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A Molecular Sensor To Characterize Arenavirus Envelope Glycoprotein Cleavage by Subtilisin Kexin Isozyme 1/Site 1 Protease

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ABSTRACT

Arenaviruses are emerging viruses including several causative agents of severe hemorrhagic fevers in humans. The advent of next-generation sequencing technology has greatly accelerated the discovery of novel arenavirus species. However, for many of these viruses, only genetic information is available, and their zoonotic disease potential remains unknown. During the arenavirus life cycle, processing of the viral envelope glycoprotein precursor (GPC) by the cellular subtilisin kexin isozyme 1 (SKI-1)/site 1 protease (S1P) is crucial for productive infection. The ability of newly emerging arenaviruses to hijack human SKI-1/S1P appears, therefore, to be a requirement for efficient zoonotic transmission and human disease potential. Here we implement a newly developed cell-based molecular sensor for SKI-1/S1P to characterize the processing of arenavirus GPC-derived target sequences by human SKI-1/S1P in a quantitative manner. We show that only nine amino acids flanking the putative cleavage site are necessary and sufficient to accurately recapitulate the efficiency and subcellular location of arenavirus GPC processing. In a proof of concept, our sensor correctly predicts efficient processing of the GPC of the newly emergent pathogenic Lujo virus by human SKI-1/S1P and defines the exact cleavage site. Lastly, we employed our sensor to show efficient GPC processing of a panel of pathogenic and nonpathogenic New World arenaviruses, suggesting that GPC cleavage represents no barrier for zoonotic transmission of these pathogens. Our SKI-1/S1P sensor thus represents a rapid and robust test system for assessment of the processing of putative cleavage sites derived from the GPCs of newly discovered arenavirus by the SKI-1/S1P of humans or any other species, based solely on sequence information.

IMPORTANCE

Arenaviruses are important emerging human pathogens that can cause severe hemorrhagic fevers with high mortality in humans. A crucial step in productive arenavirus infection of human cells is the processing of the viral envelope glycoprotein by the cellular subtilisin kexin isozyme 1 (SKI-1)/site 1 protease (S1P). In order to break the species barrier during zoonotic transmission and cause severe disease in humans, newly emerging arenaviruses must be able to hijack human SKI-1/S1P efficiently. Here we implement a newly developed cell-based molecular sensor for human SKI-1/S1P to characterize the processing of arenavirus glycoproteins in a quantitative manner. We further use our sensor to correctly predict efficient processing of the glycoprotein of the newly emergent pathogenic Lujo virus by human SKI-1/S1P. Our sensor thus represents a rapid and robust test system with which to assess whether the glycoprotein of any newly emerging arenavirus can be efficiently processed by human SKI-1/S1P, based solely on sequence information.

Arenaviruses are a large and diverse family of emerging viruses that includes several causative agents of severe hemorrhagic fever in humans. The family *Arenaviridae* is currently classified into Old World (OW) and New World (NW) virus groups (1). OW arenaviruses include the prototypic lymphocytic choriomeningitis virus (LCMV) and the highly pathogenic Lassa virus (LASV). The NW viruses are divided into three clades (A, B, and C), with clade B containing the South American hemorrhagic fever viruses Junin virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV), and Sabiá virus (SABV). New arenaviruses continue to emerge at an increasing rate, and some of them have been associated with human disease, as illustrated by Chapare virus (CHAV), which emerged in Bolivia, and Lujo virus (LUJV), isolated in a cluster of fatal hemorrhagic fever cases in southern Africa (2, 3). The advent of powerful next-generation sequencing technologies has greatly accelerated the discovery of novel arenavirus species in mammals but also in other animals, including snakes (4, 5) and, more recently, ticks (6). However, in many cases, the viruses have not been isolated and the available sequence information remains incomplete, preventing studies on viral replication in human tissue culture.

Arenaviruses are enveloped negative-strand RNA viruses with a nonlytic life cycle (7). Their genome consists of two single-

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stranded RNA species, a large segment encoding the virus polymerase (L) and a small zinc finger motif protein (Z), and a small segment encoding the virus nucleoprotein (NP) and glycoprotein precursor (GPC) (8). The arenavirus GPC is synthesized initially as a single polypeptide that is sequentially cleaved by cellular signal peptidases and then by the cellular proprotein convertase (PC) subtilisin kexin isozyme 1 (SKI-1)/site 1 protease (S1P) (9–12). Processing of GPC yields the N-terminal glycoprotein 1 (GP1), implicated in binding to cellular receptors, and the transmembrane GP2, which mediates fusion. Maturation of GPC by SKI-1/S1P is strictly required for the production of infectious particles and viral spread (9–12). The ability of newly emerging arenaviruses to hijack human SKI-1/S1P for GPC processing thus appears to be a crucial requirement for zoonotic transmission and the ability to cause human disease. Moreover, inhibitors of SKI-1/S1P show potent antiviral activity with a low probability of viral resistance, identifying SKI-1/S1P as a promising "druggable" target for therapeutic antiviral intervention (13–16).

SKI-1/S1P is initially synthesized as an inactive precursor zymogen comprising a signal peptide, an N-terminal prodomain, a catalytic domain, and a transmembrane domain followed by a basic cytosolic tail. Upon translocation into the endoplasmic reticulum (ER), SKI-1/S1P zymogen activation involves autocatalytic maturation by sequential cleavages of the N-terminal prodomain, first at sites B′ and B (**R**K**V**F ↓ **R**S**L**K_{137} ↓) and then at site C (**RR**L**L**$_{186}$ ↓) and the newly described site C′ (**RRA**S_{166} ↓) (boldface and underlining highlight conserved residues) (17). The end product, SKI-1/S1P C, represents the fully mature enzyme (18, 19). A unique feature of SKI-1/S1P is its ability to recognize and process substrates in distinct compartments of the secretory pathway. LASV GPC is already processed by SKI-1/S1P in the ER/*cis*-Golgi, whereas endogenous substrates, such as sterol regulatory element-binding proteins (SREBPs) or activation transcription factor 6 (ATF-6), are processed in the median Golgi compartment, and LCMV GPC undergoes cleavage in late Golgi compartments (20, 21). These observations suggest that the activity of the protease is somehow regulated in a compartment-specific manner.

Conventional approaches to the study of the processing of putative viral SKI-1/S1P substrates in a quantitative manner made use of homogeneous biochemical assays including synthetic peptides and soluble enzyme (22, 23). These systems have greatly contributed to our current understanding of SKI-1/S1P-mediated GPC processing. However, in some cases, these peptide-based systems failed to reproduce the SKI-1/S1P-mediated GPC processing observed in the context of viral infection, as illustrated by the lack of cleavage of peptides derived from LCMV or GTOV GPC (22, 23). Since full-length GPCs of both LCMV and GTOV are readily cleaved in a range of mammalian cells (9, 11, 12), the lack of processing suggests that these otherwise powerful *in vitro* assays do not accurately recapitulate the authentic cellular environment. Here we employ a recently developed robust and reliable cell-based assay for the detection of endogenous SKI-1/S1P activity (17) to characterize arenavirus GPC processing in a quantitative manner. First, we show that our sensor accurately reproduces key features of SKI-1/S1P-mediated processing of authentic arenavirus GPCs, including LCMV GPC, and we define the minimal structures necessary and sufficient to confer specificity and a subcellular location of cleavage. In the next step, we use our sensor to predict the efficient processing of the GPC of the newly emergent LUJV by human SKI-1/S1P and to define the cleavage site, providing a proof of concept. Last, we use our sensor to investigate the efficiency of processing of known and newly emergent pathogenic and nonpathogenic NW arenaviruses by human SKI-1/S1P in order to assess their zoonotic potential. Our SKI-1/S1P sensor represents a rapid and robust test system for assessment of the processing of putative cleavage sites derived from GPCs of newly discovered arenaviruses by the SKI-1/S1P of humans or any other species, based solely on genetic information.

