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

Shuzo Urata\(^1\) and Jiro Yasuda\(^{1,2}\)

\(^1\)Department of Emerging Infectious Diseases, Institute of Tropical Medicine (NEKKEN), Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

\(^2\)The Graduate School of Biomedical Science, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

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 co-expression of glycoprotein (GPC) altered the requirements for cholesterol but not the L-domains and host factor, Tsg101, for Z-induced virus-like particle (VLP) production. In particular, the cholesterol requirement for VLP production was cell-type-dependent. In addition, GPC was found to be important for co-localization of Z with CD63, a late endosomal marker. We also found that the N-terminal region (aa 3–10) of Z was critical for its myristoylation and VLP production. These findings will contribute to our understanding of LASV assembly and budding.

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**INTRODUCTION**

Arenaviruses are bisegmented RNA viruses, and both segments encode two viral proteins: the nucleoprotein (NP) and glycoprotein (GPC) are encoded by the S segment, whilst RNA polymerase (L) and small zinc-finger protein (Z) are encoded by the L segment (Buchmeier et al., 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., 2006, 2009). 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 virus-like particle (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 co-localization of Z with CD63, which is a late endosomal 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 aa 3 to 10 is important for myristoylation of Z and subsequent VLP production. These findings contribute to our understanding of LASV Z-mediated VLP production.

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**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, an 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 Methods. As shown in Fig. 1(a), co-expression of GPC and NP did not affect the cellular expression of Z. Co-expression of GPC together with Z decreased Z-mediated VLP production (50%), whilst 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 the reduction of Z-mediated VLP production by GPC in 293T cells. In addition, it has been reported that cholesterol presents rafts are involved in VLP production, we treated 293T cells with 8.7 mM methyl-β-cyclodextrin (MβCD), which is known to disrupt lipid rafts by chelating cholesterol, for 30 min prior to transfection with pCLV-Z or pCLV-Z+pCLV-GPC. MβCD treatment did not affect the intracellular expression of Z or GPC (Fig. 1b). MβCD treatment had little effect on Z-mediated VLP production, whilst 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 were 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 Methods. 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 panel), the levels of Z and GPC expression were equivalent between the DMSO and MβCD treatment groups. VLP production induced by Z was reduced upon GPC co-expression, 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), whilst VLP production induced by Z alone was unaffected by MβCD treatment. In Vero cells (Fig. 1c, middle panel), 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 with control DMSO treatment. In MDCK cells (Fig. 1c, right panel), Z and GPC expression levels were equivalent between the 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. 1(b) suggested that cholesterol is required for Z+GPC VLP production in 293T cells. Confocal microscopy was performed to examine whether Z and GPC are co-localized 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 h post-transfection. Z was detected by mouse anti-FLAG mAb and GPC/GP2 was detected by rabbit anti-GPC/GP2 polyclonal antibody. In addition, lipid rafts were detected using a Vybrant Alexa Fluor 555 Lipid Raft labelling kit containing cholera toxin subunit B (CT-B) to bind specifically to the plasma membrane (PM) lipid raft marker, ganglioside GM1. When LASV Z was expressed alone, Z was localized mainly at the PM, but co-localization of Z and rafts was not observed (Fig. 2a). GPC/GP2 was also localized mainly at the PM, but co-localization of GPC/GP2 and lipid rafts was not detected (Fig. 2b). Although co-localization of Z and GPC/GP2 was observed at a limited point near the PM when both were expressed (Fig. 2c, d, arrow), co-localization of lipid rafts and Z/GPC/GP2 was not observed (Fig. 2d, e). Next, we focused on CD63, which is a late endosomal marker that was shown previously to be co-localized with LASV Z in

![Image of LASV Z and GPC/GP2 localization](image)

