Cis- and cell type-dependent trans-requirements for Lassa virus-like particle production

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Summary

Lassa virus (LASV) small zinc-finger protein (Z), which contains two L-domain motifs, plays a central role in virus budding. Here, we report that coexpression of glycoprotein (GPC) alters the requirements of cholesterol, but not the L-domains and host factor, Tsg101, for Z-induced virus-like particle (VLP) production. Especially, the cholesterol requirement for VLP production was cell type-dependent. In addition, GPC was important for colocalization of Z with CD63, a late endosome marker. We also found that the N-terminal region (amino acids 3 – 10) of Z was critical for its myristoylation and VLP production. These findings will contribute to our understanding of LASV assembly and budding.

Introduction

Arenaviruses are bisegmented RNA viruses, and both segments encode two viral proteins—nucleoprotein (NP) and glycoprotein (GPC) are encoded by the S segment, while RNA polymerase (L) and small zinc-finger protein (Z) are encoded by the L segment (Buchmeier, 2013). Several arenaviruses cause hemorrhagic fever in humans. Lassa virus (LASV) causes Lassa fever, which has high morbidity and mortality rates. Therefore, LASV is a major health concern in West Africa, where several hundred thousand cases of infection are reported annually.

All arenavirus Z proteins have the same structural properties (Urata & Yasuda, 2012). The N-terminus includes Gly at position 2 (G2). G2 of Z is known to be myristoylated and is critical for cellular membrane attachment and subsequent virion release (Perez *et al.*, 2004; Strecker *et al.*, 2006). The central domain includes the RING domain (zinc finger motif), which has been shown to regulate genome replication and gene transcription (Cornu & de la Torre, 2001, 2002; Cornu *et al.*, 2004; Emonet *et al.*, 2011; Kranzusch & Whelan, 2011; Urata & de la Torre, 2011). The C-terminus includes L-domains, which are known to regulate the virus budding process. All reported arenavirus Z proteins have been shown to play a central role in virus budding (Perez *et al.*, 2003; Strecker *et al.*, 2003; Urata & de la Torre, 2011; Urata *et al.*, 2009; Urata *et al.*, 2006). Therefore, Z is considered to be an arenavirus matrix protein. The short amino acid motifs, PT/SAP, PPxY, YPXnL, and FPIV, have been reported as consensus sequences of the L-domain (Bieniasz, 2006; Chen & Lamb, 2008; Freed, 2002). PT/SAP and YPXnL interact with Tsg101 and Alix/AIP1, respectively (Martin-Serrano

et al., 2003; Strack *et al.*, 2003). These host factors are involved in the Endosomal Sorting Complex Required for Transportation (ESCRT) machinery. The PPxY motif interacts with E3 ligases of the Nedd4 family, but the role of E3 ligases in virus budding has not been determined (Martin-Serrano *et al.*, 2005). LASV Z possesses two canonical L-domain motifs, PTAP and PPPY, at its C-terminus. Both of these motifs are important for LASV budding (Perez *et al.*, 2003; Strecker *et al.*, 2003).

In the present study, we found that GPC, but not NP, influenced the Z-mediated VLP production efficiency in a cell type-dependent manner. We also examined the cholesterol requirement for Z- and Z+GPC-mediated VLP production in several cell lines, and found that the cholesterol requirement for VLP production is also cell type-dependent. In addition, we showed that GPC expression is important for colocalization of Z with CD63, which is a late endosome marker. Finally, we examined the involvement of other regions of Z than G2 and L-domains in Lassa VLP production, and found that the region from amino acids 3 – 10 is important for myristoylation of Z and subsequent VLP production. These findings contribute to our understanding of LASV Z-mediated VLP production, especially the *trans* (GPC and cellular cholesterol) and *cis* (amino acid 3 – 10 in LASV Z) elements that are important for Lassa VLP production.

Results

Effects of GPC and NP on LASV Z-mediated VLP production. To examine the effects of GPC and/or NP on LASV Z-mediated VLP production, the expression plasmid for LASV Z, pCLV-Z, was transfected into 293T cells together with expression plasmids for LASV GPC, pCLV-GPC, and/or for LASV NP, pCLV-NP-HA, and the levels of VLP production were examined as described in the Methods section. As shown in Fig. 1A, coexpression of GPC and NP did not affect the cellular expression of Z. Coexpression of GPC together with Z decreased Z-mediated VLP production (50%), while NP expression did not significantly affect Z-mediated VLP production or Z+GPC-mediated VLP production in 293T cells.

Effects of cholesterol depletion on Lassa VLP production. Previously, LASV GPC was reported to redirect Z from the basolateral side to the apical side in the polarized cell line, MDCK-II (Schlie *et al.*, 2010b). Although 293T cells are not known as a polarized cell line, it is possible that the reduction of Z-mediated VLP production by GPC in 293T cells was due to the alteration of processing or assembly/release site of VLP production in the cell. Especially, we focused on lipid rafts, which are microdomains on the cell surface that mainly consist of cholesterol and lipids (Simons & Sampaio, 2011). In fact, several viruses have been reported to utilize lipid rafts as a budding platform (Kerviel *et al.*, 2013; Takeda *et al.*, 2003). In addition, it has been reported that cholesterol presents on the envelope of LASV and is important for viral infectivity (Schlie *et al.*, 2010a). To examine whether lipid rafts are involved in VLP production, we treated 293T cells with 8.7 mM methyl-beta-cyclodextrin (MβCD),

which is known to disrupt lipid rafts by chelating cholesterol, for 30 minutes prior to transfection with pCLV-Z or pCLV-Z+pCLV-GPC. MβCD treatment did not affect the intracellular expression of Z or GP2 (Fig. 1B). MβCD treatment had little effect on Z-mediated VLP production, while Z+GPC-mediated VLP production was significantly decreased by MβCD treatment (Fig. 1B). We next examined whether the effects of MβCD treatment on Z or Z+GPC VLP production are cell type-dependent. Huh-7 and Vero cells were treated with MβCD or control DMSO, and transfected with pCLV-Z or pCLV-Z+pCLV-GPC, as described in the Methods section. As GPC/GP2 could not be detected by anti-GPC/GP2 antibody in MDCK cell lysates, pCLV-GPC-FLAG was transfected instead of pCLV-GPC and anti-FLAG antibody was used to detect LASV GP2 in MDCK cells. In Huh-7 cells (Fig. 1C, left), the levels of Z and GPC expression were equivalent between DMSO and MβCD treatment groups. VLP production induced by Z was reduced upon GPC coexpression, similar to our observations in 293T cells (Fig. 1A, lanes 1 and 2). In addition, VLP production induced by Z+GPC was reduced upon MβCD treatment in Huh-7 cells, similar to our observations in 293T cells (Fig. 1B), while VLP production induced by Z alone was unaffected by MβCD treatment. In Vero cells (Fig. 1C, middle), although there were modest reductions in Z and GPC expression upon MβCD treatment, both Z and Z+GPC induced VLP production were markedly reduced upon MβCD treatment compared to control DMSO treatment. In MDCK cells (Fig. 1C, right), Z and GPC expression levels were equivalent between DMSO and MβCD treatment groups, and MβCD treatment did not affect Z- or Z+GPC-mediated VLP production. In all cell lines tested, no or only modest cell toxicity was observed based on Z or GPC/GP2 expression levels in the cells.

Intracellular localization of LASV Z and GPC/GP2. Our data shown in Fig. 1B suggested that cholesterol is required for Z+GPC VLP production in 293T cells. Confocal microscopy was performed to examine whether Z and GPC are colocalized at lipid rafts as a membrane budding platform. 293T cells were transfected with pCLV-Z-FLAG, pCLV-GPC, or pCLV-Z-FLAG+pCLV-GPC and fixed at 24 hours posttransfection. Z was detected by mouse anti-FLAG monoclonal antibody and GPC/GP2 was detected by rabbit anti-GPC/GP2 polyclonal antibody. In addition, lipid rafts were detected using a Vybrant Lipid Raft labeling kit containing cholera toxin subunit B (CT-B) to specifically bind to the plasma membrane (PM) lipid raft marker, ganglioside GM1. When LASV Z was expressed alone, Z was mainly localized at the PM, but colocalization of Z and rafts was not observed (Fig. 2A). GPC/GP2 was also mainly localized at the PM, but colocalization of GPC/GP2 and lipid rafts was not detected (Fig. 2B). Although colocalization of Z and GPC/GP2 was observed at a limited point near the PM when both were expressed (Fig. 2C and D, arrows), colocalization of lipid rafts and Z/GPC/GP2 was not observed (Fig. 2D and 2E). Next, we focused on CD63, which is a late endosome marker that was previously shown to be colocalized with LASV Z in experiments using infectious LASV (Fehling *et al.*, 2013). Z alone or Z+GPC were expressed in 293T cells, and the intracellular localization of Z was examined by laser confocal microscopy, together with that of CD63. As shown in Fig. 3, when Z was expressed alone, colocalization with CD63 was rarely detected. On the other hand, when GPC was coexpressed with Z, colocalization of CD63 and Z was observed approximately three times more frequently than Z alone (Fig. 3C).

