Biological Characterization of Conserved Residues within the Cytoplasmic Tail of the Pichinde Arenaviral Glycoprotein Subunit 2 (GP2)

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ABSTRACT Several mammarenaviruses can cause deadly hemorrhagic fever infections in humans, with limited preventative and therapeutic measures available. Arenavirus cell entry is mediated by the viral glycoprotein (GP) complex, which consists of the stable signal peptide (SSP), the receptor-binding subunit GP1, and the transmembrane subunit GP2. The GP2 cytoplasmic tail (CT) is relatively conserved among arenaviruses and is known to interact with the SSP to regulate GP processing and membrane fusion, but its biological role in the context of an infectious virus has not been fully characterized. Using a Pichinde virus (PICV) GP expression vector and a PICV reverse genetics system, we systematically characterized the functional roles of 12 conserved residues within the GP2 CT in GP processing, trafficking, assembly, and fusion, as well as in viral replication. Except for P478A and K505A R508A, alanine substitutions at conserved residues abolished GP processing and membrane fusion in plasmid-transfected cells. Six invariant H and C residues and W503 are essential for viral replication, as evidenced by the fact that their mutant viruses could not be rescued. Both P480A and R482A mutant viruses were rescued, grew similarly to wild-type (WT) virus, and produced evidently processed GP1 and GP2 subunits in virus-infected cells, despite the fact that the same mutations abolished GP processing and membrane fusion in a plasmid-based protein expression system, illustrating the importance of using an infectious-virus system for analyzing viral glycoprotein function. In summary, our results demonstrate an essential biological role of the GP2 CT in arenavirus replication and suggest it as a potential novel target for developing antivirals and/or attenuated viral vaccine candidates.

IMPORTANCE Several arenaviruses, such as Lassa virus (LASV), can cause severe and lethal hemorrhagic fever diseases with high mortality and morbidity, for which no FDA-approved vaccines or therapeutics are available. Viral entry is mediated by the arenavirus GP complex, which consists of the stable signal peptide (SSP), the receptor-binding subunit GP1, and the transmembrane subunit GP2. The cytoplasmic tail (CT) of GP2 is highly conserved among arenaviruses, but its functional role in viral replication is not completely understood. Using a reverse genetics system of a prototypic arenavirus, Pichinde virus (PICV), we show that the GP2 CT contains certain conserved residues that are essential for virus replication, implicating it as a potentially good target for developing antivirals and live-attenuated viral vaccines against deadly arenavirus pathogens.

KEYWORDS envelope glycoprotein, protein processing, arenavirus, Pichinde virus, Lassa virus, entry, viral replication

Mammarenaviruses can cause zoonotic infections in humans, with deadly consequences (1, 2). Lassa virus (LASV) is endemic in West Africa and is responsible for approximately 300,000 cases of infections resulting in ~5,000 deaths annually (3).

Citation Shao J, Huang Q, Liu X, Di D, Dileepan M, Brisse M, Ly H, Liang Y. 2019. Biological characterization of conserved residues within the cytoplasmic tail of the Pichinde arenaviral glycoprotein subunit 2 (GP2). *J Virol* 93:e01277-19. <https://doi.org/10.1128/JVI.01277-19>.

Editor Rebecca Ellis Dutch, University of Kentucky College of Medicine

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Received 9 August 2019

Accepted 20 August 2019

Accepted manuscript posted online 28 August 2019

Published 29 October 2019

Recent outbreaks of Lassa fever in Nigeria have been reported in early 2018 and again in early 2019, with 327 reported cases and 72 deaths (case fatality rate, 22%) from 1 January through 10 February 2019 (4). In addition to LASV, several pathogenic South American arenaviruses, such as Junin virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV), Sabia virus (SABV), and Chapare virus (CHPV), can also cause sporadic outbreaks with high mortality (1). Except for the live-attenuated vaccine Candid#1, used in Argentina to prevent JUNV infection, there is currently no licensed vaccine for arenaviral pathogens. Therapeutic options are limited and depend mainly on supportive care. Ribavirin, a broad-spectrum antiviral compound, has shown some beneficial effect if it is administered at an early stage of viral infection when the symptoms are insidious, but it is known to have serious side effects (5). Favipiravir (T-705), a novel broad-spectrum inhibitor, has demonstrated efficacy in protecting against and treating LASV and JUNV in several well-established animal models (6–8), but its efficacy in treating arenavirus hemorrhagic fever patients remains to be tested in clinical trials. Development of effective vaccines and additional antivirals against arenaviral pathogens is still urgently needed.

Mammarenaviruses are enveloped, bisegmented, ambisense RNA viruses (9). Each viral genomic segment encodes two viral gene products in opposite orientations. The large (L) genomic segment encodes the viral RNA-dependent RNA polymerase protein L and the multifunctional matrix protein Z. The small (S) genomic segment encodes the viral nucleocapsid protein (NP) and the envelope glycoprotein (GP). GP is expressed as a single polypeptide that undergoes posttranslational processing and modification to form a mature tripartite glycoprotein complex, SSP–GP1–GP2, on the cell surface (10). After the cleavage of the N-terminal stable signal peptide (SSP) by signal peptidases in the endoplasmic reticulum (ER), GP is further processed by the cellular protease subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P) into the receptor-binding GP1 and transmembrane GP2 subunits (11, 12). The SSP remains associated with GP1 and GP2 to form the tripartite GP complex, which is transported through the ER and Golgi apparatus to the cell surface, where viral assembly and budding occur (13). Upon binding to the appropriate cellular receptor, the arenavirus enters the cell through receptor-mediated endocytosis, during which the low-pH environment in endosomes triggers conformational changes of the viral GP that expose the fusion peptide at the N terminus of GP2 to initiate fusion of the viral and cellular membranes. This fusion releases the viral genome into the cytoplasm, where viral RNA replication and transcription ensue. The atomic structures of GP1, GP2, and the GP1–GP2 complex in prefusion and postfusion conformations have been solved for several arenaviruses and reveal important molecular details about receptor binding and membrane fusion (14–19). However, the complete structure of the mature tripartite SSP–GP1–GP2 complex has not been determined at the atomic level.

