Cleavage of the Glycoprotein of Arenaviruses

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Abstract

The arenaviruses are a large family of emerging negative-stranded RNA viruses that include several severe human pathogens causing hemorrhagic fevers with high mortality. 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 is a key factor for zoonotic transmission and human disease potential. Apart from being an essential host factor for arenavirus infection, SKI-1/S1P is involved in the regulation of important physiological processes and linked to major human diseases. This chapter provides an overview of the mechanisms of arenavirus GPC processing by SKI-1/S1P including recent findings. We will highlight to what extent the molecular mechanisms of SKI-1/S1P cleavage of viral GPC differ from processing of SKI-1/S1P's cellular substrates and discuss the implications for virus-host interaction and coevolution. Moreover, we will show how the use of the viral GPC as a "molecular probe" uncovered novel and unusual aspects of SKI-1/S1P biosynthesis and maturation. The crucial role of SKI-1/S1P in arenavirus infection and other major human diseases combined with its nature as an enzyme makes SKI-1/S1P further an attractive target for therapeutic intervention. In the last part, we will therefore cover past and present efforts to identify specific SKI-1/S1P inhibitors.

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3.1 Arenavirus Structure, Genome Organization, and Basic Virology

The arenaviruses are a large and diverse family of emerging enveloped negative-stranded viruses that include several severe human pathogens (Buchmeier et al. 2007). The *Arenaviridae* family has been recently separated by the International Committee on Taxonomy of Viruses into the genus *Mammarenavirus* and the genus *Reptarenavirus* (Radoshitzky et al. 2015). Based on phylogenetic and serological data, the mammarenaviruses are divided into two major groups: the Old World and the New World complex. For simplicity, we will henceforth use the term "arenaviruses" synonymous for the entire family and members of the *Mammarenavirus* genus, whereas viruses of the *Reptarenavirus* genus will be specifically referred to. The Old World arenavirus lineage contains the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) with worldwide distribution. The infection of LCMV in the mouse represents one of the most powerful models in experimental virology and immunology (Oldstone 2002). LCMV is further a relevant human pathogen in pediatric and transplantation medicine (Bonthius 2009; Palacios et al. 2008). The highly pathogenic Lassa virus (LASV) is endemic in Western Africa (McCormick and Fisher-Hoch 2002), and Lujo virus (LUJV) recently emerged in Southern Africa associated with a cluster of fatal infections (Briese et al. 2009). The African arenaviruses Mopeia, Mobala, and Ippy virus have so far not been associated with human disease. The New World arenaviruses are divided into Clades, A, B, C, and D, the latter corresponding to former Clade A/B or A/rec (Radoshitzky et al. 2015). Clade B contains the human pathogenic Junin (JUNV), Machupo (MACV), Guanarito (GTOV), Sabia (SABV), and Chapare (CHAV) virus, together with the nonpathogenic Tacaribe (TCRV), Amapari, and Cupixi virus. In nature each arenavirus species has one or a limited number of closely related rodent species as reservoirs that are persistently infected, with the exception of TCRV that was isolated from bats (Buchmeier et al. 2007) and recently detected in host-seeking *Amblyomma americanum* ticks (Sayler et al. 2014). The current phylogenetic diversity of arenaviruses is likely the result of long-term coevolution between viruses and their host species, involving vertical and horizontal transfer of viruses within and between populations (Emonet et al. 2009).

Arenaviruses are enveloped negative-stranded RNA viruses, whose non-lytic life cycle is confined to the cytoplasm (De La Torre 2009). In electron microscopy, viral particles appear spherical to pleomorphic, with diameters of 50–300 nm. The arenavirus genome is comprised of two RNA segments, L (*c.* 7.3 kb) and S (*c.* 3.5 kb), containing two open reading frames in opposite orientation, separated by a noncoding intergenic region with a predicted hairpin structure. The viral S RNA encodes the nucleoprotein (NP) and the envelope glycoprotein precursor (GPC), whereas the L RNA encodes the viral RNA-dependent RNA polymerase L and the viral matrix protein Z. Synthesized as a single polypeptide chain, the viral GPC is posttranslationally cleaved by the cellular protease subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P) to yield the mature virion glycoproteins GP1 and GP2 (Fig. 3.1).

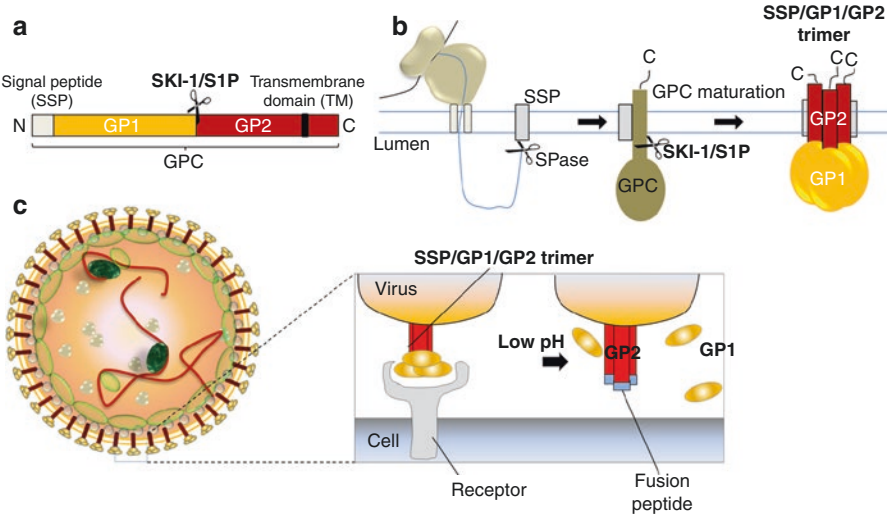

Fig. 3.1 Processing of arenavirus GPC by SKI-1/S1P. (**a**) The arenavirus GPC precursor is comprised of the stable signal peptide (SSP), GP1, and GP2. The transmembrane domain and the site of SKI-1/S1P cleavage are indicated (scissors). (**b**) Sequential processing of arenavirus GPC in the secretory pathway by signal peptidase (SPase) and SKI-1/S1P. The mature tripartite complex SSP/GP1/GP2 forms the mature trimeric GP spike. (**c**) The mature GP trimer decorates the virion surface and engages cellular receptors. Under acidic pH, GP1 dissociates and liberates the fusion peptide of GP2, triggering fusion between the viral and the cellular membrane

