

Efficient Budding of the Tacaribe Virus Matrix Protein Z Requires the Nucleoprotein[∇]

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The Z protein has been shown for several arenaviruses to serve as the viral matrix protein. As such, Z provides the principal force for the budding of virus particles and is capable of forming virus-like particles (VLPs) when expressed alone. For most arenaviruses, this activity has been shown to be linked to the presence of proline-rich late-domain motifs in the C terminus; however, for the New World arenavirus Tacaribe virus (TCRV), no such motif exists within Z. It was recently demonstrated that while TCRV Z is still capable of functioning as a matrix protein to induce the formation of VLPs, neither its ASAP motif, which replaces a canonical PT/SAP motif in related viruses, nor its YxxL motif is involved in budding, leading to the suggestion that TCRV uses a novel budding mechanism. Here we show that in comparison to its closest relative, Junin virus (JUNV), TCRV Z buds only weakly when expressed in isolation. While this budding activity is independent of the ASAP or YxxL motif, it is significantly enhanced by coexpression with the nucleoprotein (NP), an effect not seen with JUNV Z. Interestingly, both the ASAP and YxxL motifs of Z appear to be critical for the recruitment of NP into VLPs, as well as for the enhancement of TCRV Z-mediated budding. While it is known that TCRV budding remains dependent on the endosomal sorting complex required for transport, our findings provide further evidence that TCRV uses a budding mechanism distinct from that of other known arenaviruses and suggest an essential role for NP in this process.

The *Arenaviridae* are a diverse and growing family of viruses currently containing 25 recognized species (7, 16, 29, 38), including many members of considerable significance to human health. Taxonomically, the arenaviruses can be divided based on antigenicity, phylogeny, and geographical distribution into the lymphocytic choriomeningitis virus (LCMV) serocomplex or Old World arenaviruses and the Tacaribe virus (TCRV) serocomplex or New World arenaviruses (14). The New World arenaviruses can be further divided into three clades, A, B, and C, based on their phylogeny (13). This separation also corresponds to differences in receptor usage and disease phenotype (14). Among the Old World arenaviruses, both Lassa virus (LASV) and Lujo virus are capable of causing hemorrhagic fever (HF) (7, 23), while New World arenaviruses are responsible for at least five distinct HFs (13, 16). In addition, the New World arenavirus Whitewater Arroyo virus (WWAV) was originally reported as causing HF in three people in the United States, but in more recent years this finding has been the subject of debate (1, 11, 12). However, even if WWAV is excluded, arenaviruses cause at least seven distinct HFs, making them the largest family of HF-causing viruses currently known.

In terms of their structure, arenaviruses are comparatively simple, being composed of only four proteins and two ambisense genome segments. The small genome segment (S segment) encodes the surface glycoprotein (GP) and nucleoprotein (NP), while the large genome segment (L segment) encodes the polymerase (L) and a multifunctional protein

known as the small RING finger protein (Z). Arenavirus particles are enveloped and highly pleomorphic, having a diameter of 50 to 300 nm, with only a single surface protein, GP. GP is synthesized as a precursor, GPC, and is first cleaved by signal peptidase to yield a stable signal peptide (SSP), which remains noncovalently bound to GP (2, 17, 18). Association of the SSP is necessary for the further cleavage of GP into the receptor-binding ectodomain subunit, GP1, and transmembrane-spanning fusion competent subunit, GP2, by the proprotein convertase site 1 protease/subtilisin kexin isozyme 1 (S1P/SKI-1) during its transport through the secretory pathway (4, 28, 30). This mature GP complex, consisting of GP1, GP2, and the SSP, is located on the surface of virus particles (45). Together with NP and L, the two genome segments are assembled into ribonucleocapsids, which serve as the templates for transcription and replication (3, 24, 33, 37, 46). The matrix protein is located on the inner side of the viral envelope, and despite being the smallest of the arenavirus proteins, with a size of only 90 to 99 amino acids, Z has been demonstrated to take part in a number of processes and interactions. It was first identified as a regulator of genome transcription and replication (21, 33) and has been shown to interact directly with the polymerase (26, 44). Additional studies have shown numerous other interactions with cellular partners, including eukaryotic translation initiation factor 4E, promyelocytic leukemia protein, and the ribosomal P0 protein (6, 8). In addition to these regulatory functions, the Z protein of arenaviruses has, for several species, been shown to function as a matrix protein (10, 19, 36, 41, 43). Characteristic for such proteins is the ability to mediate their own release in the form of virus-like particles (VLPs), which resemble virus particles in their morphology but lack the genomic segments (25).

It has been shown for many viruses that the matrix protein is

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functionally dependent on the presence of late-domain motifs for self-budding activity (reviewed in references 5, 15, and 20). To date, four late-domain sequences have been reported, PPxY, PT/SAP, YxxL, and θ PxV (where x is any amino acid and θ is a hydrophobic amino acid) (15, 39). This also seems to be the case for several arenaviruses, although the number and sequence(s) of the late domains within Z vary. For the Old World arenavirus LCMV, a single C-terminal PPxY motif was shown to be responsible for budding (36), while for LASV both a PTAP motif and a PPxY motif were involved, with PPxY playing a dominant role (41). In both cases, this process was dependent on the ESCRT (endosomal sorting complex required for transport) pathway (36, 42). For the New World arenaviruses, a PT/SAP motif is present in all species, except for TCRV, in which only an ASAP motif is found at the corresponding position. It has been shown that JUNV Z is capable of mediating the production of chimeric VLPs containing both TCRV NP and JUNV GP and that budding of these particles is largely, although not entirely, dependent on the PTAP motif (10). However, it remains to be shown whether these observations will hold true during the budding of nonchimeric VLPs made up of only TCRV or only JUNV proteins. Interestingly, the TCRV ASAP motif was recently shown not to play a role in TCRV Z budding, although this process is still dependent on the ESCRT pathway (43). In addition, New World arenaviruses also contain a YxxL motif, but to date, no function has been associated with this sequence; indeed, a recent report indicates that for TCRV, mutation of this motif has no effect on budding (43). Cumulatively, these observations have led to the conclusion that TCRV seems to employ a budding strategy distinct from that of other arenaviruses via a process that seems to be independent of known late domains. Therefore, in this study, we have compared the budding of JUNV and TCRV Z alone and in the presence of other viral proteins in order to identify their contributions to budding and provide new insight into the mechanism of budding in TCRV.