VLP production induced by LASV Z and GPC requires Tsg101 and viral

L-domain. The data shown in Fig. 1 suggested that previously reported factors required for Z-mediated VLP production may be altered by the coexpression of GPC. Previously, we and other groups reported that Lassa Z-mediated VLP budding requires Tsg101, which is one of the key components of the ESCRT machinery, as a host factor (Perez *et al.*, 2003; Urata *et al.*, 2006). Therefore, we examined whether Tsg101 also plays an essential role in LASV Z+GPC-mediated VLP production as a host factor (Garrus *et al.*, 2001; Urata *et al.*, 2006). 293T cells were pretreated with siRNA specific for Tsg101 (siTsg101) or control siRNA (siCont) as described previously (Urata *et al.*, 2006), and then transfected with pCLV-Z and pCLV-GPC, together with siTsg101 or siCont. At 48 hours posttransfection, the VLP fraction and cell lysate were collected. Viral and cellular proteins in each fraction or lysate were separated by SDS-PAGE and then analyzed by Western blotting using the indicated antibodies (Fig. 4A). As shown in Fig. 4A, Z+GPC-mediated VLP production was decreased to 40% of control by depletion of Tsg101, suggesting that Tsg101 is involved in Z+GPC-mediated VLP budding as well as Z-mediated VLP budding (Perez *et al.*, 2003; Urata *et al.*, 2006).

We next examined whether L-domains still have an impact on LASV Z+GPC-mediated VLP production. We constructed expression plasmids for L-domain mutants of LASV Z, pCLV-Z-AAAP and pCLV-Z-PPPA, which have PTAP to AAAP or PPPY to PPPA mutation, respectively. 293T cells were transfected with pCLV-Z, pCLV-Z-AAAP, or pCLV-Z-PPPA, together with pCLV-GPC. At 48 hours posttransfection, the VLP fraction and cell lysate were collected and then analyzed by Western blotting. As shown in Fig. 4B, intracellular expression levels of Z were similar among WT and L-domain mutants. GP2 expression levels were also equivalent among cells expressing WT or

159 mutant Z proteins. The levels of VLP production induced by both Z L-domain mutants
160 were markedly decreased compared to WT (74% reduction for pCLV-Z-AAAP and 85%
161 reduction for pCLV-Z-PPPA, Fig. 4B).

162 **Identification of the region within Z critical for VLP formation.** We confirmed that
163 L-domains within Z play a critical role in Z+GPC VLP production (Fig. 4B) (Perez *et*
164 *al.*, 2003; Strecker *et al.*, 2003). The whole RING domain in LASV Z was also reported
165 to be important for VLP production (Wang *et al.*, 2012). Nevertheless, functional
166 domains other than Gly at position 2 (G2), which is known to be important for Z
167 myristoylation and subsequent virion production, the whole RING domain, and
168 L-domains, have not been identified. To examine the existence of as yet unknown
169 functional domains involved in Lassa VLP production, we constructed a series of LASV
170 Z deletion mutants (Fig. 5A), and performed VLP assay in 293T cells. As shown in Fig.
171 5B, the mutants showed various intracellular expression levels. However, VLP
172 production efficiencies of most mutants were similar to that of WT (Fig. 5C). Only the
173 Δ1 mutant with deletion of amino acids 3 – 10 showed a significant defect in VLP
174 production. Although the reasons for the differences in intracellular Z expression levels
175 of mutants are not clear, they may be related to the stabilities of the Z mutant proteins or
176 the binding affinities of Z mutants to the anti-Z polyclonal antibody used in this study.

177 Previously, LASV GPC was reported to form a VLP without Z expression in several
178 mammalian cell lines, including 293T cells (Schlie *et al.*, 2010b). Therefore, we
179 examined whether coexpression of GPC rescued the VLP production defect of Δ1
180 mutant in 293T cells. As shown in Fig. 5D, coexpression of GPC did not rescue this

181 defect. G2 of LASV Z has been reported to be important for its myristoylation and the
182 interaction with stable signal peptide (SSP) in GPC (Capul *et al.*, 2007). As the Δ1
183 region is localized next to G2, we examined whether Δ1 affected the myristoylation of Z.
184 293T cells were transfected with empty vector, pCLV-Z, pCLV-ZΔ1, or pCLV-ZG2A,
185 containing a Gly to Ala mutation at amino acid position 2, and myristoylation of each
186 protein was examined as described in the Methods section. As shown in Fig. 5E, the
187 expression levels of WT, Δ1, and G2A in cells were similar. WT was myristoylated,
188 while Δ1 and G2A were not. Next, to examine whether this VLP production defect of
189 Δ1 is due to deletion of this region, two mutants were constructed. Myristoylation of
190 HIV-1 Gag and RSV v-src at G2 were documented previously (Freed *et al.*, 1994;
191 Kaplan *et al.*, 1988; Ono & Freed, 1999). Therefore, LASV Z amino acids 3 – 10 were
192 replaced with HIV-1 Gag 3 – 10 or v-src 3 – 10 to construct pHIV-1 Gag10 LASV Z
193 (HIV-1 Gag10) or pv-src10 LASV Z (v-src10), respectively (Fig. 5F). Both mutants
194 showed reduced expression levels in the cells, and 48% and 42% reductions of VLP
195 production compared to WT based on normalized VLP production, respectively (Fig.
196 5G). The intracellular localizations of these mutant proteins were also examined. As
197 shown in Fig. 5H, these mutants were rarely colocalized with CD63, but were localized
198 at the plasma membrane the same as WT (Fig. 5H).

199 **Identification of the amino acid within positions 3 – 10 of Z critical for VLP**
200 **production.** A defect of VLP production was observed in Δ1, and replacement of LASV
201 Z 3 – 10 to both HIV-1 Gag 3 – 10 and v-src 3 – 10 recovered this defect. To further
202 analyze the importance of amino acids 3 – 10 of LASV Z, and to identify the amino
203 acids critical for VLP production, three more mutants containing a linker sequence

(GGGS) were constructed (Fig. 6A). LASV Z 3 – 10 was replaced with GGGS × 2 to construct mut1 (Fig. 6A). LASV Z 3 – 6 or 7 – 10 was replaced with GGGS to construct mut2 or mut3, respectively (Fig. 6A). As mut1 showed significant reduction of intracellular expression (Fig. 6B), only mut2 and mut3 were used to examine VLP production. Both mut2 and mut3 showed lower protein expression levels compared to WT in the cells, and reduction of VLP production ratio compared to WT (65% and 54% reduction, respectively) (Fig. 6C). To examine the contribution of each amino acid to Z-mediated VLP production, single amino acid mutations were introduced into this region and VLP assay was performed. As shown in Fig. 6D, none of these amino acid mutants showed a defect in Z-mediated VLP production. As reported previously, G2A mutant showed complete abolition of VLP production due to the lack of myristoylation (Perez *et al.*, 2004; Strecker *et al.*, 2006). Furthermore, we introduced double lysine to alanine mutation (K4, 7A) to examine the contribution of double lysines, which may affect protein folding, on VLP production. Only modest reduction of VLP production was observed in K4, 7A mutant compared to WT, suggesting that the single and double (K4, 7) mutations examined in our assay were not critical for VLP production (Fig. 6D).

