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The vOTU domain of highly-pathogenic porcine reproductive and respiratory syndrome virus displays a differential substrate preference

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ABSTRACT

Arterivirus genus member Porcine reproductive and respiratory syndrome virus (PRRSV) causes an economically devastating disease, recently exacerbated by the emergence of highly pathogenic strains (HP-PRRSV). Within the nonstructural protein 2 of PRRSV is a deubiquitinating enzyme domain belonging to the viral ovarian tumor (vOTU) protease superfamily. vOTUs, which can greatly vary in their preference for their host ubiquitin (Ub) and Ub-like substrates such as interferon stimulated gene 15 (ISG15), have been implicated as a potential virulence factor. Since various strains of PRRSV have large variations in virulence, the specificity of vOTUs from two PRRSV strains of varying virulence were determined. While both vOTUs showed de-ubiquitinating activity and markedly low deISGylating activity, HP-PRRSV demonstrated a strong preference for lysine 63-linked poly-Ubiquitin, tied to innate immune response regulation. This represents the first report of biochemical activity unique to HP-PRRSV that has implications for a potential increase in immunosuppression and virulence.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a rapidly evolving virus which emerged as a serious pathogen of swine in the late 1980s, and has since become one of the most significant concerns to the global pork industry. These concerns have only been exacerbated by the emergence in 2006/2007 of highly pathogenic PRRSV (HP-PRRSV) in China and Southeast Asia (Guo et al., 2013a; Li et al., 2007; Wu et al., 2009). Although initially characterized by a unique discontinuous 30 amino acid deletion in nonstructural protein 2 (nsp2), it was later shown that this deletion was not related to the increased virulence of these isolates (Zhou et al., 2009). More contemporary PRRSV isolates have been observed in SE Asia that show significant continuing evolution from the original 2006 isolates, but still retain the highly pathogenic phenotype (Guo et al., 2013b; Yu et al., 2012). These observations underscore the need to mechanistically understand what is responsible for the increased virulence of HP-PRRSV, which is further complicated by the complex and poorly understood virulence determinants of the broad and genetically diverse PRRSV strains.

PRRSV is a positive-sense, single-stranded RNA virus in the genus *Arterivirus* of the family *Arteriviridae*. Phylogenetic analysis of PRRSV reveals two genetically distinct genotypes: Type 1/European PRRSV (type strain Lelystad) and Type 2/North American PRRSV (type strain VR-2332), with the HP-PRRSV isolates belonging to a distinct subgroup of Type 2. It is important to note, however, that due to a high rate of mutation and recombination, it is likely that actual genetic diversity of PRRSV continuously surges ahead of our ability to discover and characterize it (Shi et al., 2010). Upon infection with PRRSV, viremia is typically established in 6–12 h and virus shedding has been observed for up to 157 days (Wills et al., 1997). It is likely that the prolonged time to virus clearance is directly related to the immunosuppressive effects of PRRSV infection. Not only does the infection of antigen presenting cells (macrophages and dendritic cells) directly impair immune activation, but the production of high levels of non-neutralizing antibody to decoy epitopes and stimulation of immunosuppressive cytokines may also contribute to pathogenesis (Charerntantanakul et al., 2006; Sang et al., 2011; Yoo et al., 2010). In addition to a subversion of the adaptive immune response, some strains of PRRSV initiate a comprehensive campaign against the innate immune response including a complex dysregulation of the type I interferon/IRF/NF-κB pathway in cultured cells (Beura et al., 2010; Fang et al., 2012; Song et al., 2010; Sun et al.,2010, 2012a; van Kasteren et al., 2013). While the mechanisms of certain aspects of this dysregulation have been determined, the role of

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PRSRV nsp2 remains convoluted. Due to the high degree of variability in nsp2, it remains likely that functional differences exist in nsp2 between various PRRSV isolates that might have consequences on strain virulence.

PRRSV nsp2 is a large, highly variable protein encoded in the ORF1a region of the genome as part of a larger polyprotein precursor (Han et al., 2006; Snijder et al., 1995; van Hemert and Snijder, 2008). Nsp2 is liberated from this precursor via cleavage of the nsp1β:nsp2 junction by the nsp1β protease and cleavage of the nsp2:nsp3 junction by the intrinsic protease activity of nsp2 (den Boon et al., 1995; Han et al., 2009a; Snijder et al., 1995; van Hemert and Snijder, 2008). This protease activity resides within an amino-terminal region of nsp2 containing a papain-like cysteine protease domain (PLP2) that cleaves at a conserved GG dipeptide between nsp2 and nsp3 (Han et al., 2009b; Mielech et al., in press; Ziebuhr et al., 2000). It has also been shown bioinformatically that this PLP2 domain is part of a larger family of mammalian proteins known as the ovarian tumor domain (OTU) proteases (Frias-Staheli et al., 2007). This subgroup of viral OTU domains (vOTU) have been biochemically shown to act as deubiquitinases (DUB) of varying specificities and activities, capable of interfering with innate immune responses via dysregulation of ubiquitin (Ub) or ISG15 related protein signaling (Akutsu et al., 2011; Capodagli et al., 2013; Capodagli et al., 2011; Frias-Staheli et al., 2007).

