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Modulation of proteolytic polyprotein processing by coxsackievirus mutants resistant to inhibitors targeting phosphatidylinositol-4-kinase IIIβ or oxysterol binding protein

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ABSTRACT

Enteroviruses (*e.g.* poliovirus, coxsackievirus, and rhinovirus) require several host factors for genome replication. Among these host factors are phosphatidylinositol-4-kinase IIIβ (PI4KB) and oxysterol binding protein (OSBP). Enterovirus mutants resistant to inhibitors of PI4KB and OSBP were previously isolated, which demonstrated a role of single substitutions in the non-structural 3A protein in conferring resistance. Besides the 3A substitutions (*i.e.,* 3A-I54F and 3A-H57Y) in coxsackievirus B3 (CVB3), substitution N2D in 2C was identified in each of the PI4KB-inhibitor resistant CVB3 pools, but its possible benefit has not been investigated yet. In this study, we set out to investigate the possible role of 2C-N2D in the resistance to PI4KB and OSBP inhibition. We show that 2C-N2D by itself did not confer any resistance to inhibitors of PI4KB and OSBP. However, the double mutant (*i.e.*, 2C-N2D/3A-H57Y) showed better replication than the 3A-H57Y single mutant in the presence of inhibitors. Growing evidence suggests that alterations in lipid homeostasis affect the proteolytic processing of the poliovirus polyprotein. Therefore, we studied the effect of PI4KB or OSBP inhibition on proteolytic processing of the CVB3 polyprotein during infection as well as in a replication-independent system. We show that both PI4KB and OSBP inhibitors specifically affected the cleavage at the 3A-3B junction, and that mutation 3A-H57Y recovered impaired proteolytic processing at this junction. Although 2C-N2D enhanced replication of the 3A-H57Y single mutant, we did not detect additional effects of this substitution on polyprotein processing, which leaves the mechanism of how 2C-N2D contributes to the resistance to be revealed.

Enteroviruses belong to *Picornaviridae* family, which is a large group of viruses with a single-stranded, positive-sense RNA genome. Members of the Enterovirus genus, such as poliovirus (PV), coxsackievirus (CV), enterovirus A71 (EV-A71), EV-D68, and rhinovirus, are causative agents of important human diseases (*e.g.*, poliomyelitis, meningitis, hand-foot-and-mouth disease, and respiratory illness) (Tapparel et al., 2013). The genome of enteroviruses encodes a single polyprotein harboring 4 structural proteins (VP4, VP2, VP3, VP1 in the P1 region) and 7 non-structural proteins (P2 region: 2A, 2B, 2C; P3 region: 3A, 3B, 3C, 3D). This polyprotein is proteolytically processed into individual proteins by viral proteases $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$. Except for the junction between P1 and P2, which is cleaved by $2A^{pro}$, the majority of cleavage events within the viral polyprotein is mediated by $3C^{pro}$ (Ypma-Wong and Semler, 1987). Processing of P2-P3 does not only generate individual viral proteins, but also various cleavage intermediates such as 2BC, 3AB, and 3CD. Together, the non-structural proteins mediate replication of the viral genome.

Enteroviruses depend on several host factors such as phosphatidylinositol-4-kinase IIIβ (PI4KB) (Hsu et al., 2010) and oxysterol binding protein (OSBP) (Arita, 2014; Strating et al., 2015) for genome replication. PI4KB is recruited to the replication sites by viral 3A protein, and generates phosphatidylinositol-4 phosphate (PI4P)-enriched membranes. PI4P likely plays a role in recruiting and concentrating cellular proteins, and possibly also viral proteins, on specific membrane sites in order to facilitate viral genome replication. Among the cellular proteins that interact with PI4P are the lipid-transfer proteins, such as OSBP (Levine and Munro, 2002). In uninfected cells, OSBP creates membrane contact sites between endoplasmic reticulum and PI4P-enriched *trans*-Golgi membranes, and shuttles cholesterol in exchange for PI4P between these organelles (Mesmin et al., 2013). In a similar manner, OSBP is recruited to enterovirus replication organelle (RO) membranes that are enriched in PI4P by PI4KB, and causes an influx of cholesterol to ROs (Strating et al., 2015). Disruptions of cholesterol homeostasis by specific inhibitors inhibited PV (Ilnytska et al., 2013) and CVB3

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(Albulescu et al., 2015b) infection, which revealed the importance of cholesterol for enterovirus replication.