Several excellent reviews cover different steps of the arenavirus life cycle (Fehling et al. 2012; Grant et al. 2012; Urata and de la Torre 2011; Emonet et al. 2011; Torriani et al. 2017; Nunberg and York 2012; Wolff et al. 2013; Loureiro et al. 2012), and only a short summary will be given here. The first step of arenavirus infection requires attachment of the viral particle to cellular receptor(s). Most Old World and Clade C New World arenaviruses use dystroglycan, a ubiquitously expressed receptor for proteins of the extracellular matrix (ECM) as a high-affinity receptor (Cao et al. 1998; Oldstone and Campbell 2011). The cellular receptor for the pathogenic Clade B New World arenaviruses was identified as human transferrin receptor 1 (TfR1) (Radoshitzky et al. 2007), a highly conserved cargo receptor involved in iron metabolism. The ability of a Clade B New World arenavirus to use human TfR1 is crucial for its potential to cause zoonotic infection and hemorrhagic fever in man, whereas nonpathogenic viruses use TfR1 orthologues from other species (Helguera et al. 2012; Radoshitzky et al. 2011). More recently, the Tyro3/Axl/Mer (TAM) receptor tyrosine kinases Axl and Tyro3/Dtk, T cell immunoglobulin mucin (TIM) proteins 1 and 4, as well as the C-type lectins DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) and LSECtin have been identified as novel candidate receptors for arenaviruses (Shimojima and Kawaoka 2012; Shimojima et al. 2012; Jemielity et al. 2013; Goncalves et al. 2013; Martinez et al. 2013). Upon initial attachment to the target cell, arenavirus particles are taken up by

receptor-mediated endocytosis. Consistent with the use of TfR1 as a receptor, Clade B New World viruses enter via clathrin-mediated endocytosis (Martinez et al. 2007), whereas Old World arenaviruses use a pathway resembling macropinocytosis (Iwasaki et al. 2014; Oppliger et al. 2016; Torriani et al. 2017). The virus passes through the multivesicular endosome and reaches the late endosome (Pasqual et al. 2011b), where low pH triggers fusion of the viral membrane with the limiting membrane of the late endosome by the fusion-active GP2, creating a "fusion pore." At the late endosome, LASV GP1 undergoes a unique "receptor switch" and engages the late endosomal/lysosomal resident protein LAMP1 for efficient fusion (Jae et al. 2014). The dependence of LASV, but not other arenaviruses including the closely related LCMV, on LAMP1 as a late endosomal entry factor represents an interesting analogy to the filoviruses Ebola virus, whose fusion depends on the late endosomal protein Niemann-Pick C1 (Jae and Brummelkamp 2015).

By an unknown mechanism of "uncoating," the arenavirus ribonucleoprotein (RNP) comprised of viral RNA, NP, and L is released into the cytosol. Viral transcription is initiated at the incoming polymerase complex, resulting in expression of NP and L. As NP accumulates, the viral polymerase shifts to a replicase mode, generating full-length antigenomic RNAs serving as templates for the transcription of GPC and Z as well as synthesis of genomic RNA. Newly synthesized NP assembles the viral replication-transcription complexes that are membrane-associated structures that contain cellular lipids and proteins (Baird et al. 2012; Knopp et al. 2015). In the final stages of the arenavirus life cycle, progeny particles assemble and are released by budding from the plasma membrane. The key factor in the budding process is the small RING finger Z protein that functions as a bona fide matrix protein in arenavirus particle assembly (Urata and De La Torre 2011; Perez et al. 2003). As with other matrix proteins of enveloped viruses, arenavirus Z interacts with the cytosolic tail of GP2 (Capul et al. 2007) and specific cellular factors of the endosomal/multiple vesicle body pathway to drive the budding of viral particles from "budding zones" (Wolff et al. 2013; Urata and De La Torre 2011; Fehling et al. 2012; Perez et al. 2003).

Recent studies isolated, identified, and characterized novel and highly divergent arenaviruses from snakes associated with boid inclusion body disease (Bodewes et al. 2013; Hetzel et al. 2013; Stenglein et al. 2012). The genome organization of these viruses corresponds to arenaviruses, and they show a high degree of divergence. Notably, the GPC of the viral envelope seems more related to filoviruses. No cases of infections in other species have been reported so far, though reptarenaviruses are capable of infecting mammalian and arthropod cells *in vitro* (Hepojoki et al. 2015). Interestingly, reptarenavirus infection of mammalian cells occurred efficiently at 30 °C but was markedly reduced at mammalian body temperature, likely highlighting adaptation to their reptile hosts (Hepojoki et al. 2015).

The most prevalent human pathogen among the arenaviruses is the Old World arenavirus LASV that causes a severe viral hemorrhagic fever with high mortality in humans. Every year LASV causes over 300,000 infections in Western Africa (Mccormick and Fisher-Hoch 2002) and has been declared one of the eight top emerging pathogens by WHO in 2015 (Sweileh 2017). There is currently neither an

efficient cure nor a licensed vaccine, resulting in case fatality rates of 15–30% (Yun and Walker 2012). In the USA, JUNV, MACV, GTOV, and SABV have emerged as causative agents of hemorrhagic fevers with high case-fatality rates with JUNV representing the most important public health problem (Grant et al. 2012). Novel arenaviruses emerge on the average every 2 years and can be associated with severe diseases (Briese et al. 2009; Delgado et al. 2008). Highly pathogenic arenaviruses have been included in the list of Category A pathogens by the Centers for Disease Control and Prevention. Global climate changes may influence population dynamics of natural rodent host populations, likely increasing exposure of humans to these pathogens.

The pathophysiology of fatal arenavirus infection is not well understood and involves viral and host immune factors (Yun and Walker 2012; Prescott et al. 2017). A highly predictive parameter for disease outcome is the viral load, indicating a close competition between viral spread and replication and the patient's immune system (Prescott et al. 2017). Drugs targeting specific steps of the viral life cycle can reduce multiplication and spread of the virus. This may provide the patient's immune system a window of opportunity to develop an antiviral immune response. An in-depth understanding of the molecular mechanisms underlying arenavirus multiplication and virus-host cell interaction is therefore of great importance to develop novel and efficacious strategies for antiviral therapeutic intervention.

3.2 Arenavirus GP Structure and Function

The arenavirus GPC is synthesized initially as a single polypeptide precursor that is sequentially cleaved by cellular signal peptidases and then by SKI-1/S1P (Lenz et al. 2001; Rojek et al. 2008; Beyer et al. 2003) (Fig. 3.1a, b). Processing of GPC by SKI-1/S1P yields the N-terminal GP1, which is implicated in binding to the cellular receptors (Borrow and Oldstone 1992) and the transmembrane GP2 that mediates fusion and resembles class I viral fusion proteins (Eschli et al. 2006; Igonet et al. 2011; Parsy et al. 2013). Arenavirus GPC contains a remarkably stable signal peptide (SSP) of 58 amino acids that contains two hydrophobic domains and undergoes myristoylation at its N-terminus (Eichler et al. 2003a, b; York et al. 2004; Froeschke et al. 2003). The SSP becomes part of a mature tripartite complex SSP/GP1/GP2 where it interacts with the GP2 subunit (Fig. 3.1b). Recent electron cryomicroscopy combined with tomography revealed that SSP/GP1/GP2 complexes of LASV assemble into a trimeric spike that is 9 nm high and 10 nm wide and undergoes significant changes when exposed to low pH (Li et al. 2016). Structural studies on the GP1 of LASV, MACV, and JUNV revealed a similar compact α/β fold, despite significant sequence deviation (Bowden et al. 2009; Cohen-Dvashi et al. 2015; Mahmutovic et al. 2015). Structural studies on the complex of MACV GP1 with its cellular receptor hTfR1 revealed that the GP1 monomer represents the functional unit of receptor recognition and that trimerization is not required for receptor binding (Abraham et al. 2010; Radoshitzky et al. 2011). Notably, MACV GP1 binds to the apical surface of hTfR1 without competing with transferrin binding.