The Ub polychain formation, also known as polyubiquitination, forms via isopeptide bond between a Ub C-terminus with either the N-terminus, or one of seven other lysines, of another Ub molecule. Both mono- and polyubiquitination of host proteins act as key regulatory steps in multiple distinct cell signaling pathways (Komander et al., 2009; Kulathu and Komander, 2012). One relevant example of this is the use of K63-linked poly-Ub in the activation of retinoic acid-inducible gene I/mitochondrial antiviral signaling protein (RIG-I/MAVS) (Zeng et al., 2010), linear poly-Ub-dependent activation of inhibitors (IκB kinases) of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Tokunaga et al., 2009), and K48-linked poly-Ub to trigger degradation of IκB and activation of NF-κB signaling (Alkalay et al., 1995; Chen et al., 1995). Alternative forms of ubiquitination (K6, K11, K27 and K33) have been implicated in other cellular functions, including lysosome/vesicle trafficking, DNA damage response, inhibition of protein degradation, mitochondrial damage/autophagy, endoplasmic reticulum (ER) associated protein degradation, T-cell activation, and tumor necrosis factor-alpha (TNFα) signaling (Kulathu and Komander, 2012). Further, there exist multiple Ub-like proteins, such as interferon (IFN) stimulated gene 15 (ISG15), for which there are diverse functions and regulatory pathways including anti-viral host defense (Zhao et al., 2013). It is clear from this diverse and interwoven set of processes that it is insufficient to simply define a protease as a vOTU or DUB and subsequently ascertain the global picture of downstream cellular effects.

Herein, we describe detailed biochemical characterization of the vOTU activity of the PLP2 domain from a HP-PRRSV strain (JXwn06), and contrast that activity to that from the PLP2 domain of the avirulent modified live virus vaccine strain Ingelvac PRRS® (MLV). To do this, we utilized purified vOTU domains in an *in vitro* activity assay with chemically defined, purified substrates. This system provides significant advantages to *in cellulo* and *in vivo* activity assays, including the ability to directly query substrate preference and observe simplified reaction kinetics. Further, these assays avoid complications due to off-target effects or effects on complex cellular pathways which can make data interpretation problematic. Using this approach, we demonstrate that both the MLV and JXwn06 vOTU/PLP2 domains showed DUB activity on K48-Ub and K63-Ub substrates, respectively. Interestingly, the PLP2 domain from HP-PRRSV strain JXwn06 displayed significantly greater activity against K63-Ub substrates than the PLP2 domain of MLV. This represents the first report of a biochemical activity unique to HP-PRRSV that has potential implications for increased immunosuppression and may contribute to the increased virulence exhibited by this Asian HP-PRRSV strain.

Results

Sequence comparison of PRRSV strain JXwn06 and MLV vOTU domains

The vOTU domains of very few strains of PRRSV have been directly examined for suppression of innate immunity in cell culture. Two such studies utilized SD01-08 (a Type 1 isolate) (Sun et al., 2010) or VR-2332 (van Kasteren et al., 2012), which both cause only mild disease *in vivo*, and neither of which included an assessment of enzyme kinetics. Another study examined the ability of HP-PRRSV strain 07HBEZ vOTU to suppress IFN-β induction *in vitro* (Li et al., 2010). To more precisely examine and compare vOTU domain enzymatic activity and specificity for PRRSV strains of differing pathogenicity, the vaccine derivative Ingelvac PRRS® MLV and HP-PRRSV strain JXwn06 were chosen for analysis. A vOTU domain alignment of these two PRRSV strains compared to a related arterivirus (equine arteritis virus; EAV) and CCHFV was produced, using the catalytic and zinc-binding residues as alignment signature amino acids (Fig. 1). The two PRRSV strains display 83.7% identity/84.2% similarity within the designated vOTU region, while this domain displays ~21% identity between both PRRSV strains and EAV, and only ~10% identity between PRRSV and CCHFV.