MATERIALS AND METHODS

Viruses and cells. TCRV and JUNV (strain Rumero) were kindly provided by the University of Geneva and the Public Health Agency of Canada, respectively. All work with JUNV was carried out in the biosafety level 4 (BSL-4) laboratory at the Philipps University Marburg. 293 (human embryonic kidney) and Vero E6 (African green monkey kidney) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; PAN Biotech), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen) and grown at 37°C and 5% CO₂. *Escherichia coli* XL-1 Blue was used for all routine cloning procedures.

Virus propagation and RNA isolation. Both TCRV and JUNV stocks were grown in Vero E6 cells. Fresh virus stocks were prepared by diluting the provided virus stocks 1:100 in 20 ml of serum-free DMEM and infecting 80% confluent Vero E6 cell monolayers in 162-cm² flasks for 60 min at 37°C in a 5% CO₂ atmosphere. After infection, the inoculum was removed and fresh DMEM containing 2% FBS, 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen) was added. Supernatant was harvested after 5 (TCRV) or 7 (JUNV) days and clarified by low-speed centrifugation at 1,000 \times g. For RNA isolation, the virus supernatant was inactivated in a guanidinium isothiocyanate-based buffer (AVL buffer, RNeasy kit; Qiagen) and RNA was extracted according to the manufacturer's directions.

Growth kinetics of TCRV and Junin virus (JUNV) in Vero E6 cells. Vero E6 cell monolayers at 80 to 90% confluence in six-well plates were infected with TCRV or JUNV at a multiplicity of infection of 0.01, 0.1, or 1 in 1 ml of serum-free DMEM for 60 min at 37°C in a 5% CO₂ atmosphere. Following absorption, the inoculum was removed and the cells were washed three times

with DMEM to remove any unbound virus. Cells were placed in fresh DMEM containing 2% FBS, 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen) and incubated for 6 days. Samples were collected every 24 h for analysis of progeny virus release by plaque assay.

Plaque assay for titer determination. Vero E6 cell monolayers at 80 to 90% confluence in six-well plates were infected with 1 ml of a 10-fold dilution series of TCRV or JUNV supernatants in serum-free DMEM for 60 min at 37°C in a 5% CO₂ atmosphere. Following absorption, the inoculum was removed and the cells were washed three times with DMEM to remove any unbound virus. Monolayers were then overlaid with 4 ml of minimum essential medium containing 0.7% (TCRV) or 0.9% (JUNV) Bacto agar (BD) in addition to 2% FBS, 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). Plaques were allowed to develop for either 6 days (JUNV) or 8 days (TCRV) before being stained with a 0.1% crystal violet solution (10% formaldehyde, 0.1% crystal violet). After 24 h, the agar was removed and the plaques were counted.

Plasmid construction. The open reading frames (ORFs) for TCRV or JUNV NP, GPC, and Z were amplified from viral RNA using reverse transcription-PCR and cloned into pCAGGS (35) for eukaryotic expression. Additionally, a Flag tag (DYKDDDDKK) was inserted into each ORF as a molecular tag at either the N (NP) or the C (GPC and Z) terminus using a conventional PCR-based approach. The use of an N-terminal NP tag was based on a previous report indicating that incorporation of the Flag tag at this position in TCRV NP does not affect NP function in an infectious VLP (iVLP) assay (10). Similarly, Z proteins from both JUNV and TCRV containing C-terminal tags have been shown to be functional in iVLP and VLP assays, respectively (10, 43). In the case of TCRV Z, it was further necessary to encode a nontranslated linker upstream of the ORF to resolve the formation of a secondary structure which was found to block both the expression and the sequencing of this construct (data not shown). All constructs were validated by sequencing. For JUNV, the sequences of all of the ORFs matched those published in GenBank (accession numbers AY619640 and AY619641), while for TCRV (NC_004292 and NC_004293), the following changes in the NP ORF were identified: A216G, C385T, C504T, T731C, G797C, A1071G, A1120G, +G1164, A1172T, Δ T1175, T1469A, T1557C, G1627A, and G1647A. These changes were found to correspond to the sequence of the virus stock from which the RNA was cloned (data not shown).

Expression of virus proteins. For expression of the various JUNV and TCRV proteins, transfection into 293 cells was carried out using TransIT (Mirus) according to the manufacturer's instructions using 3 μ l TransIT/ μ g DNA. In all experiments, 1 μ g of each plasmid to be transfected was used, with any remaining variation in total DNA transfected made up by the transfection of empty pCAGGS. Supernatants and cell lysates were harvested for VLP purification and Western blot analysis, respectively, at 48 h posttransfection unless otherwise indicated.

Purification of VLPs. In order to purify and concentrate VLPs released into the transfected cell culture supernatant, crude supernatants were first precleared by centrifugation at 800 \times g for 10 min at 4°C. These cleared supernatants were then pelleted through a 20% sucrose/TNE cushion at 164,000 \times g for 3 h at 4°C in either an SW41 or an SW32 rotor. Pelleted VLPs were then drained and resuspended in 150 μ l phosphate-buffered saline (PBS) for analysis by protease K digestion or 150 μ l PBS and 50 μ l 4 \times sodium dodecyl sulfate (SDS) gel loading buffer with β -mercaptoethanol for Western blot analysis. Samples for Western blot analysis were additionally boiled at 99°C for 5 min.

Protease K digestion. In order to determine if viral proteins were present on the surface or in the interior of the VLP membrane, purified VLPs were produced as described above. VLPs were harvested from cells transfected with JUNV plasmids after 48 h and from cells transfected with TCRV after 96 h, as insufficient material was produced at earlier time points to allow a clear analysis. After purification, 40- μ l aliquots of VLPs were combined with 12 μ l PBS, 7.2 μ l PBS and 4.8 μ l protease K (150 μ g/ml), or 7.2 μ l PBS containing 0.1% Triton X-100 and 4.8 μ l protease K. Samples were incubated for 1 h at 37°C. The protease K was then inactivated by heating at 99°C for 5 min prior to the addition of 20 μ l of 4 \times SDS gel loading buffer with β -mercaptoethanol preheated to 99°C for analysis by Western blotting and denaturation by further boiling at 99°C for 5 min.