Discussion

Here, we showed that LASV GPC, but not NP, decreased Z-mediated VLP production in 293T cells (Fig. 1A), and GPC also influenced the sensitivity of Z-mediated VLP production to MβCD treatment (Fig. 1B). Interestingly, the sensitivity against MβCD treatment for VLP production was cell type-dependent (Fig. 1B and 1C). Huh-7 cells showed similar results to 293T cells (Fig. 1B and 1C). Z+GPC induced VLP production was reduced upon MβCD treatment, but Z-induced VLP production was unaffected (Fig. 1C, left). In Vero cells, both Z alone and Z+GPC-induced VLP production were reduced upon MβCD treatment (Fig. 1C, middle). In MDCK cells, both Z alone and Z+GPC-induced VLP production were unaffected by MβCD treatment (Fig. 1C, right). These results suggested that the cholesterol requirement for Z or Z+GPC mediated VLP production is cell type-dependent. It was reported that both GP in LASV-infected MDCK cells and GP-transfected MDCK cells exhibited the same apical surface expression pattern, suggesting that the transient GP expression distribution showed the same pattern as LASV-infected GP (Schlie *et al.,* 2010b).

Previous studies on other arenaviruses, including JUNV, Mopeia virus, Pichinde virus, and Tacaribe virus, as well as the present study indicated that other viral proteins could modulate Z-mediated VLP production and affect the efficiency of VLP production (Casabona *et al.,* 2009; Groseth *et al.,* 2010; Shtanko *et al.*, 2010; Wang *et al.*, 2012). Therefore, we propose that coexpression of GPC together with Z is necessary to mimic LASV budding.

Based on the results shown in Fig. 1 indicating that cholesterol is required for

Z+GPC-mediated VLP production in 293T cells, we examined whether lipid rafts, in which cholesterol is one of the main components, on the PM act as platforms for Z+GPC budding in 293T cells. Although colocalization of Z/GPC and lipid rafts was not detected, colocalization of Z and GPC/GP2 was sometimes detected (Fig. 2). As cholesterol is involved in the virion membrane and plays a critical role in infection (Schlie *et al.*, 2010a), and the results presented here indicating that Z or Z+GPC-mediated VLP production were reduced on MβCD treatment, but in a cell type-dependent manner, cholesterol appears to be an important *trans* factor for producing an infectious LASV. In addition, the observations that LASV Z and GPC are localized at detergent-soluble membrane regions in CHO-K1 cells (transfection system) and in Huh-7 cells (infection system) (Schlie *et al.*, 2010a) and that LASV Z and GPC do not colocalize with lipid rafts (Fig. 2) indicate that these proteins are mainly localized at non-lipid rafts, detergent-soluble membrane areas. Considering these results, it is possible that once LASV Z and GPC reach the PM, they localize at non-lipid rafts, detergent-soluble membrane areas, but relocalize to the cholesterol-rich domains, lipid rafts, just before budding and incorporate cholesterol into the virion, as proposed for JUNV (Agnihothram *et al.*, 2009).

It has been shown that LASV Z colocalizes with CD63 and M6PR, both of which are late endosome markers (Fehling *et al.*, 2013). Therefore, we examined whether LASV Z alone or LASV Z+GPC colocalized with CD63. In fact, we found that GPC relocalized Z to CD63 (Fig. 3), indicating that GPC is important to mimic Z-mediated VLP assembly and budding.

It was demonstrated that GPC expression did not alter the requirements for Tsg101 and L-domains in LASV Z and Z+GPC on VLP budding (Fig. 4A and B). These results agreed with a previous report using the LASV infection system (Strecker *et al.*, 2003) and suggested the involvement of LASV Z L-domains in virion production. These results (Figs. 1 and 4) suggested that both Z and GPC are required to mimic Lassa virus assembly/budding using the VLP system.

All arenavirus Z proteins reported to date have been shown to be matrix proteins with bona fide budding activity. To determine if there are other regions that regulate Z-mediated VLP production than G2 or L-domains, which are well characterized, we constructed a series of deletion mutants and examined VLP production. We showed that the region consisting of amino acids 3 – 10 (Δ1) of LASV Z was critical for VLP production (Fig. 5A – C), and this was due to the lack of myristoylation (Fig. 5E). To examine whether LASV Z myristoylation and subsequent VLP production require specific amino acids 3 – 10 of LASV Z, these residues of the original LASV Z sequence were replaced with HIV-1 Gag (HIV-1 Gag10) or RSV v-src (v-src10). Both proteins are myristoylated at G2 (Fig. 5F) (Freed *et al.*, 1994; Ono & Freed, 1999; Schultz *et al.*, 1985). Both mutants showed lower protein expression levels compared to WT in the cells, suggesting that amino acids 3 – 10 are at least partially required for protein expression or stability. In addition, although the VLP production ratios were reduced compared to WT, both mutants rescued VLP production, suggesting that LASV Z does not require a specific amino acid in the region comprised of amino acids 3 – 10 for VLP production (Fig. 5G). These mutants did not exhibit differences in intracellular distribution compared to WT (Fig. 5H). Next, to further explore the importance of this 3

– 10 amino acid sequence for LASV Z-mediated VLP production, we generated a mutant (mut1) in which amino acids within the Δ1 (3 – 10) region were replaced with the GGGS × 2 linker sequence (Fig. 6A and B). We observed significant reduction in expression of mut1 compared to WT in cells. These observations, together with the results shown in Fig. 5G, supported the suggestion that the specific sequence of amino acids 3 – 10 in LASV Z is important for protein expression or stability. To narrow down the region important for LASV Z-mediated VLP production in LASV Z 3 – 10, two other mutants were constructed and VLP production was examined (Fig. 6C). Both mutants exhibited less cellular expression and showed lower VLP production ratios than WT. These results indicated that both the sequences of amino acids 3 – 6 and 7 – 10 have some roles, but are not critical, for cellular expression or stability as well as VLP production. The reason for stable expression of the whole deletion LASV Z 3 – 10 (Δ1) is not clear. Finally, to identify the specific amino acid that regulates VLP production with the region of amino acids 3 – 10 in LASV Z, single amino acid mutations were introduced, and VLP assays were performed (Fig. 6B). In addition, double lysine mutations (K4, 7A) were also examined for VLP production efficiency (Fig. 6C). None of the single or double mutation constructs showed reduction of VLP production, indicating that these single or double mutations were not sufficient for determining the amino acids responsible for VLP production in the Δ1 region. All arenaviral Z and several retroviral Gag proteins have been reported to be myristoylated at G2 for attachment to the cellular membrane (Bryant & Ratner, 1990; Gottlinger *et al.*, 1989; Pal *et al.*, 1990; Urata & Yasuda, 2012; Urata *et al.*, 2009), and this attachment is critical for the assembly and production of infectious progeny virions. To produce Lassa

VLP, the 3 – 10 amino acid sequence does not have to be specific for LASV Z, as substitution of this region with HIV-1 Gag and RSV v-src recovered the defect of Δ1 VLP production, although the degree of recovery did not completely reach the WT level (Fig. 5G). The ratios of VLP production induced by mut2 and mut3 were reduced compared to WT. These results suggested that a specific amino acid of LASV Z 3-10 is required to produce VLP efficiently. Based on these results, we concluded that the whole region of amino acids 3 – 10 in LASV Z is critical for myristoylation and is important for efficient protein expression, stability, and subsequent VLP production.

In conclusion, we described several important aspects of the molecular mechanisms of LASV Z and Z+GPC-mediated VLP production. As Z plays a central role in arenavirus assembly and budding, these findings will contribute to our understanding of LASV assembly and budding.