The SSP of arenavirus is 58 amino acids (aa) long and is unique in that it is an essential component of the mature GP complex and plays important roles in regulating GP intracellular trafficking, proteolytic maturation, and membrane fusion (20–23), as well as viral virulence (22). GP1 contains receptor-binding sites and therefore is responsible for host cell attachment. GP2 is a type I membrane fusion protein that consists of an ectodomain containing fusion peptides and heptad repeats, a transmembrane (TM) domain, and a short cytoplasmic tail (CT) (16, 24). The GP2 CT contains multiple highly conserved residues, including six invariant C and H residues, which form two zinc-binding clusters and are involved in SSP interaction (25, 26). Studies have demonstrated an important role of the GP2 CT in GP processing for several arenaviruses, including lymphocytic choriomeningitis virus (LCMV), JUNV, and LASV (26–29). Interaction between the GP2 CT and the SSP regulates intracellular trafficking, SKI-1/S1P-mediated GP1–GP2 cleavage, and membrane fusion (23, 30). Accordingly, it has been shown that efficient virus replication and propagation require the presence of SSP and the GP2 TM and/or CT from a homologous virus (31). By use of recombinant LCMV (rLCMV) or rLCMV expressing LASV GP, two mutations in the GP2 CT have been shown to contribute to viral virulence in mice, suggesting an important role for the GP2 CT in viral

FIG 1 Sequence and structure of the arenavirus GP2 CT. (A) Sequence alignment of the GP2 CT regions of 22 arenaviruses. The positions of the highly conserved residues are indicated at the top (numbered according to the PICV GP sequence); numbers indicating the positions of zinc-binding residues are shown in blue. The two zinc-binding clusters identified by Briknarova et al. (25) are indicated at the bottom. Cluster I includes H447, H449, C455, and H485, while cluster II includes H459, C467, C469, and presumably a water molecule and readily accepts C33 from the SSP as the fourth ligand (25) (numbered according to the JUNV GP sequence). MOBV, Mobala virus; MOPV, Mopeia virus; IPPYV, Ippy virus; FLEV, Flexal virus; PARV, Parana virus; ALLV, Allpahuayo virus; PIRV, Pirital virus; TCRV, Tacaribe virus; CPXV, Cupixi virus; AMAV, Amapari virus; OLVV, Oliveros virus; LATV, Latino virus; WWAV, Whitewater Arroyo virus; BCNV, Bear Canyon virus. (B) The ribbon model of the JUNV GP2 CT (PDB structure 2L0Z) is shown in blue, and the superimposed PICV model built by SWISS-MODEL is shown in green. Zinc ions are depicted as gray spheres. The side chains of the highly conserved C and H residues, as well as that of W503, are shown. The image was prepared using PyMOL.

fitness and virulence (32, 33). However, the biological roles of the conserved GP2 CT region have not been comprehensively characterized in the context of an infectious arenavirus.

In this study, we systematically characterized the functional roles of the conserved residues within the GP2 CT in GP processing, trafficking, assembly, and fusion, as well as their biological roles in viral replication, using the Pichinde virus (PICV) GP expression vector and reverse genetics system available in our laboratory (34). PICV is a nonpathogenic biosafety level 2 (BSL2) prototypic arenavirus, and its infection of guinea pigs has been used as a safe and economical small-animal model for Lassa fever (35, 36). Our study has identified the conserved GP2 CT residues that are essential for viral replication and has revealed intriguing differences in GP processing between a plasmid-based protein expression system and viral infection.

RESULTS

Characterizing the effects of GP2 CT mutations on PICV GP processing, cell surface expression, and membrane fusion. Sequence alignment of the GP2 cytoplasmic tail (CT) among 22 different arenaviruses shows a highly conserved membrane-proximal region with multiple invariant amino acid residues, including several positively charged residues (R470, R482, R505 or K505), two Pro residues (P478 and P480), and one Trp residue (W503), in addition to six invariant Cys and His residues (H469, H471, C477, H481, C489, and C491) (numbered according to the PICV sequence) (Fig. 1A). The published nuclear magnetic resonance (NMR) spectroscopy of JUNV GP2 residues 445 to 485 shows a novel fold containing two zinc ions, one of which is coordinated by H447, H449, C455, and a nonconserved H485, while the other is coordinated by H459, C467, C469, and presumably a water molecule as the fourth ligand, which can be readily supplied by C57 from the SSP (numbered according to the JUNV sequence) (25). We modeled the folding of the PICV GP2 CT on this published JUNV structure, the ribbon model of which is presented in Fig. 1B, with the side chains of the conserved C

and H residues, as well as that of W503, shown. Since the PICV GP2 CT lacks a C-proximal H residue corresponding to JUNV H485, the fourth ligand to coordinate the first zinc ion is unknown.

