and 169 ($p < 0.0001$), 169GG and MT ($p = 0.0032$), 169GG and MTGG ($p = 0.0015$), MTGG and 169 ($p < 0.0001$), and MTGG and MT ($p < 0.0001$).

expression of the CCHFV OTU domain cannot completely antagonize the effects of ISG15 in this system. Nevertheless, these data show that expression of a catalytically active viral OTU domain can antagonize the antiviral effects of ISG15 in vivo.

Negative Regulation of the NF-κB Pathway by Viral OTU Domains

While the data above indicate that a viral OTU domain protease can counter the antiviral activities of ISG15, they do not address the possibility that the DUB activity of these proteins might also play a role in immune evasion. To address this hypothesis, we evaluated the effects of the CCHFV-L and EAV-nsp2 OTU domains on the NF-κB signaling pathway. Expression of the OTU domains of CCHFV-L and of EAV-nsp2 decreased in a dose-dependent manner the activation of an NF-κB-responsive promoter (Fujita et al., 1992) after TNFα treatment. This inhibition was similar to that mediated by A20, an OTU domain-containing inhibitor of the NF-κB pathway (Figure 7A). Inhibition was about 10-fold greater in the presence of the L(1–169) domain than the L(1–169)2A mutant, indicating a role for the OTU domain protease activity. These results were further confirmed by the ability of CCHFV-L(1–169) to inhibit NF-κB activation as measured by the inhibition of endogenous p65 nuclear translocation upon TNFα treatment (Figures 7B and 7C). The slight inhibition of NF-κB activation by the L(1–169)2A protein could be caused by some residual binding of this mutant to ubiquitinated substrates or by the presence of some other regulatory motifs within this protein. Nevertheless, the p65 nuclear translocation inhibition by the L(1–169) protein was significantly higher when compared to its mutant counterpart ($p = 0.0044$). Overall, these results demonstrate the ability of viral OTU domains to affect immune pathways that are regulated by ubiquitination.

DISCUSSION

Here we show that viral OTU domain-containing proteins are proteases that hydrolyze Ub and ISG15 from conjugated proteins. This dual deconjugating activity provide an elegant example of the economy of viral evolution, since both Ub and ISG15 rely on a conserved conjugation motif. Furthermore, the protease activity by the viral OTU domains has the physiologic capacity to evade two different cytokine pathways, IFNαβ and TNFα, that are fundamentally important for antiviral immunity.