Methods

Plasmids, siRNAs, and antibodies. The expression plasmids for Lassa virus Z (pCLV-Z) and GPC (pCLV-GPC) (both Josiah strain) were generated previously (Sakuma *et al.*, 2009; Urata *et al.*, 2006). pCLV-Z-FLAG and pCLV-GPC-FLAG, which express Z and GPC with FLAG-tags at their C-termini, were also constructed by insertion of FLAG-tag sequence into pCLV-Z and pCLV-GPC, respectively. The NP gene of Josiah strain was cloned into pcDNA3.1 (–) (Invitrogen, Carlsbad, CA) and the HA-tag was fused to its C-terminus (pCLV-NP-HA). The expression plasmids for Z mutants were constructed with a QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) or KOD Plus Mutagenesis Kit (Toyobo, Osaka, Japan) according to the respective manufacturer's instructions. Human immunodeficiency virus 1 (HIV-1) *gag* and Rous sarcoma virus (RSV) *v-src* genes were referenced from GenBank accession numbers AF324493.2 and K01644.1, respectively. Polyclonal antibodies against LASV Z or GPC/GP2 were described previously (Sakuma *et al.*, 2009; Urata *et al.*, 2006). Anti-HA (#2367, 6E2) and Streptavidin-HRP (#3999) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-FLAG M2 antibody to detect pCLV-GPC-FLAG on Western blotting was purchased from Sigma (St. Louis, MO). Anti-CD63 antibody was obtained from Santa Cruz (sc-5275; Santa Cruz Biotechnology, Santa Cruz, CA). siRNA and antibody against Tsg101 were described previously (Garrus *et al.*, 2001; Urata *et al.*, 2006). Second antibodies against rabbit-IgG (W401B) and mouse IgG (A2304), both conjugated with peroxidase, were purchased from Promega (Madison, WI) and Sigma, respectively. The signals were detected using ECL Prime Western Blotting Detection Reagents (GE Healthcare,

345 Waukesha, WI) according to the manufacturer's instructions.

346 **Cells and MβCD treatment**. 293T, Huh-7, MDCK, and Vero cell lines were
347 maintained with Dulbecco's modified Eagle's medium (DMEM) (D6429; Sigma)
348 containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Life
349 Technologies, Carlsbad, CA). Cells were treated with MβCD (C4555; Sigma, 8.7 mM
350 as a final concentration) for 30 minutes to chelate cellular cholesterol, and culture media
351 were replaced with fresh media before application of the transfection mixture.

352 **VLP assay.** Detection of LASV Z-mediated VLP production was described previously
353 (Urata *et al.*, 2006). Briefly, 293T cells were transfected with pCLV Z (or mutants) and
354 related plasmids with Trans-IT LT-1 (Mirus Bio Corp., Madison, WI). At 48 hours
355 posttransfection, cell debris was removed by centrifugation, and media were loaded on
356 top of a 20% sucrose cushion, followed by ultracentrifugation (195000 × *g*, 30 minutes,
357 4°C) to collect VLPs. Cells were lysed with lysis A buffer (1% Triton X-100, 25 mM
358 Tris-HCl, pH 8.0, 50 mM NaCl, and 10% Na-deoxycholate), and cell debris was
359 removed by centrifugation (13000 × *g*, 10 minutes, 4°C). VLPs and cell lysate samples
360 were separated by SDS-PAGE followed by Western blotting. Transfection of Huh-7,
361 MDCK, and Vero cells was performed with Lipofectamine 2000 (Invitrogen), and at 6
362 hours posttransfection, media were replaced with fresh media. Relative VLP production
363 was calculated as total VLP-associated Z/cell-associated Z and normalized to wild-type
364 (WT) or control treatment as 1.0.

365 **Immunofluorescence microscopy.** At 24 hours after transfection with pCLV-Z-FLAG
366 and/or pCLV-GPC, 293T cells were fixed with 4% paraformaldehyde (Wako, Osaka,

Japan) for 30 minutes at room temperature (RT). Fixed cells were treated with blocking buffer [10% FBS diluted with dilution buffer consisting of 3% BSA+0.3% Triton-X100 in PBS (–)] for 1 hour. After blocking, monoclonal antibody against FLAG tag (M2; Sigma) and/or polyclonal antibody against LASV GPC/GP2, as well as Vybrant component A (V-34404, Vybrant Lipid Raft labeling kit 555; Life Technologies), were used to stain LASV Z, GPC/GP2, and lipid rafts (GM1), respectively. After 2 hours of incubation at RT for the 1st staining, cells were washed twice with PBS (–), and goat anti-mouse IgG FITC (ab7064; Abcam) or goat anti-rabbit IgG Alexa Fluor 647 (ab150079; Abcam) was used for labeling the 1st antibodies for 2 hours at RT. After the 2nd staining, cells were washed twice with PBS (–), and DAPI was used to stain the nuclei for 30 minutes at RT. Finally, after three washes with PBS (–), cells were incubated with Vybrant component B for 15 minutes at 4°C to crosslink the cholera toxin subunit B (CT-B)-labeled lipid rafts (Vybrant component A) with anti-CT-B antibody (Vybrant component B), and then washed three times with PBS(–), covered with slide glasses, and observed by confocal microscopy (LSM780; Zeiss, Oberkochen, Germany). In the CD63 localization experiment, LASV Z and mutants were detected by anti-LASV Z polyclonal antibody, and CD63 was detected by anti-CD63 monoclonal antibody. Goat anti-mouse IgG FITC (ab7064; Abcam) or goat anti-rabbit IgG TRITC (T5268; Sigma) was used as the respective second antibody.

Detection of myristoylated protein. Click-iT myristic acid azide (C10268; Invitrogen), biotin alkyne (B10185; Invitrogen), and Click-iT protein reaction buffer kit (C10276; Invitrogen) were used to detect LASV Z myristoylation according to the manufacturer's instructions. Briefly, 293T cells (1×10^6 cells) were seeded and incubated for 6 hours

under 5% CO_2 at 37°C. After incubation, cells were transfected with 0.5 μg of plasmids using LT-1 and then cultured for a further 18 hours. Culture media were replaced with fresh media containing click-iT myristic acid azide at a final concentration of 10 μM. After 6 hours of incubation, cells were lysed with lysis buffer (50mM Tris-HCl, pH 8.0, 1% SDS) containing protease inhibitor (80-6501-23; GE Healthcare) and benzonase (E1014; Sigma). After centrifugation (1300 × *g*, 5 minutes, 4°C) to remove cell debris, samples were used for click reaction using biotin alkyne, and methanol/chloroform protein precipitation was performed to prepare the samples for SDS-PAGE. Samples of the same volume were loaded and detected by Western blotting with either rabbit anti-LASV Z polyclonal antibody followed by HRP anti-rabbit IgG to detect LASV Z WT/mutants or HRP-streptavidin to detect myristoylated proteins.

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524

FIGURE LEGENDS

Figure 1. *Trans* factors that affect Lassa VLP production. (A) Effects of GPC and NP on LASV Z-mediated VLP release. 293T cells were transfected with pCLV-Z alone or with a combination of pCLV-GPC and/or pCLV-NP-HA. The empty vector, pCAGGS, was transfected to adjust the total amount of plasmid. At 48 hours posttransfection, culture supernatants were collected, and VLPs were collected by ultracentrifugation as described previously (Urata *et al.*, 2006). Cell lysates were prepared with Lysis A buffer. Cell lysates and VLPs were subjected to SDS-PAGE, followed by Western blotting using polyclonal antibodies against LASV-Z, LASV GP2, as well as HA to detect LASV NP (Left). The intensities of the bands were quantified by LAS3000 (Fujifilm, Tokyo, Japan). The efficiencies of VLP production were calculated as VLP-associated Z/Cell-associated Z. The efficiency of LASV Z alone was set to 1.0 and relative VLP production is shown in the right panel. The data are averages and standard deviations from three independent experiments. (B and C) The involvement of cholesterol in Lassa VLP production in several cell lines. 293T cells (B), Huh-7 cells, and Vero cells (C) were pretreated with either DMSO or MβCD (8.7 mM) for 30 minutes, washed once with PBS (–), followed by transfection with pCLV-Z and pCAGGS (empty vector) or pCLV-Z and pCLV-GPC. In the case of MDCK cells, pCLV-GPC-FLAG was transfected instead of pCLV-GPC. At 48 hours posttransfection, VLP and cell lysates were analyzed as described above.

Figure 2. LASV Z and GPC/GP2 do not colocalize at lipid rafts. 293T cells were cultured on poly-L-lysine-coated cover glasses, and transfected with pCLV-Z-FLAG (A),

pCLV-GPC (B), or both pCLV-Z-FLAG and pCLV-GPC (C – E). All samples were fixed 24 hours posttransfection. Z-FLAG was detected by mouse anti-FLAG monoclonal antibody followed by anti-mouse IgG-FITC antibody; GPC/GP2 was detected by rabbit anti-GPC/GP2 polyclonal antibody followed by anti-rabbit IgG-Alexa Fluor 647 antibody. Lipid rafts were stained with a Vybrant Lipid Raft labeling kit 555, and nuclei were stained with DAPI. Z stack was also captured. In (C), the dotted square shows a higher magnification view of the bottom right. The white arrow indicates the colocalization of LASV Z and GPC/GP2 (C and D).