More recent crystallographic studies resolved the structure of the pre-fusion conformation of the mature envelope GP of the prototypic Old World arenavirus LCMV (Hastie et al. 2016). Within the pre-fusion trimer, LCMV GP1 and GP2 undergo extensive interactions, involving ionic bonds. In contrast to the New World arenaviruses, monomeric LCMV GP1 is unable to bind the receptor dystroglycan with high affinity, suggesting that either avidity or the quaternary structure of the pre-fusion trimer is required. Once delivered to the late endosome, low pH sets off a series of conformational changes leading to shedding of GP1 and triggering of fusion of the viral and cellular membrane mediated by GP2 (Fig. 3.1c). The post-fusion conformation of arenavirus GP2 is similar to the six-helix bundle conformation common to a number of class I fusion proteins of enveloped viruses (Igonet et al. 2011; Parsy et al. 2013). The SSP is crucial for transport and processing of arenavirus GPC (Messina et al. 2012; York and Nunberg 2007; York et al. 2004; Eichler et al. 2003a, b). Both N- and C-termini of SSP are located in the cytosol (Agnihothram et al. 2007), and SSP associates non-covalently with a zinc-binding domain within the cytoplasmic tail of GP2 (Agnihothram et al. 2006; Briknarova et al. 2011). The SSP-GP2 interactions critically modulate pH-induced activation of membrane fusion (York and Nunberg 2006, 2009) and are targeted by a range of potent arenavirus fusion inhibitors (Shankar et al. 2016; York et al. 2008), pinpointing this unique feature of arenavirus fusion as a target for the development of antiviral therapeutics.

3.3 The Proprotein Convertase SKI-1/S1P Cleaves Arenavirus GPC

A crucial step of arenavirus infection is the maturation of the envelope glycoprotein precursor GPC. With the exception of the Crimean-Congo hemorrhagic fever virus which belongs to the *Bunyavirus* family, mammarenaviridae are the only viral pathogens known to hijack the proprotein convertase SKI-1/S1P to process their envelope GP. Proprotein convertases (PC) are 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 (Seidah and Prat 2007, 2012). The PCs share homology to the kexin subfamily of subtilases with a distinctive "Ser/His/Asp" catalytic triad that mediates peptide bond scission (Seidah and Prat 2002). Furin, PC5/6B, PC7, and SKI-1/S1P are membrane-anchored, while the remaining enzymes are secreted (PC4, PC5/6A, PACE4, and PCSK9) or retained in granules (PC1/3, PC2) (Seidah 2011). Basic PCs have similar but not identical *consensus* sequences K/RXnR↓ that may result in overlapping patterns of substrate cleavage. In contrast, SKI-1/S1P and PCSK9 cleave after hydrophobic or small residues, BX(hydrophobic)X↓ (Pasquato et al. 2006) and VFAQ↓, respectively (Benjannet et al. 2004). Processing by PC is essential for the proper function of a plethora of cellular proteins, including prohormones, growth factor precursors, transcription factors, proteases, and adhesion molecules.

The convertase SKI-1/S1P has been co-discovered by the laboratory of Nabil Seidah, working on the biosynthesis of brain-derived neurotrophic factor (Seidah et al. 1999), and the group of Brown and Goldstein, investigating the regulation of cholesterol metabolism (Sakai et al. 1998). SKI-1/S1P is a type I membrane protein synthesized as an inactive precursor of 1052 amino acids, comprised of a signal peptide, an N-terminal prodomain, and a catalytic domain (Fig. 3.2a). The transmembrane domain of SKI-1/S1P is followed by a basic cytosolic tail (amino acids 1023–1052). As all PCs, SKI-1/S1P activation requires removal of an N-terminal prodomain that assists the correct folding of the protease (Fig. 3.2a). Upon translocation into the ER, SKI-1/S1P undergoes autocatalytic maturation by sequential cleavages of the N-terminal prodomain first at sites B′/B (**R**K**V**F↓**R**S**L**K_{137}↓), followed by site C (**R**R**L**L_{186}↓) and the newly described site C′ (**R**R**A**S_{166}↓) (Da Palma et al. 2014). The end product, the C form of SKI-1/S1P, represents the fully mature enzyme (Toure et al. 2000; Elagoz et al. 2002). In contrast to basic PCs, maturation of SKI-1/S1P is unique because fragments of the truncated prodomain remain attached to the catalytic subunit of the protease (Fig. 3.2b). While retention of the prodomain prevents catalytic activation of basic PCs, the complexes of SKI-1/S1P with the attached prodomain fragments are enzymatically active and may differently interact with cellular and viral substrates, as detailed below (Da Palma et al. 2014) (Fig. 3.2b). Using the GPC of arenaviruses as "molecular probes," recent studies revealed that the prodomain of SKI-1/S1P has a modular structure. Specifically, the N-terminal AB fragment represents an autonomous structural and functional unit that is necessary and sufficient for SKI-1/S1P folding and partial activation (Da Palma et al. 2016). In contrast, the C-terminal BC fragment of the prodomain lacks a defined structure but seems crucial for autoprocessing and full activation. The AB sequence of the prodomain is evolutionary highly conserved, whereas the BC fragment shows considerable variation and is even missing in some species. Phylogenetic and functional studies suggest that primordial SKI-1/S1P may have contained a simpler prodomain consisting of the conserved AB fragment, whereas the BC region appears as a later evolutionary acquisition possibly allowing subtle regulation of the maturation process (Da Palma et al. 2016).