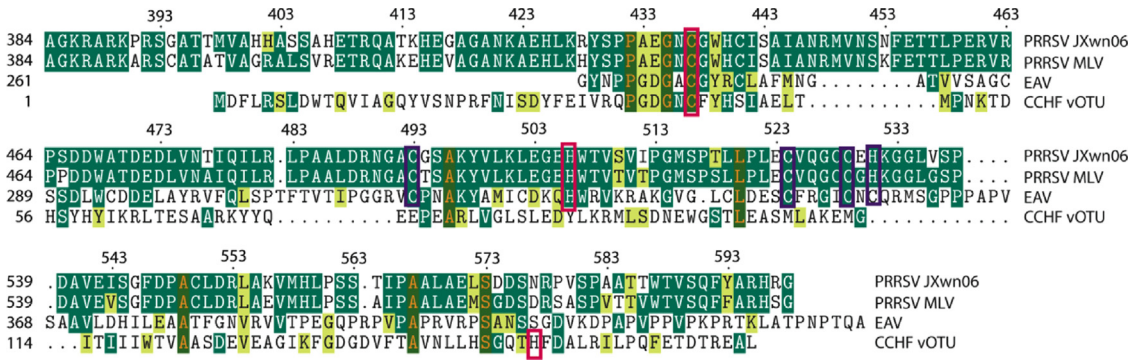

Fig. 1. Sequence alignment of vOTUs. Sequence similarity is indicated as non-conserved (white background, black text), similar (yellow background, black text), conserved (green background, white text), or all matching (dark green background, orange text). Catalytic residues are boxed in red, and zinc-binding residues are boxed in purple. PRRSV sequence numbering is based on the residue number within nsp2.

Characterization of strain specificity

In order to investigate potential functional differences between vOTU domains from different PRRSV strains, we obtained biochemical data on the activity and specificity of PRRSV vOTU domains from strains JXwn06 and MLV. Initially, enzymatic activity was assessed utilizing Ub and ISG15 substrates, as well as a peptide substrate comprised of five C-terminal amino acids common to Ub and ISG15, RLRGG (Fig. 2). By monitoring the release of the aminomethylcoumarin fluorophore from the C-terminus of these substrates, rate and substrate preference data was obtained. The vOTU domains originating from JXwn06 and MLV possess a noteworthy ability to cleave human Ub-AMC (hUb-AMC) conjugates, at 3.67 ± 0.04 and $2.30 \pm 0.04\ \mathrm{min}^{-1}$, respectively. However, when these vOTUs were assessed for activity against human ISG15-AMC (hISG15-AMC), the vOTUs of JXwn06 and MLV exhibited activity 2500 and 320 times less than their activities toward Ub-AMC, respectively. Interestingly, when assessed using the peptide substrate ZRLRGG-AMC, which is comprised of the last five conserved amino acids between Ub and ISG15, a large deviation in activity was observed between the vOTUs from MLV and JXwn06. Specifically, the activity of the MLV vOTU was $0.40 \pm 0.04\ \mathrm{min}^{-1}$, whereas JXwn06 vOTU was only $2.05 \pm 0.01 \times 10^{-2}\ \mathrm{min}^{-1}$. This result, as well as the dramatic difference between cleavage of Ub-AMC and hISG15-AMC, suggest a more complex interaction with the protein substrate beyond simple recognition of peptide sequence.

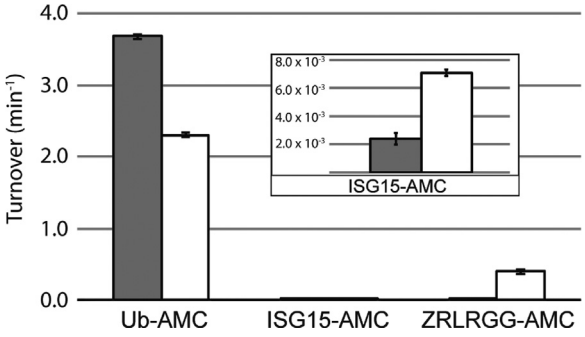

Fig. 2. Kinetic data for fluorescence based assay. JXwn06 vOTU data is shown in gray and MLV vOTU data is shown in white. ISG15-AMC data is shown in the insert for clarity. Turnover number of substrate is measured as increase in fluorescence as AMC is cleaved off of the substrate molecule.