We targeted each of the 12 conserved residues in the PICV GP2 CT with Ala (A) substitutions in the pCAGGS-PICV-GP protein expression vector. After sequence confirmation, the plasmids were individually transfected into human embryonic kidney epithelial (293T) cells. The transfected-cell lysates were used to assess GP expression and were processed by Western blotting using a guinea pig anti-PICV antiserum, which has been shown previously to be able to detect both the polyprotein precursor GP (GPC) and its cleaved products GP1 and GP2 but not the SSP (22). Only two mutants (the P478A mutant and a double mutant targeting the positively charged C-terminal residues R505 and K508) were processed (Fig. 2A, lanes 7 and 14) at levels similar to those of the wild-type (WT) GP (lane 2). All the other mutants (the H469A, R470A, H471A, C477A, P480A, H481A, R482A, C489A, C491A, and W503A mutants) were present as precursor proteins and did not produce detectable levels of GP1 and GP2 products, suggesting that these mutations severely impaired GP processing, likely through various mechanisms such as oligomerization and folding in the ER. Taking these findings together, it appears that all of the GP2 CT conserved residues except P478, R505, and K508 play important roles in regulating GP processing.

We next determined the levels of GP complex assembly at the cellular membrane by flow cytometry after immunostaining the GP plasmid-transfected 293T cells with an anti-PICV antiserum and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. To normalize for the levels of transfection efficiency and protein expression, we also detected the level of intracellular GP by conducting immunostaining in the presence of 0.5% saponin to permeabilize the cells. The levels of intracellular and cell surface expression of GP were quantified as the percentage of cells that were positive for GP or the mean fluorescence intensity (MFI), with and without saponin. Cell surface expression levels differed significantly among the GP mutants despite a relatively consistent level of intracellular GP expression, calculated by the percentage of GP-positive cells (Fig. 2B, top). The normalized GP surface expression, calculated by the ratio of surface GP-positive cells to intracellular GP-positive cells (R_s/i), is shown for each of the GP constructs in Fig. 2C (top). The WT and the two GP mutants with competent GP1–GP2 cleavage (the P478A and R505A K508 mutants) had similar R_s/i levels (0.7 to 0.9), suggesting that these glycoproteins were efficiently transported and assembled at the cell surface. Three other mutants (the H469A, P480A, and R482A mutants) had reduced yet still significant levels of normalized surface expression (R_s/i , ~0.3) (Fig. 2C, top). In contrast, the other seven mutants (the R470A, H471A, C477A, H481A, C489A, C491A, and W503A mutants) showed very low levels of normalized surface expression ($R_s/i < 0.15$) (Fig. 2C, top), suggesting that these mutations severely blocked the intracellular trafficking of the GP complex to the cell membrane. A similar trend was observed by the analysis of MFI (Fig. 2B and C, bottom), even though the R_s/i values of the H469A, P480A, and R482A mutants were not obviously higher than those of the other seven mutants (Fig. 2C, bottom).

We next evaluated the effects of each individual mutation on GP complex-mediated membrane fusion activity by a syncytium formation assay. For this purpose, 293T cells were cotransfected with an enhanced green fluorescent protein (eGFP) expression plasmid and either an empty plasmid as a control or the WT or individual mutant GP expression plasmid. The transfected cells were exposed to a low-pH environment and were observed for syncytium formation via fluorescence microscopy. In contrast to the control (vector), WT GP expression led to the efficient formation of syncytia, as evidenced by fused and multinucleated cells (Fig. 3A). Syncytium formation was also observed for the P478A and R505A K508A mutants but not for any of the other GP2 CT mutants (Fig. 3A), suggesting that most of the GP2 CT mutants are deficient in GP complex-mediated membrane fusion. To quantify the levels of GP-mediated fusion activities, we used a luciferase (LUC)-based fusion assay as described previously (22). GP-mediated membrane fusion allows for the transfer of T7 RNA polymerase from

FIG 2 Effects of GP2 CT mutations on the intracellular expression, processing, and cell surface expression of GP. (A) Expression and processing of WT and mutant PICV GPs. Expression vectors of the WT GP CT or GP2 CT mutants were transfected into 293T cells. Cell lysates were analyzed by Western blotting with guinea pig anti-PICV antisera. Actin expression was used to ensure equal sample loading efficiency. (B) Cell surface and intracellular expression of WT and mutant PICV GPs. WT or mutant GP2 CT expression vectors were transfected into 293T cells, each in triplicate. Cells were stained with guinea pig anti-PICV antisera and an FITC-labeled goat anti-guinea pig antibody, with and without saponin, to detect intracellular and surface expression, respectively, and the results were analyzed by flow cytometry. The percentage of GP-positive cells (top) and the mean fluorescence intensity (MFI) (bottom) were quantified by FlowJo. (C) The ratio of surface to intracellular GP expression (Rs/i) was calculated for each GP construct, based on the percentage of GP-positive cells (top) and the MFI (bottom). At least three independent experiments were conducted. Statistical analysis comparing the WT with each mutant was conducted using Student's *t* test.