MATERIALS AND METHODS

Antibodies. Mouse monoclonal antibody (MAb) 83.6 against LCMV GP has been described previously (24). The rabbit anti-*Gaussia* luciferase (anti-GLuc) antibody was from New England BioLabs, and the mouse anti-α-tubulin MAb was obtained from Sigma. Polyclonal rabbit anti-mouse and polyclonal goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Dako.

Plasmids and constructs. Plasmids coding for wild-type (WT) full-length SKI-1/S1P and the mutant SKI-1/S1P-KDEL have been described previously (19), as have expression plasmids for LASV GPC, LCMV GPC, and LCMV GPC containing the cleavage sequence RRLL (LCMV-RRLL GPC) (11, 14, 25). An expression plasmid containing the full-length cDNA of LUJV was provided by the Viral Special Pathogens Branch of the U.S. Centers for Disease Control and Prevention, Atlanta, GA. The SKI-1/S1P sensors containing the 9-mer recognition sequence of LASV GPC, IYISRRLL ↓ G, and the uncleavable sequence (U-LASV) IYISEELL ↓ G have been described in a previous report (17). Sensors containing 9-mer peptides derived from arenavirus GPCs were generated by the insertion of specific oligonucleotides coding for the designed sequence flanked by unique restriction sites at the 3′ and 5′ ends, as reported previously (17). This cassette insertion method allows for the rapid generation of constructs containing different cleavable motifs. Sequences of the specific oligonucleotides will be provided upon request.

Cell culture and transfection. HEK293T human embryonic kidney cells and A549 human lung epithelial cells were maintained in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 100 U/ml penicillin and 0.1 mg/ml streptomycin (P/S). Chinese hamster ovary (CHO) K1 cells were maintained in DMEM–Ham's F-12 medium (1:1; Biochrom AG) supplemented with 10% FBS and P/S. SKI-1/S1P-deficient CHO-K1-derived SRD12B cells (26), provided by Michael S. Brown and Joseph L. Goldstein (University of Texas Southwestern Medical Center, Dallas, TX), were grown in CHO-K1 medium supplemented with 5 μg/ml cholesterol (Sigma), 20 μM sodium oleate (Sigma), and 1 mM sodium mevalonate (Sigma). All cell lines were kept at 37°C under 5% CO_2. SRD12B and CHO-K1 cells were transfected with Lipofectamine as reported elsewhere (27). Sensor transfections were performed using polyethylenimine (PEI) obtained from Polysciences as described previously (17). At 4 h posttransfection, solutions were replaced by fresh medium. Transfection efficiencies were evaluated after the time points indicated on the figures by detection of the enhanced green fluorescent protein (EGFP) reporter.

Western blot analysis. Media were collected, and cells were washed twice with cold phosphate-buffered saline (PBS), followed by lysis in cell lytic buffer (Sigma) supplemented with cOmplete, Mini, protease inhibitor cocktail (Roche) according to the manufacturer's instructions. Cell lysates were cleared by centrifugation (15,000 rpm, 10 min) and supernatants transferred to new tubes. Conditioned media were centrifuged twice (1,500 rpm, 5 min) to remove cellular debris, and supernatants were likewise transferred to new tubes. Samples were mixed 1:1 with 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 100 mM dithiothreitol (DTT) and were boiled for 5 min at 95°C. Samples were separated by SDS-PAGE and were blotted onto nitrocellulose membranes. Membranes were blocked in 3% (wt/vol) skim milk in PBS containing 0.2% (wt/vol) Tween 20 (Sigma), and proteins were detected by overnight incubation at 4°C with mouse MAb 83.6 against LCMV GP (1:1,000), an anti-V5 MAb (1:5,000), a rabbit anti-GLuc antibody (1:

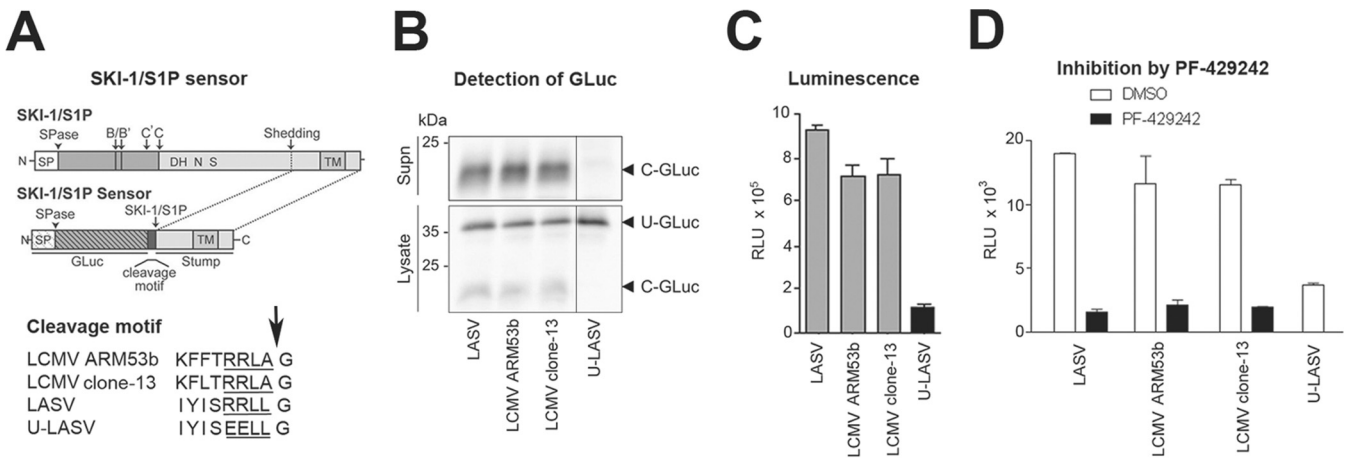

FIG 1 Efficient processing of a cell-based sensor of SKI-1/S1P bearing the recognition sequences of LASV and LCMV GPCs. (A) Schematic representation of the SKI-1/S1P sensor. The SKI-1/S1P-derived stump region and GLuc, as well as the peptide representing the cleavage motif, are indicated. The sequences of the 9-mer peptides representing the cleavage motifs of LASV GPC and the GPCs of LCMV ARM53b and its variant clone-13, as well as the noncleavable control (U-LASV), are presented. (B and C) Efficient processing of LASV and LCMV sensors by endogenous human SKI-1/S1P. The indicated sensors were transfected into HEK293T cells using PEI. (B) After 48 h, GLuc activity was detected in the supernatants (Supn) and cell lysates by Western blotting. The samples loaded for the supernatants and cell lysates represent 1% and 20% of the total amounts, respectively. Signals presented within the same box are from the same blot. Where bands have been cut and moved for clarity of presentation, this is indicated by narrow vertical lines. The uncleaved sensor (U-Gluc) and cleaved GLuc (C-GLuc) are indicated. (C) GLuc activity was detected in the supernatant by a luciferase assay. Data are mean relative light units (RLU) ± standard deviations ($n = 3$). (D) Inhibition of sensor processing by a specific SKI-1/S1P inhibitor. The indicated sensors were expressed in HEK293T cells, followed by treatment with the SKI-1/S1P inhibitor PF-429242 (20 μM). At 48 h, GLuc activity was detected in the supernatant as described for panel C. Results are means ± standard deviations ($n = 3$). DMSO, dimethyl sulfoxide.