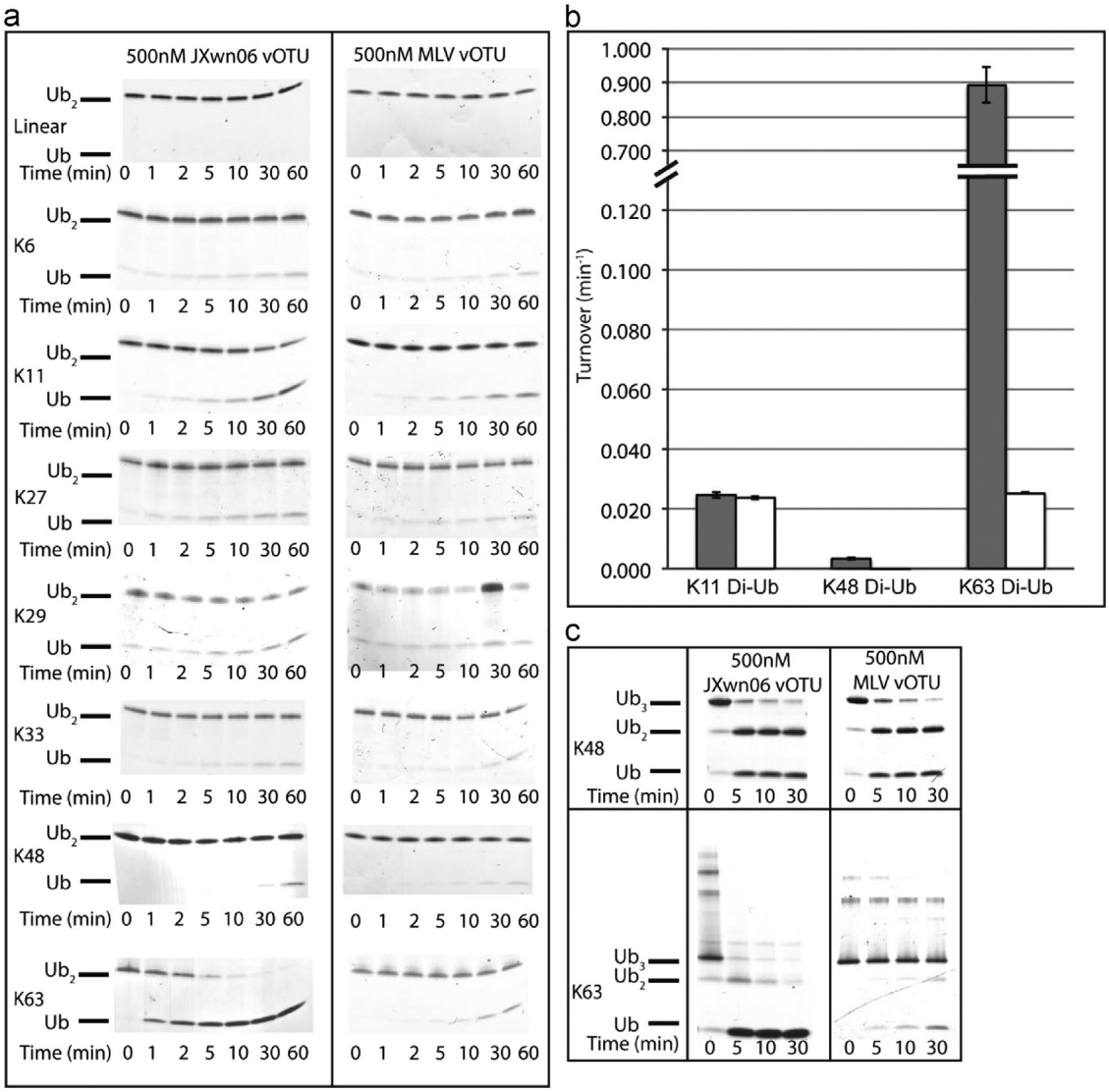

Fig. 3. Cleavage of di- and tri- Ub. (a) Cleavage of various di-Ub linkages by JXwn06 and MLV vOTUs. (b) Kinetic data for FRET based Di-Ub assay. JXwn06 vOTU data is shown in gray and MLV vOTU data is shown in white. (c) Cleavage of tri-Ub by JXwn06 vOTU and MLV vOTU.

Di-ubiquitin specificity divergence

In some cases, vOTU domains have been shown to have higher turnover rates for poly-Ub than for Ub-AMC due to an increase in the number of favorable contacts to which the enzyme can bind the substrate (Capodagli et al., 2013). Although the activity for mono-Ub was found to be relatively similar between JXwn06 and MLV vOTUs, differences in rates of cleavage for di-Ub may exist between strains. To more precisely differentiate the di-Ub specificities of the JXwn06 and MLV vOTU domains, a panel of varying di-Ub substrates (composed of K6, 11, 27, 29, 33, 48, 63 and linear di-Ub linkages; Fig. 3a) was screened for activity. Both vOTU domains had an overall trend of highest activity towards K63 linked di-Ub, followed by (in descending order) K11, K29, K6, K27, and K48-linkage, with no detectable activity toward linear linked di-Ub. Interestingly, while the observed activity was similar for most di-Ub linkages, JXwn06 vOTU was markedly more active toward K63 di-Ub, cleaving nearly all of the substrate within 10 min, compared to the MLV vOTU, for which a significant proportion of uncleaved K63 substrate remained after 60 min. In order to quantify the difference in activity towards di-Ub substrates, cleavage of K11, K48, and K63 di-Ub FRET substrates was assessed. These results aligned with the di-Ub gel shift assays, with both vOTU domains displaying low activity towards K48 linked di-Ub, and disparate activities utilizing K63 linked di-Ub. The activity of the JXwn06 vOTU towards K63 linked di-Ub was determined to be approximately 40-fold higher than that of MLV vOTU (Fig. 3b).