SKI-1/S1P plays a key role in regulation of lipid metabolism and other physiological processes and is linked to a wide range of human disorders, including hypercholesterolemia, vascular diseases, cancer, and viral infections (Fig. 3.3a). Its proven role in human diseases and the nature as an enzyme make SKI-1/S1P an interesting target for therapeutic intervention. The activity of SKI-1/S1P was first linked to cholesterol and fatty acid biosynthesis, where it was implicated in the activation of the sterol regulating protein factors (SREBP) (Sakai et al. 1998). Other transcription factors were then shown to be activated in a SKI-1/S1P-dependent manner, including activating transcription factor (ATF) 6 that senses ER stress (Ye et al. 2000) and members of the cAMP response element-binding proteins (CREB) family (Kondo et al. 2005). All these substrates share a similar mechanism of activation which involves processing by SKI-1/S1P followed by cleavage by site-2 protease (S2P). The initial, rate-limiting SKI-1/S1P processing occurs in the lumen of the Golgi compartment of the secretory pathway. The SKI-1/S1P cleavage unmasks a

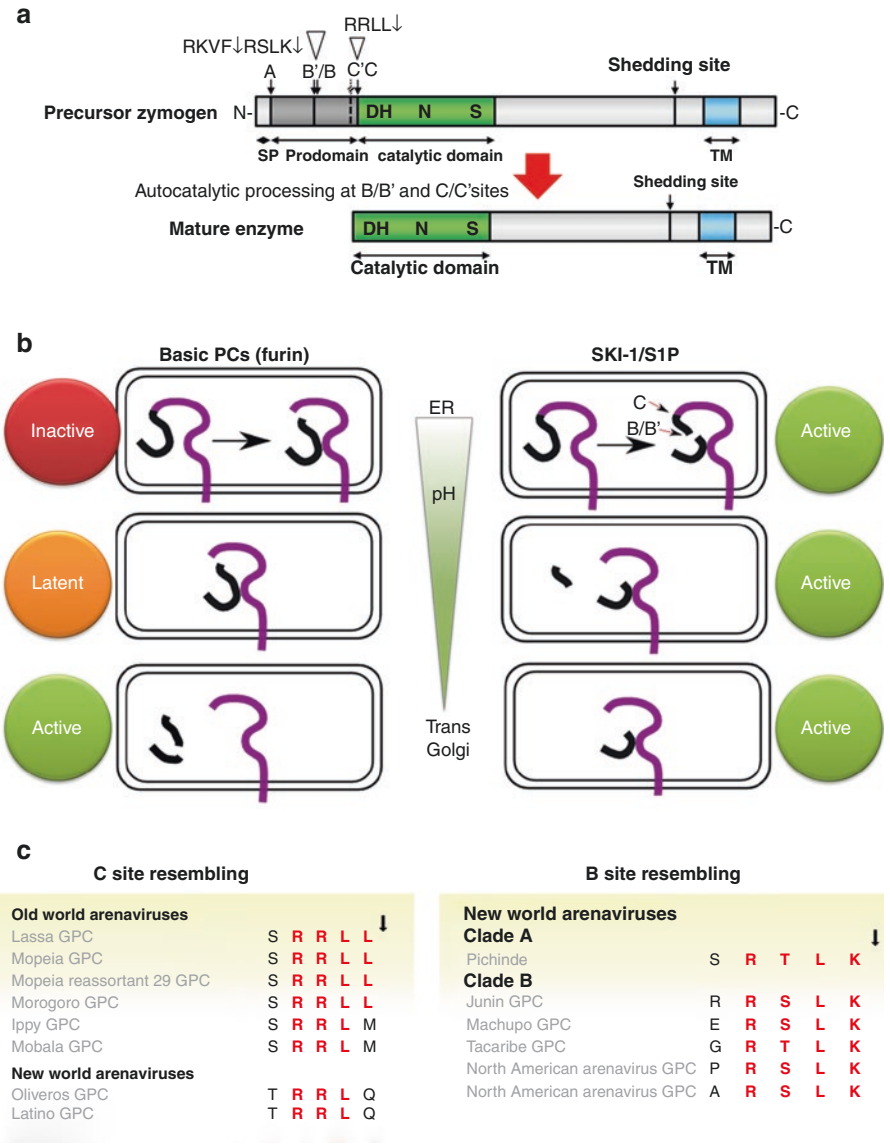

Fig. 3.2 The unusual mechanism of SKI-1/S1P maturation. (**a**) SKI-1/S1P is synthesized as an inactive zymogen precursor that undergoes autocatalytic cleavage to remove the prodomain. The signal peptide (SP), prodomain, catalytic domain, shedding site, and transmembrane domain, as well as autoprocessing sites A, B′/B, and C′/C and their corresponding amino acid sequences, are indicated. Autoprocessing at site C generates the mature enzyme. (**b**) Schematic representation of the maturation of the prototypic basic PC furin (left) and SKI-1/S1P (right). Initial autoprocessing of the furin prodomain results in a catalytically inactive latent complex between prodomain and enzyme. Complete removal of the prodomain is required to liberate the active enzyme late in the secretory pathway. In contrast, immature forms of SKI-1/S1P containing prodomain fragments of different lengths present all along the secretory pathway are catalytically active. (**c**) The GP1/GP2 SKI-1/S1P processing sites resemble sites B and C of autoprocessing. For details, please see text

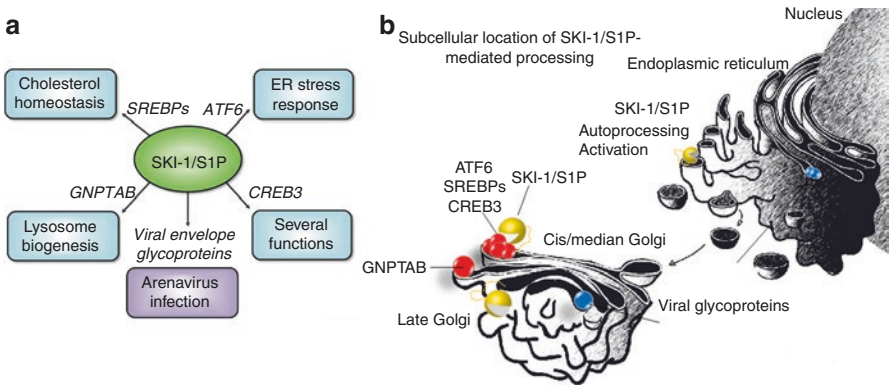

Fig. 3.3 Cellular substrates of SKI-1/S1P. (**a**) Cellular substrates of SKI-1/S1P are linked to major human disorders. (**b**) Subcellular location of SKI-1/S1P autocatalytic activation and SKI-1/S1P-mediated processing of the major cellular substrates. For details, please see text

second site of processing located close to the cytosolic face of the membrane, which is subsequently cleaved by the metalloprotease S2P. Processing by S2P releases a soluble fragment into the cytosol that subsequently enters the nucleus and acts in transcriptional regulation. In addition to transcription factors, SKI-1/S1P is implicated in processing of pro-brain-derived neurotrophic factor (Seidah et al. 1999); *N*-acetylglucosamine-1-phosphotransferase (GNPTAB), which is responsible for the correct sorting of lysosomal proteins (Marschner et al. 2011); and the renin receptor (Nakagawa et al. 2016). SKI-1/S1P further plays a role in bone and muscle formation (Gorski et al. 2011, 2016), ECM signaling, and axial development (Achilleos et al. 2015), as well as fur pigmentation (Rutschmann et al. 2012). However, the exact SKI-1/S1P substrates involved in these latter processes have not yet been clearly identified.