Since all enteroviruses rely on PI4KB and OSBP for efficient viral replication, several PI4KB inhibitors (Arita et al., 2009, 2011; Heinz and Vance, 1995, 1996; Hsu et al., 2010; van der Schaar et al., 2012; Wikel et al., 1980), including BF738735 (van der Schaar et al., 2013), and OSBP inhibitors (Arita et al., 2013; Strating et al., 2015), including OSW-1 (Albulescu et al., 2015a), have been found to exert broad-spectrum antiviral activity. PV and CVB3 mutants that are resistant to PI4KB inhibitors were found to possess single amino acid substitutions in the viral 3A protein (Arita et al., 2009; De Palma et al., 2009; Heinz and Vance, 1995, 1996). Previously, we showed that CVB3 3A mutations confer resistance by bypassing the need for PI4KB activity and PI4P accumulation (van der Schaar et al., 2012), but the exact resistance mechanism remains to be revealed. The same mutations were also shown to confer resistance to OSBP inhibitors, consistent with the proposed role of PI4P in recruiting OSBP (Albulescu et al., 2015a, 2017; Strating et al., 2015).

Previously, three independent CVB3 cultures were generated which were resistant to the PI4KB inhibitor BF738735 (van der Schaar et al., 2013). Genome analysis of these isolates revealed two single amino acid substitutions in the 3A protein (H57Y from two isolates and I54F from one isolate). In addition, another substitution (N2D) in the 2C protein appeared in all three isolates, which indicates that it may play an important role in resistance development. However, since the 3A mutants alone were sufficient to provide resistance (van der Schaar et al., 2013), the importance of this substitution in 2C has not been investigated yet. In this study, we set out to investigate the role of the 2C-N2D substitution in the resistance mechanism to PI4KB and OSBP inhibition. Possibly, this substitution provides resistance to PI4KB inhibition by itself, or it may support the resistance mechanism of the 3A substitution. To test this, we employed a previously established recombinant CVB3 that encodes *Renilla* luciferase (Rluc). Rluc is located upstream of the P1 region in the viral genome and can be used as a sensitive measure for genome replication. CVB3-Rluc (Lanke et al., 2009) and CVB3-Rluc 3A-H57Y viruses (van der Schaar et al., 2012) are derived from the full length infectious clones pRluc-53CB3/T7(3A-H57Y). CVB3-Rluc carrying 2C-N2D alone or a combination of 2C-N2D and 3A-H57Y were obtained in a similar manner as described elsewhere (van der Schaar et al., 2012). Cells were infected with these different viruses for 30 min, after which the inoculum was removed and fresh medium lacking or containing PI4KB or OSBP inhibitors was added. At 8 h post-infection, the cells were lysed to determine the intracellular luciferase activity. In agreement with our previous observations, CVB3 3A-H57Y was resistant to both PI4KB inhibitor BF738735 (van der Schaar et al., 2013) and OSBP inhibitor OSW-1 (Albulescu et al., 2015a) (Fig. 1). Compared to wild-type virus, replication of the 3A-H57Y mutant was enhanced by ~100-fold under PI4KB or OBSP inhibition. The substitution in 2C alone did not provide resistance to either PI4KB or OSBP inhibitors (Fig. 1). The 2C-N2D/3A-H57Y double mutant rescued virus replication in the presence of BF738735 to an even greater extent. At all the concentrations of BF738735 tested, the level of replication of 2C-N2D/3A-H57Y was ~3-fold higher than that of 3A-H57Y (Fig. 1A). The synergistic effect of 2C-N2D and 3A-H57Y was also observed upon treatment with OSW-1 (Fig. 1B). These results show that 2C-N2D provided small additive benefits to CVB3 replication only when accompanied by the substitution in 3A.