5,000), and a mouse anti-α-tubulin MAb (1:10,000) as primary antibodies, combined with HRP-conjugated polyclonal rabbit anti-mouse (1:3,000) and polyclonal goat anti-rabbit (1:6,000) secondary antibodies. Membranes were developed by chemiluminescence using a LiteAblot kit (Euroclone). Signals were acquired by an ImageQuant LAS 4000 Mini system (GE Healthcare Life Sciences) or by exposure to X-ray films. Western blot results were quantified with ImageQuant TL software (GE Healthcare Life Sciences).

Detection of SKI-1/S1P sensor processing by a luciferase assay. Sensors were transfected into HEK293T cells using PEI as described above. After the indicated time posttransfection, conditioned medium was harvested and cleared by centrifugation, and secreted GLuc activity was detected as described in reference 17. Briefly, conditioned medium (3.5 μl) was manually distributed in a white half-volume 96-well plate and was mixed with 60 μl of a substrate solution composed of coelenterazine stock (Molecular Probes) diluted in PBS (1:5,000). A stock solution was prepared by dissolving coelenterazine in acidified methanol at 160 μg/ml and was stored at −80°C. A TriStar LB 941 microplate reader (Berthold) was used to measure the luminescence of cell medium samples. Substrate injection was performed automatically and was immediately followed by a luminescence reading. The substrate solution was freshly prepared each time.

Retroviral pseudotypes. A recombinant Moloney murine leukemia virus (MLV) containing a luciferase reporter was pseudotyped with LUJV GPC, LASV GPC, or the G protein of vesicular stomatitis virus (VSV) as described previously (25). Pseudotypes were serially diluted and were then added to cell monolayers in 96-well plates. After 1 h, the inoculum was removed, and cells were first washed briefly and then incubated for 48 h. Infection was quantified using the Steady-Glo high-sensitivity luciferase reporter gene assay (Promega) in a TriStar LB 941 microplate reader (Berthold). Retroviral pseudotypes were then concentrated by ultracentrifugation at 25,000 rpm and 4°C for 2 h using a SW28 rotor. Supernatants were discarded after centrifugation and pellets resuspended for 16 h in DMEM–20 mM HEPES (pH 7.5) at 4°C as described previously (28).

Statistical analysis. Assays were carried out in biological triplicates, and statistical analysis was performed using the GraphPad Prism software package. One-way analysis of variance (ANOVA) was used for multiple comparisons, and a *P* value of 0.01 was set as the threshold for significance.

RESULTS

A cell-based SKI-1/S1P sensor recapitulates the specificity and subcellular location of SKI-1/S1P processing of LASV and LCMV GPCs. Previous studies with a range of mammalian cells revealed that the GPCs of LCMV and LASV both undergo efficient SKI-1/S1P-dependent processing throughout the viral life cycle (9–11, 29). However, studies on the processing of the bona fide SKI-1/S1P cleavage sites of LASV and LCMV GPCs using established *in vitro* assays based on chromogenic peptides gave mixed results. A methyl coumaride (MCA)-conjugated-peptide derived from the LASV GPC sequence from residue P1 to P8 (IYISRRLL) proved to be an excellent substrate, whereas the corresponding sequences from LCMV ARM53b GPC (KFFTRRLA) and its variant clone-13 (KFLTRRLA) were resistant to *in vitro* cleavage (22, 23). Subsequent attempts by our laboratory using longer peptides covering as many as 20 amino acids surrounding the SKI-1/S1P cleavage site of LCMV GPC combined with sensitive mass spectrometry to assess cleavage failed to show any detectable processing (data not shown), suggesting an inherent problem with the assay at hand. To overcome this limitation, we employed a newly developed cell-based sensor for SKI-1/S1P activity to study the processing of LCMV GPC (17). This assay is based on a chimeric protein composed of a *Gaussia* luciferase (GLuc) reporter anchored to the membrane by the stump region of SKI-1/S1P through a cleavable 9-mer peptide derived from residues P1 to P8 and P1′ surrounding the sessile peptide bond of the SKI-1/S1P recognition sites of choice (Fig. 1A). A suitable restriction cassette allows rapid insertion of any given 9-mer amino acid sequence into our sensor using synthetic oligonucleotides (17). Upon processing, this sensor releases GLuc to the medium, where it can be

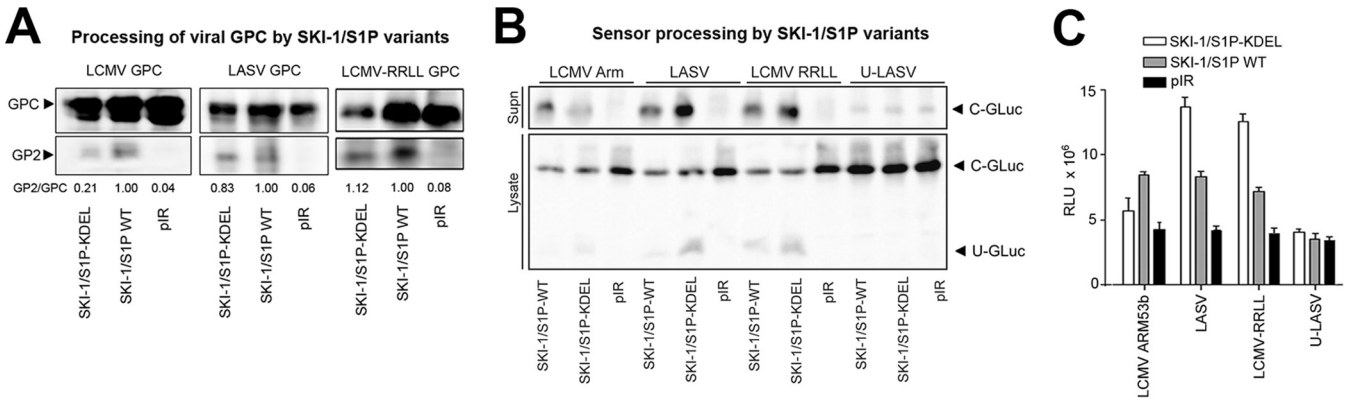

FIG 2 LCMV and LASV sensors recapitulate the compartment-specific processing of full-length GPCs. (A) Processing of recombinant LCMV and LASV GPCs by SKI-1/S1P variants. SKI-1/S1P-deficient SRD12B cells were cotransfected with LCMV GPC, LASV GPC, or LCMV-RRLL GPC and the indicated SKI-1/S1P variant or an empty vector (pIR). The processing of GPC was assessed by Western blotting using MAb 83.6 against LCMV GP, whose epitope is conserved and present in the GPC and mature GP2 of LCMV and LASV (24). The positions of GPC and mature GP2 are indicated. Signals presented within the same box are from the same blot. To quantify the extent of cleavage, the signal intensities for GP2 were measured and normalized to those for GPC by densitometry as reported previously (27). The GP2/GPC ratio of WT SKI-1/S1P was set at 1. The diffuse bands observed above and below the GP2 band represent different forms of glycosylation and were included in the densitometric analysis. The relatively inefficient processing of GPC is a consequence of the limited cotransfection rate. (B and C) Processing of sensors by SKI-1/S1P variants. The indicated sensors were transfected into SRD12B cells in combination with either SKI-1/S1P-KDEL, WT SKI-1/S1P, or an empty vector (pIR). (B) After 48 h, processing was monitored by detection of cleaved GLuc (C-GLuc) in supernatants (Supn) and cell lysates by Western blotting. The uncleaved sensor (U-Gluc) and C-GLuc are indicated. Signals presented within the same box are from the same blot. (C) GLuc activity was measured in the supernatant by a luciferase assay as for Fig. 1C. Results of one experiment representative of several independent experiments are shown. Data are means ± standard deviations ($n = 3$).