**Fig. 2.** LASV Z and GPC/GP2 do not co-localize at lipid rafts. 293T cells were cultured on poly-l-lysine-coated cover slips and transfected with pCLV-Z-FLAG (a), pCLV-GPC (b), or both pCLV-Z-FLAG and pCLV-GPC (c–e). All samples were fixed at 24 h post-transfection. Z–FLAG was detected by mouse anti-FLAG mAb 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 Alexa Fluor 555 Lipid Raft labelling kit, and nuclei were stained with DAPI. The Z stack was also captured. In (c), the dotted square is shown at a higher magnification in the bottom right. The white arrows (c, d) indicate the co-localization of LASV Z and GPC/GP2.
experiments using infectious LASV (Fehling et al., 2013). Z alone (Fig 3(a)) or Z+GPC (Fig 3(b)) 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, co-localization with CD63 was rarely detected. On the other hand, when GPC was co-expressed with Z, co-localization of CD63 and Z was observed approximately three times more frequently than with Z alone (Fig. 3c).

**VLP production induced by LASV Z and GPC requires Tsg101 and the 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 co-expression of GPC. Previously, we and other groups reported that LASV 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 pre-treated with small interfering RNA (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 h post-transfection, the VLP fraction and cell lysate were collected. Viral and cellular proteins in each fraction or lysate were separated by SDS-PAGE and analysed by Western blotting using the indicated antibodies (Fig. 4a). As shown in Fig. 4(a), Z+GPC-mediated VLP production was decreased to 40% of that of the 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 had 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 a PTAP→AAAP or PPPY→PPPA mutation, respectively. 293T cells were transfected with pCLV-Z, pCLV-Z-AAAP or pCLV-Z-PPPA, together with pCLV-GPC. At 48 h post-transfection,
the VLP fraction and cell lysate were collected and analysed by Western blotting. As shown in Fig. 4(b), intracellular expression levels of Z were similar among the WT and L-domain mutants. GP2 expression levels were also equivalent among cells expressing WT or mutant Z proteins. The levels of VLP production induced by both Z L-domain mutants were markedly decreased compared with that of WT (74% reduction for pCLV-Z-AAAP and 85% reduction for pCLV-Z-PPPA; Fig. 4b).

Identification of the region within Z critical for VLP formation

We confirmed that L-domains within Z play a critical role in Z + GPC VLP production (Fig. 4b) (Perez et al., 2003; Strecker et al., 2003). The whole RING domain in LASV Z has also been reported to be important for VLP production (Wang et al., 2012). Nevertheless, functional domains other than G2, which is known to be important for Z myristoylation and subsequent virion production, the whole RING domain and L-domains have not been identified. To examine the existence of as-yet-unknown functional domains involved in Lassa VLP production, we constructed a series of LASV Z deletion mutants (Fig. 5a), and performed VLP assay in 293T cells. As shown in Fig. 5(b), the mutants showed various intracellular expression levels. However, VLP production efficiencies of most mutants were similar to that of the WT (Fig. 5c). Only the Δ1 mutant with deletion of aa 3–10 showed a significant defect in VLP production. Although the reasons for the differences in intracellular Z expression levels of mutants are not clear, they may be related to the stabilities of the Z mutant proteins or the binding affinities of Z mutants to the anti-Z polyclonal antibody used in this study.

Previously, LASV GPC was reported to form a VLP without Z expression in several mammalian cell lines, including 293T cells (Schlie et al., 2010b). Therefore, we examined whether co-expression of GPC rescued the VLP production defect of the Δ1 mutant in 293T cells. As shown in Fig. 5(d), co-expression of GPC did not rescue this defect. G2 of LASV Z has been reported to be important for its myristoylation and the interaction with stable signal peptide in GPC (Capul et al., 2007). As the Δ1 region is localized next to G2, we examined whether Δ1 affected the myristoylation of Z. 293T cells were transfected with empty vector, pCLV-Z, pCLV-ZA1 or pCLV-ZG2A, containing a Gly—Ala mutation at aa 2, and myristoylation of each protein was examined as described in Methods. As shown in Fig. 5(e), the expression levels of WT, Δ1 and G2A in cells were similar. WT was myristoylated, whilst Δ1 and G2A were not. Next, to examine whether this VLP production defect of Δ1 was due to deletion of this region, two mutants were constructed. Myristoylation of human immunodeficiency virus type 1 (HIV-1) Gag and Rous sarcoma virus (RSV) v-src at G2 have been documented previously (Freed et al., 1994; Kaplan et al., 1988; Ono & Freed, 1999). Therefore, LASV Z amino aa 3–10 were replaced with HIV-1 Gag aa 3–10 or v-src aa 3–10 to construct pHIV-1 Gag10 LASV Z (HIV-1 Gag10) or pv-src10 LASV Z (v-src10), respectively (Fig. 5f). Both mutants showed reduced expression levels in the cells, and 48 and 42% reductions in VLP production compared with WT based on normalized VLP production, respectively (Fig. 5g). The intracellular localizations of these mutant proteins were also examined. As shown in Fig. 5(h), these mutants were rarely co-localized with CD63 but were localized at the PM the same as the WT (Fig. 5h).