Alterations in lipid homeostasis have been shown to affect the proteolytic processing of the PV polyprotein (Arita, 2016; Ford Siltz et al., 2014; Ilnytska et al., 2013). Depletion of free cholesterol or disruption of cholesterol within membranes stimulated processing at the 3C-3D junction in PV-infected cells (Ilnytska et al., 2013). Upon PI4KB inhibition, accumulation of P2P3 and 3D as well as decrease of 2C and 3CD were observed in an *in vitro* translation setting (Ford Siltz et al., 2014). In another study, 3AB accumulation was found in PV-infected cells in the presence of a PI4KB inhibitor, while the level of other proteins was unaffected (Arita, 2014). Hence, no clear and consistent picture of the role of lipids in polyprotein processing has emerged yet.

In this study, we first investigated the effects of PI4KB inhibition on proteolytic processing of the CVB3 polyprotein in virus-infected cells. HeLa R19 cells were infected with wild-type, 3A-H57Y, or 2C-N2D/3A-H57Y viruses for 30 min, after which the inoculum was removed and fresh medium lacking or containing PI4KB inhibitor BF738735 was added. At 8 h post-infection, the cells were lysed and subjected to Western blot analysis using antibodies against CVB3 2C, 3A, and 3D proteins (Fig. 2). In the absence of inhibitor (*i.e.*, DMSO treatment), both 3A-H57Y and 2C-N2D/3A-H57Y virus already showed a better cleavage efficiency at the 3A-3B junction compared to wild-type. The levels of 3CD, 3D, 2BC, and 2C remained comparable between the wild-type and the mutants. Unfortunately, viral protein production of the wild-type virus was impaired already at a very low concentration of PI4KB inhibitor (*i.e.*, 20 nM, which is below the IC_{50}). This impairment in viral protein production is likely due to the inhibitory effect of BF738735 on viral RNA replication. It is well known that the rate of polyprotein processing changes over time during replication. Therefore, it was not possible to achieve a fair comparison between the wild-type virus and the mutant viruses in the presence of inhibitor in infected cells.

To overcome this problem, we set up a replication-independent system, which allows the production of equal amounts of viral protein independent of genome replication. This system exploits a previously established CVB3(-Rluc) cDNA that is rendered replication-deficient through modifications in the cloverleaf (p53CB3/T7-CL-6+5 or pRluc-53CB3/T7-CL-6+5) (Langereis et al., 2014). Since RNA synthesis is under the control of T7 polymerase, which is stably expressed in the cell line Huh7-Lunet/T7 (Backes et al., 2010), it is unlikely that the viral protein production is affected by the presence of inhibitors. To verify this, Huh7-Lunet/T7 cells were transfected with pRluc-53CB3/T7-CL-6+5 cDNA in the presence or absence of inhibitors, and were lysed 7 h later to quantify the luciferase activity. Figure 3A shows that the luciferase levels were affected neither by PI4KB inhibition nor by OSBP inhibition. These results corroborate that inhibition of PI4KB or OSBP did not affect the CVB3 polyprotein production.