detected using a robust and sensitive luciferase assay. The SKI-1/S1P membrane anchor ensures correct cellular targeting and optimal substrate-enzyme recognition (17). To construct the corresponding sensors, we inserted peptides representing the SKI-1/S1P recognition site (P1 to P4), the upstream flanking residues (P5 to P8), and residue P1′ of LCMV ARM53b (KFFTRRLA ↓ G) and clone-13 (KFLTRRLA ↓ G), as well as LASV (IYISRRLL ↓ G) (Fig. 1A). As a control, we included a sensor bearing a mutated, noncleavable sequence derived from LASV (IYISEELL ↓ G) (underlining highlights the mutated sites). Upon expression in HEK293T cells, the sensors for LCMV and LASV GPCs underwent efficient processing, as assessed by detection of cleaved GLuc in the supernatants by Western blotting, whereas only background levels of cleavage were detected for the control sensor (Fig. 1B). By probing the total protein obtained from cell lysates, we were able to detect the uncleaved precursor (U-GLuc) and traces of processed GLuc. However, the amounts of sensor protein in the intracellular fraction detected at the time of lysis corresponded to <5% of the amounts detected in the supernatants. While the relative amounts of accumulated cleaved GLuc in the supernatants were highly consistent between experiments, the ratios of cleaved to uncleaved sensor in the intracellular pool tended to show more variation. Quantitative determination of GLuc activity in the supernatant by a luciferase assay, which represents the actual readout for efficiency of processing, revealed comparable cleavage of the sensors for LASV and LCMV GPCs (Fig. 1C), in line with the extent of processing detected by Western blotting (Fig. 1B). To validate the specific cleavage of our sensors by SKI-1/S1P, we used the small-molecule inhibitor PF-429242, which is specific for SKI-1/S1P (30, 31). As shown in Fig. 1D, treatment with PF-429242 reduced sensor processing to the level of the noncleavable probe, verifying SKI-1/S1P-mediated processing of the LCMV sensors. The efficient processing of sensors bearing the 9-mer peptides derived from LCMV ARM53b and clone-13 GPC indicated that the lack of cleavage observed in previous assays (22, 23) was an artifact of the assay format and not an inherent property of the substrate sequence. The comparable efficiencies of processing of LCMV and LASV GPC sensors in human cells match well with the observed cleavage of full-length GPC either upon expression as a recombinant protein or during viral infection (9–11, 29).

Previous studies revealed that LASV GPC is processed by SKI-1/S1P in the ER/*cis*-Golgi, whereas LCMV GPC is cleaved in a late Golgi compartment, indicating specific processing dependent on the subcellular location (9, 10). When expressed in SKI-1/S1P-null SRD12B cells, an SKI-1/S1P variant retained in the ER (SKI-1/S1P-KDEL) can efficiently cleave LASV GPC but not LCMV GPC (Fig. 2A). Introduction of the RRLL recognition site into LCMV GPC (LCMV GPC-RRLL) redirects processing to the ER/*cis*-Golgi, as evidenced by efficient cleavage by SKI-1/S1P-KDEL (Fig. 2A) and as shown in earlier studies (27). To assess the compartment specificity of our sensors, we likewise coexpressed our sensors for LASV and LCMV GPCs with SKI-1/S1P-KDEL and WT protease in SRD12B cells and monitored processing. In addition, we included a sensor bearing the sequence KFFTRRLL ↓ G, corresponding to the recognition sequence present in LCMV GPC-RRLL, and the uncleavable sensor variant (U-LASV) as a negative control. Detection of cleaved GLuc by Western blotting and a luciferase assay revealed more-efficient cleavage of the sensors for LASV and LCMV GPC-RRLL by SKI-1/S1P-KDEL than of the LCMV sensor (Fig. 2B and C). In sum, the data indicate that our sensor bearing 9-mer peptides derived from LCMV and LASV GPCs recapitulates key features of the specificity, relative efficiency, and subcellular location of SKI-1/S1P processing of the authentic viral GPCs.

In the next step, we sought to define the relative contributions of the actual SKI-1/S1P recognition site (P1 to P4) and flanking region (P5 to P8) to the specificity, efficiency, and subcellular location of SKI-1/S1P processing. For this purpose, we generated

sensors for LASV and LCMV containing only 5-mer peptides representing residues P1 to P4 and P1′. While these truncated sensors for LASV and LCMV GPC were processed efficiently, Western blot analysis revealed additional bands, indicating aberrant processing (data not shown). Upon coexpression with SKI-1/S1P-KDEL and WT SKI-1/S1P, we could not observe the compartment specificity revealed by sensors containing the 9-mer substrate peptide (data not shown). These data suggested that the sequence comprising P1 to P8 and P1′ was necessary and sufficient to define the specificity, efficiency, and subcellular location of the processing of OW arenavirus GPCs.

Prediction of SKI-1/S1P processing of LUJV GPC using the SKI-1/S1P sensor. New arenaviruses are rapidly emerging, and in many cases, viruses could not be isolated and only genetic information is available. Considering the variation at the known and putative SKI-1/S1P cleavage sequences found in the GPCs of arenaviruses from different clades, a defined consensus sequence cannot be easily recognized (22, 32). Moreover, the recent discovery of arenaviruses that are only distantly related to the major clades raises the question of whether these viruses depend on SKI-1/S1P for GPC processing at all. The data obtained with our sensor suggest that knowledge of the sequence comprising P1 to P8 and residue P1′ of the putative GP1/GP2 cleavage site would be necessary and sufficient for testing whether the GPC of any newly emerging arenavirus can be efficiently processed by human SKI-1/S1P, which appears to be a prerequisite for productive infection in humans.

In 2008, a novel arenavirus was found associated with a cluster of fatal human hemorrhagic fever cases in southern Africa, with a high case fatality rate of 80% (2). The signs and symptoms of the disease resemble those of Lassa fever (33), and the new virus, LUJV, was identified as a phylogenetically distant member of the *Arenaviridae* branching from the ancestral node of the OW arenaviruses (2). Sequence alignments with GPCs from other African arenaviruses revealed that the putative GP1/GP2 cleavage sequence found in LUJV, RKLM ↓ K, is only distantly related to the sequences of other African arenaviruses, such as LASV and Mopeia virus (RRLL ↓ G), Mobala virus (RRLM ↓ S), or Ippy virus (RRLM ↓ S) (Fig. 3A). In particular, the K residue at P1′ was rather unexpected, since this amino acid had so far never been found in this position in any cellular or viral SKI-1/S1P substrate, and very rarely in the substrates of other proprotein convertases. As a proof of concept, the putative cleavage sequence of LUJV was evaluated for SKI-1/S1P processing in our sensor. To this end, the 9-mer sequence HYKVRKLM ↓ K was inserted into our sensor (Fig. 3B), and processing was assessed; the LASV sensor and the uncleavable control sensor were used for comparison. When expressed in HEK293T cells, the LUJV sensor underwent efficient processing, similar to that of the LASV sensor, as assessed by detection of cleaved GLuc by Western blotting (Fig. 3C) and a luciferase assay (Fig. 3D). Upon expression in SKI-1/S1P-deficient SRD12B cells, processing of the LUJV sensor was abrogated, while efficient cleavage was observed in WT CHO-K1 cells, pinpointing SKI-1/S1P (Fig. 3C and E). The results obtained with our sensor suggest that LUJV GPC may be cleaved by human SKI-1/S1P with an efficiency comparable to that for LASV GPC. Interestingly, replacement of K at P1′ by A failed to markedly enhance the processing of our LUJV sensor by SKI-1/S1P (data not shown), suggesting that human SKI-1/S1P can tolerate a positively charged amino acid at P1′ in the context of the LUJV recognition sequence.