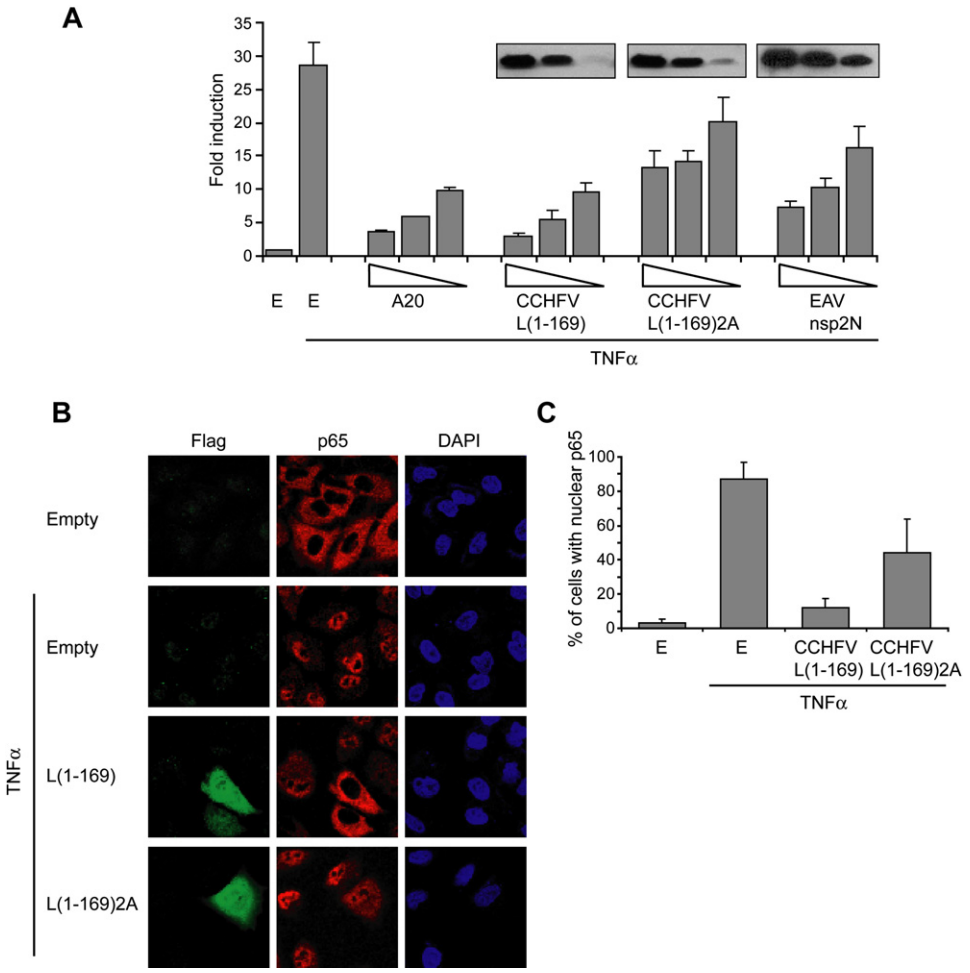

Figure 7. CCHFV-L and EAV-nsp2 OTU Domains Inhibit TNFα-Mediated NF-κB Activation

(A) NF-κB reporter assay in 293T cells transfected with OTU domains and treated with TNFα. Results shown are an average of three independent experiments. The western blot indicates expression of viral OTU proteins as detected with anti-HA [CCHFV-L(1–169) and CCHFV-L(1–169)2A] or anti-Flag antibodies (EAV-nsp2N). E, empty plasmid.

(B) A549 cells were transfected with indicated plasmids, stimulated with TNFα, and stained for p65 (red) and L(1–169) or L(1–169)2A (green). Nuclei were stained with DAPI (blue). The result shown is a representative of three independent experiments.

(C) L(1–169) or L(1–169)2A transfected cells in (B) were scored according to subcellular distribution of p65. Differences in p65 nuclear accumulation in TNFα-treated cells were analyzed by Student's t test: E and L(1–169) ($p < 0.0001$), E and L(1–169)2A ($p = 0.0007$), and L(1–169) and L(1–169)2A ($p = 0.0045$). E, empty plasmid. The result shown is a representative of three independent experiments.

Data in (A) and (C) are presented as mean ± SD (n = 3).

Viral DUB and DeISGylating Enzymes: A Unique Strategy for Immune Evasion?

Biochemical and genetic evidence supports the concept that protein ubiquitination plays a critical role in the induction of both the innate and the adaptive cellular immune system (Liu et al., 2005). For example, in addition to NF-κB signaling, Ub regulates several aspects of antiviral immunity, such as MHC class I and II antigen presentation (Loureiro and Ploegh, 2006; Shin et al., 2006), TLR/IL1 signaling (Chen, 2005), and induction of type I IFN by the cellular viral sensor RIGI (Gack et al., 2007). Inhibition of protein ubiquitination might also affect other cellular processes that can be subverted by viruses for their own advantage, such as the proteasome-mediated protein degradation system, multiple signal transduction events, or cell cycle progression. Given the effects that we observed on NF-κB signaling, it seems likely that viral OTU domain-containing proteases may be able to target these and other Ub-dependent pathways.

While the biochemical effects of ISGylation have been studied far less extensively than those of Ub, ISG15 is an important antiviral protein (Lenschow et al., 2005, 2007; Okumura et al., 2006). Thus, it is not surprising that viruses may use multiple strategies to counter the antiviral effects of ISG15. The work presented here provides a viral strategy for decreasing expression of bona fide ISG15 conjugates in cells. The first such strategy reported is the direct association of the NS1 protein of influenza B virus with ISG15. This association inhibits protein ISGylation by blocking the ISG15-UBE1L interaction (Yuan et al., 2002; Yuan

and Krug, 2001), while the viral OTU domain proteases analyzed here accomplish a similar effect via deconjugation.

The Ub and ISG15 deconjugation activities by the viral OTU domains contrast with the specific Ub deconjugation activity by the OTU domain-containing cellular proteins tested in this study. We speculate that other viral proteases, perhaps including some that do not have OTU domains, will be found to target both Ub- and ISG15-dependent processes. To date, viral DUB activities and in vitro cleavage of ISG15 fusion proteins have been demonstrated for the adenoviral protease adenain (Balakirev et al., 2002) and the papain-like proteases from the severe acute respiratory syndrome coronavirus (SARS-CoV) (Barretto et al., 2005; Lindner et al., 2005; Ratia et al., 2006).