FIG 3 Effects of GP2 CT mutations on membrane fusogenic activity. (A) GFP-based fusion assay. 293T cells were transfected with a plasmid expressing eGFP, together with either an empty vector or a plasmid expressing WT PICV GP or an individual mutant GP, and the transfected cells were treated with a low-pH medium to trigger membrane fusion. Images were taken by fluorescence microscope. (B) LUC-based fusion assay. Luciferase activity was determined for each construct in the fusion assay as described in Materials and Methods. RLuc, *Renilla* luciferase. Statistical analysis comparing results for the vector and each construct was conducted using Student's *t* test. ns, not statistically significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

BSRT7-5 cells to transcribe the T7 promoter-driven LUC reporter gene from the transfected 293T cells. Relative to the vector control, WT GP led to an increase in LUC activity by ~ 2 log units, at a level similar to that for the positive control, vesicular stomatitis virus glycoprotein G (VSV-G) (Fig. 3B). Except for the P478A and R404A K508A mutants, which induced LUC activity levels similar to that of WT GP, all mutants produced much lower levels of LUC activity, some of which (those for the H469A and C491A mutants) were not even significantly higher than that for the vector control (Fig. 3B). These results are consistent with those of the syncytium formation assays (Fig. 3A), and together, they demonstrate that most of the GP2 CT mutants are defective in fusogenic activities.

Characterizing the effects of GP2 CT mutations on Pichinde viral growth. To examine the biological roles of the conserved residues of the GP2 CT in the viral replication cycle, we introduced the same series of Ala substitutions into the GP gene on the S segment of a PICV reverse genetics system that we have reported previously (34). Each viral S segment expression plasmid containing either the WT or a mutant GP gene, along with the viral L segment expression plasmid, was transfected into BSRT7-5 cells in order to rescue recombinant viruses. As expected, recombinant viruses with the P478A or R505A K508A mutations, which did not affect GP processing, cell surface expression (Fig. 2), or GP complex-mediated membrane fusion activity (Fig. 3), were

FIG 4 Rescue of wild-type and GP2 CT mutant recombinant PICVs. (A) Plaque assays of recombinant PICVs that have been successfully rescued. Viral mutants that cannot be rescued are listed at the bottom. (B) Viral growth curve analysis in BHK-21 cells. BHK-21 cells were infected (MOI, 0.01) with the WT or one of the recombinant PICV mutants for which results are shown in panel A. Viral titers in the supernatants at various time points postinfection were quantified by plaque assays on Vero cells. The results shown are averages and standard deviations from three independent experiments. Statistical analyses were conducted using Student's *t* test. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

successfully rescued and formed plaques similar in size to WT virus plaques (Fig. 4A). Despite multiple attempts, we could not rescue viruses with an individual mutation at W503 (W503A) or any of the six zinc-binding H and C residues (H469A, H471A, C477A, H481A, C489A, and C491A), implicating these seven invariable residues as indispensable for the viral life cycle, a notion consistent with the detrimental effects of their mutations on GP processing, cell surface expression, and membrane fusion activity (Fig. 2 to 4). Unexpectedly, recombinant viruses with an R470A, P480A, or R482A mutation, which caused significant defects in GP processing (Fig. 2) and GP complex-mediated membrane fusion (Fig. 3), were successfully rescued. The R470A mutant virus formed small plaques, whereas the P480A and R482A mutant viruses formed large plaques similar to those of the WT virus (Fig. 4A). We sequenced the viruses in order to confirm that all virus stocks (the WT and the R470A, P478A, P480A, R482A, and R505A K508A mutants) contained the desired mutations in the GP2 CT. When the R470A virus was passaged in BHK-21 cells, it rapidly generated a single C-to-T nucleotide change to result in an Ala (GCG)-to-Val (GTG) amino acid substitution mutation, indicating that the R470A mutant is severely defective in viral replication and thus cannot be stably maintained in a viral population, which is consistent with its negative effects on GP processing and membrane fusion. On the other hand, both the P480A and R482A mutant viruses replicated stably in cell cultures even after multiple rounds of passaging without any sequence changes. Full-genome sequencing of the P480A and R482A mutants confirmed that the viral genome did not contain any mutation other than the engineered mutation.

Growth curves for recombinant virus mutants were analyzed in BHK-21 cells at a multiplicity of infection (MOI) of 0.01. Except for the R470A mutant virus, which grew

FIG 5 The P480A and R482A mutant GPs are processed in virus-infected cells and on virion particles. (A) Analysis of GP processing in virion particles. WT and GP2 CT mutant viruses were lysed and were analyzed by Western blotting with a guinea pig anti-PICV serum. Both the NP and processed GP1–GP2, but not the unprocessed GP precursor, were detected. (B and C) Analysis of GP processing in virus-infected BHK-21 (B) and 293T (C) cells. Cells were either mock infected or infected with the respective viruses at an MOI of 0.01. At 24 h postinfection, cell lysates were analyzed by Western blotting with guinea pig anti-PICV antisera and an anti-actin antibody.

to titers 1 to 1.5 log units lower than those of the WT virus, all mutant viruses showed growth kinetics similar to that of the WT virus (Fig. 4B). It is worth noting that although the original viral stock contained the R470A mutation, the supernatants from the viral growth curve experiment and from subsequent passages contained a mixture of R470A and R470V mutations, making it difficult to conduct follow-up studies with the R470A mutant. Taken together, these results suggest that R470 is not essential, but is still important, for optimal viral multiplication, whereas the invariable Pro residues (P478 and P480) and the conserved, positively charged residues at R482, R505, and K508 are not critically required for virus replication.