Next, we analyzed whether the PI4KB and OSBP inhibitors influence the proteolytic processing of the CVB3 polyprotein in this system. Huh7-Lunet/T7 cells were transfected with wild-type, 3A-H57Y, or 2C-N2D/3A-H57Y replication-deficient cDNAs and viral protein levels were assessed by Western blot analysis after 7 h (Fig. 3B). The ratio of 3AB:3A for wild-type was prominently perturbed under both PI4KB inhibition and OSBP inhibition, indicating a defect in proteolytic processing at the 3A-3B junction. While the ratio of 3AB:3A was close to 1 in DMSO-treated cells, it increased 4- to 5-fold in the presence of PI4KB and OSBP inhibitors due to a decrease in the 3A level. Both the 3A-H57Y single mutant and the 2C-N2D/3A-H57Y double mutant rescued the impaired proteolytic processing at the 3A-3B junction upon PI4KB and OSBP inhibition. Similar as in infected cells, no effects of the BF738735 or OSW-1 on 3CD:3D or 2BC:2C ratios were observed in this replication-independent system. The observation that levels of 2BC, 2C, 3CD, and 3D did not change upon PI4KB or OSPB inhibition implies that proteolytic processing at the 2A-2B, 2B-2C, 2C-3A, 3B-3C, and 3C-3D cleavage junctions was unaffected. Together, the data demonstrate that inhibition of either PI4KB or OSBP specifically hinders proteolytic processing at the 3A-3B junction and that the substitution in 3A recovers impaired proteolytic processing at this junction.

In summary, we studied the role of 2C-N2D and 3A-H57Y substitutions in the resistance mechanism of CVB3 to PI4KB and OSBP inhibitors. We showed that the CVB3 3A-H57Y mutant can overcome the absence of essential lipids (*i.e.*, PI4P and cholesterol) in membranes by rescuing the impaired proteolytic processing at the 3A-3B junction. A recent study on PV showed that PI4KB inhibition also resulted in the accumulation of 3AB during infection with low concentrations of a

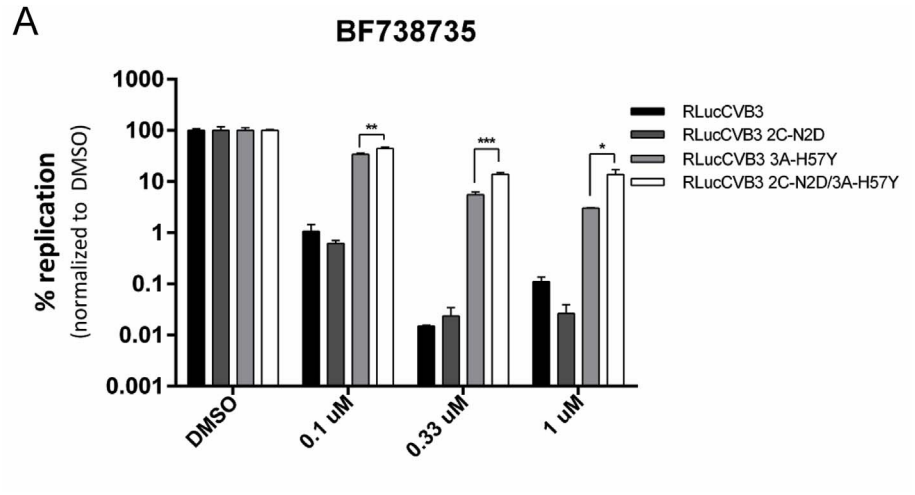
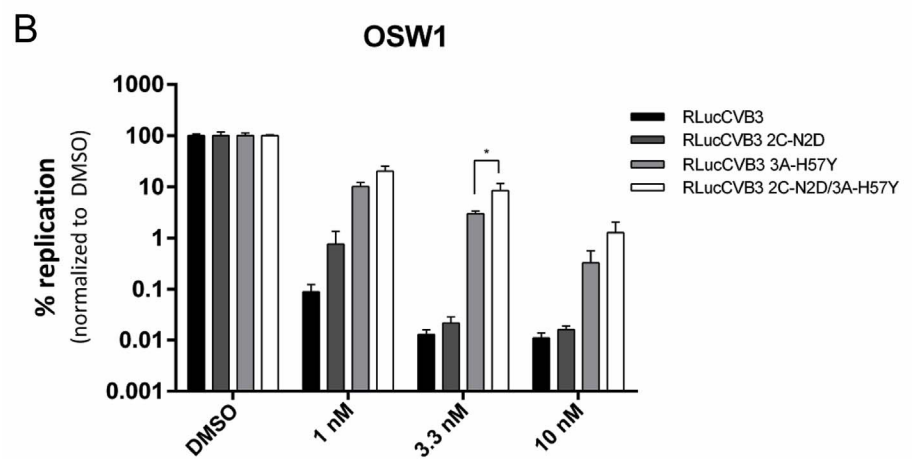