To assess potential differences in vOTU domain activity on substrates similar to poly-Ub, activity assays were performed using K48 and K63 linked tri-Ub (Fig. 3c). In stark contrast to the K48 linked di-Ub substrate, both vOTU domains of MLV and JXwn06 rapidly cleaved the majority of K48 tri-Ub into di- and mono-Ub within 5 min. However, consistent with the previous results, the resulting K48 linked di-Ub appeared refractory to subsequent vOTU cleavage. As in the di-Ub cleavage assay, the K63 linked tri-Ub substrate was rapidly and completely cleaved to mono-Ub by the JXwn06 vOTU domain within 10 min. Unlike the surge in activity observed using K48 linked tri-Ub for the MLV vOTU domain, its cleavage of K63 linked tri-Ub proceeded at the same modest rate observed for K63 di-Ub.

proISG15 activity

While Ub is highly conserved among species, ISG15 is more divergent, with human and porcine ISG15 sharing 67% sequence homology (Fig. 4a). Nairovirus vOTUs such as the CCHFV vOTU have previously been demonstrated to possess species specificity for ISG15 (Capodagli et al., 2013). To determine if the low hISG15-AMC activity observed for JXwn06 and MLV vOTU domains was also related to species specificity, cleavage of the pro-form of human (hproISG15) and porcine (pproISG15) were examined, using the CCHFV vOTU as a control. First, cleavage of hproISG15 was examined. At the low enzyme concentration of 20 nM, CCHFV

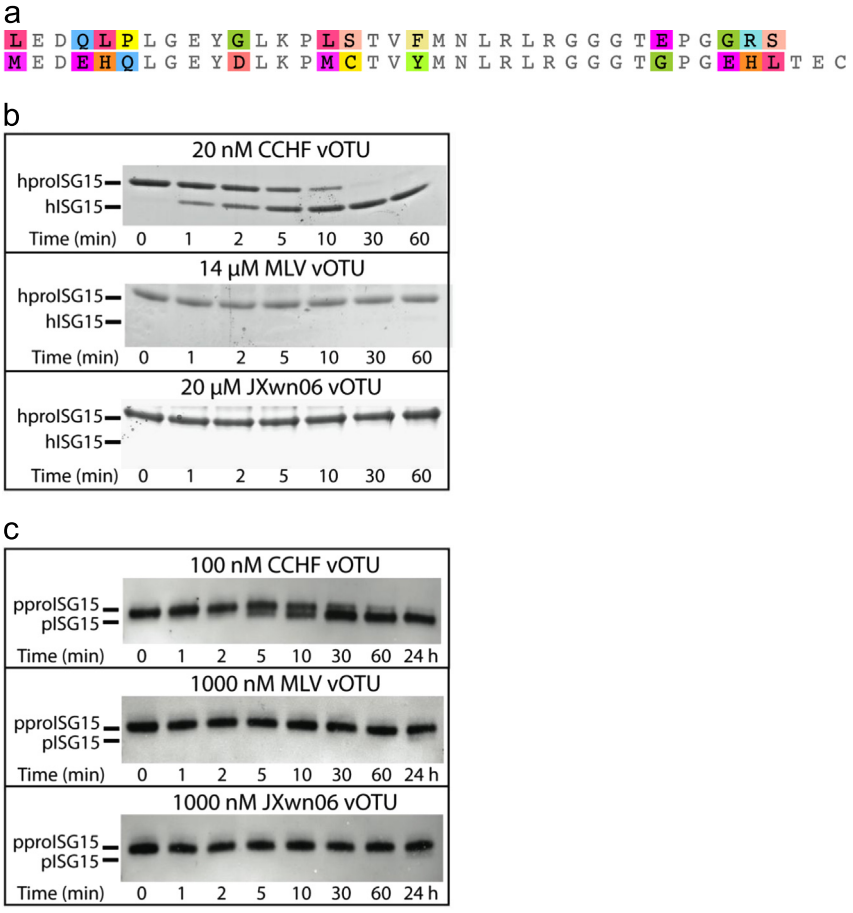

Fig. 4. (a) Sequence alignment of C-terminal end of human (top) and porcine (bottom) ISG15. (b) ProISG15 gel shift assay. Cleavage of human (h) proISG15 by CCHFV, JXwn06, or MLV vOTU is shown over a one-hour time course and visualized by coomasie blue staining. (c) Cleavage of porcine (p) proISG15 by varying concentrations of CCHFV, JXwn06 and MLV vOTUs are shown over a one hour time course as well as a 24 h time point. Cleavage products were visualized by anti-FLAG® western blot of tagged-pproISG15.