P480A and R482A mutant GPs are properly processed in virus-infected cells and on virion particles. Since P480A and R482A GP mutant proteins failed to show efficient GP processing in plasmid-transfected 293T cells (Fig. 2A) but recombinant mutant viruses carrying these individual mutations could be rescued and grew similarly to the WT virus (Fig. 4), we wondered whether these mutant proteins were processed on virion particles and in virus-infected cells. Western blot analysis of virion particles with a guinea pig anti-PICV antiserum detected both the viral nucleoprotein (NP) and the GP1 and GP2 subunits, but not the unprocessed GP precursor (Fig. 5A). Similar observations were made for the WT and all viable mutant viruses, including the P480A and R482A viruses. To address whether the mutant GPs could be expressed and properly processed in virus-infected cells, we infected BHK-21 cells with the respective recombinant viruses at an MOI of 0.1 for 24 h and performed Western blot assays of the virus-infected cell lysates using the guinea pig anti-PICV antiserum (Fig. 5B). For some unknown reason, the WT and R505A K508A mutant viruses appeared to express lower levels of viral proteins than the other four mutant viruses. Nevertheless, significant levels of the GP1 and GP2 subunits were detected in all cell lysates, suggesting that the respective mutant GPs could undergo efficient processing in virus-infected cells. Similar results were obtained in virus-infected 293T cells (Fig. 5C), indicating that the effect was cell culture independent. The GP1 and GP2 subunits were the predominant forms in 293T cells infected with the WT virus or the R505A K508A, R470A, or P478A mutant virus and were also readily detectable in cells infected with the P480A or R482A mutant virus (Fig. 5C). Therefore, it appears that the P480A and R482A mutant GPs, though processed less efficiently than the WT GP, are nonetheless present as properly cleaved products in virus-infected cells and that their reduced processing efficiency does not have an obvious impact on the levels of virus replication. Taken together, our data suggest that several GP2 CT mutants, including the R470A, P480A, and R482A mutants, can be processed more efficiently in virus-infected cells than in cells transfected with GP expression plasmids, illustrating the importance of using the proper cell-based systems for studying viral glycoprotein expression and function.

PICV infection does not facilitate the proper processing of GP expressed from a plasmid. The differential processing efficiencies of several GP mutants when expressed by plasmid transfection and during viral infection raised an interesting possibility, that viral infection might alter the intracellular protein-processing pathway such that the mutant proteins normally blocked at the early ER could be transported to the late ER and Golgi apparatus for processing. To test this hypothesis, we determined whether PICV infection could increase the processing efficiencies of the mutant GPs expressed from plasmids in the same cells. To distinguish the GPs expressed from plasmids and those from the PICV viral genome during infection, we generated C-terminally hemagglutinin (HA)-tagged versions of the WT and mutant GPs in the pCAGGS protein expression plasmid. We first tested whether the HA tag would alter GP processing by transfecting the respective plasmids into BSRT7-5 cells, the same cells that were used to rescue the recombinant viruses. Western blotting of cell lysates using an anti-HA monoclonal antibody showed that only HA-tagged WT, P478A, and R505A K508A proteins were efficiently processed in plasmid-transfected cells, while the rest of the GP2 CT mutants showed very low levels of protein cleavage products (Fig. 6A). These observations are consistent with those for untagged GP expression in plasmid-transfected 293T cells (Fig. 2A), demonstrating that the HA tag does not alter the protein-processing feature of the respective mutant GPs. We also determined whether the GP mutants can exit from the ER and move to the Golgi apparatus for further processing by performing an immunofluorescence assay (IFA). For this purpose, HA-tagged WT and mutant GP plasmids were each transfected into BHK-21 cells, together with plasmid pmScarlet_Giantin_C1, which expressed a Golgi protein fused with mScarlet red fluorescent protein, and plasmid pBFP-KDEL, which expressed blue fluorescent protein (BFP) with an ER-localizing signal. The subcellular locations of the HA-tagged GPs were detected by IFAs using a mouse anti-HA antibody followed by an FITC-conjugated anti-mouse antibody. The WT and all mutant GPs were detected in the ER (Fig. 6B). Colocalization with the Golgi bodies was also detected for all the GPs (Fig. 6B), suggesting that all GP2 mutants tested can exit from the ER to be transported to the Golgi apparatus.

We next transfected 293T cells with a plasmid expressing HA-tagged WT or mutant (R470A, P480A, or R482A) GP, since these mutations showed differential GP processing efficiency between plasmid transfection and virus infection, and 1 h later, we either infected those cells with recombinant WT PICV (strain P18) or performed a mock infection. Western blotting was conducted 48 h later to detect the unprocessed HA-tagged GP (and its cleavage product, GP2-HA) expressed from plasmids by use of an anti-HA monoclonal antibody and to detect all forms of the GP, including the cleaved GP1–GP2 subunits, by use of the anti-PICV serum (Fig. 6C). As expected, in the absence of viral infection, the HA-tagged mutant GPs, in sharp contrast to the HA-tagged WT GP, had almost undetectable levels of processed products, as demonstrated by both the anti-HA antibody and the anti-PICV antiserum (Fig. 6C, lanes 3 to 6). PICV infection did not increase the levels of GP processing of any of the HA-tagged mutant GPs (Fig. 6C, lanes 10 to 13, center [anti-HA antibody]). As a control, the anti-PICV antiserum could effectively detect both the unprocessed and the processed forms of GP, as well as the viral NP, in all WT PICV-infected cells (Fig. 6C, lanes 8 to 13, top). The relatively high levels of the unprocessed GP precursor protein in plasmid-transfected and virus-infected cells (Fig. 6C, lanes 10 to 13, top) reflected the increased expression levels of the GPs resulting from both plasmid transfection and viral infection. Taking our findings together, PICV infection does not increase the level of GP1–GP2 cleavage products for mutant GPs expressed from plasmid transfection, indicating that the differential GP1–GP2 cleavage efficiency observed for some GP2 mutant proteins between plasmid transfection and viral infection is not due to overall changes in the cellular protein-processing pathway upon viral infection.