Fig. 1. The 2C-N2D substitution enhances the resistance of 3A-H57Y mutant to PI4KB or OSBP inhibitors. HeLa R19 cells were infected with CVB3-Rluc wild-type, 3A-H57Y, 2C-N2D, or 2C-N2D/3A-H57Y at an MOI of 1 for 30 min. After removal of the inoculum, Dimethyl sulfoxide (DMSO), BF738735 **(A)**, or OSW-1 **(B)** was added to the cells. After 8 h, cells were lysed to determine *Renilla* luciferase activity. Bars represent the mean of triplicate values ± SEM. Significant differences were calculated by paired *t*-test. *, = $P < 0.05$; **, = $P < 0.01$; ***, = $P < 0.001$.



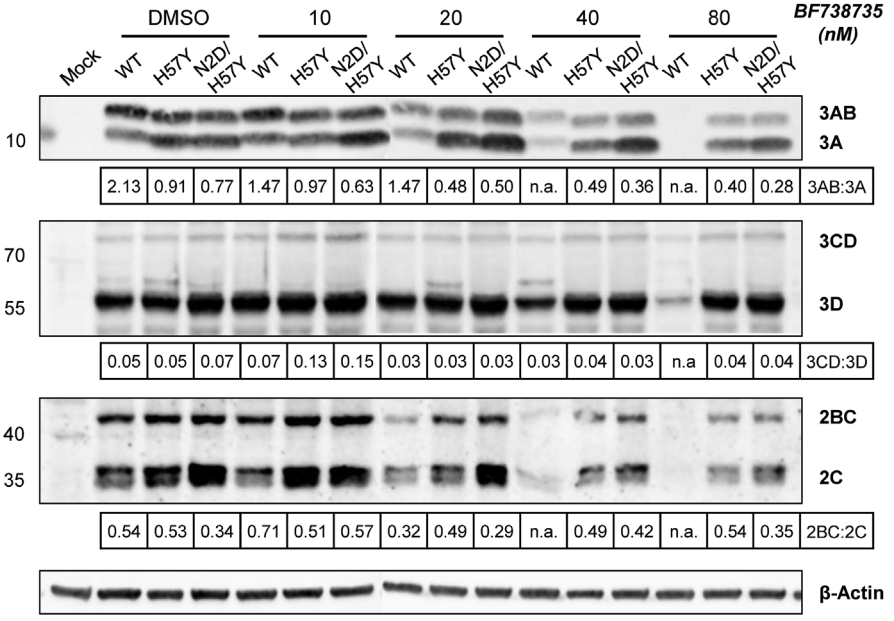


Fig. 2. The CVB3 mutants exhibit better cleavage efficiency at the 3A-3B junction compared to the wild-type. HeLa R19 cells were infected with CVB3 wild-type, 3A-H57Y, or 2C-N2D/3A-H57Y at an MOI of 10 for 30 min. After removal of the inoculum, DMSO or BF738735 was added to the cells. After 7 h, cells were lysed and the lysates were subjected to Western blot analysis to examine effects on viral polyprotein processing. Antibodies against 3A, 3D, and 2C were used to detect viral proteins. Actin was used as a loading control. Densitometry of bands was done with LI-COR® Image Studio™ software.