Identification of the amino acid within positions 3–10 of Z critical for VLP production

A defect in VLP production was observed in mutant Δ1, and replacement of LASV Z aa 3–10 with either HIV-1 Gag 3–10 or v-src 3–10 recovered this defect. To analyse further the importance of aa 3–10 of LASV Z, and to identify the amino acids critical for VLP production, three more mutants containing a linker sequence (GGGS) were constructed (Fig. 6a). LASV Z aa 3–10 were replaced with GGGS × 2 to construct mut1 (Fig. 6a). LASV Z aa 3–6 and 7–10 were replaced with GGGS to construct mut2 and mut3, respectively (Fig. 6a). As mut1 showed a 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 with WT in the cells and reduction of the VLP production ratio compared with 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 a VLP assay was performed. As shown in Fig. 6(d), none of these amino acid mutants showed a defect in Z-mediated VLP production. As reported previously, a 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 the K4,7A mutant compared with that of the WT, suggesting that the single and double (K4,7) mutations examined in our assay were not critical for VLP production (Fig. 6e).

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, c). Huh-7 cells showed similar results to 293T cells (Fig. 1b, c). Z + GPC-induced VLP production was reduced upon MβCD treatment, but Z-induced VLP production was unaffected (Fig. 1c, left.
**Fig. 5.** Cis factors that affect Lassa VLP production. (a) Schematic representation of LASV Z deletion mutants used in this study. Two L-domains (PTAP and PPPY) are shown at the top. (b) 293T cells were transfected with pCLV-Z (WT) or the expression plasmid for each LASV Z deletion mutant. VLP production was analysed as described in Fig. 1(a). (c) The efficiencies of VLP production of each Z mutant were calculated as described in Fig. 1(a). The efficiency of the WT was set to 1.0. The data are means ± SD from three independent experiments. (d) Effects of GPC expression on VLP production mediated by the Δ1 mutant. 293T cells were transfected with pCLV-Z or pCLV-ZΔ1 together with pCLV-GPC. VLP production was analysed as described in Fig. 1(a). (e) Myristoylation of the Δ1 mutant. 293T cells were transfected with empty plasmid, pCLV-Z, pCLV-ZΔ1 or pCLV-ZG2A. At 18 h post-transfection, the culture medium was replaced with fresh medium containing Click-iT myristic acid azide (10 μM). At 6 h after medium exchange, cell lysates were prepared and used to perform a Click reaction.
with biotin alkyne according to the manufacturer’s instructions. Purified proteins were detected with either rabbit anti-LASV Z polyclonal antibody followed by HRP-conjugated anti-rabbit IgG antibody or HRP-conjugated streptavidin. (f) Schematic representation of HIV-1 Gag10 and RSV v-src10. LASV Z amino acid sequence aa 3–10 was replaced with the aa 3–10 sequence of HIV-1 Gag or v-src. The asterisk (*) indicates the consensus myristoylation amino acid (Ser) at position 6. (g) 293T cells were transfected with pCLV-Z (WT) or pHIV-1 Gag10 LASV Z (HIV-1 Gag10) of pv-src10 LASV Z (v-src10). VLP production was analysed as described in Fig. 1(a). (h) LASV Z (WT), HIV-1 Gag10 and v-src10 were expressed in 293T cells, and stained together with CD63. Nuclei were stained with DAPI. Bars, 5 μm.