FIG 6 Viral infection does not facilitate the processing of GP that is expressed from a plasmid. (A) Effects of GP2 CT mutations on the expression and processing of HA-tagged GP in plasmid-transfected BSRT7-5 cells. Expression vectors carrying HA-tagged WT or mutant GPs were transfected into BSRT7-5 cells. Cell lysates were analyzed by Western blotting with anti-HA and anti-actin antibodies. (B) Subcellular localization of HA-tagged GPs. BHK-21 cells were transfected with individual HA-tagged GPs together with the pmScarlet_Giantin_C1 plasmid, expressing a Golgi apparatus-localized protein fused with mScarlet, and the pBFP-KDEL plasmid, expressing blue fluorescent protein with an ER localization signal. Cells were stained with a mouse anti-HA antibody and an FITC-conjugated anti-mouse antibody and were detected by confocal microscopy. Representative fluorescence images of the ER (blue), Golgi apparatus (red), GPs (green), and an overlay are shown for each construct. (C) Effects of PICV infection on GP processing. 293T cells were either mock transfected or transfected with an empty expression vector or the respective HA-tagged GP constructs, and 1 h later, they were either mock infected (lanes 1 to 6) or infected with recombinant PICV (strain P18) at an MOI of 1 (lanes 8 to 13) for 48 h. Cell lysates were analyzed by Western blotting with a guinea pig anti-PICV serum (top), an anti-HA antibody (center), and an anti-actin antibody (bottom).

DISCUSSION

In this study, we examine the functional and biological importance of all conserved amino acid residues within the PICV GP2 CT. Our data suggest that the arenavirus GP2 CT plays an essential role in viral replication by regulating GP processing and membrane fusion, and they reveal an intriguing difference in GP processing between a plasmid-based protein expression system and viral infection.

We have shown that the GP2 CT plays an important role in GP processing, surface expression, and membrane fusion. Ala substitutions at several conserved residues of the PICV GP2 CT, except for P478A and R505A K508A, lead to defective GP processing (Fig. 2A and Fig. 6) and significantly reduce the cell surface expression of the viral glycoproteins (Fig. 2B). Although the WT and all GP2 mutants can exit the ER to the Golgi apparatus for further processing (Fig. 6B), the block of cell surface expression may occur at a later step(s) of intracellular trafficking, the mechanism of which warrants further investigation. Our results validate the results of some previous studies on the important role of the GP2 CT in GP processing (26–29). In addition to the six invariant C and H residues (26, 28), we have identified in the current study several other conserved residues (R470, P480, R482, and W503) that are required for efficient GP processing. However, the roles of the GP2 CT in mediating viral glycoprotein cell surface expression appear to differ among the different arenaviruses. LCMV and LASV GP2 CT mutants do not affect GP transport and cell surface expression despite defective GP processing (27, 28), whereas most of our PICV GP2 CT mutants show significantly reduced levels of cell surface expression (Fig. 2B). The reasons for the discrepancy are unclear, but it is possibly due to the potential differences between these viral GPs in intracellular trafficking. In fact, differential effects of the SSP F49A and C57A substitution mutations on GP intracellular trafficking between PICV (22), JUNV, and LCMV (23, 37) have been reported. Therefore, these arenavirus GPs, despite a generally conserved mechanism of protein processing and trafficking, can show some variations. The reduced levels of cell surface expression of PICV GP2 CT mutants are not likely due to defective GP processing, since GP1–GP2 cleavage itself is not essential for cell surface expression of PICV GP (11, 22). In addition, several PICV GP2 CT mutants (the H469A, P480A, and R482A mutants) have much higher levels of cell surface expression than others (the R70A, H471A, C477A, H481A, C489A, C491A, and W503A mutants) with similarly defective GP processing (Fig. 2C). We also show that most PICV GP2 CT mutants exhibit defective membrane fusogenic activity (Fig. 3), correlating with their GP-processing defects, likely because these unprocessed GP mutants have reduced levels of cell surface assembly and may not be able to expose the fusion peptides within the N-terminal region of the GP2 subunit when exposed to a low-pH condition.