3.4 The Mechanism of SKI-1/S1P Processing of Arenavirus GPC Differs from Cellular Substrates

Alignment of the putative GP1/GP2 cleavage sites in arenavirus GPC reveals extensive sequence variation (Table 3.1). Due to this unusual residue pattern at the processing site, the protease responsible for GPC cleavage was found long after the identification of a *consensus* motif. So far, SKI-1/S1P has been implicated in processing of all mammarenavirus GPCs tested, including the Old World viruses LASV (Lenz et al. 2000, 2001), LCMV (Pinschewer et al. 2003; Beyer et al. 2003), the distantly related LUJV (Oppliger et al. 2015; Urata et al. 2015), as well as New World arenaviruses of different Clades (Rojek et al. 2008; Pasquato et al. 2011; Oppliger et al. 2015). Alignment of the sequences surrounding the putative cleavage site (P10-P10′ positions) reveals the presence of a highly conserved Arg and hydrophobic residue at P4 and P2 positions, respectively (Table 3.1). Interestingly, for the Old World and Clade C New World viruses, the sequences upstream from the scissile bond are of hydrophobic character.

Table 3.1 Amino acid sequences at the GP1/GP2 cleavage sites of arenavirus GPC

ID	Name	Sequence at cleavage site
>YP_009141003.1	Mariental	RSIYISRRIL GTFTWTLSDS
>ADX32840.1	Menekre	KSIYISRRLL GTFSWTLSDN
>AFU54705.1	Middle Pease River	RQAVGIRKLQ AFFSWTLSDN
>YP_516226.1	Mobala	REIYISRRLM GTFTWTLSDS
>AEO89355.1	Mopeia	RNFYISRRLL GLFTWTLSDS
>ABC71134.1	Mopeia	RSSYISRRLL GLFTWTLSDS
>AFY05576.1	Mopeia Lassa reassortant 29	RDIYISRRLL GTFTWTLSDS
>AFY05594.1	Mopeia Lassa reassortant 29	RDMYISRRLL GTFTWTLSDS
>AFY05619.1	Mopeia virus AN20410	RNFYISRRLL GLFTWTLSDS
>YP_003090214.	Morogoro	KNFYISRRLL GLFTWTLSDS
>AGT56422.1	Natoeduori	RTRFIARKLA GTFSWTLSDD
>AFU54675.1	North America	GQTAGIRKLQ AFFSWTLSDN
>ABW96598.1	North America	KQVIKVRKLL AFFTWSLSDA
>ABW96600.1	North America	KQMIKPRSLK SFFSWSLSDA
>ABW96602.1	North America	KQMIGARSLK AFFTWSLSDA
>AFD98839.1	Ocozocoautla de Espinosa	KNMFTRRTLK AFFSWSLTDS
>AJZ76770.1	Okahandja	RSIYLSRRLR SVFSWTLTDA
>AKG54821.1	Oliveros	GQSFITRRLQ AFLTWTLSDS
>YP_001936017.1	Parana	AYSSVSRKLL GFFTWDISDS
>AIN76883.1	Patawa	AYSSVSRKLM GFFTWDISDS
>AER45493.1	Pichinde	AYSSVSRKLL GFFTWDLSDS
>ACD71458.1	Pirital	AYSSVSRKLL GFFTWDISDS
>AAT88084.1	Pirital	AYGSVSRKLL GFFTWDISDS
>YP_089665.1	Sabia	GRSSGSRRPL GIFSWTITDA
>ABW96596.1	Skinner Tank	SQIVRARKLH AFFTWSLTDS
>AHW46355.1	Tacaribe	KSIAVGRTLK AFFSWSLTDP
>ACC99352.1	Tamiami	TQVVRARRIL SFFTWSLSDA
>ABU94341.1	Tonto creek	NQVIRARKLH AFFTWSLTDS
>AFU54702.1	Whitewater Arroyo	SQMIKARRLQ NFFSWSLSDA
>YP_001911113.1	Whitewater Arroyo	KQMIKSRTLK SFFAWSLSDA

The GenBank ID, name of virus species, and amino acids at the GP1/GP2 cleavage site are displayed. The conserved R residues in position 4 and hydrophobic residues in P2 position are highlighted

Maturation of GPC by SKI-1/S1P is strictly required for the production of infectious particles and viral cell-to-cell spread (Beyer et al. 2003; Lenz et al. 2001; Rojek et al. 2008). A crucial role for SKI-1/S1P for arenavirus dissemination *in vivo* is further suggested by the observation that mice bearing the "wood rat" mutation Y496C in SKI-1/S1P show enhanced resistance to infection with LCMV due to impairment of GPC processing (Popkin et al. 2011). Accordingly, proof-of-concept studies with protein- and peptide-based SKI-1/S1P inhibitors revealed that targeting GPC maturation represents a novel and promising antiviral strategy (Maisa et al. 2009; Rojek et al. 2010) as will be further developed below. Inhibition of SKI-1/S1P in infected cells results in the formation of noninfectious "naked" particles that contain viral RNP but lack GP (Lenz et al. 2001; Kunz et al. 2003; Rojek et al. 2008), indicating specific incorporation of fully mature, processed GP. How the arenavirus budding machinery is capable to achieve this specificity is currently unknown. Notably, SKI-1/S1P processing is not required for cell-surface transport of arenavirus GPC (Kunz et al. 2003; Schlie et al. 2010a), and small amounts of uncleaved GPC can be detected at the surface of infected cells (Kunz et al. 2003). However, in contrast to trimeric mature SSP/GP1/GP2 complexes, the uncleaved GPC forms monomers and oligomers spanning a wide size range, indicating that SKI-1/S1P processing is critical for the correct oligomeric state (Schlie et al. 2010a). Moreover, mutations in the cytosolic tail of LCMV and LASV GP2 affect SKI-1/S1P processing of the ectodomain (Schlie et al. 2010b; Kunz et al. 2003), suggesting some sort of transmission of structural information through the membrane. Since viral budding requires interactions of the cytosolic domain of GP2 with the matrix protein Z (Capul et al. 2007), processing by SKI-1/S1P may be required for targeting mature GP to putative "budding domains" and/or unmasking GP2 binding domains to Z.