To verify the SKI-1/S1P dependence of processing in the context of the authentic GPC, full-length LUJV GPC was expressed in SRD12B, CHO-K1, and HEK293T cells, and processing was monitored by Western blotting. Expression in CHO-K1 cells, but not in SRD12B cells, resulted in processing of LUJV GPC to an extent comparable to that of LASV GPC (Fig. 3F), indicating dependence on SKI-1/S1P. In human cells, the LUJV and LASV GPCs were processed with similar efficiencies (Fig. 3G). Treatment with the SKI-1/S1P inhibitor PF-429242 markedly reduced LUJV GPC processing, again pinpointing SKI-1/S1P (Fig. 3G). In both CHO-K1 and HEK293T cells, LUJV GPC migrated slightly faster than the LASV proteins. Lastly, we sought to confirm that SKI-1/S1P cleavage was crucial for the infectivity mediated by LUJV GP. Because LUJV is a biosafety level 4 (BSL4) pathogen, work with the live virus is restricted to high-containment facilities. Since viral cell attachment and the entry of OW arenaviruses are mediated exclusively by the viral GP, we used a well-established retroviral pseudotype platform to assess the biological function of LUJV GPC as a function of SKI-1/S1P processing. Recombinant Moloney murine leukemia virus (MLV) containing a luciferase reporter was pseudotyped with LUJV or LASV GPC as described previously (25). As positive and negative controls, we generated MLV pseudotypes bearing the G protein of vesicular stomatitis virus (VSV) and pseudotypes lacking a GP, respectively. To address the requirement of SKI-1/S1P processing for the formation of infectious particles, LUJV pseudotypes were generated in the presence or absence of the SKI-1/S1P inhibitor PF-429242. Briefly, a HEK293-derived packaging cell line expressing MLV Gag and Pol was transfected with the MLV genomic plasmid and expression constructs for the viral GPC, followed by treatment with PF-429242 during the production phase of the pseudotypes. Supernatants were harvested, and fresh monolayers of A549 cells were infected with serial dilutions of pseudotypes. Detection of infection after 48 h indicated that LUJV and LASV pseudotypes produced in cells treated with PF-429242 showed markedly reduced infectivity (Fig. 3H), indicating a requirement for SKI-1/S1P-mediated cleavage for the biological function of LUJV GPC. As expected, VSV pseudotypes were not affected by treatment with the SKI-1/S1P inhibitor. Detection of LUJV GP species in MLV pseudotypes concentrated by ultracentrifugation revealed the absence of GPC and GP2 in particles produced in the presence of PF-429242 (Fig. 3I). Notably, despite high expression levels of GPC in total-cell lysates, MLV pseudotypes produced in cells with active SKI-1/S1P contained only mature GP2 (Fig. 3I). This situation is reminiscent of those of LASV, LCMV, and JUNV, where GPC processing by SKI-1/S1P is likewise required for incorporation into virion particles, and infection of SKI-1/S1P-deficient cells results in the formation of "nude" virion particles devoid of GP1/GP2 spikes (10–12). The correct prediction of SKI-1/S1P processing of the GPC of a distantly related arenavirus such as LUJV illustrates the potential of our sensor to assess the SKI-1/S1P dependence and relative efficiency of cleavage of the GPCs of novel arenaviruses.

Processing of the SKI-1/S1P recognition sequences of clade B NW arenaviruses does not correlate with virulence. All currently known human-pathogenic NW arenaviruses—JUNV, MACV, GTOV, SABV, and CHAV—belong to clade B, where they do not form a separate sublineage but are phylogenetically interspersed with nonpathogenic viruses, such as Tacaribe virus (TACV); vi-