How the GP2 CT contributes to GP maturation, trafficking, and membrane fusion activity is not completely understood. Biochemical and genetic analyses support the functional association between the GP2 CT and the SSP in regulating viral glycoprotein processing, trafficking, and GP-mediated membrane fusion (25, 26, 30). For example, the JUNV GP2 CT has been shown to interact with the SSP, likely through the invariant C and H residues forming two intersubunit zinc-binding centers together with the C57 residue of the SSP (25, 26, 30). Since the arenaviral SSP has been shown to be necessary for the intracellular trafficking of GP through the ER and Golgi apparatus for its proper processing and surface expression (22, 30, 38, 39), amino acid substitution mutations at the zinc-binding residues of the GP2 CT may disrupt its association with the SSP and thus can impair GP processing and membrane fusogenic activity (26). A functional interaction between the SSP and GP2 has also been supported by earlier studies demonstrating that the SSP and GP2 transmembrane and CT domains from the same virus are needed for proper GP maturation and production of infectious viruses (31) and that an epistatic interaction between the attenuated mutation F427I in GP2 and the SSP K33S mutation of JUNV Candid#1 is required for regulating membrane fusion activity (40). Taking these data together, most of the conserved residues of the GP2 CT are

probably required for its proper folding and interaction with the SSP, which facilitates GP1–GP2 cleavage, intracellular trafficking, cell surface expression, and fusion activity.

Using a PICV reverse genetics system, we demonstrate that the six zinc-binding C and H residues (H469, H471, C477, H481, C489, and C491), as well as the W503 residue, are essential for viral replication, as evidenced by the fact that the recombinant viruses containing these respective Ala substitution mutations are nonviable (Fig. 4A). In addition, the R470 residue is not essential but is still important for viral replication, as evidenced by the fact that although a recombinant virus containing R470A can be rescued, it replicates at a significantly lower level than WT virus, and the Ala can quickly mutate to an alternative residue (R470V) (Fig. 4). Other highly conserved residues, such as P478, P480, R482, and R505, do not seem to play a major role in viral replication; recombinant viruses carrying those respective Ala substitution mutations can still replicate at kinetics similar to that of the WT virus in cell cultures (Fig. 4B). Thus, we hypothesize that the invariant C and H amino acid residues and the W503 residue are essential for the proper folding of the GP2 CT and its association with the SSP, while some other conserved residues, such as two P residues (P478 and P480) and two R residues (R482 and R505), may facilitate the folding of the GP2 CT, but their role in viral replication may be redundant.

A surprising discovery from our study is that recombinant PICVs carrying a P480A or R482A mutation, which show defective GP processing (Fig. 2A) and membrane fusogenic activity (Fig. 3) similar to those of other nonviable mutants in GP plasmid-transfected cells, can nevertheless be efficiently rescued and that these two mutations do not negatively impact viral replication in cell cultures (Fig. 4B). We have conducted full-genome sequencing analysis of the recombinant P480A and R482A viruses and confirmed that they do not carry any other (compensatory) mutations. The viability of P480A and R482A mutant viruses suggests that relatively small amounts of functional GP are sufficient for viral multiplication, which is in line with previous studies showing dramatic downregulation of GP expression in persistently arenavirus-infected cells *in vitro* and *in vivo* (41) as well as a recombinant LCMV exhibiting WT-like growth in cells despite barely detectable GP expression levels (42). Further characterization of the GP processing in virus-infected cells demonstrates that the P480A and R482A GPs are evidently cleaved into the GP1 and GP2 subunits, though at lower levels than those for the WT glycoprotein (Fig. 5B). Moreover, our study reveals an intriguing difference in the efficiency of arenaviral GP processing between plasmid-based protein expression systems and viral infection. Several mutant PICV GPs (P470A, P480A, and R482A) are processed much more efficiently in virus-infected cells than in plasmid-transfected cells. This is not likely due to the overall alteration of cellular trafficking and protein-processing pathways induced by virus infection, since PICV infection does not appear to increase the processing efficiency of HA-tagged mutant GP expressed from plasmids (Fig. 6C). While further studies are required in order to address this intriguing discrepancy in the different cell-based systems, our current study highlights the necessity of evaluating viral glycoprotein functions in the context of an infectious virus.

In summary, we have shown that the six invariant C and H residues, as well as the W503 residue, of the PICV GP2 CT play an essential biological role in viral replication, possibly by maintaining proper folding and interaction with the SSP. Thus, this essential interaction between the SSP and GP2 can serve as a potentially good target for developing much-needed antivirals and as a specific site at which to attenuate the virus for the generation of candidate vaccines against the highly pathogenic arenaviruses.

MATERIALS AND METHODS

Cell lines. Human embryonic kidney 293T cells, baby hamster kidney (BHK-21) cells, and human airway epithelial (A549) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 mg penicillin and streptomycin. BSRT7-5 cells, which are BHK-21 cells stably expressing T7 RNA polymerase, were obtained from K. Conzelmann (Ludwig-Maximilians-Universität, Germany) and were cultured in DMEM supplemented with 10% FBS, 1 mg Geneticin per ml, and 50 mg penicillin and streptomycin per ml. African green monkey kidney (Vero) cells were maintained in Eagle's minimum essential medium (MEM) with 10% FBS plus 50 mg penicillin and streptomycin per ml.

Plasmids and transfection methods. Ala substitution mutations in the PICV GP gene were created by overlapping PCR mutagenesis reactions in the protein expression vectors (pCAGGS-PICV-GP and pCAGGS-PICV-GP-HA) as well as in the GP gene of the S-segment-containing plasmid of the PICV P18 reverse genetics system (34, 43). All mutations were confirmed by sequencing. The plasmids used to rescue recombinant PICV mutant viruses have been described previously (34, 43). Plasmids were transfected into 293T cells by using the conventional calcium phosphate transfection method. Plasmids were transfected into BSRT7-5 cells by using the Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen). pmScarlet_Giantin_C1 was a gift from Dorus Gadella (44) (Addgene plasmid 85048 [www.addgene.org/85048/]). pBFP-KDEL was a gift from Gia Voeltz (45) (Addgene plasmid 49150 [www.addgene.org/49150/]).