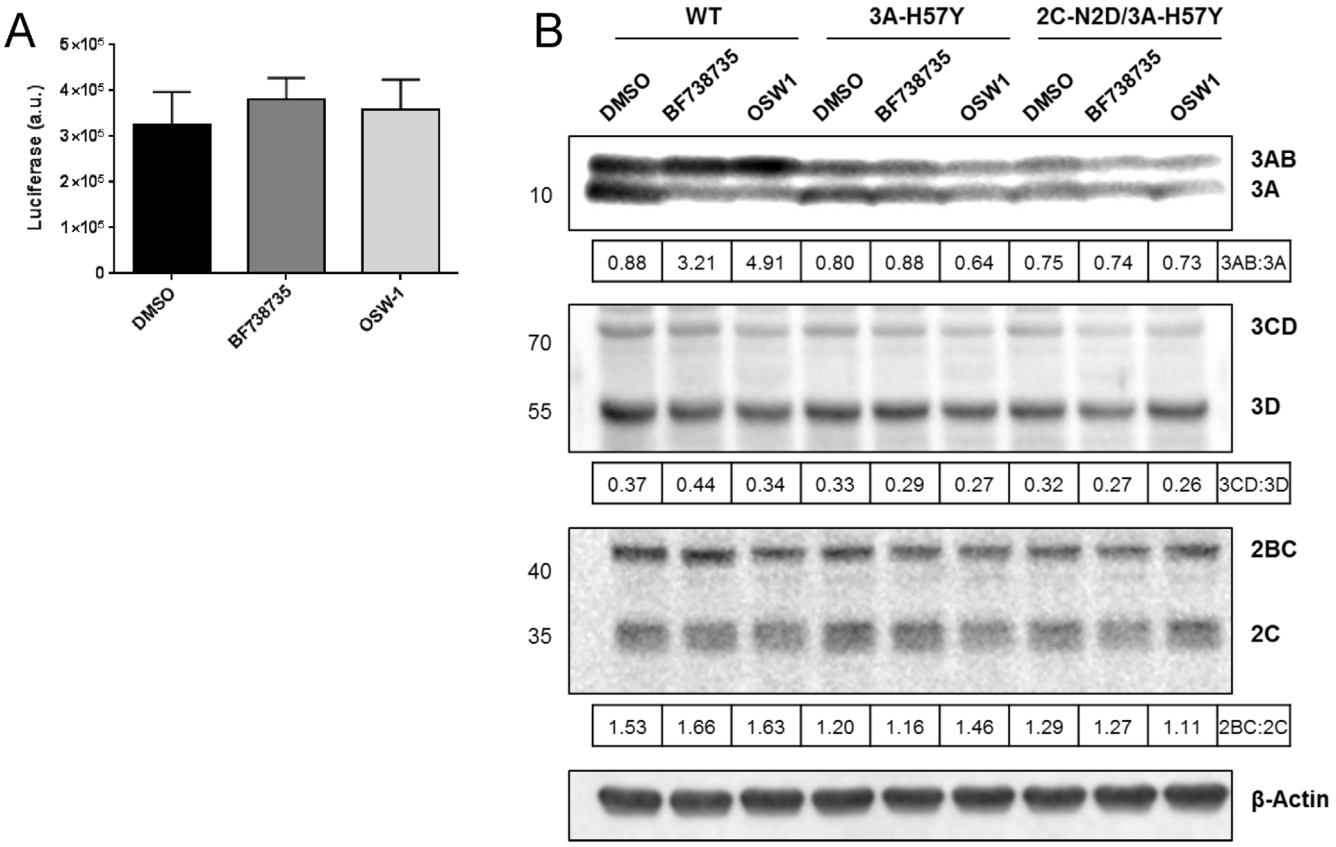


Fig. 3. The 3A-H57Y substitution rectifies the impaired polyprotein processing upon PI4KB or OSBP inhibition. (A) Luciferase levels produced upon transfection of RLuc-CVB3-Δ1-6+5 cDNA were determined to test whether the presence of inhibitors interferes with polyprotein production. Huh7-Lunet/T7 cells were transfected with linearized plasmid in the presence of DMSO, 1 μM BF738735, or 10 nM OSW-1. Inhibitors were added prior to transfection. After 7 h, cells were lysed to measure *Renilla* luciferase activity. **(B)** Huh7-Lunet/T7 cells were transfected with p53-CVB3-Δ1-6+5 wild-type, p53-CVB3-Δ1-6+5 3A-H57Y, or p53-CVB3-Δ1-6+5 2C-N2D/3A-H57Y cDNA in the presence of DMSO, 1 μM BF738735, or 10 nM OSW-1. After 7 h, cells were lysed and the lysates were subjected to Western blot analysis to examine effects on viral polyprotein processing. Antibodies against 3A, 3D, and 2C were used to detect viral proteins. Actin was used as a loading control. Densitometry of bands was done with LI-COR® Image Studio™ software.

PI4KB inhibitor (Arita, 2016). The PV 3A-A70T mutant, which is resistant to PI4KB inhibition, also compensated for this processing impairment by restoring the ratio of 3AB:3A. Notably, this mutant already showed enhanced 3A production in the absence of inhibitor, as also observed in our CVB3 mutant infected cells (Fig. 2). Yet, in the replication-independent system, this enhanced processing at the 3A-3B junction was only noticeable upon PI4KB or OSBP inhibition (Fig. 3B). The reasons for these discrepant results are unknown. Clearly, the infection system best represents the natural condition. However, it is known that the rate of proteolytic processing of viral proteins changes over time during replication. Therefore, it cannot be excluded that subtle differences in growth kinetics exist between wild-type and mutants, and that these may (partly) account for the differences in proteolytic processing that we observed. This potential drawback was circumvented by the replication-independent system, yet in this system some subtle effects from genome replication on polyprotein processing may be lacking. Notwithstanding the differences found between the two systems, both showed that PI4KB and OSBP inhibitors specifically impair proteolytic processing at the 3A-3B junction and that a single point mutation can rectify this defect.