OTU Domain Specificity and Deconjugating Activity, a Target for Antiviral Drug Development?

We found that the CCHFV-L OTU domain processed Ub and ISG15 conjugates and pro-ISG15 and pro-Nedd8 in vitro but did not have activity against any SUMO isoforms. Ub, ISG15, and Nedd8 differ from SUMO in their exposed C-terminal motifs: LRLRGG for Ub and ISG15 and LALRGG for Nedd8 versus QQQTGG for SUMO-2 and SUMO-3. This raises the interesting possibility that sequences similar to the LRLRGG motif may play an important role in substrate recognition and specific cleavage by CCHFV-L OTU and perhaps other viral proteases. Interestingly, the nsp2 of arteriviruses cleaves the nsp2/3 junction at FRLIGG (EAV) or GRLLGG (PRSSV) (Allende et al., 1999; Snijder et al., 1996; Ziebuhr et al., 2000), sequences similar to the LRLRGG motif. Thus, arterivirus OTU proteases have dual functions: performing essential viral polyprotein processing and targeting host substrates to modulate the antiviral response. This is analogous to the hepatitis C virus NS3-4A protease, which is involved both in viral polyprotein processing and in cleaving the cellular antiviral proteins TRIF and IPS-1 (Li et al., 2005; Lin et al., 2006).

The characterization of the CCHFV-L OTU domain and the development of in vitro assays for its enzymatic activities as described in this study will make it feasible to screen for potential inhibitors specific for CCHFV-L and other OTU domain-containing viral proteins. High-throughput screening of chemical compound libraries has proven to be a valuable tool in the identification of small-molecule inhibitors of other viral proteases such as HCV-NS3-4A (Sudo et al., 2005) and SARS-3CLpro (Blanchard et al., 2004). Since the viral OTU domains have a unique capacity to target both Ub and ISG15 conjugates, the design of specific inhibitors might be possible. For this reason, structural studies will be of great value to understand both the molecular basis of the unique biochemical activities of these viral proteins and the potential for development of antiviral compounds.

Physiologic Importance of Viral OTU Domain Protease DUB and DeISGylating Activities

Our demonstration that viral OTU domain-containing proteases can decrease both Ub and ISG15 conjugates in cells does not alone constitute proof that protease activity directed toward these substrates plays a role in viral infection. We were limited in performing the obvious experiment of assessing the role of viral OTU domain protease activity during infection by two things. First, CCHFV is a biosafety level 4 pathogen lacking a good animal model and whose molecular biology is not well enough developed to allow generation of mutant viruses. Second, the viral OTU domain proteases evaluated here either have, or are likely to have, important effects on processing of viral polyproteins (see above). Given this, we felt that it would be difficult to prove that these proteases have effects during infection that are solely attributable to their DUB and deISGylating activities. However, in transgenic mice, recombinant chimeric Sindbis viruses, and transfected cells, we found that viral OTU domain-containing proteases have significant effects on Ub- and ISG15-dependent host processes of known importance for innate immunity. We therefore conclude that these proteins have bona fide immune evasion properties. It will be interesting to further investigate the activities of these and other viral proteases that target Ub- and ISG15-dependent processes during viral infection.

EXPERIMENTAL PROCEDURES

Expression Plasmids

Plasmids pCAGGS-6HismISG15, pCAGGS-hUBE1L-HA, pFLAGCMV2-UbcM8, and pcDNA3.1-UbcM8 were provided by Dong-Er Zhang (Scripps Research Institute, La Jolla, CA) (Giannakopoulos et al., 2005). Herc5 was provided by Motoaki Ohtsubo (Kurume University, Fukuoka-ken, Japan). pcDNA 3.1^{+}-HA-Ub was provided by Dr. Domenico Tortorella (Mount Sinai School of Medicine, NY) (Treier et al., 1994). Peak10-Flag-A20 plasmid was provided by Dr. Adrian Ting (Mount Sinai School of Medicine). The construction of all other plasmids is described in the Supplemental Experimental Procedures.