Figure 3. LASV GPC increases the colocalization of Z with CD63. A. LASV Z-FLAG was expressed in 293T cells, and LASV Z and CD63 were stained. B. LASV Z-FLAG and LASV GPC were coexpressed in 293T cells, and LASV Z and CD63 were stained. Nuclei were stained with DAPI. Bar, 5 μm. C. Cell number of LASV Z and CD63 colocalized cells were divided by LASV Z-positive cell number (100 cells) in both LASV Z alone and LASV Z+GPC expressing samples, and indicated with percent (%) in y-axis.

Figure 4. The involvement of Tsg101 and L-domains in Lassa VLP production. (A) 293T cells were transfected with scramble siRNA (siCont) or siRNA targeting Tsg101 (siTsg101). At 24 hours posttransfection, culture media were replaced with fresh media and cells were transfected with each siRNA and both pCLV-Z and pCLV-GPC. VLP production was analyzed as described above (Fig. 1A) (Left). The efficiency of VLP production was calculated as described in Fig. 1A and the results are shown on the right. (B) 293T cells were transfected with pCLV-Z (WT), pCLV-Z-AAAP (PTAP>AAAP), or

569 pCLV-Z-PPPA (PPPY>PPPA), together with pCLV-GPC. VLP production was analyzed
570 as described in Fig. 1A (Left). The efficiency of VLP production was also calculated as
571 described in Fig. 1A (Right). The data are averages and standard deviations from three
572 independent experiments.

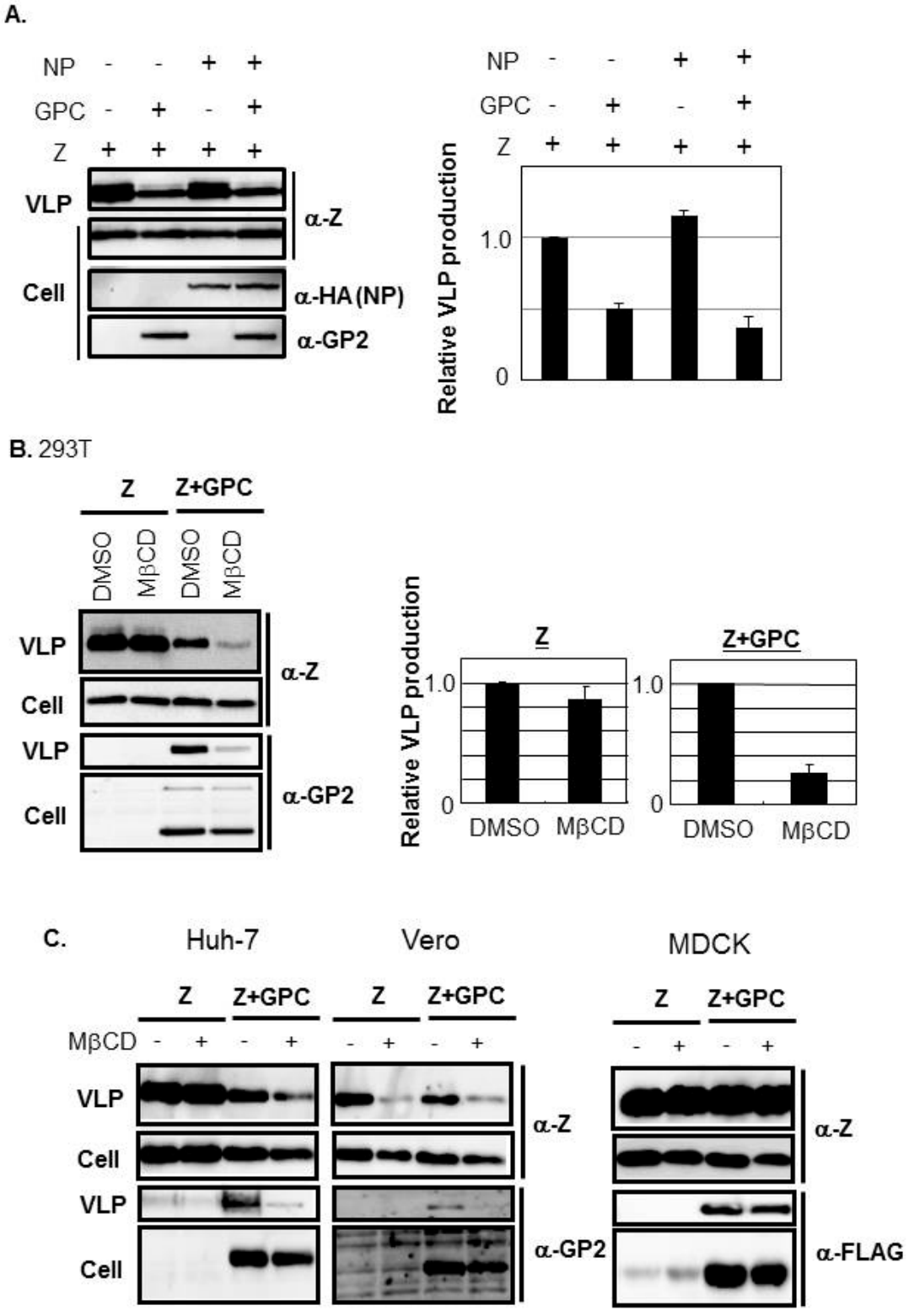
573 **Figure 5. *Cis* factors that affect Lassa VLP production.** (A) Schematic representation
574 of LASV Z deletion mutants used in this study. Two L-domains (PTAP and PPPY) are
575 shown at the top. (B) 293T cells were transfected with pCLV-Z (WT) or the expression
576 plasmid for each LASV-Z deletion mutant. VLP production was analyzed as described
577 in Fig. 1A. (C) The efficiencies of VLP production of each Z mutant were calculated as
578 described in Fig. 1A. The efficiency of WT was set to 1.0. The data are averages and
579 standard deviations from three independent experiments. (D) Effects of GPC expression
580 on VLP production mediated by Δ1. 293T cells were transfected with pCLV-Z or
581 pCLV-ZΔ1 together with pCLV-GPC. VLP production was analyzed as described in Fig.
582 1A. (E) Myristoylation of Δ1. 293T cells were transfected with empty plasmid, pCLV-Z,
583 pCLV-Z Δ1, or pCLV-ZG2A. At 18 hours posttransfection, culture media were replaced
584 with fresh media containing Click-iT myristic acid azide (10 μM). At 6 hours after
585 medium exchange, cell lysates were prepared and used to perform click reaction with
586 biotin alkyne according to the manufacturer's instructions. Purified proteins were
587 detected with either rabbit anti-LASV Z polyclonal antibody followed by
588 HRP-conjugated anti-rabbit IgG antibody or HRP-conjugated Streptavidin. (F)
589 Schematic representation of HIV-1 Gag10 and RSV v-src10. LASV Z 3 – 10 amino acid
590 sequence was replaced with the 3 – 10 sequence of HIV-1 Gag or v-src. The asterisk (*)
591 indicates the consensus myristoylation amino acid (Ser) at position 6. (G) 293T cells

592 were transfected with pCLV-Z (WT) or pHIV-1 Gag10 LASV Z (HIV-1 Gag10) of
593 pv-src10 LASV Z (v-src10). VLP production was analyzed as described in Fig. 1A. (H)
594 LASV-Z (WT), HIV-1 Gag10, and v-src10 were expressed in 293T cells, and stained
595 together with CD63. Nuclei were stained with DAPI. Bar, 5 μm.