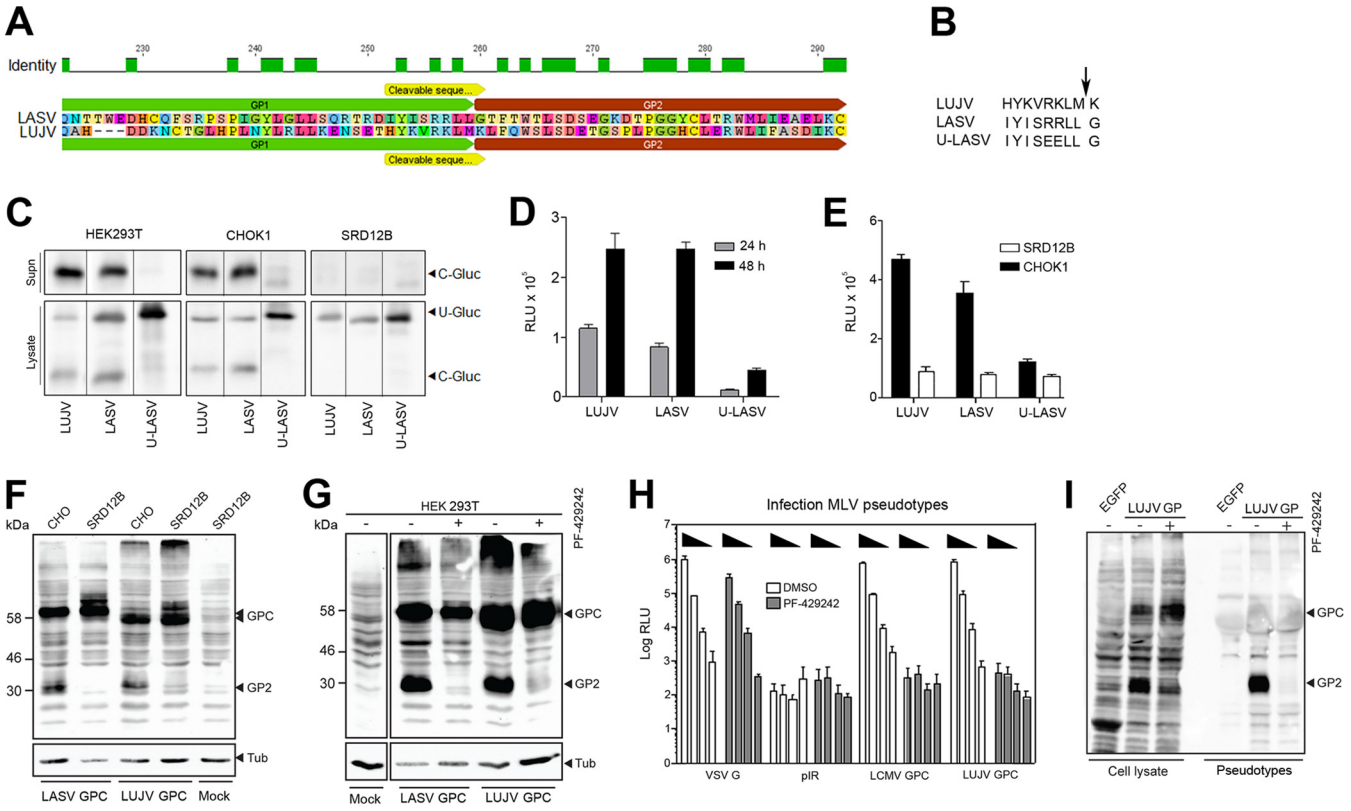

FIG 3 Prediction of SKI-1/S1P processing of LUJV GPC by using the SKI-1/S1P sensor. (A) Sequence alignment of LUJV and LASV GPCs. Green and red arrows indicate sequences corresponding to GP1 and GP2, respectively, and a yellow arrow indicates the cleavable 9-mer sequence. (B) The cleavable 9-mer sequences present in the sensors. (C to E) Efficient and specific processing of the LUJV sensor by SKI-1/S1P in human and hamster cells. The indicated sensors were transfected into HEK293T, CHO-K1, and SKI-1/S1P-deficient SRD12B cells. (C) Processing by sensors was detected by Western blotting as described for Fig. 1B. The uncleaved sensor (U-Gluc) and cleaved GLuc (C-GLuc) are indicated. Signals presented within the same box are from the same blot. Rearrangement of bands for clarity is indicated by narrow vertical lines. (D and E) For quantitative assessment of sensor cleavage, GLuc activity was measured in the supernatants of transfected HEK293T cells sampled at the indicated time points (D) and in the supernatants of CHO-K1 and SRD12B cells after 48 h (E) as described for Fig. 1C. Results are means ± standard deviations ($n = 3$). (F) Specific processing of LUJV GPC by SKI-1/S1P. Full-length LUJV and LASV GPCs were expressed in SRD12B and CHO-K1 cells, and an empty vector (Mock) was used as a control. After 48 h, total-cell lysates were prepared, and GPC processing was examined by Western blotting as described for Fig. 2A. The GPC precursor and mature GP2 are indicated, and tubulin (Tub) was detected as a loading control. Signals presented within the same box are from the same blot. (G) Cleavage of LUJV GPC in human cells depends on SKI-1/S1P. HEK293T cells were transfected with the LASV or LUJV GPC, followed by treatment with the SKI-1/S1P inhibitor PF-429242 (20 μM) throughout the experiment. After 48 h, total-cell lysates were probed for GPC processing by Western blotting as for panel F. Signals presented within the same box are from the same blot. (H) Processing of LUJV GPC is required for productive cell entry. LUJV GPC, LASV GPC, or VSV G was incorporated into a retroviral pseudotype bearing a luciferase reporter in its genome in the presence and absence of PF-429242 (20 μM) as described previously (25). An empty vector (pIR) served as a negative control. After 48 h, pseudotypes were harvested, and serial dilutions were prepared to infect fresh monolayers of A549 cells. Pseudotype infection was measured after 48 h by a Steady-Glo high-sensitivity luciferase reporter gene assay. Results are means ± standard deviations ($n = 3$). (I) Detection of GPC processing in pseudotypes. MLV pseudotypes containing LUJV GPC and pseudotypes devoid of GPC (EGFP) were produced as described for panel H in the presence and absence of PF-429242 (20 μM). Supernatants were cleared of cellular debris and were then subjected to ultracentrifugation through a sucrose cushion as described in Materials and Methods. Pellets containing the pseudotypes were solubilized, and the presence of GPC was detected by Western blotting as for panel F. The total-cell lysate was used for comparison. The GPC precursor and mature GP2 are indicated. Note the absence of specific GPC or GP2 signals in the pseudotype preparations produced in the presence of the SKI-1/S1P inhibitor.

ruses so far not associated with human disease, such as Amapari virus (AMPV) and Cupixi virus (CPXV); and viruses whose disease potential remains unknown, such as Ocozocoautla de Espinosa virus (OCEV), a novel, uncultured arenavirus (Fig. 4A). The capacity of JUNV, MACV, GTOV, SABV, and CHAV to cause disease in humans has been linked to their ability to use human transferrin receptor 1 (hTfR1) (34). In contrast, the nonpathogenic clade B viruses use TfR1 derived from their reservoir hosts and other species, but not from humans (35). In addition to the ability to recognize human TfR1, the efficiency of GPC processing by human SKI-1/S1P likely represents an important host determinant for virulence. Therefore, using our sensor platform, we tested if the human disease potential of clade B viruses correlates with their efficiency at using human SKI-1/S1P for GPC processing. First, we generated a sensor containing a 9-mer sequence corresponding to the known SKI-1/S1P recognition site of JUNV (QLPRRSLK ↓ A) (the underlined sequence is the canonical recognition site). In order to validate the SKI-1/S1P specificity of processing, we expressed the sensors for JUNV and LASV in the presence and absence of the inhibitor PF-429242 in HEK293T and HeLa cells. The uncleavable U-LASV sensor was included as a negative control. Detection of sensor processing by Western blotting (Fig. 4B) and measurement of GLuc activity in the cell culture supernatant (Fig. 4C) consistently re-