Antibodies

Antibodies against Flag (M2 and rabbit polyclonal, Sigma, St. Louis, MO), HA (HA.7 [Sigma] HA.11 [Covance Research, Berkeley, CA]), Ub (P4D1, Cell Signaling, Danvers, MA,) NF-κB p65 (F-6, Santa Cruz Biotech, Santa Cruz, CA), and actin (AC-74, Sigma) were used following manufacturer's protocol. Anti-mouse ISG15 monoclonal (3C2 and 2D12) and polyclonal antibodies (Lenschow et al., 2005) and antiserum recognizing EAV-nsp2 (Snijder et al., 1994) have been previously described.

Purification of CCHFV L(1–169) from *E. coli*

BL-21 cells (Stratagene, La Jolla, CA) were transformed with pGEX-L(1–169) or pGEX-L(1–169)1A CCHFV, cultured to an OD_{600} of 0.6 in 2xYT medium and induced for 6 hr at 30°C with 0.1 mM IPTG. Bacteria were resuspended in lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 1 mM DTT, 200 mM NaCl, and 0.1% NP-40), and purification of the GST fusion proteins was performed using GSH Sepharose resin (Amersham) according to the manufacturer's protocol. GST was cleaved using PreScission Protease (Pharmacia, Uppsala, Sweden) in cleavage buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1 mM EDTA, and 1 mM DTT).

Assays for DeISGylation in Cultured Cells

Initially, 293T cells cultured in 12-well dishes were cotransfected with 0.4 μg of pCAGGS-6HismISG15, 0.4 μg of pCAGGS-hUBE1L-HA, and 0.2 μg of pFLAGCMV2-UbcM8 along with OTU domain expression plasmids or empty pCAGGS plasmid using Lipofectamine 2000. In

subsequent experiments testing eukaryotic and viral OTU constructs, 293T cells in 12-well dishes were cotransfected with OTU domain expression plasmids and 0.5 μg pCAGGS-6His mISG15, 0.5 μg pCAGGS-mUBE1L-HA, 0.5 μg of plasmid encoding Herc5, and 0.2 μg pFLAGCMV2 UbcM8 or pCDNA3.1-UbcM8. Twenty-four hours posttransfection, cells were lysed in Laemmli sample buffer, boiled, and analyzed by immunoblot using anti-ISG15 mAb 3C2 as previously described (Lenschow et al., 2005). Each transfection experiment was performed a minimum of three times.

Assay for Deubiquitination in Cultured Cells

293T cells cultured in 12-well dishes were cotransfected with 0.5 μg of pcDNA3.1-HA-Ub and various OTU domain expression plasmids or empty pCAGGS plasmid using Lipofectamine 2000. Twenty-four hours posttransfection, the cells were lysed in Laemmli sample, boiled, and immunoblotted with anti-HA antibody. Each transfection experiment was performed a minimum of three times.

Generation of ISG15 Conjugates

Fourteen 10 cm dishes of 293T cells were transfected with 6 μg pCAGGS-6HismISG15, 3 μg pCAGGS-hUBE1L-HA, and 3 μg pFLAGCMV2-UbcM8. Twenty-four hours later, cells were harvested, resuspended in 20 mM Tris-HCl (pH 8.0) with 300 mM NaCl, and lysed by three cycles of freeze-thaw. Lysates were centrifuged for 15 min at 14,000 rpm. His-tagged ISG15 conjugates were purified over a His-Select Spin Column (SIGMA) following the manufacturer's directions. Column-bound conjugates were washed extensively with washing buffer (20 mM Tris-HCl [pH 8.0], 300 mM NaCl, and 5 mM Imidazole) and eluted with 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 250 mM Imidazole. Protein concentration was measured by Bradford assay (Bio-Rad).

In Vitro Deconjugation Assays

K^{48} Ub_{2-7}, K^{63} Ub_{3-7}, SUMO-2_{2-8}, SUMO-3_{2-8}, pro-ISG15, pro-Nedd8, pro-SUMO-1, USP5/Isopeptidase T, NEDP1, UBP43, $A20_{CD}$, and His6-$SENP2_{CD}$ were purchased from Boston Biochem (Cambridge, MA). All reactions were performed in 50 mM Tris-HCl (pH 7.6), 5 mM MgCl2, and 2 mM DTT at 37°C for 2 hr. Serial 10-fold dilutions of L(1–169) or L(1–169)1A (ranging from 2.5 μM to 2.5 nM) were incubated with either 2.5 μg Ub chains or SUMO chains; 2.5 μg pro-ISG15, pro-Nedd8, or pro-SUMO-1; 10 μl *Ubp43*$^{-/-}$ lysate; or 3 μg of 6HisISG15 conjugates. Positive control for deconjugation was incubation with 100 mM USP5 (Ub), $SENP2_{CD}$ (SUMO), NEDP1 (Nedd8), or UBP43 (ISG15). Negative control was incubation of chains or conjugates in assay buffer alone. Reactions were terminated by addition of Laemmli sample buffer and separated by SDS-PAGE electrophoresis on a 4%–20% gradient gel (BioRad). Proteins were visualized by SimplyBlue Safestain (Invitrogen) staining (Ub and SUMO) or by anti-ISG15 western blot.