The cleavage sites of arenavirus GPC differ from cellular substrates and resemble the B and C autoprocessing motifs of SKI-1/S1P (Fig. 3.2c). The GPC of JUNV contains the sequence RSLK↓ (B site), whereas LASV and LCMV GPCs are cleaved at the motifs RRLL↓ and RRLA↓ (C site). LASV GPC with the recognition sequence RRLL undergoes SKI-1/S1P processing early in the secretory pathway (Lenz et al. 2001), whereas LCMV GPC containing RRLA is processed in late Golgi or post-Golgi compartments (Wright et al. 1990; Beyer et al. 2003). Membrane-associated SKI-1/S1P is found predominantly in the early Golgi where cellular SKI-1/S1P substrates are cleaved (Pullikotil et al. 2007). Thus, the data at hand indicate that SKI-1/S1P is active in at least three different sub-compartments of the secretory pathway, ER/*cis*-Golgi (LASV GPC), median Golgi (SREBPs, ATF6, CREBs, GNPTAB), and late Golgi (LCMV GPC) (Fig. 3.3b). How arenaviruses selected specific subcellular compartments for SKI-1/S1P-mediated GPC maturation is still not fully understood, but recent studies gave some hints. Subtle changes of the sequence at the cleavage site can have drastic effects on the location and efficiency of GPC maturation, despite maintaining the RXLX↓ *consensus* motif. As an example, processing of an LCMV GPC mutant containing the cleavage site RRLL derived from LASV GPC is redirected from late Golgi to the ER/*cis*-Golgi. In contrast, introduction of the LCMV GPC cleavage motif RRLA into the

LASV GPC backbone results in an uncleavable protein (Burri et al. 2012). The unusual mechanism of zymogen activation and maturation of SKI-1/S1P described above (Fig. 3.2b) may contribute to this phenomenon. As mentioned above, autocatalytic processing is necessary but not sufficient to remove the SKI-1/S1P prosegment, resulting in different already active forms of the enzyme still bearing prodomain fragments of distinct lengths (Fig. 3.2b). It is conceivable that such enzyme/prodomain complexes located in defined sub-compartments of the secretory pathway may show differential specificity for viral and cellular substrates. Further evidence for differential recognition of viral and cellular substrates by SKI-1/S1P comes from the observation that the mutations R130E and R134E within the B′/B autoprocessing site result in selective impairment of viral GPC processing, but not cleavage of cellular substrates (Burri et al. 2012).

Due to their non-lytic strategy of replication, arenaviruses can establish persistent infections *in vitro* and *in vivo* without causing overt signs of pathology. Considering the multiple roles of SKI-1/S1P in maintaining cellular functions, the largely nonoverlapping subcellular localization of viral vs. cellular substrates (Fig. 3.3b) may be a consequence of the extensive coevolution of the viruses with their reservoir hosts. Accordingly, high expression levels of GPC during acute arenavirus infection do not interfere with the SKI-1/S1P-mediated processing of ATF6 involved in the host cell's ER stress response (Pasqual et al. 2011a). As a consequence, arenavirus infection results in specific and transient activation of the ATF6-regulated branch of the cellular ER stress response that includes upregulation of chaperones, adjusting the folding capacity to the increased demand. The differential subcellular location of SKI-1/S1P processing of viral and cellular substrates may therefore allow extensive viral replication and gene expression without causing overt cytopathic effects (Oldstone 2002).

3.5 Optimized Recognition of Arenavirus GPC by SKI-1/S1P: Viral Advantage and Achilles' Heel

Comparison of the currently known sequences of arenavirus GPCs revealed the presence of a highly conserved aromatic residue at position P7 relative to the SKI-1/S1P recognition sites in Old World and Clade C New World arenaviruses, but not in New World viruses of Clades A and B or cellular substrates (Burri et al. 2013) (Table 3.1). Early experimental evidence already supported the notion that an aromatic amino acid at P7 somehow promotes SKI-1/S1P cleavage of both LASV (Pasquato et al. 2006) and LCMV GPC (Beyer et al. 2003). Subsequent molecular modeling allowed docking of the LASV GPC-derived peptides into the putative catalytic pocket of SKI-1/S1P (Burri et al. 2013). These studies revealed that the aromatic "signature residue" in position P7 of some viral GPC recognition sequences interacts with residue Y285 located in the extended substrate-binding pocket of SKI-1/S1P (Burri et al. 2013). Indeed, introduction of the mutation Y285A into SKI-1/S1P gives an enzyme that is markedly impaired in processing of LASV GPC, but not GPC of New World arenaviruses or cellular substrates. During coevolution

with their mammalian hosts, the GPCs of Old World and Clade C New World viruses apparently expanded the molecular contacts with SKI-1/S1P beyond the classical four amino acid recognition sequences, resulting in an enlarged binding surface. The concept that critical residues flanking the classical recognition sites can modulate PC processing is also supported by findings with basic PCs and their cellular substrates. A comparative study showed the influence of surrounding amino acids on the relative PC cleavage efficiency (Remacle et al. 2008). The presence of an N at position P1′ after the scissile bond in the substrate growth differentiation factor-11 markedly reduces cleavage by PACE4, furin, and PC7 while being selectively permissive to PC5/6 (Essalmani et al. 2008). Moreover, Constam and colleagues demonstrated that L and G at P2′ and P3′ positions in nodal, a regulator of the fate of pluripotent cells, dramatically enhance basic PC processing, further supporting this concept (Constam and Robertson 1999). The specificity of the interaction between Y285 of SKI-1/S1P and aromatic P7 residues for LASV GPC processing, but not cleavage of cellular substrates, makes this interaction a promising target for the development of specific antiviral drugs against this important human pathogen. Perturbation of the interaction of the highly conserved aromatic side chain in position P7 of LASV GPC with the contact residue Y285 of SKI-1/S1P, e.g., by a small molecule, is not expected to affect the SKI-1/S1P catalytic triad, limiting unwanted side effects. The markedly reduced processing of LASV GPC bearing a Y to A mutation in position P7 (Burri et al. 2013) suggests that viral escape variants lacking the aromatic signature residue at P7 may have impaired fitness, making the P7/Y285 interaction a true "Achilles' heel" of the virus.

3.6 The Processing of Reptarenavirus GPC Is Largely Unknown

For the mammarenaviridae, several lines of evidence suggest that the maturation of the viral GPC by SKI-1/S1P is a crucial step in the virus life cycle. In contrast, little is known about the biosynthesis, maturation, and processing of reptarenavirus GPC. The sequences of reptarenavirus GPC seem to deviate from the canonical SSP/GP1/GP2 composition of mammarenaviruses and bear some resemblance to filovirus GP (Li et al. 2016; Stenglein et al. 2012). In contrast to arenavirus GPC, whose processing critically depends on SKI-1/S1P, the GP of the filovirus Ebola undergoes processing by furin, the prototypic member of the basic PC family, although this cleavage seems dispensable for virus infection and propagation (Neumann et al. 2002) (see also Chap. 5). Further studies are required to identify the specific cellular protease(s) involved in reptarenavirus GPC processing and to see if this step is crucial for functional maturation. A very different mechanism cannot be ruled out, including the use of another class of host-derived proteases that may function in the secretory pathway during biosynthesis, at the cell surface, or during the entry process, as illustrated by the use of endosomal cathepsins by filoviruses (Hunt et al. 2012) or coronaviruses that use multiple proteases, including cathepsins, cell surface transmembrane protease/serine proteases, furin, and trypsin (Millet and Whittaker 2015, see also Chap. 4).