vOTU cleaved the hproISG15 at a rate that was readily observed in the one-hour time trial. Since the PRRSV vOTUs exhibited much lower turnover numbers for hISG15-AMC, the highest allowable concentrations of MLV and JXwn06 were utilized. However, even at the increased concentrations for MLV and JXwn06 vOTUs, no cleavage of the hproISG15 was observed for either in the one-hour time span (Fig. 4b). When examining cleavage of pproISG15, while CCHFV vOTU was seen to cleave the pproISG15 at a significantly reduced rate to that of hproISG15, no cleavage of pproISG15 by JXwn06 and MLV vOTU domains was detected within a one-hour time course. It was only after an extended 24-hour incubation that a trace of cleaved product was observed (Fig. 4b and c).

Discussion

The identification of a vOTU domain in nsp2 of the arterivirus PRRSV sets it among a number of viruses containing vOTUs, including nairoviruses, tymoviruses, a tenuivirus, and other nidoviruses. Recently, vOTUs from nairovirus representatives CCHFV, Dugbe virus and Erve virus (ERVEV), as well as the tymovirus prototypical member turnip yellow mosaic virus (TYMV) were probed extensively, and illustrated vOTUs from different species can vary greatly in their preference for substrate (Capodagli et al., 2013). The vOTUs from JXwn06 and MLV follow this trend. Relative to previously characterized vOTU domains of viruses, JXwn06 and MLV vOTU domains displayed moderate deubiquitinating activity. The vOTUs previously probed have deubiquitinating activity which ranges from approximately 0.03 to 27 min^{-1} (Capodagli et al., 2013). The PRRSV vOTUs fall into the lower end of this range, with deubiquitinating activity closest in value to that of ERVEV vOTU, which is considered a poor deubiquitinating enzyme relative to other nairoviruses (Capodagli et al., 2013). However, relative to nairovirus counterparts, such as vOTUs from CCHFV and ERVEV, that clearly possess deISGylating activity, PRRSV vOTUs deISGylating activity is extremely low. Comparatively, other vOTUs have activity ranging from ERVEV vOTU at the high end, at 15 min^{-1}, to TYMV vOTU at the low end with activity of only 0.005 min^{-1}. The PRRSV vOTUs hISG15 activities are near the lower end of these reported vOTU activities, with similar values to the TYMV vOTU, whose plant host lacks an ISG15 homolog. Upon comparing the proteolytic activity of the PRRSV vOTUs toward the last five amino acids common to both Ub and ISG15 as measured by cleavage of ZRLRGG-AMC, a divergence in their comparative activities was found. The activity of the MLV vOTU is 20 times greater than that of the JXwn06 vOTU and 30% greater than the highest activity observed in the recent survey of representative vOTU domains reported by (Capodagli et al., 2013).

Further comparison and analysis of the vOTU domain from the highly pathogenic JXwn06 strain of PRRSV with the vOTU domain of the less pathogenic North American MLV strain revealed an intriguing result. The less virulent MLV vOTU maintained a better proteolytic activity, as measured by fold change of relative cleavage of ZRLRGG-AMC, and the two vOTUs had similar moderate Ub-AMC activity. However, upon examining the activity of the two strain's vOTUs towards poly-linked Ub, rather than the mono-Ub-AMC, a large divergence in their activities was uncovered. The JXwn06 vOTU exhibited K63 linked Ub activity over 40 times greater than that of MLV, exemplifying how vOTUs with similar activity against mono-Ub may have divergent activity towards different di-Ub linkages. Suppression of K63 linked poly-Ub early in infection by JXwn06 may contribute to the increased disease severity in highly pathogenic PRRSV infection.

During initial examination of vOTU domains, such as that from CCHFV, it was theorized that all vOTUs might possess dual deubiquitinating and deISGylating activity. However, further work demonstrated that some vOTUs likely serve primarily as deubiquitinases, while others may have a stronger preference for ISGylated substrates (Capodagli et al., 2013). For PRRSV vOTU domains from Jxwn06 and MLV, both were found to not only have very low activity against hISG15-AMC, but were unable to appreciably cleave either pproISG15 or hproISG15. The latter experiment eliminates a species-specificity effect, as seen in CCHFV vOTU, as the primary driver for the absence of activity (Fig. 4b and c). As a result, the lack of deISGylating activity suggests that PRRSV core domain may be more suited as a deubiqutinase than a deISGylase. The lack of deISGylating activity is surprising given that PRRSV nsp2 has been previously suggested to play a direct role in reducing the amount of ISGylation in cells (Sun et al., 2012b). This may indicate additional protein requirements for cleavage of ISG15 by PRRSV vOTUs. These additional requirements may come in the form of accessory proteins, or in a requirement for a larger domain within the nsp2 protein, as it has previously been demonstrated that larger proteolytically active domains of nsp2 were required for *trans* vs. *cis* cleavage specificities (Han et al., 2009a). Alternatively, the observed cleavage of ISG15 modified proteins in cell culture may be due to an upstream effect of nsp2, rather than a direct effect of vOTU domain activity. Also, the extended time course inherent in cellular testing, coupled with the observed trace activity of PRRSV vOTUs for ISGylated substrates, may have permitted the gradual buildup of deISGylated substrates thereby exaggerating the magnitude of PRRSV vOTU's deISGylating activity. This is of particular importance, given the kinetics of the antiviral response, particularly in the early stages of viral infection.