Viruses

Sindbis viruses were generated from a cDNA clone by in vitro transcription and RNA transfection of BHK-21 cells as previously described (Levine et al., 1996; Lenschow et al., 2005). Recombinant virus stocks were produced and titered on BHK-21 cells as previously described (Lenschow et al., 2005). Sindbis virus AR86 was a kind gift of Dr. Mark Heise (University of North Carolina, Chapel Hill) (Heise et al., 2000).

NF-κB Reporter Gene Assay

293T cells were cotransfected with 3.3-fold dilutions (starting at 100 ng) of A20, CCHFV-L(1–169), CCHFV-L(1–169)2A, EAV-nsp2N, or empty plasmid along with the firefly luciferase gene construct under the control of the NF-κB-binding sites) (Wang et al., 2000) and pRL-TK (*Renilla* luciferase; Promega, WI). The total amount of transfected DNA was kept constant by adding the pCAGGS empty vector. Twenty-four hours posttransfection, the cells were stimulated with TNFα (10 ng/ml) for 6 hr, and luciferase activities were measured using the Dual-Luciferase Reporter (DLR) Assay System (Promega). Final NF-κB luciferase values were normalized with the *Renilla* luciferase values as internal control.

Immunofluorescence

Two hundred nanograms of empty plasmid or Flag-tagged L(1–169) and L(1–169)2A were transfected into A549 cells. Twenty-four hours later, cells were stimulated with 10 ng/ml TNFα for 2 hr. Cells were fixed and permeabilized for 30 min at room temperature with 2.5% formaldehyde and 0.5% Triton X-100, washed extensively with PBS, and stained with anti-p65 and anti-Flag antibodies. Following PBS washes, cells were stained with anti-mouse (p65) or anti-rabbit (Flag) and secondary antibodies, and then mounted in medium containing an antifade reagent. Nuclear localization of p65 was scored in 100 to 400 transfected cells for each experimental condition.

Mouse Studies

IFNαβR$^{-/-}$ mice on the 129/SV/Pas background were initially obtained from M. Aguet, Swiss Institute of Experimental Cancer Research (Epalinges, Switzerland) (Behr et al., 2001; Dunn et al., 2005). CCHFV-L(1–1325) transgenic mice were generated at the WUSM Pathology Microinjection Core by microinjecting a linearized construct derived from pCAGGS-HA-L(1–1325) into B6 oocytes. Oocytes were implanted into pseudopregnant mice, and resulting litters were genotyped using PCR (primer sequences available upon request). Individual embryo MEFs from transgenic lines 1836, 1854, and 2929 were generated as described previously (Weck et al., 1999). Uninfected transgenic brain was homogenized in 1 ml of DMEM with protease inhibitors using 100 μl 1.0 mm diameter zirconia-silica beads in a MagNa Lyser (Roche, Indianapolis, IN). To assess transgene expression, 4 × 10^5 MEFs or 12 μl of brain homogenate were immunoblotted with HA.11 and anti-actin antibodies. Eight- to ten-week-old male *IFNαβR*$^{-/-}$ mice were infected subcutaneously (s.c.) in the left hind footpad with 5 × 10^6 PFU of virus diluted in 50 μl of Hank's balanced salt solution (HBSS). Four- to five-week old L(1–1325) transgenic mice were infected s.c. in the left hind footpad with 5000 PFU of Sindbis virus AR86 diluted in 50 μl of HBSS. Mice were bred and maintained at Washington University School of Medicine in accordance with all federal and university guidelines.

Statistical Analysis

All data were analyzed with Prism software (GraphPad, San Diego, CA). Survival data were analyzed by the log-rank (Mantel-Haenzsel) test, with death as the primary variable. Single-step growth curves were analyzed by one-way analysis of variance (ANOVA).

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures and two supplemental figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/2/6/404/DC1/.

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