Western blotting. 293T cells were lysed at 48 h posttransfection using lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40 [pH 7.4]) with a protease inhibitor cocktail (Roche). Protein lysates were separated by SDS-PAGE and were transferred to a nitrocellulose (NC) membrane. The NC membrane was blocked with 5% nonfat powdered milk in Tris-HCl buffer (Tris-buffered saline [TBS]) for 1 h at room temperature. The membrane was incubated with an anti-PICV guinea pig serum or an anti-HA monoclonal antibody (Sigma) at 4°C overnight, washed three times with TBS (50 mM Tris-HCl [pH 7.4]), probed with an IRDye-labeled donkey anti-guinea pig antibody (Li-Cor Biosciences) or a horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (PerkinElmer), and visualized and quantified by an Odyssey infrared imaging system (Li-Cor Biosciences) and myECL (Thermo Scientific), respectively.

Detection of cell surface and intracellular expression of the GP complex by flow cytometry. 293T cells were transfected with either an empty pCAGGS vector or an individual GP mutant construct. Forty-eight hours posttransfection, cells were collected, fixed with 4% paraformaldehyde (PFA) for 5 min, washed once with phosphate-buffered saline (PBS), and blocked with 1% bovine serum albumin (BSA) for 1 h at 4°C. To detect surface expression of the GP complex, cells were incubated with a guinea pig anti-PICV serum overnight at 4°C, washed with PBS three times, and incubated with an FITC-labeled goat anti-guinea pig antibody (Santa Cruz) for 1 h at ambient temperature. Cells were washed three times with PBS and were analyzed for GP-positive cells by flow cytometry using a FACSCelesta flow cytometer (BD Biosciences). To detect the intracellular expression level of the GP complex, cells were permeabilized with 0.5% saponin when immunostained with antibodies for flow cytometry as described above. The percentage of GP-positive cells and the mean fluorescence intensity (MFI) of 293T cells were analyzed by FlowJo software. The ratio of surface to intracellular levels of the GP complex is shown for each GP construct in Fig. 2 as the average of triplicates, with error bars representing standard deviations.

Fusion assay. For the enhanced green fluorescent protein (eGFP)-based syncytium formation assay, 293T cells were transfected with the eGFP plasmid together with the pCAGGS vector or individual GP constructs as described previously (22). Thirty-six hours posttransfection, cells were treated for 5 min with a pH-adjusted medium (pH 5), which was then replaced with fresh complete DMEM for an additional 12 h of culturing. The cell cultures were visualized by fluorescence microscopy to detect syncytium formation.

For the luciferase-based fusion assay, 293T cells were transfected with the T7 promoter-driven firefly luciferase reporter plasmid together with either an empty pCAGGS vector or individual GPC constructs. Twenty-four hours posttransfection, the transfected 293T cells were detached by trypsinization and mixed with BSRT7-5 cells, which constitutively express T7 RNA polymerase. At 36 h posttransfection, cells were treated for 5 min with a pH-adjusted medium (pH 5), which was then replaced with fresh complete DMEM for an additional 12 h of cultivation. Cells were lysed 12 h later and were analyzed with the luciferase assay kit (Promega).

Immunofluorescence assay to detect the subcellular localization of GP. BHK-21 cells seeded on chamber slides were transfected with individual HA-tagged WT and mutant GPs, together with pmScarlet_Giantin_C1, which expresses a Golgi protein fused with mScarlet (red fluorescence), and pBFP-KDEL, which expresses an ER signal sequence fused with BFP (blue fluorescence). At 48 h posttransfection, the cells were fixed with 4% paraformaldehyde and stained with a mouse anti-HA antibody and an FITC-conjugated goat anti-mouse antibody in a blocking solution (PBS supplemented with 1% BSA, 1% goat serum, and 0.2% saponin). After extensive washing, the cells were mounted with Vectashield mounting medium and were examined by confocal microscopy (FluoView FV1000/BX61). Images were processed using Fiji software (46).

Generation of recombinant PICV mutants and viral growth curve analysis. WT and mutant P18 PICVs were rescued as described previously (34, 43). The L plasmid, together with the S plasmid containing either a WT or a mutated GP gene, was transfected into BSRT7-5 cells. At various time points posttransfection, virus supernatants were collected for plaque assays to determine whether any infectious virus particles were generated. Individual plaques were then picked and were amplified once in BHK-21 cells to prepare virus stocks. Each viral mutant was confirmed by sequence analysis after reverse transcription (RT)-PCR. For viral growth curve analysis, BHK-21 cells were infected with either the WT or an individual mutant virus at an MOI of 0.01, each in triplicate. At different time points postinfection, supernatants were collected and were analyzed for infectious viral particles by plaque assays in Vero cells as described previously (34).

ACKNOWLEDGMENTS

We thank K. Conzelmann (Ludwig-Maximilians-Universität, Germany) for the BSRT7-5 cells.

This work was supported by NIH R01 grants AI083409 (to Y.L.), AI093580 (to H.L.),

and A1131586 (to Y.L. and H.L.). M.B. was supported in part by NIH predoctoral fellowship T32 DA007097.

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