PRRSV virulence and attenuation have been studied extensively, with most studies implicating multiple regions of the virus in contributing to virulence, consistent with the observation that individual PRRSV vaccines appear to have become attenuated by different mechanisms (An et al., 2011; Brockmeier et al., 2012; Ellingson et al., 2010; Han et al., 2013; Kim et al., 2010; Kwon et al., 2008; Ni et al., 2013; Nielsen et al., 2001; Shi et al., 2013; Song et al., 2010; Storgaard et al., 1999; Wang et al., 2008; Yuan et al., 2001). Narrowing the focus to the PLP2/vOTU region of nsp2, previous reports have generally demonstrated deubiquitinating activity of certain strains, leading to subsequent suppression of innate immune responses in infected cells (Frias-Staheli et al., 2007; Li et al., 2010; Sun et al., 2010; van Kasteren et al., 2012), suggesting a mechanistic role in PRRSV virulence. In this report, we have shown that the core vOTU domain (215 residues), which varies only 17% (amino acid identity) between different strains of PRRSV, was responsible for marked differences in K63-linked deubiquitination, reinforcing the role of the PLP2/vOTU domain in virulence variation between PRRSV strains. It is important to note that this core domain must function primarily as a viral protease, placing significant constraints on evolution of secondary functions. This also underscores the complexities of PRRSV virulence, since MLV and its parental field isolate (VR-2332) share the identical vOTU domain sequence while exhibiting differential pathogenicity in swine. Therefore it is crucial to recognize that while differential vOTU activity may contribute to virulence, it remains one of a constellation of potential virulence factors. Mechanistically, several critical innate immune sensors and cellular anti-viral pathways utilize K63 ubiquitination as an essential activation step, such as RIG-I/MAVS, MDA5, and MITA/STING (Jiang et al., 2012; Oshiumi et al., 2013). Antagonism of these pathways by a vOTU domain with preference for K63-polyubiquitin could lead to a decrease in type I interferon production and NF-κB signaling, thereby abrogating or significantly delaying establishment of the anti-viral state. If K63 preference and increased deubiquitination activity of the JXwn06 vOTU domain is a shared characteristic of all highly-pathogenic PRRSV isolates, then it is possible that this unique activity contributes to the increased virulence of these strains.

Methods

Construction of OTU domains and porcine proISG15

Ingelvac PRRS® nsp2 nucleotides 1342–1986, coding for nsp2 aa 1-215, were inserted downstream of a 6× histidine tag, codon optimized for expression in E. coli, and used to synthesize the viral OTU polypeptide (MLV vOTU) (GenScript, Piscataway, NJ). A JXwn06 vOTU polypeptide representing nsp2 aa 1-215 was produced in a like manner. The coding sequence for porcine proISG15 (NM_0011286469) was codon optimized for expression in *E. coli*, and internal restriction endonuclease recognition sequences were altered by silent mutagenesis for cloning purposes (Huang et al., 2009). This optimized gene was synthesized with an amino terminal 3× FLAG-Tag and 10× His-Tag, as well as NcoI and BamHI sites for cloning purposes at the 5′ and 3′ ends, respectively (GenScript). This synthetic gene was subcloned into pUC57 and subsequently moved to pET26b (EMD Millipore) for bacterial expression and purification.

Protein purification

Porcine proISG15 (pproISG15) was purified from *E. coli* BL21 (DE3) cells harboring the gene for pproISG15 grown at 37 °C in 6 L of LB broth containing 100 μg/mL of ampicillin until the optical density at 600 nm reached 0.6. Expression of pproISG15 was induced by the addition of IPTG to a final concentration of 0.5 mM at 20 °C overnight. The cells were resuspended in cold Buffer A (50 mM Tris [pH 7.5], 1 mM EDTA, and 20% (w/v) sucrose), and then centrifuged at 6700 × *g* for 30 min. The cells were then resuspended in 5 mM $MgCl_2$. The suspension was centrifuged at 6700 × *g* for 30 min, and the protein was purified from the supernatant via Ni-NTA affinity, followed by size-exclusion chromatography on a Superdex75 using Buffer B (200 mM NaCl, 20 mM HEPES [pH 7.5], 2 mM DTT). The protein was further purified by cation exchange using a 10/100 MonoS column. The protein was eluted using a gradient from Buffer C (100 mM NaCl, 50 mM ammonium acetate [pH 4.5]) to Buffer D (1000 mM NaCl, 50 mM ammonium acetate [pH 4.5]). The vOTU from Crimean Congo hemorrhagic fever (CCHFV vOTU) was purified as described previously (Capodagli et al., 2011).