Western blot analysis. Samples were separated for Western blot analysis on a 15% polyacrylamide gel by electrophoresis and transferred to nitrocellulose via semidry transfer. Membranes were then blocked overnight in 10% skim milk at 4°C in PBS. Detection of all of the constructs used in these experiments was possible using a mouse anti-Flag M2 monoclonal antibody (Sigma) at a dilution of 1:500 and a secondary Alexa Fluor 680-coupled goat anti-mouse secondary antibody (Molecular Probes) diluted 1:5,000. Fluorescent signals were detected

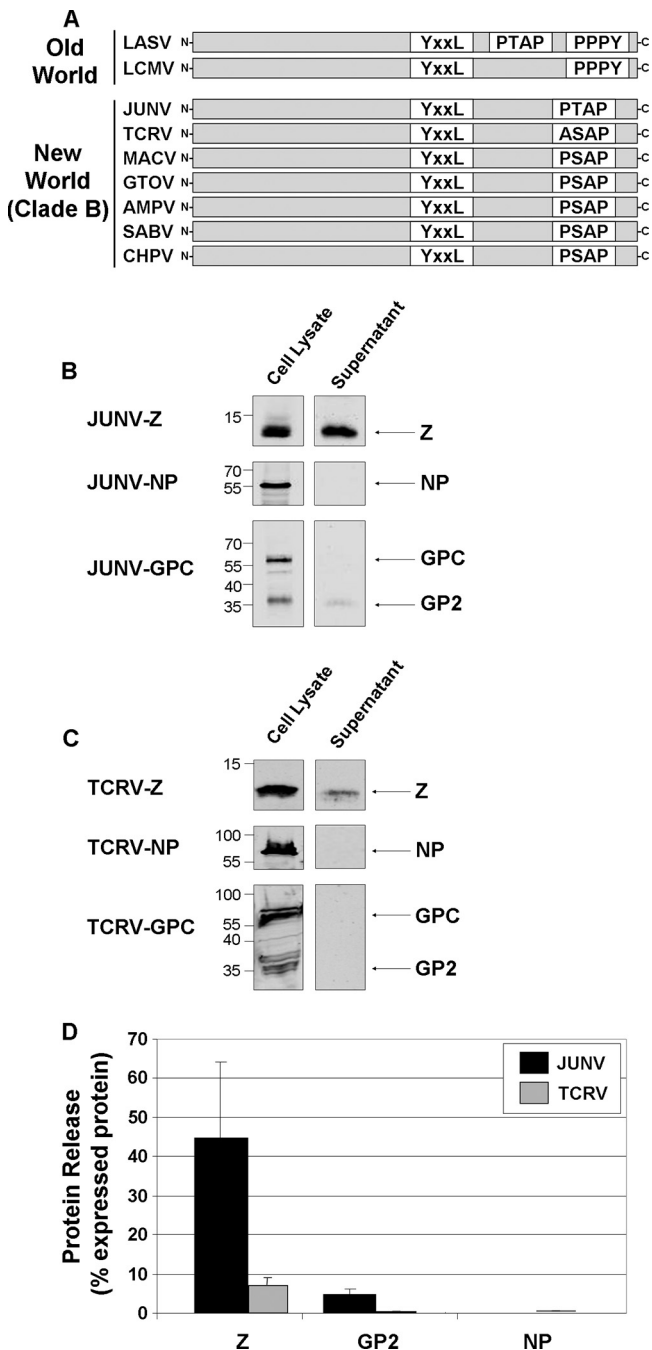


FIG. 1. Expression and VLP release by JUNV and TCRV proteins. (A) Schematic representation of the linear structure of the Z proteins of the Old World arenaviruses LCMV and LASV, as well as the clade B New World arenaviruses, including JUNV and TCRV. The relative positions of known and putative late-domain motifs, as well as the late-domain-like ASAP motif of TCRV, are illustrated. (B) Budding of JUNV NP, GP, and Z. 293 cells were transfected with 1 μ g of eukaryotic expression plasmid pCAGGS-JUNV Z (C-Flag), pCAGGS-JUNV NP (N-Flag), or pCAGGS-JUNV GPC (C-Flag). After 48 h, cell lysates were collected and analyzed by SDS-PAGE and Western blotting. Cell supernatants were purified over a 20% sucrose cushion before also being analyzed by SDS-PAGE and Western blotting. All proteins were detected using a mouse anti-Flag M2 monoclonal antibody diluted 1:500 and an Alexa Fluor 680-coupled goat anti-mouse secondary antibody diluted 1:5,000. (C) Budding of TCRV NP, GP, and Z. 293 cells were transfected with 1 μ g of eukaryotic expression

and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences).

RESULTS

Production of both JUNV and TCRV VLPs is driven by Z.

To examine the influence of the various numbers and types of late domains found among different arenaviruses (Fig. 1A), we first generated eukaryotic expression vectors containing JUNV NP, GP, and Z, as well as TCRV NP, GP, and Z. These constructs were then singly transfected into 293 cells, and after 48 h, both supernatants and cell lysates were harvested. To purify VLPs, cell culture supernatants were further pelleted through a 20% sucrose cushion. Following examination by Western blotting, we observed that all proteins were expressed (Fig. 1B and C). However, for both JUNV and TCRV, the matrix protein Z is the only protein capable of mediating significant self-release into cell culture supernatants when expressed alone. Interestingly, we also observed that while budding of JUNV Z is very robust, with approximately 45% of the total protein being released in the form of VLPs after 48 h, the release of TCRV Z was much weaker, approximately 8%, under these conditions (Fig. 1D). This was despite the use of pCAGGS as a vector for all JUNV and TCRV proteins and strong expression of TCRV Z in transfected cell lysates, as shown in Fig. 1C. In contrast, we did not see significant differences in the growth kinetics or virus titers produced during the infection of Vero E6 cells with TCRV and JUNV, indicating that additional viral factors may be required for efficient TCRV budding (data not shown).

NP and GP are independently recruited into Z VLPs.

In order to better understand the contributions of the other viral proteins to the budding process, and in particular to identify factors which may facilitate efficient budding of the New World arenaviruses, we further analyzed the incorporation of NP and GP into Z-derived VLPs. For this purpose, pCAGGS-JUNV Z, together with either pCAGGS-JUNV NP or pCAGGS-JUNV GP, or all three constructs together were transfected into 293 cells and then both cell lysates and supernatants, for VLP purification, were harvested after 48 h. When they were cotransfected, we observed the incorporation of JUNV NP and JUNV GP either independently or in combination into VLPs produced by JUNV Z (Fig. 2A). Similarly, either TCRV NP alone or TCRV NP together with TCRV GP could be incorporated into TCRV Z VLPs (Fig. 2B). Release of TCRV Z together with TCRV GP occurred very inefficiently, making it difficult to make definitive observations on the incorporation of TCRV GP into Z-induced VLPs (Fig. 2B).