3.7 Targeting SKI-1/S1P-Mediated GPC Processing as an Anti-arenaviral Strategy

A major challenge for the development of drugs against human pathogenic arenaviruses is the limited structural information available on the pathogens. As all viruses, arenaviruses critically depend on the molecular machinery of the host cell for their multiplication. This is particularly true for the biosynthesis of the envelope GP that involves a complex interplay with cellular factors, including SKI-1/S1P. So far, all human pathogenic arenaviruses seem to be SKI-1/S1P-dependent, and SKI-1/S1P activity is crucial for productive infection. In the absence of SKI-1/S1P activity, infected cells produce noninfectious naked particles devoid of GP, due to a yet-unknown mechanism of selective incorporation of cleaved GP. Studies have been carried out to investigate the ability of arenaviruses to escape SKI-1/S1P. Recombinant LCMV containing the furin cleavage site RRRR↓ instead of RRLA↓ was found to be replication competent and behaved similarly to the wild-type virus in cell culture (Rojek et al. 2010). Although this suggested that arenaviruses may, at least in principle, use other proteases than SKI-1/S1P for GPC processing, persistent virus infection of SKI-1/S1P null cells or inhibitor treatment so far never resulted in the emergence of SKI-1/S1P-independent viral escape variants (Rojek et al. 2008, 2010; Pasquato et al. 2012b). Moreover, complete inhibition of SKI-1/S1P seems not required to restrict arenavirus infection *in vivo*, since the partially active "wood rat" variant of the enzyme conferred significant protection and prevented persistent viral infection with LCMV (Popkin et al. 2011). In sum, inhibition of SKI-1/S1P appears as a promising therapeutic approach to combat arenavirus infection (Pasquato et al. 2012a).

Direct inhibition of the mature protease is a widely used approach to interfere with the normal enzymatic activity. Although this approach often gives excellent results, a catalytically dead enzyme results in a general loss of activity toward all substrates, cellular and viral alike. Unwanted side effects must be carefully taken into consideration. Considering the crucial role of SKI-1/S1P in major physiological processes and disorders, including infection with highly pathogenic arena- and bunyaviruses, efforts have been made to develop a panel of protein-based and small molecule inhibitors that will be covered below.

3.7.1 Protein-Based Strategies

The naturally occurring serpin α_1-antitrypsin (AT) had been long known as a potent suicide inhibitor for trypsin-like proteases, entrapping the enzyme in a stable complex following cleavage at the reactive site loop. Subsequently, mutations have been inserted into the reactive site loop of α_1-AT to introduce the $B(X)_nB\downarrow$ motif of basic PCs, yielding a potent protein-based furin inhibitor (α_1-PDX) (Anderson et al. 1993). Based on the homology of the catalytic sites of basic and nonbasic PCs, the reactive site loop of α_1-AT was further mutated to introduce the SKI-1/S1P BX(hydrophobic)X↓ motif RRVL. In cell culture experiments, α_1-AT RRVL

efficiently blocked SKI-1/S1P-mediated processing of SREBPs. Overexpression of α_1-AT RRVL inhibited LASV GPC maturation and had a strong antiviral effect by markedly reducing cell-to-cell spread and infectious viral particle production (Maisa et al. 2009), providing proof of concept.

3.7.2 Peptide-Based Compounds and Small Molecules

Analogous to peptide chloromethyl ketones (CMK) developed to inhibit furin-like proteases, two CMK peptides were designed, containing the IYISRRLL and RRLL motifs derived from LASV GPC. Both CMK peptides act as irreversible inhibitors of SKI-1/S1P at the level of substrate maturation (Pasquato et al. 2006). Proof-of-principle studies showed that these small molecules potently block infection of LCMV (Rojek et al. 2010). However, due to their toxicity, CMK inhibitors are not suitable as therapeutic agents.

The aminopyrrolidine amide compound PF-429242 is a reversible, competitive inhibitor of SKI-1/S1P discovered by Pfizer Inc. that efficiently blocks processing of endogenous cellular substrates (Hay et al. 2007). Following PF-429242 administration *in vivo*, SREBP-2 activation in mice is dramatically reduced resulting in a marked drop of plasma cholesterol levels (Hawkins et al. 2008). Pharmacological inhibition of SKI-1/S1P activity by PF-429242 also blocks LASV and LCMV GPC maturation reducing cell-to-cell propagation with only mild off-target effects (Urata et al. 2011; Pasquato et al. 2012b). Cells persistently infected with LCMV were efficiently cleared by treatment with PF-429242 without emergence of drug-resistant viral escape variants (Pasquato et al. 2012b). Initial evaluation of PF-429242 *in vivo* in a murine model raised concerns about applications against chronic diseases, such as familial hypercholesterolemia (Hawkins et al. 2008). However, human pathogenic arenaviruses cause acute diseases, limiting antiviral treatment to a time window of a few weeks. Considering these relatively short periods of treatment, the toxicological and pharmacokinetic profile of PF-429242 makes it still an interesting experimental drug candidate (Hawkins et al. 2008).

The current standard of care for treatment of human arenavirus infection is an off-label use of the nucleoside analogue ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (Parker 2005). Early administration of ribavirin reduces the fatality in human Lassa fever (Mccormick et al. 1986) and experimental infections with MACV (Kilgore et al. 1995) and JUNV (Weissenbacher et al. 1987) in animals. However, to achieve high efficacy, ribavirin needs to be administered early during infection intravenously and is often associated with side effects. Novel anti-arenaviral drugs may be used individually or in combination with therapy and ribavirin to combat human pathogenic arenaviruses, allowing lower doses of ribavirin. Indeed, the combination of PF-429242 with ribavirin revealed stronger than additive effect of the two drugs (Pasquato et al. 2012b). The basis for this apparent synergism may lie in the distinct underlying antiviral mechanisms of the two drugs. Depending on the concentration used, ribavirin inhibits arenavirus infection at the level of replication (Ruiz-Jarabo et al. 2003) and shows drug

action as a mutagen (Moreno et al. 2011). In contrast, PF-429242 affects the biosynthesis of the viral GP, blocking the formation of infectious progeny virus from infected cells.