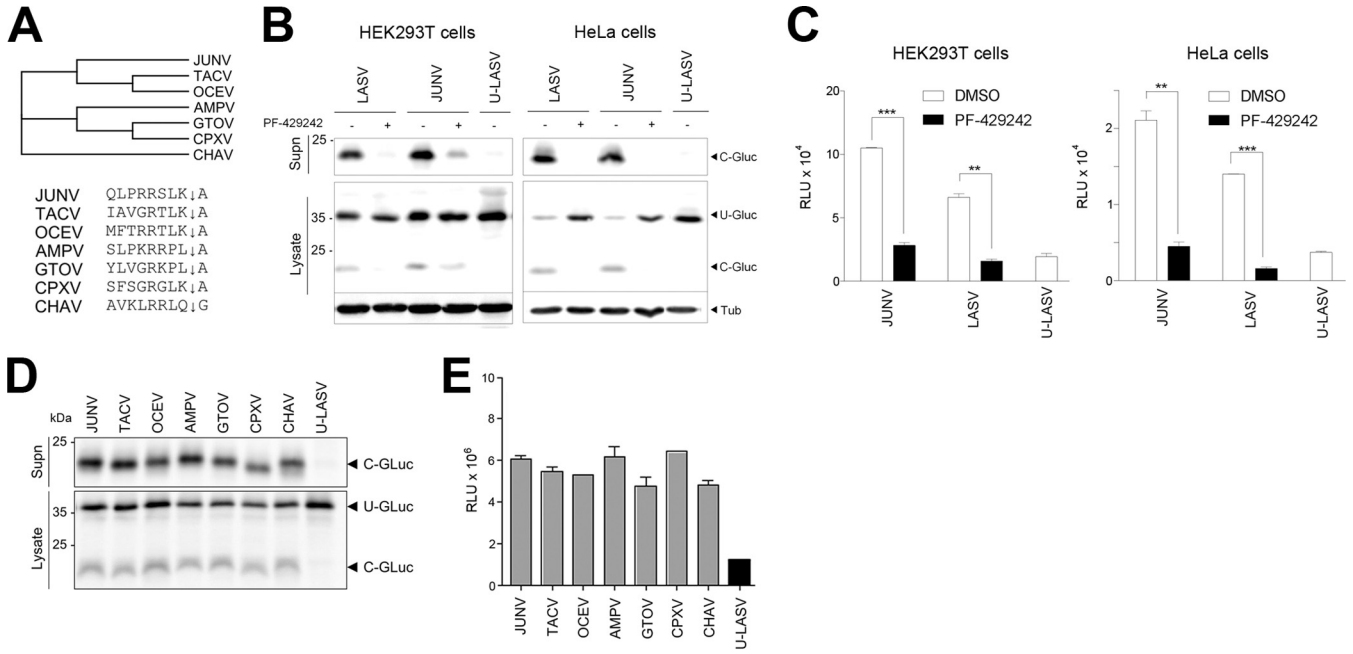

FIG 4 The efficiency of SKI-1/S1P processing of clade B New World arenavirus GPCs does not correlate with virulence. (A) Schematic of the phylogenetic relationships of the clade B NW viruses studied. The neighbor-joining tree is based on the amino acid sequences of the GPCs of the indicated viruses. The sequences of the 9-mer peptides present in the sensors are indicated. (B and C) Processing of the JUNV sensor compared to that of the LASV sensor. A sensor containing a 9-mer peptide derived from JUNV GPC, the LASV sensor, and the uncleavable sensor (U-LASV) were transfected into HEK293T and HeLa cells, followed by treatment with the SKI-1/S1P inhibitor PF-429242 (20 μM). (B) At 48 h, GLuc was detected by Western blotting. The uncleaved sensor (U-Gluc) and cleaved GLuc (C-GLuc) are indicated, and tubulin (Tub) was used as a loading control. (C) GLuc activity was detected in the supernatant by a luciferase assay. Results, expressed in relative light units (RLU), are means ± standard deviations ($n = 3$). Data were analyzed by one-way ANOVA (***, $P < 0.01$; **, $P < 0.05$). (D and E) Efficient processing of the sensors for clade B NW arenaviruses by human SKI-1/S1P. The indicated sensors were transfected into HEK293T cells. (D) After 48 h, sensor processing was examined by Western blotting. The uncleaved sensor and cleaved GLuc are indicated. (E) GLuc activity in the supernatant was detected by a luciferase assay as for Fig. 1C. Results of one experiment representative of several independent experiments are shown. Data are means ± standard deviations ($n = 3$).

vealed slightly more efficient cleavage of the JUNV sensor than of the LASV sensor. The processing of both sensors was markedly reduced in the presence of PF-429242, validating the specificity of SKI-1/S1P (Fig. 4B and C).

In order to compare the efficiencies of SKI-1/S1P processing of different clade B viruses, we generated sensors containing the highly divergent 9-mer sequences surrounding the putative SKI-1/S1P sites of the pathogenic viruses MACV, GTOV, SABV, and CHAV, the nonpathogenic viruses TACV, AMPV, and CPXV, and the virus of unknown pathogenicity OCEV (Fig. 4A). Upon expression in HEK293T cells, all sensors underwent efficient processing, yielding GLuc fragments of the expected size (Fig. 4D). Quantitative assessment of sensor processing by detection of GLuc release in the supernatant revealed only minor differences between pathogenic and nonpathogenic clade B NW arenaviruses across several experiments (Fig. 4E). Our data indicate that the efficiency of NW arenavirus GPC processing by human SKI-1/S1P does not correlate with virulence. Unlike the human form of TfR1 at the level of cell entry, human SKI-1/S1P apparently does not represent a barrier for the zoonotic transmission of clade B NW viruses.

The existing SKI-1/S1P recognition sites of LASV and JUNV GPCs appear suboptimal. Previous studies revealed that the SKI-1/S1P recognition sites of LASV and other OW arenaviruses mimic the RRLL sequence of the C maturation site, whereas clade B NW viruses resemble the B autoprocessing site RSLK (22). Moreover, aromatic "signature" residues at position P7 of OW arenavirus GPCs, but not NW arenavirus GPCs, enhance SKI-1/S1P processing (32). Intrigued by the lack of the aromatic residue at P7 in clade B NW viruses and the very distinct motifs at P1 to P4, we generated chimeric sensors with cleavable peptides comprising LASV P1 to P4 combined with LCMV P5 to P8 (LCMV-LASV) or JUNV P5 to P8 (JUNV-LASV), as well as JUNV P1 to P4 combined with LASV P5 to P8 (LASV-JUNV) (Fig. 5A). Upon expression of the sensors in human cells, we observed consistently enhanced processing of the chimeric sensor JUNV-LASV and, to a lesser extent, of LCMV-LASV and LASV-JUNV relative to that of sensors containing the parental sequences (Fig. 5B and C). These findings provide the first hints that existing viral GPCs may not contain the most cleavable sites for human SKI-1/S1P.

DISCUSSION

Subtilisin/kexin type proprotein convertases (PCs) constitute a family of nine conserved calcium-dependent serine endoproteases and include the basic PCs (PC1/3, PC2, furin, PC4, PACE4, PC5/6, and PC7) as well as the nonbasic PCs (SKI-1/S1P and PCSK9) (36). A wide range of emerging human-pathogenic viruses have evolved to hijack cellular PCs for the processing of their envelope glycoproteins, which is essential for productive infection. Based on their crucial roles in transmission and subsequent virus-host interaction, PCs appear to be important determinants for the host range and tissue tropism, and hence for the disease

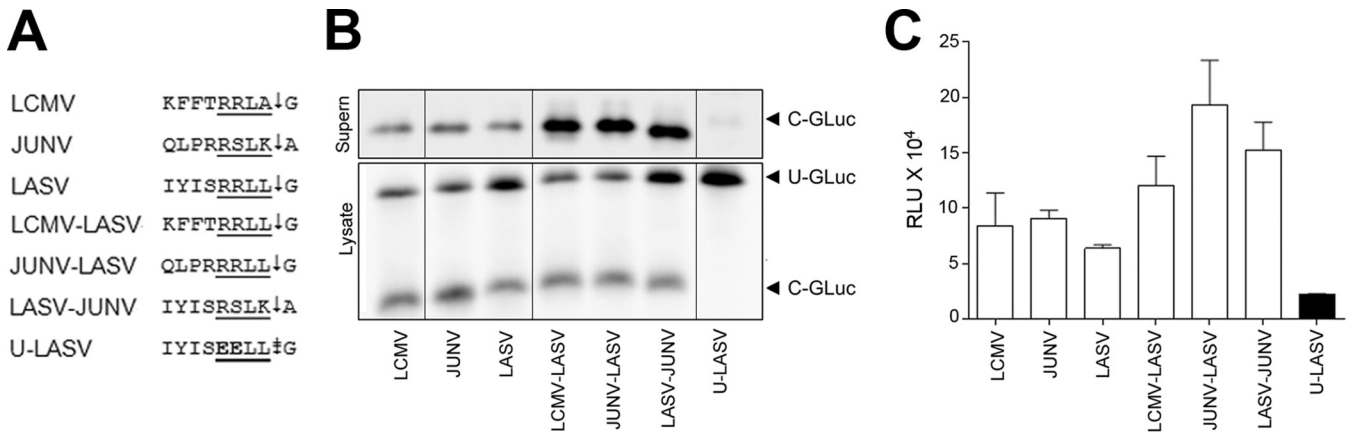

FIG 5 The existing SKI-1/S1P recognition sites of LASV and JUNV GPC appear suboptimal. (A) The 9-mer recognition sequences present in the WT and chimeric sensors. (B and C) Sensors containing chimeric 9-mer sequences show enhanced processing. (B) The indicated sensors were transiently transfected into HEK293T cells, and processing was detected by Western blotting. The uncleaved sensor (U-Gluc) and cleaved GLuc (C-GLuc) are indicated. (C) GLuc activity in the supernatant was measured as described for Fig. 1C. Results of one experiment representative of several independent experiments are shown. Data are means ± standard deviations ($n = 3$).

potential, of a virus. As essential cellular factors for viral infection, and as enzymes, they also represent promising "druggable" targets for antiviral therapeutics. In contrast to basic PCs, which are used by a wide variety of viruses to process their envelope glycoproteins (37), the eighth member of the PC family, SKI-1/S1P, is hijacked by only two families of emerging viruses, the *Arenaviridae* and some members of the *Bunyaviridae* (9, 10, 12, 38). The processing sites of arenavirus GPCs are unique and differ from cellular substrates because of their mimicry of the autoprocessing motifs of SKI-1/S1P (22). Moreover, LASV GPC undergoes SKI-1/S1P processing early in the secretory pathway, while LCMV GPC has been shown to be processed in a late Golgi compartment (9, 10, 27). Interestingly, membrane-associated SKI-1/S1P is found predominantly in the median Golgi compartment, where most cellular SKI-1/S1P substrates are cleaved (39). The apparently nonoverlapping subcellular localizations of viral and cellular substrates may have evolved to avoid perturbation of the endogenous function of SKI-1/S1P. Indeed, SKI-1/S1P-mediated processing of ATF-6 in the context of the host cell's unfolded protein response is not perturbed by arenavirus infection, despite high levels of GPC expression (29).