596

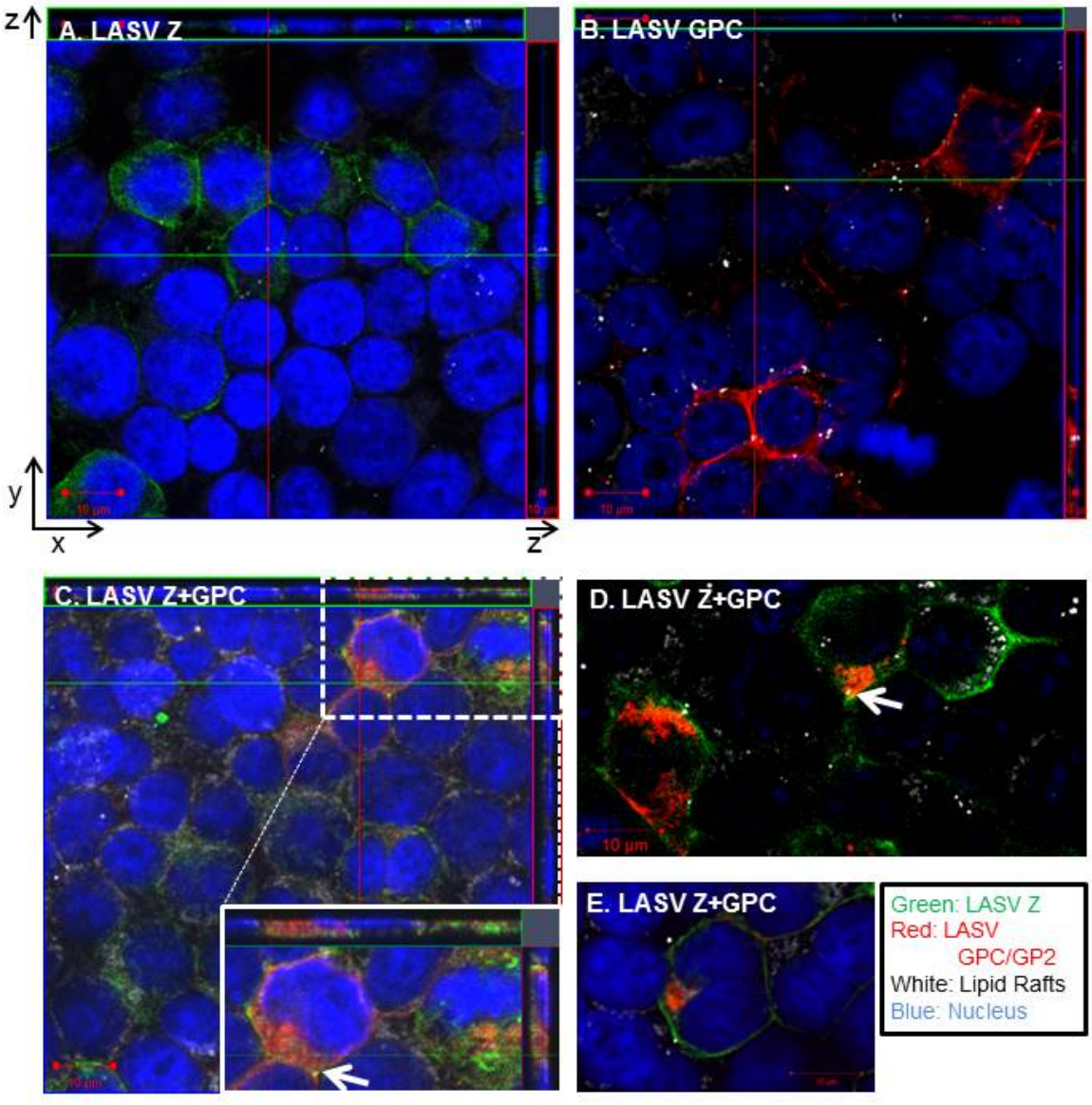
597 **Figure 6. Analysis of VLP production in LASV Z 3 – 10 amino acid mutants.** (A)
598 Alignment of the N-terminal amino acid sequences of Z from three LASV strains
599 (Josiah strain, NP_694871.1, NL strain, AAO59510, CSF strain, AAO59514.1), three
600 LASV Z mutants (mut1, mut2, and mut3) and the conserved N-myristoylation motif.
601 The amino acid sequences of positions 1 – 10 are indicated. (B) Cellular expression of
602 mut1. Either WT or mut1 was expressed in 293T cells, and cellular expression was
603 examined by Western blotting. (C) VLP assay was performed to examine the efficiency
604 of mut2 and mut3 for VLP production in 293T. The levels of VLP production of each
605 single point mutant (D) or double lysine mutant (E) of LASV Z were analyzed as
606 described in Fig. 1A.