3.7.3 A Novel Cell-Based Sensor for SKI-1/S1P as a Platform for High-Throughput Drug Screening

As outlined above, the currently available SKI-1/S1P inhibitors are potent and specific and represent invaluable experimental drugs for proof-of-concept studies. However, they face considerable restrictions regarding therapeutic use in clinical medicine. The protein nature of α_1-AT RRVL makes cell permeability and *in vivo* drug delivery challenging. The smaller SKI-1/S1P-specific decanoylated CMK peptides show good cell permeability, but due to their toxicity profile and short half-life, their use is restricted. PF-429242 has low cytotoxicity and shows *in vitro* IC_{50} in the low micromolar range. However, the compound has an unfavorable pharmacokinetic profile *in vivo* and has to the best of our knowledge not yet entered the clinical test phase. Considering the promise of SKI-1/S1P as a drug target and the limitations of the candidate inhibitors at hand, the identification of novel small molecule inhibitors for SKI-1/S1P is of high priority.

Conventional approaches to study substrate processing by SKI-1/S1P use homogeneous biochemical assays including synthetic chromogenic peptides and purified soluble enzyme (Pasquato et al. 2006). These systems have greatly contributed to our current understanding of the biochemistry of SKI-1/S1P and lead to the discovery of candidate drugs like PF-429242. However, as mentioned above, evidence is accumulating that the interaction of SKI-1/S1P with its substrates is more complex and may also be regulated at the level of subcellular location. Robust and quantifiable cell-based assays are therefore needed to screen for inhibitors of SKI-1/S1P processing of specific substrates in the authentic cellular context. To close this gap, a novel reliable cell-based assay allowing the quantitative detection of the enzymatic activity of endogenous SKI-1/S1P has been developed, exploiting key findings on the processing of viral GPC (Da Palma et al. 2014). The 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 virus-derived cleavable peptide sequence (Fig. 3.4). The SKI-1/S1P-cleavable 9mer sequence IYIS**RRLL**↓G used in the prototypic sensor is derived from LASV GPC, which is one of the best substrates currently known (Pasquato et al. 2006, Lenz et al. 2001), assuring optimal sensitivity and specificity of the sensor. The membrane anchor of the sensor mimics that of SKI-1/S1P, allowing correct cellular targeting and favoring optimal substrate-enzyme recognition. Upon processing, this sensor releases the soluble reporter GLuc to the medium, where it can be easily detected using a sensitive and cost-efficient luciferase assay. The sensor recapitulates the key features of the viral substrate from which the processing site has been derived, both in terms of subcellular localization and efficiency of cleavage. The robust and reliable nature of this novel cell-based sensor assay allows implementation in high-throughput screening (HTS).

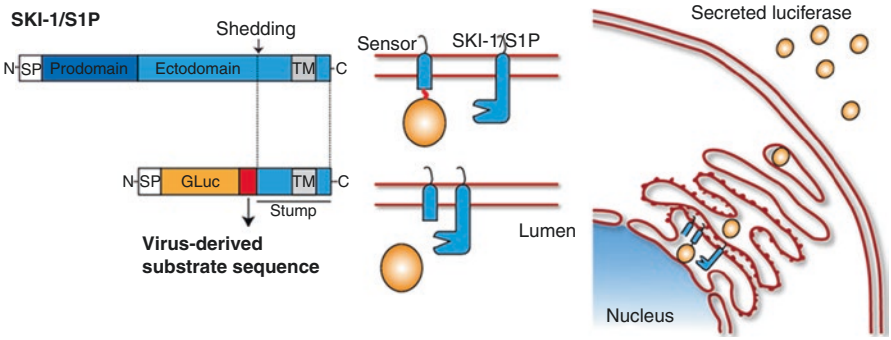

Fig. 3.4 A cell-based sensor for the detection of endogenous SKI-1/S1P activity. Schematic of the SKI-1/S1P sensor. The SKI-1/S1P-derived stump region and the GLuc reporter, as well as the virus-derived peptide comprising the cleavage motif, are indicated. Processing of the sensor by endogenous SKI-1/S1P releases the GLuc reporter (sphere) that is secreted into the tissue culture supernatant, where it can be detected *via* luminescence assay. For details, please see text

PCs are essential for normal cell functions during development and in adults (Seidah and Prat 2012); nonetheless several studies showed that specific inhibition of a definite PC is not detrimental in case of *in vivo* short-term treatment (Hay et al. 2007; Shiryaev et al. 2007). Thus, targeting viral GP cleavage is a novel promising therapeutic approach to fight against virulent pathogens such as hemorrhagic arenaviruses. Our modern globalized world increasingly faces the threat of emerging viruses due to human migration, rapidly progressing urbanization, almost free global trade, exposure to animals, and climatic changes. The development of novel broadly specific antivirals is therefore important to help meeting these unmatched medical problems.

3.7.4 Use of the SKI-1/S1P Sensor to Predict Protease Use of Newly Emerging Arenaviruses

New arenaviruses are rapidly emerging and are in some cases associated with severe human diseases. With the advent of powerful next-generation sequencing approaches, we expect to see accelerated discovery of many new arenavirus species, pathogenic or not, in the years to come. However, in many cases viruses may not be isolated, and only genetic information will become available. Considering the extensive variation at the known and putative SKI-1/S1P recognition sequences in known arenavirus GPC, defined *consensus* sequences cannot easily be found (Burri et al. 2013; Pasquato et al. 2011). Data at hand indicate that knowledge of the sequence P1–8 and residue P1′ of the putative GP1/GP2 cleavage site of a novel arenavirus GPC would be necessary and sufficient to test if the new virus can hijack human SKI-1/S1P, which is a prerequisite for productive infection and hence disease potential in man. In a recent study, the cell-based SKI-1/S1P sensor was applied to make a first prediction if the recently emerged LUJV GPC is processed by human

SKI-1/S1P. Phylogenetic analysis identified LUJV as an outlier within the Old World arenaviruses as the sequence RKLM↓K at the putative GP1/GP2 border differed significantly from the known *consensus* (Burri et al. 2013). Despite these important differences, a sensor containing a 9mer peptide derived from LUJV GPC underwent efficient processing by human SKI-1/S1P, which was then validated with authentic full-length GPC (Oppliger et al. 2015).

Considering the promise of SKI-1/S1P as therapeutic target for novel antiviral drugs to combat human pathogenic arenaviruses, SKI-1/S1P dependence of a newly emerging pathogenic arenavirus assessed by this new sensor may open the possibility for rapid intervention. The SKI-1/S1P sensor can further be used to assess the processing of arenavirus GPCs by SKI-1/S1P orthologues derived from other species, shedding light on the complex ecology of arenaviruses. Conceptually, the sensor platform developed for SKI-1/S1P based on lessons learned from arenavirus GPC cleavage may be applicable for other human proteases that are responsible for processing of a plethora of viral envelope GPs in a wide range of species. For the virology research community such a platform may serve as a rapid and cost-effective evaluation of viral GP processing by human proteases that may contribute to our preparedness against the threat of emerging viruses.

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