As all of the constructs used in this study were tagged with

plasmid pCAGGS-TCRV Z (C-Flag), pCAGGS-TCRV NP (N-Flag), or pCAGGS-TCRV GPC (C-Flag). The lysates and supernatants were harvested and analyzed as described for panel B. (D) Quantification of JUNV and TCRV protein release. Following Western blotting, protein bands for both supernatants and cell lysates were quantified using an Odyssey Imager and the protein release was calculated as the amount of protein released in the total supernatant as a percentage of the total protein in the supernatant and cell lysate fractions. The values to the left of panels B and C are molecular sizes in kilodaltons.

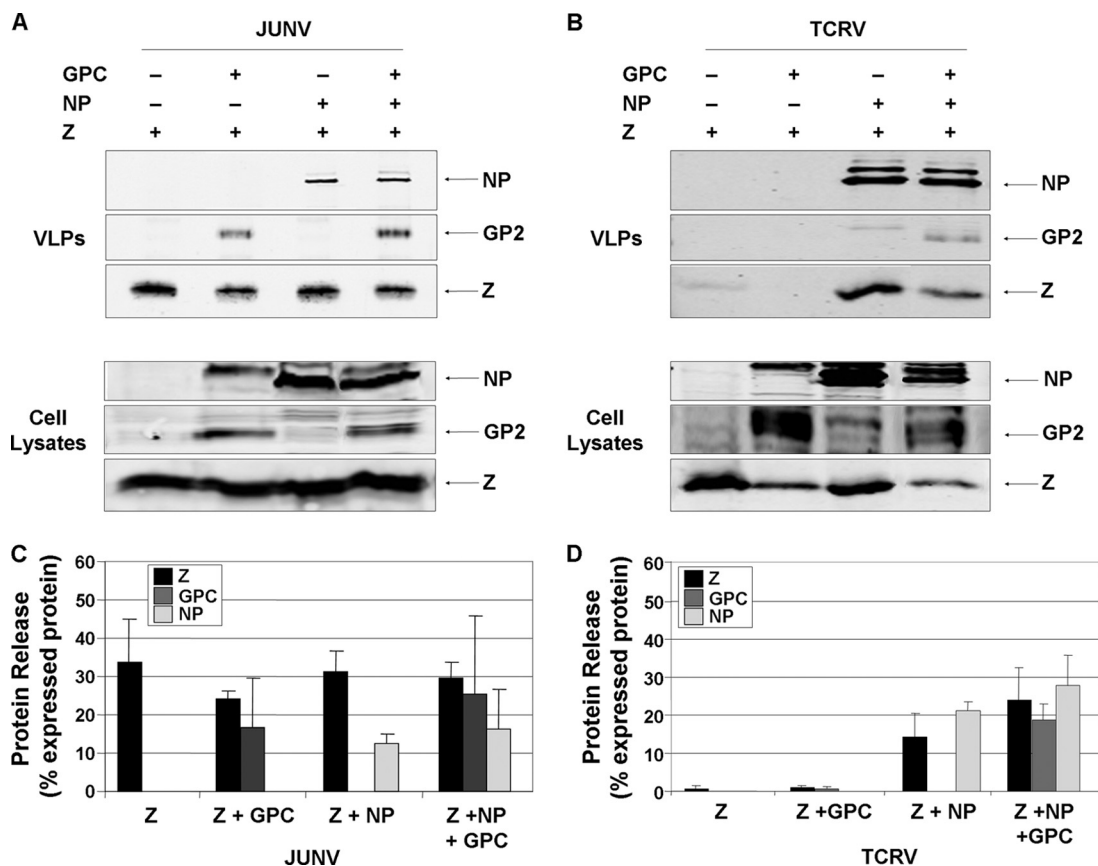


FIG. 2. Incorporation of other viral components into Z VLPs. (A) Incorporation of JUNV NP and GPC into JUNV Z-induced VLPs. 293 cells were transfected with 1 μ g of eukaryotic expression plasmid pCAGGS-JUNV Z (C-Flag) alone or together with 1 μ g of pCAGGS-JUNV NP (N-Flag), pCAGGS-JUNV GPC (C-Flag), or both. Differences in the absolute plasmid masses transfected were compensated for by the addition of empty pCAGGS vector. After 48 h, cell lysates were collected and analyzed by SDS-PAGE and Western blotting (lower panel). Cell supernatants were purified over a 20% sucrose cushion before also being analyzed by SDS-PAGE and Western blotting (upper panel). All proteins were detected using a mouse anti-Flag M2 monoclonal antibody diluted 1:500 and an Alexa Fluor 680-coupled goat anti-mouse secondary antibody diluted 1:5,000. (B) Incorporation of TCRV NP and GPC into TCRV Z-induced VLPs. Experiments were carried out using pCAGGS-TCRV NP (N-Flag), pCAGGS-TCRV GPC (C-Flag), and pCAGGS-TCRV Z (C-Flag) as described above. (C and D) Quantification of JUNV and TCRV protein incorporation into VLPs. Following Western blotting, protein bands for both supernatants and cell lysates were quantified using an Odyssey Imager and the protein release was calculated as the amount of protein released in the total supernatant as a percentage of the total protein in the supernatant and cell lysate fractions.

a Flag epitope, this allowed direct comparisons of samples on a single blot for the purpose of quantification, thus allowing us to assess the impact of protein coexpression on VLP production and incorporation of the individual proteins. With JUNV, we did not identify any significant differences in either the amount of Z VLPs released when NP or GP alone was transfected or when NP and GP were cotransfected (Fig. 2C). Neither did cotransfection of NP notably increase the incorporation of GP or vice versa. For TCRV, we observed a dramatic increase in the production of Z VLPs when NP was coexpressed (Fig. 2D). Due to the low production of VLPs in the absence of NP, it was difficult to quantitatively compare the incorporation of GP into VLPs produced in the absence or presence of NP. However, once the amount of each protein released was standardized to reflect the increased overall levels of budding observed in the presence of NP, no significant enhancement of GP incorporation as a result of NP expression was observed for TCRV. Similarly, the presence of GP had no significant influence on the incorporation of NP.