In the present study, we evaluated a recently developed cell-based molecular sensor of SKI-1/S1P activity for the quantitative assessment of SKI-1/S1P-mediated cleavage of arenavirus GPCs derived from different viral clades, including viruses that have not yet been isolated. Using the sequences of the well-studied LASV and LCMV GPCs as an experimental paradigm, we demonstrated that in the context of our sensor, a 9-mer substrate peptide representing positions P1 to P8 and P1′ of the SKI-1/S1P cleavage site is necessary and sufficient to recapitulate the specificity, efficiency, and subcellular location of processing observed in the authentic GPC. Moreover, our sensor allowed study of the processing of viral SKI-1/S1P recognition sequences that were not processed in classical *in vitro* assays, as illustrated by peptides derived from LCMV GPC (23) and GTOV GPC (22). Our data show that subtle changes in the 9-mer sequence at the cleavage site have drastic effects on the efficiency and subcellular localization of SKI-1/S1P-mediated maturation. Adaptations enabling the virus to efficiently hijack human SKI-1/S1P will therefore likely result in amino acid changes within the crucial 9-mer peptide surrounding the SKI-1/S1P cleavage site. As a consequence, our sensor may be a suitable tool for making a first prediction as to whether any novel arenavirus GPC is processed by human SKI-1/S1P and, if so, to what extent the virus will be capable of using the human form of the protease. For a proof of concept, we used our sensor to predict SKI-1/S1P processing of the GPC of LUJV, which was recently genetically characterized in a cluster of fatal hemorrhagic fever cases in southern Africa (2). Phylogenetic analysis identified LUJV as an outlier within the OW arenaviruses, and the sequence RKLM ↓ K at the putative GP1/GP2 border deviated significantly from the SKI-1/S1P processing sites of other arenaviruses (32). Despite these important differences, our LUJV sensor underwent efficient processing by human SKI-1/S1P, similarly to our sensor for LASV. Subsequent validation in the context of the authentic full-length GPC using a pseudotype platform confirmed the SKI-1/S1P dependence of LUJV GPC processing for viral entry into human cells. Interestingly, replacement of the unusual residue K at P1′ by A, which is more common in cellular and viral substrates, did not further enhance the SKI-1/S1P processing of our LUJV sensor. This suggests that the existing 9-mer sequence flanking the SKI-1/S1P recognition site present in LUJV GPC has already undergone adaptation enabling it to hijack human SKI-1/S1P efficiently, explaining, in part, its virulence in humans (2, 33). Our sensor allows an assessment of the effects of mutations occurring within the 9-mer peptide on SKI-1/S1P cleavage. However, mutations in other parts of the GPC or changes in posttranslational modifications, in particular N- and O-glycosylation, may affect GPC processing in a way that cannot be detected in our system.

Since the 1950s, the clade B NW arenaviruses JUNV, MACV, GTOV, SABV, and CHAV have emerged and caused outbreaks of human hemorrhagic fevers with high mortality. Pathogenic NW viruses occur in sublineages together with nonpathogenic viruses, e.g., JUNV with the closely related nonpathogenic virus TACV. Important differences in the host adaptation of arenaviruses are linked to their zoonotic disease potential. All pathogenic NW arenaviruses can use hTfR1 for cell attachment, whereas nonpathogenic viruses are restricted to TfR1 orthologues of other species (34). The ability to use hTfR1 is absolutely predictive of the po-

tential to cause severe disease in humans, and structural studies suggest that minor changes in GP1 could allow nonpathogenic viruses to recognize hTfR1 (35, 40). Once the virus is inside the cell, productive infection critically depends on the ability of the virus to hijack human SKI-1/S1P for efficient GPC processing (9–12). Intriguingly, several attempts to select for SKI-1/S1P-independent escape variants of LCMV and JUNV have failed (12, 14), suggesting that these arenaviruses are unlikely to adapt rapidly to use another cellular protease for GPC processing, and thus revealing human SKI-1/S1P as an important host factor that critically influences the efficiency of viral multiplication. Clinical and experimental studies show that high viral titers early in disease are strong predictors of fatal outcomes in arenaviral hemorrhagic fevers (41, 42). Since GPC maturation is crucial for infectious virus production, rather subtle quantitative differences in the efficiency of processing of distinct arenavirus GPCs by human SKI-1/S1P may result in different viral loads and may influence disease outcome. Using our sensor, we performed a quantitative analysis of the processing of the 9-mer peptides derived from GPCs of clade B NW arenaviruses with distinct disease potential. Despite the wide diversity of sequences, our studies revealed very similar efficiencies of processing by human SKI-1/S1P. These data suggest that once the barrier of human receptor use is overcome, nonpathogenic NW viruses could efficiently use human SKI-1/S1P, which does not represent a species barrier.

Previous studies by us and others have revealed that amino acid residues within the flanking region comprising P5 to P8 can affect the efficiency of arenavirus GPC processing (23, 32). In particular, OW GPC sequences contain an aromatic "signature" residue at P7 that promotes more-efficient interaction with the protease, enhancing processing (32). The aromatic "signature" residue at P7 is absent from most NW arenavirus GPCs, except for those of OCWV and CPXV. However, in this context, the F at position P7 does not enhance SKI-1/S1P processing. Using our sensor, we combined the P1-to-P4 sequence of LASV with the P5-to-P8 sequence of JUNV, and vice versa. Rather unexpectedly, the newly generated chimeric sequence JUNV-LASV was cleaved significantly more efficiently than the parental viral sequences, which belong to the best-known substrates for SKI-1/S1P. We are, of course, aware of the artificial nature of our sensor system containing these chimeric sequences. Nevertheless, the data provide a first hint that the 9-mer SKI-1/S1P recognition sequences of existing arenavirus GPCs have not yet evolved maximum cleavability. The reasons for this are currently unclear, but one might speculate that having "suboptimal" SKI-1/S1P recognition sites in their GPCs may limit the multiplication of viruses in their natural reservoir species, thereby preventing overt pathology.

In sum, our SKI-1/S1P sensor represents a rapid and robust test system with which to assess the processing of putative cleavage sites derived from the GPCs of newly discovered arenaviruses by human SKI-1/S1P, based solely on sequence information. Considering the promise of SKI-1/S1P as a "druggable" target for antivirals (13, 14) and the availability of small-molecule inhibitor candidates (15, 16), the SKI-1/S1P dependence of a newly emerging pathogenic arenavirus may open the possibility for therapeutic antiviral intervention. Furthermore, in SKI-1/S1P-null cells, our sensor can be used to assess the processing of arenavirus GPCs by SKI-1/S1P orthologues derived from other species. While our present study focused on SKI-1/S1P and arenaviruses, we are currently developing sensors of a similar design for the basic PCs that are responsible for the processing of a plethora of viral envelope GPs in a wide range of species. Our goal is to provide our sensor platform to the virology research community for rapid and cost-effective evaluation of viral GP processing by human PCs, which may contribute to current research and preparedness to confront the threat of emerging viruses.

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