Fluorescent vOTU deubiquitination and deISGylation assays

All assays were performed in duplicate in Buffer E (100 mM NaCl, 50 mM HEPES [pH 7.5], 0.01 mg/mL bovine serum albumin (BSA), 5 mM DTT) using a Corning Costar half-volume black 96-well plate with a reaction volume of 50 μL. The rates of the reactions were observed using an Infinite M1000 series plate reader (Tecan, Inc.). Specifically, the increase in fluorescence (excitation λ, 360 nm; emission, 460 nm) of 7-amino-4-methylcourmarin (AMC) upon cleavage from hUb-AMC, hISG15-AMC, (Boston Biochem, MA) and ZRLRGG-AMC (Bachem) substrates was monitored for JXwn06 and MLV vOTU (GenScript). To calculate turnover rates for 1 μM hISG15-AMC, 1 μM hUb-AMC, and 50 μM ZRLRGG-AMC, 1 μM, 4 nM, and 4 μM of enzyme were used against hISG15-AMC, hUb-AMC, and ZRLRGG-AMC, respectively.

The turnover rates for poly-Ub fluorescence resonance energy transfer (FRET) linkage substrates K11, K48, and K63 (Boston Biochem, MA) at 1 μM were determined by monitoring the increase in fluorescence (excitation λ, 544 nm; emission, 572 nm) resulting by the separation of a FRET TAMRA/QXL pair. Cleavage of three commercially available FRET TAMRA/QXL pair configurations per K48 and K63 poly-Ub linkage FRET substrates were assessed. Each di-Ub FRET substrate at 1 μM was evaluated against an enzyme concentration of 500 nM.

Gel shift assays

Poly-Ub linked by the different isopeptide bonds (K6, K11, K29, K33, K48, and K63), as well as the N-terminal peptide bond (linear), were purchased from Boston Biochem, MA. K27 linked di-Ub was purchased from Ubiquigent. Dimeric-Ub substrates (10 μM) were incubated with 500 nM JXwn06 or MLV vOTU in reaction Buffer F (100 mM NaCl, 50 mM HEPES [pH 7.5], and 2 mM DTT) at 37 °C. Reactions were stopped at various times over 1 h by mixing 9 μL of each reaction with 2× SDS-Tricine sample buffer and heating at 95 °C for 5 min. Results were visualized on 10–20% Mini-PROTEAN® Tris–Tricine Precast Gels (Bio-Rad, CA). For vOTU cleavage of trimeric K48 and K63 linkages, 20 μM of tri-Ub substrates were tested and analyzed in the same manner as di-Ub.

10 μM hproISG15, (Boston Biochem, MA) was incubated with 20 nM CCHFV vOTU, 14 μM MLV, or 20 μM JXwn06 vOTU. 10 μM pproISG15 was incubated with 20 nM CCHFV vOTUs. ProISG15 gel shift assays were tested and analyzed in the same manner as di-Ub. The concentrations of MLV and JXwn06 used were the maximum allowable concentration possible for this assay.

Assay for vOTU cleavage of porcine proISG15 by anti-FLAG® western blotting

The pproISG15 cleavage assays were assembled as described above in reaction buffer F, containing 10 μM pproISG15, and either 100 nM CCHFV vOTU or 1000 nM PRRSV vOTU domain. Reactions were incubated at 37 °C, and aliquots were removed at time 0, 1, 2, 5, 10, 30, and 60 min, as well as at 24 h. Reaction progress was halted by addition of the removed aliquots to an equal volume of SDS-PAGE loading buffer and storage at −20 °C until electrophoresis.

Timepoint samples for pproISG15 cleavage assays were separated by 10% Tris–Tricine SDS–PAGE, and resolved proteins were transferred to a PVDF membrane using the iBlot transfer apparatus (Life Technologies). The membrane was blocked with 3% milk in PBS and immunoblotted with a 1:3000 dilution of mouse α-FLAG® monoclonal antibody (Sigma-Aldrich; F1804). Proteins were detected using a 1:15000 dilution of peroxidase conjugated goat α-mouse IgG antibody (Zymax, Life Technologies) and the ECL Plus substrate (Pierce). Immunoblots were visualized using blue light fluorescence in a G:BOX imager (Syngene).

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