Arrangement of NP, GP, and Z in multicomponent VLPs. In order to determine if proteins incorporated into Z VLPs were arranged in an authentic fashion, we determined the location of each protein with respect to the lipid membrane using protease K digestion. For this approach, aliquots of either JUNV or TCRV VLPs containing combinations of the structural proteins NP, GP, and Z were treated with either protease K alone or protease K in the presence of detergent. We could show that JUNV Z in the supernatant is protected against protease K digestion, indicating its localization inside VLPs (Fig. 3, upper panels). Further, JUNV Z can recruit NP proteins into the interior of VLPs as well. This recruitment occurs regardless of whether or not GP is also expressed (Fig. 3, upper panels). In contrast, the transmembrane GP remained, in all cases, subject to partial degradation by protease K, consistent with its large ectodomain, which protrudes from the surface of VLPs (Fig. 3, upper panels). For TCRV, neither VLPs containing Z alone nor particles containing Z plus GP were obtained in a sufficient quantity under the conditions used to allow a clear result

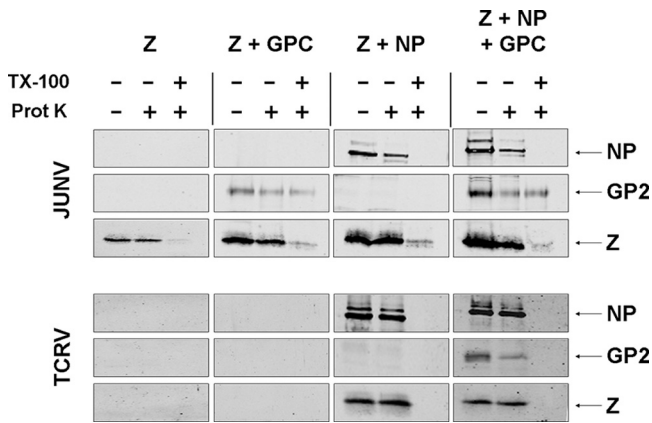


FIG. 3. Organization of JUNV and TCRV Z, NP, and GP inside multicomponent VLPs. 293 cells were transfected with 1 μ g of eukaryotic expression plasmid pCAGGS-JUNV Z (C-Flag) alone or together with 1 μ g of pCAGGS-JUNV NP (N-Flag), pCAGGS-JUNV GPC (C-Flag), or both (upper panel). Alternatively cells were transfected with 1 μ g of pCAGGS-TCRV Z (C-Flag) alone or together with 1 μ g of pCAGGS-TCRV NP (N-Flag), pCAGGS-TCRV GPC (C-Flag), or both (lower panel). Differences in the absolute plasmid mass transfected were compensated for by the addition of empty pCAGGS vector. After 48 h, cell supernatants were purified over a 20% sucrose cushion before being resuspended in PBS alone, PBS plus protease K (Prot K), or PBS plus Triton X-100 (TX-100) plus protease K. Samples were incubated at 37°C for 1 h before being analyzed by SDS-PAGE and Western blotting. All proteins were detected using a mouse anti-Flag M2 monoclonal antibody diluted 1:500 and an Alexa Fluor 680-coupled goat anti-mouse secondary antibody diluted 1:5,000.

following protease K digestion (Fig. 3, lower panels). However, samples expressing NP in addition to Z or Z plus GP were produced in significantly higher quantities. These particles demonstrate that NP incorporated either alone or together with GP is contained inside Z-driven VLPs (Fig. 3, lower panels). In contrast, TCRV GP, which, like JUNV GP, has a large ectodomain, is subject to protease K digestion in the absence of detergent treatment (Fig. 3, lower panels). These data indicate that VLPs are formed by both the JUNV and TCRV Z proteins and that NP and GP are recruited within multicomponent VLPs into locations consistent with authentic virus architecture.

NP enhances TCRV but not JUNV VLP production. Given the observation that NP expression seems to promote the release of TCRV Z VLPs, we next were interested in quantitating the influence of NP on JUNV and TCRV budding and comparing the kinetics of VLP release. First we analyzed the relative amounts of Z expressed in cell lysates either following transfection of pCAGGS-Z alone or pCAGGS-Z plus pCAGGS-NP. Using this approach, we could exclude an influence of NP on the intracellular levels of Z (Fig. 4A). We then examined the release of Z VLPs from cells from 24 to 96 h after transfection with Z alone or Z plus NP (Fig. 4B). For JUNV, NP expression resulted in a slight increase in budding at 24 h posttransfection but had no prominent effects on either the kinetics of VLP production nor the absolute amount of VLP produced. In contrast, for TCRV, the additional expression of NP resulted in an acceleration of the kinetics of VLP release, with significant VLP release occurring already at 24 h, as opposed to 48 to 72 h for Z alone (Fig. 4B). For