607



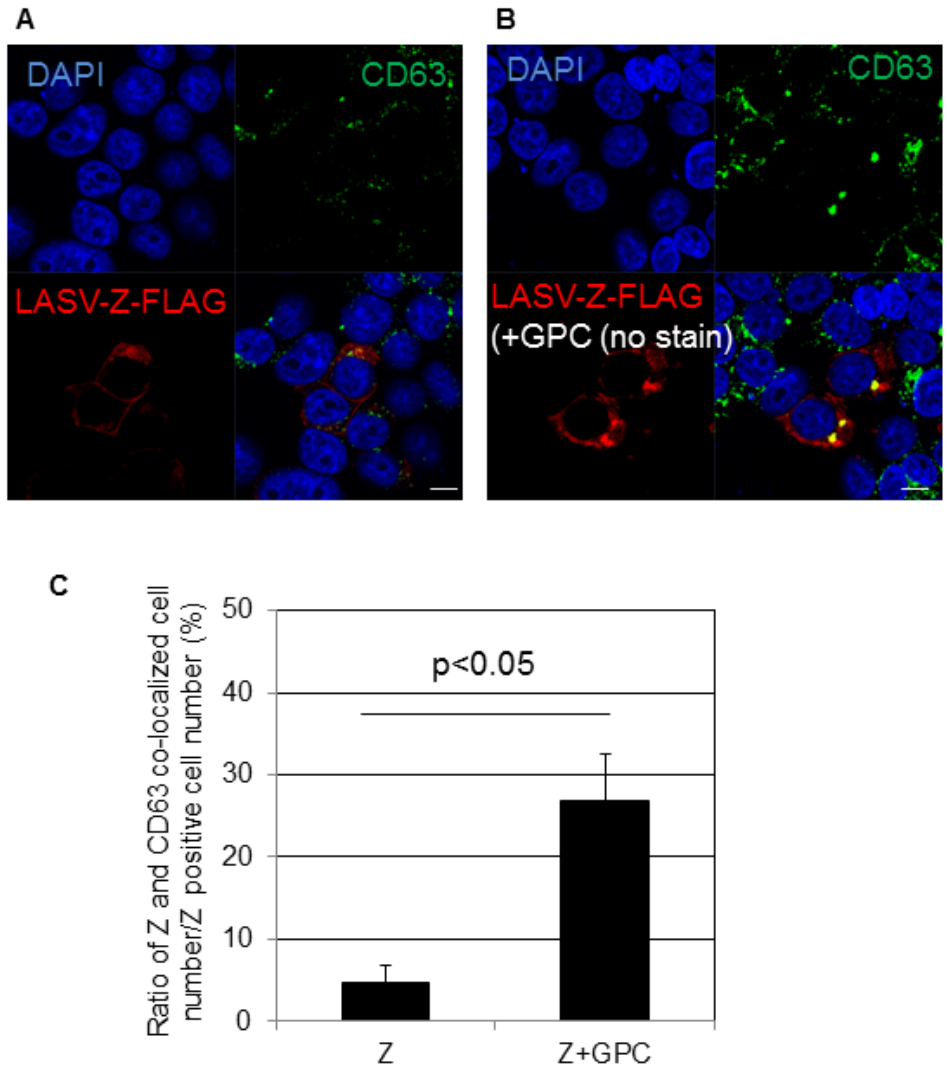
608 **Figure 1. Urata and Yasuda**

610

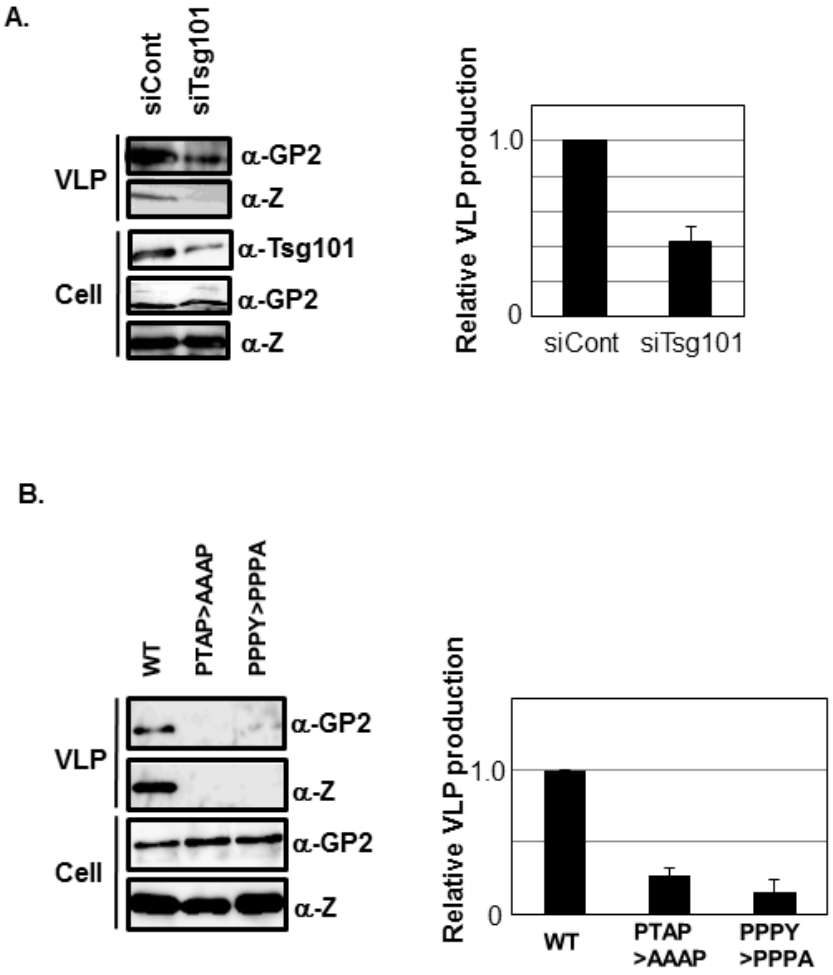


611 **Figure 2. Urata and Yasuda**

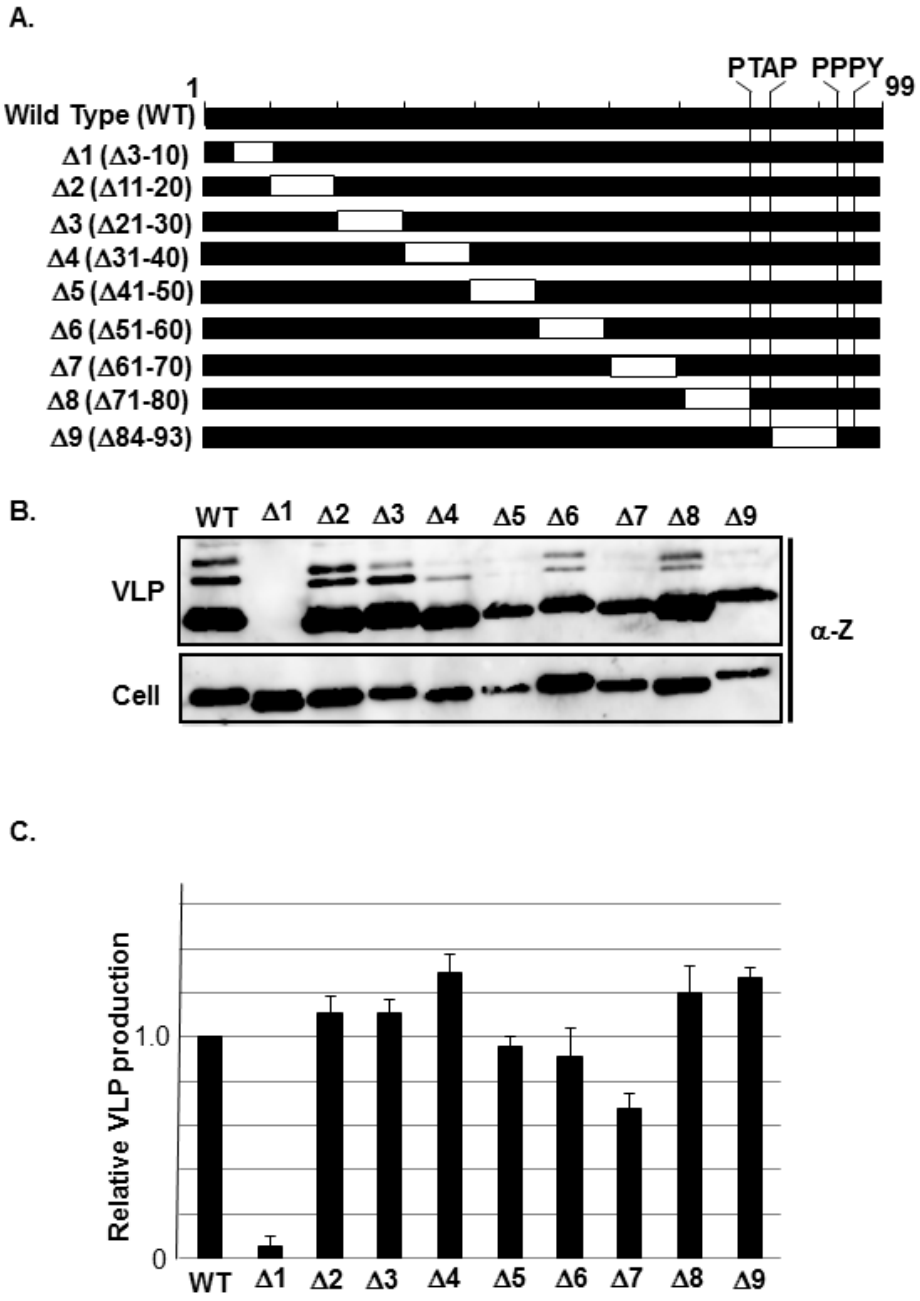
612



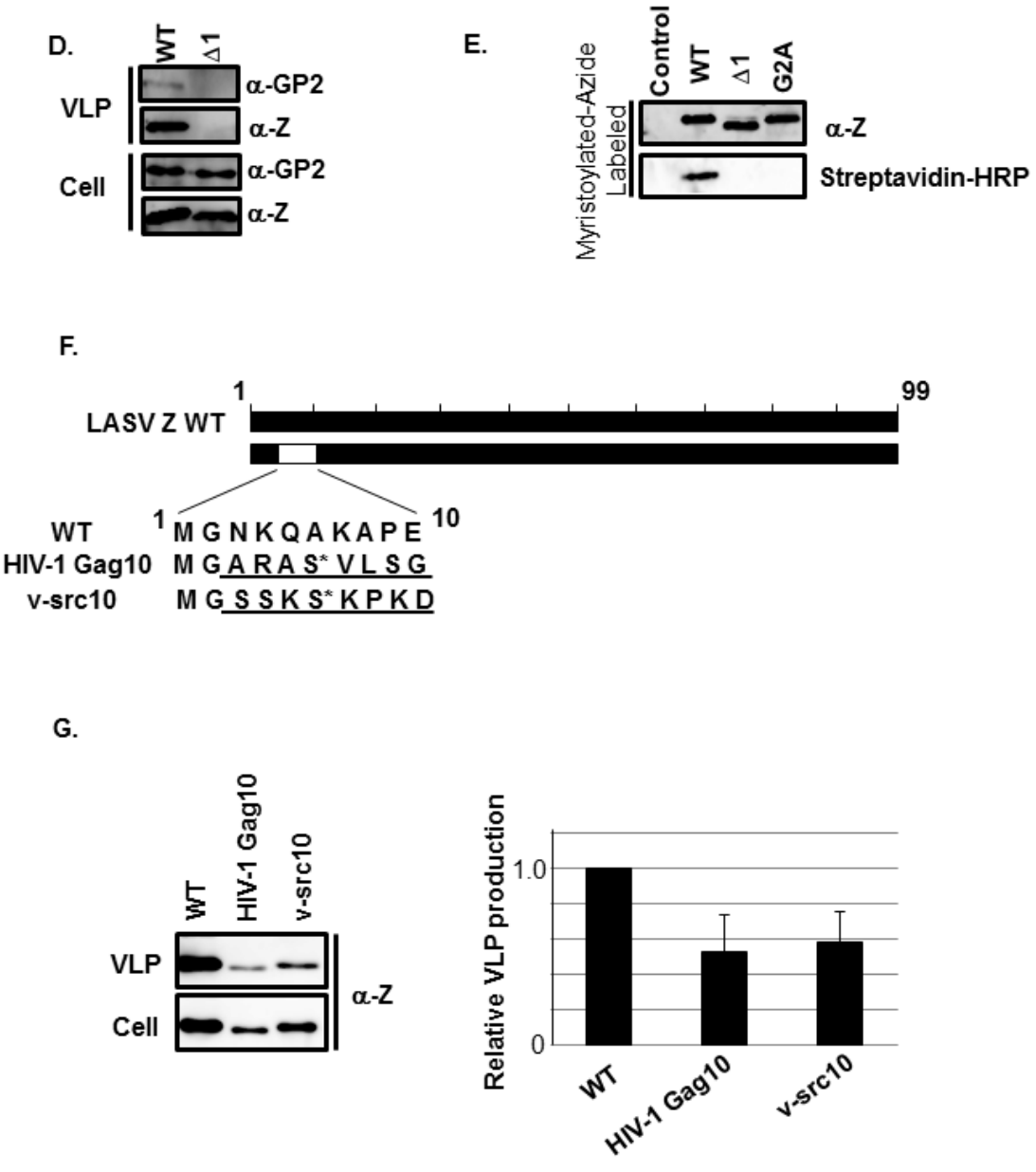
613 **Figure 3 Urata and Yasuda**