It is not clear how the single amino acid substitutions A70T in PV 3A and H57Y in CVB3 3A can rescue the inefficient cleavage at the 3A-3B junction. One possible explanation is that they impose an altered conformation of 3A in PI4P/cholesterol depleted membranes, resulting in a better exposure and accessibility of the 3A-3B cleavage site to $3C(D)^{pro}$. Our observation that 3A-3B cleavage is impaired by OSW-1, which inhibits OSBP but has no effect on PI4KB activity (Albulescu et al., 2015a), points to an important role of cholesterol in determining the 3A conformation and 3A-3B cleavage efficiency.

What then is the role of 2C-N2D in the resistance mechanism? By itself, the 2C-N2D mutant did not confer resistance, but in combination with the 3A mutation it provided better resistance to PI4KB and OSBP inhibition, albeit to a marginal extent. This result suggests that the substitution in 2C plays a synergistic role with 3A in the resistance mechanisms rather than a distinctive resistance mechanism. Since the N2D substitution is near the 2B-2C junction, it may alter the cleavage efficiency at this junction. Although no such effect was observed, this possibility cannot be excluded as subtle effects may be difficult to detect by Western blot analysis. Another possible scenario is that the substitution in 2C provides additive resistance through a mechanism other than polyprotein processing. Biochemical as well as genetic data point to a functional interaction between viral proteins 2C and 3A (Teterina et al., 2006, 2011; Yin et al., 2007). For example, poliovirus acquired resistance to brefeldin A with substitutions in both 2C and 3A (Crotty et al., 2004; Viktorova et al., 2015). Hence, 2C-N2D may increase virus fitness by modulating functional interactions between viral proteins 2C and 3A-H57Y. The exact mechanism of how the 2C-N2D mutation increases viral replication upon PI4KB or OSBP inhibition remains to be elucidated.

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