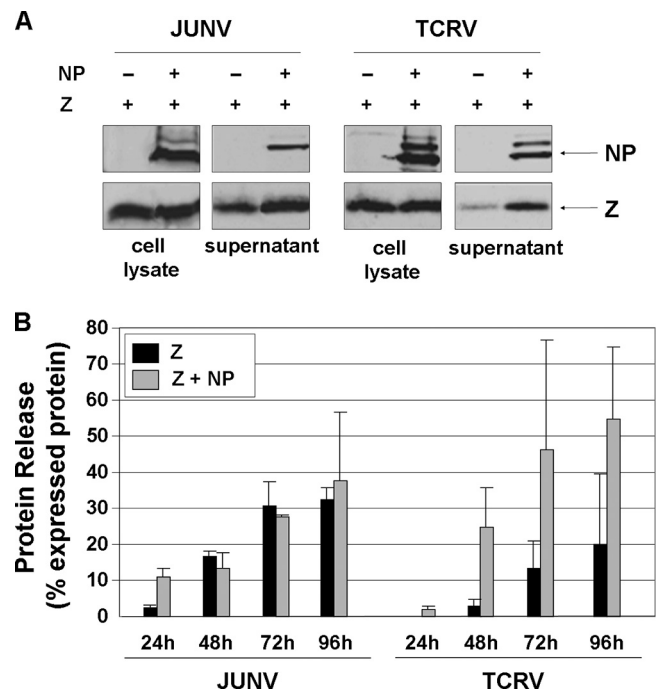


FIG. 4. Enhancement of Z VLP budding in the presence of NP. (A) Release of TCRV and JUNV Z in the presence or absence of NP. 293 cells were transfected with 1 μ g of eukaryotic expression plasmid pCAGGS-JUNV Z (C-Flag) and 1 μ g of either pCAGGS-JUNV NP (N-Flag) or pCAGGS vector. Similarly, cells were also transfected with pCAGGS-TCRV Z (C-Flag) and 1 μ g of either pCAGGS-TCRV NP (N-Flag) or pCAGGS vector. After 48 h, cell lysates were collected and analyzed by SDS-PAGE and Western blotting. Cell supernatants were purified over a 20% sucrose cushion before also being analyzed by SDS-PAGE and Western blotting. All proteins were detected using a mouse anti-Flag M2 monoclonal antibody diluted 1:500 and an Alexa Fluor 680-coupled goat anti-mouse secondary antibody diluted 1:5,000. (B) Kinetics of JUNV and TCRV protein release. Samples were transfected as described for panel A, and samples were harvested at 24 h, 48 h, 72 h, and 96 h posttransfection. Following Western blotting, protein bands for both supernatants and cell lysates were quantified using an Odyssey Imager and the protein release was calculated as the amount of protein released in the total supernatant as a percentage of the total protein in the supernatant and cell lysate fractions.

TCRV, this corresponded to an increase in the rate of VLP release per 24-h period from 6% (Z only) to 17% (Z plus NP) of the total protein. In contrast, this value remained unchanged for JUNV (12% [Z only] versus 10% [Z plus NP] of the total protein released per 24-h period). In addition, the most noticeable effect of NP cotransfection was a prominent increase in the maximum amount of TCRV VLP formation. This increased from a maximum of 20% of the Z protein released in the form of VLPs to a maximum of 55%. This value is slightly higher than the levels of VLP formation resulting from the expression of JUNV Z alone.

Enhancement of TCRV VLP budding by NP is dependent on late-domain motifs in Z. We next wanted to determine if any of the proposed late domains in TCRV Z had a role in either the budding of TCRV Z alone or in the enhancement of budding by NP. Mutations were generated that changed the TCRV ASAP motif to PSAP, ASAA, or AAAA or altered the

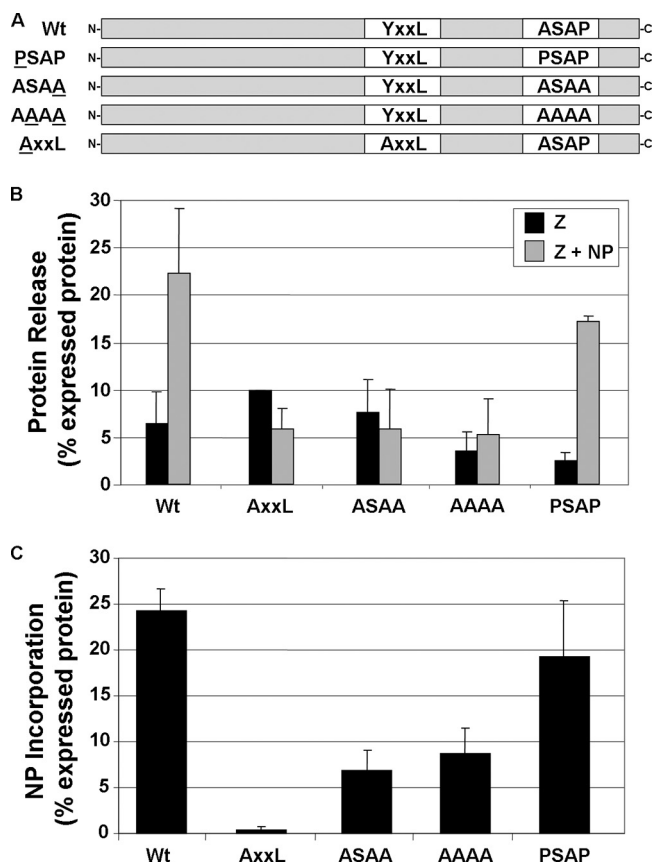


FIG. 5. Effects of mutations in putative late-domain motifs on TCRV Z budding, enhancement of budding by NP, and NP incorporation into VLPs. (A) Schematic representation of the of TCRV Z mutants analyzed. The relative positions of putative late-domain motifs, as well as the late-domain-like ASAP motif of TCRV, are shown, and the mutations made in these motifs are indicated. (B) Budding of TCRV Z late-domain mutants. 293 cells were transfected with 1 μ g of eukaryotic expression plasmid pCAGGS-TCRV Z (C-Flag) (wild-type [Wt]) or the various mutants indicated in panel A together with either 1 μ g of empty pCAGGS vector or 1 μ g of pCAGGS-NP (N-Flag). After 48 h, cell lysates were collected and analyzed by SDS-PAGE and Western blotting. Cell supernatants were purified over a 20% sucrose cushion before also being analyzed by SDS-PAGE and Western blotting. All proteins were detected using a mouse anti-Flag M2 monoclonal antibody diluted 1:500 and an Alexa Fluor 680-coupled goat anti-mouse secondary antibody diluted 1:5,000. Subsequently, bands for both supernatants and cell lysates were quantified for Z release using an Odyssey Imager and the protein release was calculated as the amount of protein released in the total supernatant as a percentage of the total protein in the supernatant and cell lysate fractions. (C) Samples prepared as described for panel B were also quantified with respect to the release of NP in VLPs as described for panel B.

YxxL motif to AxxL (Fig. 5A). We observed that mutation of the YxxL motif had no significant effect on the release of TCRV Z VLPs; however, this mutation resulted in the loss of any budding enhancement by NP (Fig. 5B, AxxL). This loss of NP-mediated enhancement of budding was associated with an almost complete loss of NP incorporation into Z VLPs (Fig. 5C). Further, mutation of ASAP to ASAA or AAAA, which would be expected to abolish any budding activity mediated by this motif, also had no significant effect on the levels of VLP release by Z alone (Fig. 5B). However, these mutations com-

pletely blocked the enhancement of budding through NP and decreased NP incorporation into Z and NP VLPs by approximately 75% (Fig. 5B and C). Contrary to expectations, we found that mutation of ASAP to PSAP, rather than increasing budding due to the generation of a known late domain, resulted in no significant change in the budding activity of Z. If anything, a slight decrease in the budding of TCRV Z VLPs was observed with the PSAP mutant (Fig. 5B, PSAP). Interestingly, budding of the PSAP mutant could also be enhanced if NP was cotransfected and resulted in levels of VLP release comparable to that achieved with wild-type TCRV Z and NP. Consistent with this, the PSAP mutant was still able to recruit NP into VLPs in amounts that were comparable to those of wild-type Z. Taken together, these data indicate that both the ASAP and YxxL motifs are necessary for the recruitment of NP into VLPs and that this activity correlates with the ability of NP to enhance the production of VLPs by TCRV Z.