Panel). In Vero cells, both Z alone and Z + GPC-induced VLP production were reduced upon MβCD treatment (Fig. 1c, middle panel). In MDCK cells, both Z alone and Z + GPC-induced VLP production were unaffected by MβCD treatment (Fig. 1c, right panel). 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 Junin virus (JUNV), Mopeia virus, Pichinde virus and Tacaribe virus, as well as the present study, indicate that other viral

![Figure 6](https://example.com/figure6.png)

**Fig. 6.** Analysis of VLP production in LASV Z aa 3–10 mutants. (a) Alignment of the N-terminal amino acid sequences of Z from three LASV strains (Josiah strain, GenBank accession no. NP_694871.1; NL strain, AAO59510; CSF strain, AAO59514.1), three LASV Z mutants (mut1, mut2 and mut3) and the conserved N-myristoylation motif. The amino acid sequences of positions 1–10 are indicated. (b) Cellular expression of mut1. WT or mut1 was expressed in 293T cells, and cellular expression was examined by Western blotting. (c) A VLP assay was performed to examine the efficiency of mut2 and mut3 for VLP production in 293T cells. (d, e) The levels of VLP production of each single point mutant (d) or double lysine mutant (e) of LASV Z were analysed as described in Fig. 1(a).
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 co-expression 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 co-localization of Z/GPC and lipid rafts was not detected, co-localization 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 upn M, presented here indicating that Z- or Z-mediated VLP production and budding. Based on the results shown in Fig. 1 indicating that cholesterol is required for Z-mediated VLP production, we generated a mutant (mut1) in which amino acids within the Δ1 (aa 3–10) region were replaced with the GGGS × 2 linker sequence (Fig. 6a, b). We observed a significant reduction in expression of mut1 compared with the WT in cells. These observations, together with the results shown in Fig. 5(g), supported the suggestion that the specific sequence of aa 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 aa 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 the WT. These results indicated that the sequences of aa 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 of LASV Z aa 3–10 (Δ1) is not clear. Finally, to identify the specific amino acid that regulates VLP production with in the region of aa 3–10 in LASV Z, single amino acid mutations were introduced, and VLP assays were performed (Fig. 6d). In addition, double lysine mutations (K4,7A) were also examined for VLP production efficiency (Fig. 6e). 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 arenavirus Z and several retroviral Gag proteins have been reported to be myristoylated at G2 for attachment to the cellular membrane (Bryant & Ratner, 1990; Göttlinger 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 aa 3–10 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 with that of the WT. These results suggested that a specific amino acid within LASV Z aa 3–10 is required to produce VLP efficiently. Based on these results, we concluded that the whole region of aa 3–10 in LASV Z is critical for...
myristoylation and is important for efficient protein expression, stability and subsequent VLP production.

In conclusion, we have 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 LASV 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 the FLAG-tag sequence into pCLV-Z and pCLV-GPC, respectively. The NP gene of Josiah strain was cloned into pCDNA3.1 (+) (Invitrogen) and the haemagglutinin (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) or KOD Plus Mutagenesis kit (Toyobo) according to the respective manufacturer’s instructions. HIV-1 gag and RSV v-src genes were referenced from GenBank accession numbers AF324493.2 and K01644.1, respectively. The polyclonal antibodies against LASV Z or GPC/GP2 have been described previously (Sakuma et al., 2009; Urata et al., 2006). Anti-HA (clone 6E2) and streptavidin–HRP were purchased from Cell Signaling Technology. Anti-FLAG M2 antibody to detect pCLV-GPC-FLAG on Western blotting was purchased from Sigma. Anti-CD63 antibody was obtained from Santa Cruz Biotechnology. The siRNA and antibody against Tsg101 have been described previously (Garrus et al., 2001; Urata et al., 2006). Secondary antibodies against rabbit IgG and mouse IgG, both conjugated with HRP, were purchased from Promega and Sigma, respectively. The signals were detected using ECL Prime Western blotting Detection Reagents (GE Healthcare) according to the manufacturer’s instructions.