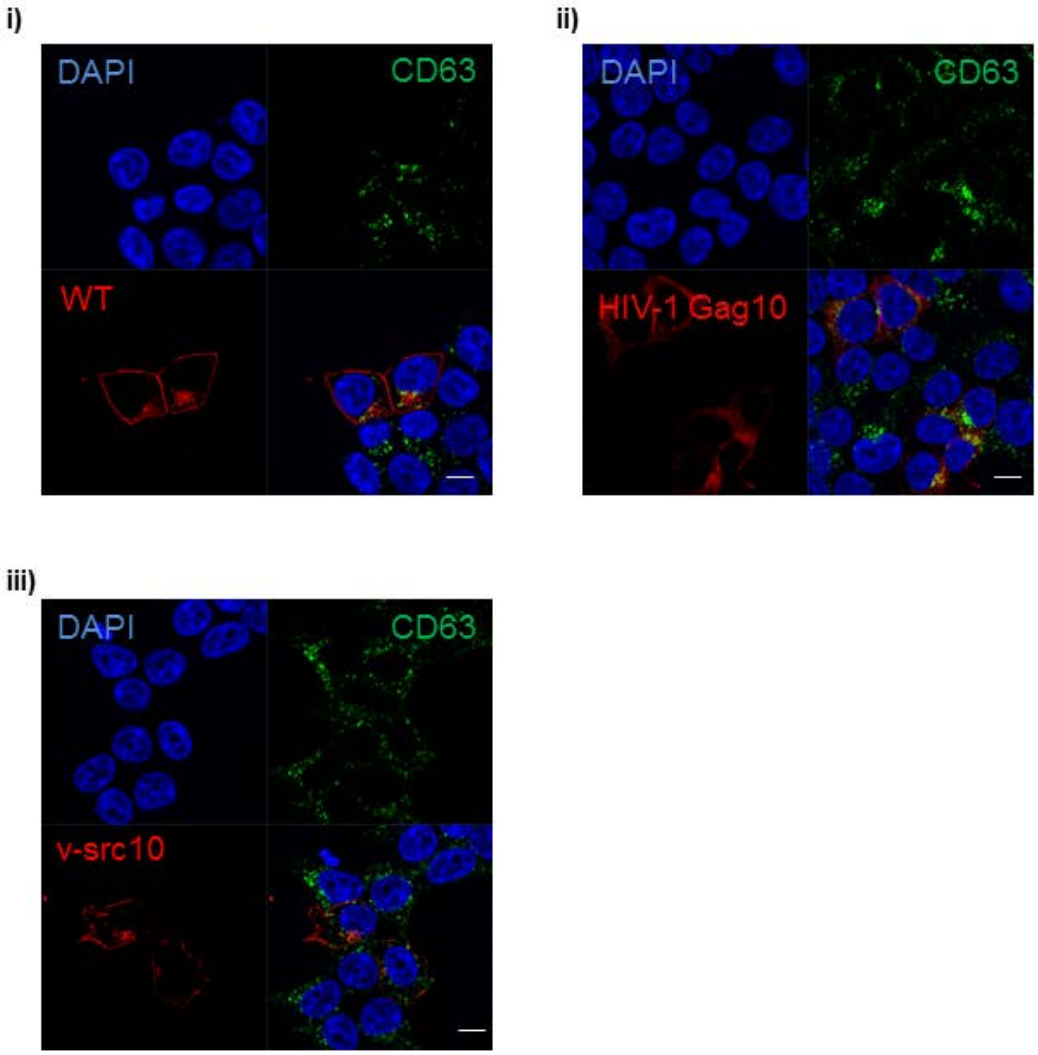
614 **Figure 4. Urata and Yasuda**



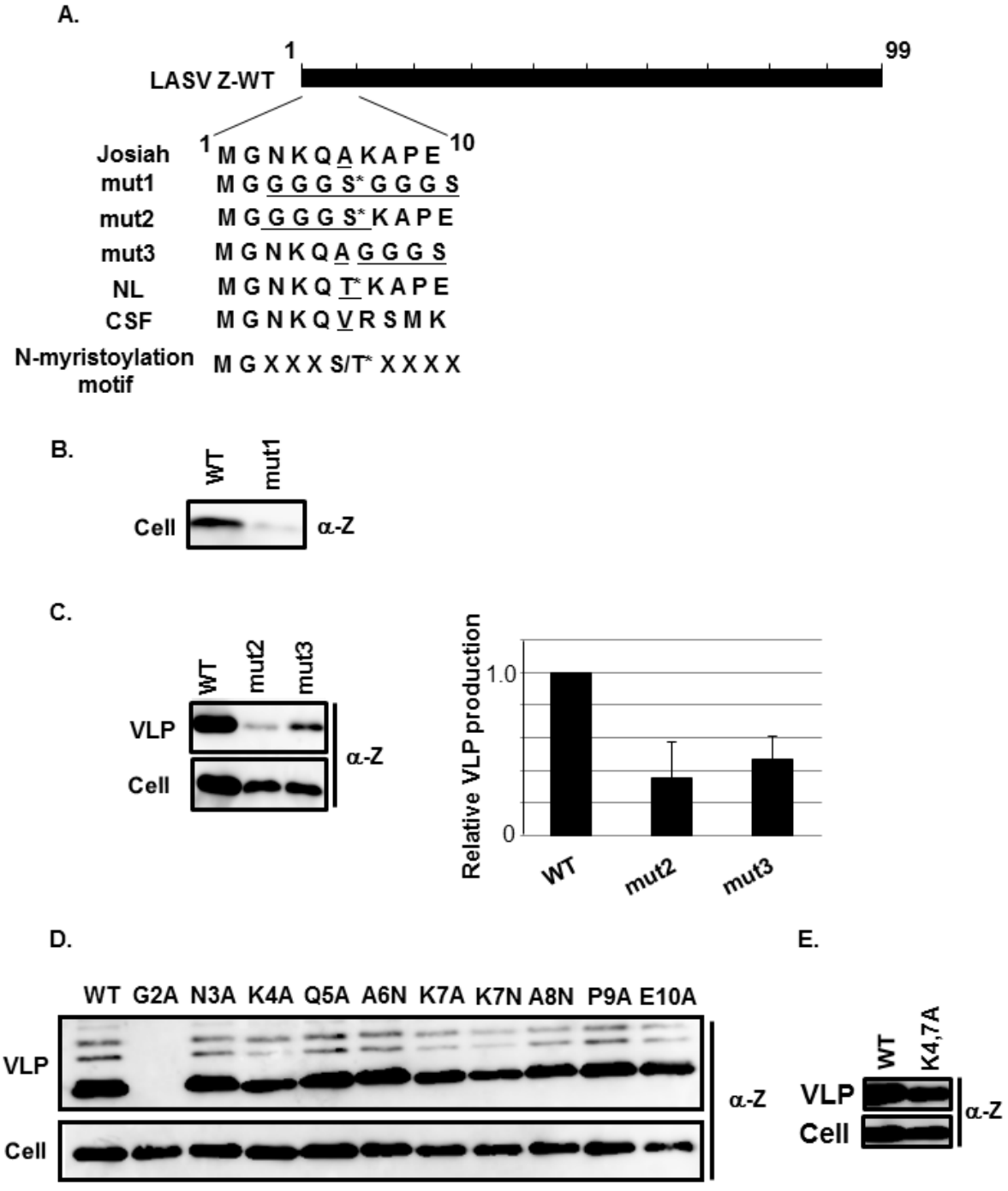
615 **Figure 5 (A-C). Urata and Yasuda**



616 **Figure 5 (D-G). Urata and Yasuda**



617 **Figure 5H. Urata and Yasuda**



618 **Figure 6. Urata and Yasuda**