DISCUSSION

For Old World arenaviruses, particularly LCMV and LASV, budding of virus particles has been shown to be mediated by their matrix protein Z and the dominant late-domain motif for budding has been identified as the PPxY motif (36, 41). Interestingly, this motif is absent in the Z proteins of all New World arenaviruses, most of which contain a single C-terminal PT/SAP late domain (Fig. 1A). However, an exception is found in TCRV, where Z completely lacks any proline-rich late-domain motif. Given the surprising nature of this observation, we wanted to confirm that this did not constitute an error in the previously reported sequence of this virus. This was particularly important since the arenavirus late domains are located at the extreme 3' end of the ORF, in an area which would usually be derived from primer sequences during PCR amplification. Thus, in order to confirm that, indeed, no proline-rich late domain exists in TCRV, we prepared RNA directly from TCRV stocks and amplified a region encompassing the entire Z ORF for sequencing using primers located entirely outside the ORF itself. Using this approach, we could confirm that TCRV Z does not contain any proline-rich late domains but instead contains a late-domain-like ASAP motif (data not shown).

Having confirmed these differences in both the number and types of late domains present in the matrix proteins of arenaviruses, it was of interest for us to establish whether the matrix proteins of New World arenaviruses would be able to function independently in driving budding or whether additional viral factors would be required and what the contributions to budding by the individual late-domain motifs found in these viruses would be. To study these questions, we selected JUNV as a representative member of the New World arenaviruses and TCRV as the exception to what is otherwise the common late-domain arrangement in New World arenaviruses.

We observed that, as has recently been reported elsewhere for TCRV (43), both the JUNV and TCRV Z proteins are able to bud from transfected cells when expressed alone (Fig. 1B and C). Importantly, the expression of neither NP nor GP alone leads to a significant amount of VLP release. This observation supports the identification of Z as the driving force for virus release for New World arenaviruses. However, when

we compared the budding activity of TCRV Z with that of closely related JUNV Z, we observed that the percentage of expressed protein released as VLPs was very low (8% versus 45%) at 48 h posttransfection (Fig. 1D), although both proteins showed comparable and high levels of intracellular expression and their ORFs display a high degree of homology. A kinetic analysis of budding by TCRV Z showed that release continued to increase over time after transfection (Fig. 4B), with maximum levels reached after 96 h, at which time VLP levels were similar to those seen for JUNV budding at the standard harvest time of 48 h. This indicates that it is more likely kinetic differences, rather than an inherent defect in the TCRV budding process, that lead to variations in the efficiency of VLP release between TCRV and JUNV. This may also suggest that the efficient budding of TCRV VLPs, in comparison to LASV, reported recently by Urata et al. (43), stems from differences in the level or kinetics of protein expression from the TCRV Z constructs used in our respective studies. This is supported by our observation that efficient budding by TCRV Z still occurs at late time points posttransfection.

While a previous study has examined the incorporation of viral components into chimeric New World arenavirus VLPs (10), no attempts have been made to examine viral protein recruitment in a homologous context, an approach that could provide important insights into the contribution of these proteins to the budding process. To address this, we examined the incorporation of the relevant NP and GP proteins into either JUNV or TCRV Z VLPs. We could show that NP and GP can be incorporated into Z-induced VLPs, either individually or together (Fig. 2A and B), a finding that supports the report of Casabona et al., who used chimeric JUNV/TCRV VLPs (10). However, we could not identify a significant increase in GP incorporation into Z VLPs in the presence of NP when protein sets were derived entirely from either JUNV or TCRV. This is in contrast to increases in GP incorporation of 5- to 10-fold in the presence of NP reported when using chimeric VLPs (10), suggesting that a requirement for NP for efficient GP incorporation may be specific to chimeric VLP assembly.

We could further confirm, using protease K digestion, that for both TCRV and JUNV, when NP, GP, and Z from the same species are cotransfected, the resulting VLPs contain both Z and NP inside viral particles, while GP is partly present on the surface of VLPs (Fig. 3). Further, the lack of viral RNA in these preparations indicates that incorporation of NP is likely independent of ribonucleoprotein complex formation. This finding is consistent with previous reports indicating that the LCMV, LASV, and JUNV Z proteins all interact with GP directly (9, 10). Similarly, interactions between LCMV and LASV Z and NP (19, 36) and between JUNV Z and TCRV NP (10) have also been shown to occur. The existence of separate Z-NP and Z-GP interactions in TCRV VLPs supports the structure of authentic TCRV particles, as determined by cryo-electron microscopy tomography (34), and indicates that the VLPs used in this study reflect the product of an authentic budding process.

Of great interest to us was the observation that upon the coexpression of TCRV Z and NP, budding was dramatically increased and reached levels comparable to those observed in JUNV Z-expressing cells (Fig. 4B). Also, cotransfection of Z and NP resulted in an earlier release of detectable levels of

TCRV VLPs. Interestingly, no significant effect on the kinetics of JUNV VLP release or on the maximum level of VLP production was observed. Together with a previous report which indicates that expression of TCRV NP together with JUNV Z also does not affect VLP release (10), these data indicate that TCRV NP is not a general enhancer of arenavirus budding but that its effect on budding is specific to TCRV Z. In order to exclude an influence of NP directly on Z expression, we examined the cell lysates produced from cells transfected with either Z alone or Z and NP but found that there were no changes in the levels of intracellular Z protein expression when NP was cotransfected. Further, over the course of the experiment, intracellular concentrations of TCRV or JUNV Z differed by less than twofold. Thus, differences in the kinetics and/or level of Z expression between these constructs are unlikely to be responsible for the low level of TCRV budding that we observed in this study, compared to that of JUNV. On this basis, we suggest that, for TCRV, NP plays a direct role in enhancing the budding of Z, which then not only leads to an earlier onset of budding but increases the amount of VLPs produced at all time points. This finding is consistent with our observation that infectious JUNV and TCRV revealed similar growth kinetics and suggests an important role for other viral factors in addition to Z. The observation that a similar effect does not occur with JUNV then also suggests that TCRV is using a mechanism of budding distinct from that of other New World arenaviruses, which can be expected to bud efficiently through their PT/SAP motif, as has been shown for JUNV (10). Such an idea would support recent work which has indicated that budding by TCRV Z alone is not dependent on the ASAP or YxxL motif but still occurs through an ESCRT-mediated pathway and thus is sensitive to Vps4 mutants (43).