Cells and MjCD treatment. 293T, Huh-7, MDCK and Vero cell lines were maintained with Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) containing 10% FBS and 1% penicillin/streptomycin (Life Technologies). Cells were treated with MJ/CD (Sigma, 8.7 mM final concentration) for 30 min to chelate cellular cholesterol, and the culture medium was replaced with fresh medium before application of the transfection mixture.

VLP assay. Detection of LASV Z-mediated VLP production was as described previously (Urata et al., 2006). Briefly, 293T cells were transfected with pCLV-Z (or mutants) and related plasmids with Trans-IT LT-1 (Mirus Bio). At 48 h post-transfection, cell debris was removed by centrifugation, and the medium was loaded on top of a 20% sucrose cushion, followed by ultracentrifugation (195 000 g, 30 min, 4 °C) to collect VLPs. Cells were lysed with lysis A buffer [1% Triton X-100, 25 mM Tris/HCl (pH 8.0), 50 mM NaCl, 10% sodium deoxycholate], and cell debris was removed by centrifugation (13 000 g, 10 min, 4 °C). VLPs and cell lysate samples were separated by SDS-PAGE followed by Western blotting. Transfection of Huh-7, MDCK and Vero cells was performed with Lipofectamine 2000 (Invitrogen), and at 6 h post-transfection, the medium was replaced with fresh medium. Relative VLP production was calculated as total VLP-associated Z/cell-associated Z and normalized to the WT or control treatment as 1.0.

Immunofluorescence microscopy. At 24 h after transfection with pCLV-Z-FLAG and/or pCLV-GPC, 293T cells were fixed with 4% paraformaldehyde (Wako) for 30 min at room temperature. Fixed cells were treated with blocking buffer [10% FBS diluted with dilution buffer consisting of 3% BSA + 0.3% Triton X-100 in PBS (−) (PBS lacking Mg2+ and Ca2+)] for 1 h. After blocking, mAb against FLAG tag (clone M2; Sigma) and/or polyclonal antibody against LASV GPC/GP2, as well as Vybrant component A (Vybrant Alexa Fluor 555 Lipid Raft labelling kit; Life Technologies), were used to stain LASV Z, GPC/GP2 and lipid rafts (GM1), respectively. After 2 h of incubation at room temperature for the first staining, cells were washed twice with PBS (−), and goat anti-mouse IgG–FITC (Abcam) or goat anti-rabbit IgG–Alexa Fluor 647 (Abcam) was used to detect the primary antibodies for 2 h at room temperature. After the second staining, cells were washed twice with PBS (−), and DAPI was used to stain the nuclei for 30 min at room temperature. Finally, after three washes with PBS (−), cells were incubated with Vybrant component B for 15 min at 4 °C to cross-link the CT-B-labelled lipid rafts (Vybrant component A) with anti-CT-B antibody (Vybrant component B), and then washed three times with PBS (−), covered with glass coverslips and observed by confocal microscopy (LSM780; Zeiss). In the CD63 localization experiment, LASV Z and mutants were detected by anti-LASV Z polyclonal antibody, and CD63 was detected by anti-CD63 mAb. Goat anti-mouse IgG–FITC (Abcam) or goat anti-rabbit IgG–TRITC (Sigma) was used as the respective secondary antibody.

Detection of myristoylated protein. Click-iT myristic acid azide (Invitrogen), biotin alkyne (Invitrogen) and Click-iT protein reaction buffer kit (Invitrogen) were used to detect LASV Z myristoylation according to the manufacturer’s instructions. Briefly, 293T cells (1 × 106) were seeded and incubated for 6 h under 5% CO2 at 37 °C. After incubation, the cells were transfected with 0.5 μg plasmids using LT-1 and then cultured for a further 18 h. Culture medium was replaced with fresh medium containing Click-iT myristic acid azide at a final concentration of 10 μM. After 6 h of incubation, the cells were lysed with lysis buffer [50 mM Tris/HCl (pH 8.0), 1% SDS] containing protease inhibitor (GE Healthcare) and benzoxan (Sigma). After centrifugation (13 000 g, 5 min, 4 °C) to remove cell debris, samples were used for a 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-conjugated anti-rabbit IgG to detect LASV Z WT mutants or HRP–streptavidin to detect myristoylated proteins.

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