NP expression has been shown for several other viruses in different families to have a positive influence on budding. For Ebola virus, it has been shown that while VP40 is the major matrix protein and allows efficient formation of VLPs when expressed alone, production is significantly increased in the presence of NP (32). Also, in paramyxoviruses it has been shown for both simian virus 5 and mumps virus that efficient VLP formation requires M, as well as NP and a surface GP (31, 40). The basis of a role for TCRV NP in promoting VLP budding is not currently understood, but the observation that such a phenomenon occurs in several virus families suggests a central role. For instance, NP may influence the formation of conformations of Z that are capable of budding more efficiently or may be required for efficient transport of Z to the budding site.

Mutations in the ASAP and YxxL motifs of TCRV had been shown to be unimportant for budding of TCRV Z alone (43). However, we have shown that Z and NP together are necessary for efficient budding of TCRV. This is in contrast to the budding of closely related JUNV, which appears to be representative of the budding strategy of all other New World arenaviruses, as indicated by their common late-domain motif arrangement. Therefore, in light of a previous report indicating that TCRV Z budding is still occurring via the ESCRT pathway (43), we were interested in whether mutations in either the ASAP or the YxxL motif would affect budding during the cotransfection of Z and NP. Since previous work with retroviruses has shown that mutation of the motif PSAP to ASAP

results in only a threefold decrease in budding efficiency, indicating residual activity of this motif (22), we further mutated the ASAP motif of TCRV to ASAA or AAAA. While this mutation had no effect on the amount of VLPs produced by Z alone, it completely blocked the enhancement of budding by coexpression of NP (Fig. 5B). Supporting this observation, the incorporation of NP into VLPs by this mutant was decreased by about 75% (Fig. 5C). A complementary Z mutant in which the ASAP motif was replaced with PSAP was also generated. Surprisingly, this mutant was not significantly affected in its budding activity when expressed alone, as might have been expected given previous work indicating that this late domain is responsible for JUNV budding (10). Importantly, this mutant's budding was also still efficiently enhanced when it was coexpressed with NP and under these conditions it showed levels of budding equal to those of wild-type Z and NP. Also, consistent with this observation, levels of NP incorporation into VLPs by the PSAP mutant remained comparable to those of the wild type, further demonstrating that this mutant retains largely wild-type function. Taken together, our data suggest that the ASAP motif, and the proline residue in particular, may be important for enhancement of TCRV Z budding by NP, possibly via interaction with NP.

In order to further explore a possible contribution to budding by the arenavirus Z YxxL domain, for which no role in arenavirus budding has yet been shown, we generated a mutant in which the YxxL motif was changed to AxxL. Surprisingly, this mutant resulted in a complete loss of budding enhancement when NP was coexpressed and an almost complete loss of NP incorporation into VLPs. However, the budding activity of Z alone was not affected by the mutation, suggesting that the mutation did not cause major structural changes in Z, which would render it biologically inactive. Thus, the YxxL motif seems to show a clear influence on the enhancement of budding by NP, as well as NP incorporation into VLPs, suggesting that this motif is somehow important for interaction with NP in both a physical and a functional context. Based on secondary structural predictions (SSpro; Institute for Genomics and Bioinformatics, University of California, Irvine), the YxxL motif is expected to form part of an alpha-helical structure (data not shown), which also could play a role in the recognition of or interaction with NP through this motif during the budding process. It is known that the RING domain, which spans the central two-thirds of the arenavirus Z protein, is critical for the *in vitro* self-assembly of Z (27). However, it is unclear to what extent the RING domain is needed for the budding of Z VLPs. For Old World arenavirus VLPs, it was reported to be required (36), but recent findings showed that for the budding of JUNV Z VLPs, integrity of the RING domain was not required (10). Further, a recent report has shown that the arenavirus RING domain influenced Z-NP interaction and NP incorporation into Z-induced iVLPs (10). Thus, we cannot exclude the possibility that loss of interaction of TCRV Z with NP in our studies may be occurring indirectly due to perturbation of the RING domain by the YxxL mutation, which occurs in this region. However, since we see similar effects due to mutation of the ASAP motif, which occurs at the extreme C terminus of Z, outside of the RING domain, it seems likely that other factors are responsible for the contribution of these motifs. Also, the fact that the budding activity of Z alone remains

unchanged speaks against major structural disturbances due to these mutations.

In summary, we have shown that, unlike NP and GP, both the TCRV and JUNV Z proteins are capable of budding independently. However, TCRV Z does this much less efficiently than JUNV Z, although the two are phylogenetically closely related. By analyzing the formation of multicomponent VLPs to determine the effects of the other viral proteins on Z-induced budding, we observed a strong enhancement of TCRV budding as a result of the coexpression of NP. This effect was unique to TCRV Z. Since NP expression could be shown to have no effect on the level of intracellular Z expression, we conclude that NP is involved in mediating the budding of Z directly. Further, mutants were generated in which the putative YxxL late-domain motif and the atypical late-domain-like ASAP motif of TCRV were mutated to AxxL and ASAA or AAAA, respectively. Analysis of these mutants indicated that both of these motifs are involved in the enhancement of Z budding by NP, as well as the incorporation of NP into Z-induced VLPs, with the YxxL motif showing the stronger effect. Thus, we can conclude not only that both Z and NP are required for efficient TCRV budding but that this function is dependent on the involvement of both the ASAP and YxxL motifs. Although the mechanism by which enhancement of budding of Z by NP occurs is unknown, to our knowledge, this is the first time that a function could be ascribed to the YxxL motif in the Z protein of any arenavirus. Work is currently in progress to address the precise role of the ASAP and YxxL motifs in the structural organization of Z and to establish the mechanism by which NP enhances budding.

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