Antiviral Research 99 (2013) 49–60

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Review

Viral envelope glycoprotein processing by proprotein convertases

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ARTICLE INFO

Article history:
Received 22 February 2013
Revised 9 April 2013
Accepted 14 April 2013
Available online 20 April 2013

Keywords:
Viral glycoprotein processing
Protease
Proprotein convertase
Enveloped virus
Antiviral therapy

ABSTRACT

The proprotein convertases (PCs) are a family of nine mammalian enzymes that play key roles in the maintenance of cell homeostasis by activating or inactivating proteins *via* limited proteolysis under temporal and spatial control. A wide range of pathogens, including major human pathogenic viruses can hijack cellular PCs for their own purposes. In particular, productive infection with many enveloped viruses critically depends on the processing of their fusion-active viral envelope glycoproteins by cellular PCs. Based on their crucial role in virus-host interaction, PCs can be important determinants for viral pathogenesis and represent promising targets of therapeutic antiviral intervention. In the present review we will cover basic aspects and recent developments of PC-mediated maturation of viral envelope glycoproteins of selected medically important viruses. The molecular mechanisms underlying the recognition of PCs by viral glycoproteins will be described, including recent findings demonstrating differential PC-recognition of viral and cellular substrates. We will further discuss a possible scenario how viruses during co-evolution with their hosts adapted their glycoproteins to modulate the activity of cellular PCs for their own benefit and discuss the consequences for virus-host interaction and pathogenesis. Particular attention will be given to past and current efforts to evaluate cellular PCs as targets for antiviral therapeutic intervention, with emphasis on emerging highly pathogenic viruses for which no efficacious drugs or vaccines are currently available.

Contents

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http://dx.doi.org/10.1016/j.antiviral.2013.04.013

1. Introduction

The proprotein convertases (PCs) are a family of nine mammalian enzymes that play key roles in maintaining cellular and systemic homeostasis by activating or inactivating proteins by limited proteolysis in a spatially and temporally controlled manner. A wide range of pathogens, including major human pathogenic viruses, evolved to hijack cellular PCs and to (ab)use them for their own needs. In particular, productive infection with many enveloped viruses critically depends on the processing of their fusion-active viral envelope glycoproteins by cellular PCs. Based on their crucial role in virus-host interaction, PCs appear as important determinants for the host-range, tissue tropism, and the disease potential of a virus. As essential cellular factors for viral infection they further represent promising targets for antiviral therapeutics.

The present review discusses basic aspects and recent developments in the PC-mediated maturation of viral envelope glycoproteins, in the context of selected viruses of medical importance. We will describe the molecular mechanisms underlying the recognition of PCs by viral glycoproteins and highlight recent findings demonstrating differential recognition of viral and cellular substrates by PCs. In this context, we will propose a possible scenario of how viruses during co-evolution with their hosts have adapted their glycoproteins to modulate the activity of cellular PCs for their own benefit, and discuss the consequences for virus–host interaction and pathogenesis. A last part will cover past and current efforts to evaluate cellular PCs as targets for antiviral drugs, in particular in the context of emerging highly pathogenic viruses for which no efficacious drugs or vaccines are currently available.

2. PCs are crucial for normal cell function and homeostasis

The basic biology of the proprotein convertases (PCs) and their therapeutic potentials have been covered by an excellent recent review (Seidah and Prat, 2012), and only a short summary will be given here. PCs are calcium-dependent serine endoproteases with currently 9 identified family members – PC1/3, PC2, furin, PC4, PACE4, PC5/6, PC7 (basic amino acid (aa)-specific PCs), SKI-1/S1P, and PCSK9 (non basic PCs) (Seidah and Prat, 2002, 2007). They share homology to the yeast kexin subfamily of subtilases with a distinctive "Ser–His–Asp" catalytic triad, which mediates peptide bond scission of substrates that dock into their catalytic pocket (Seidah, 2011).

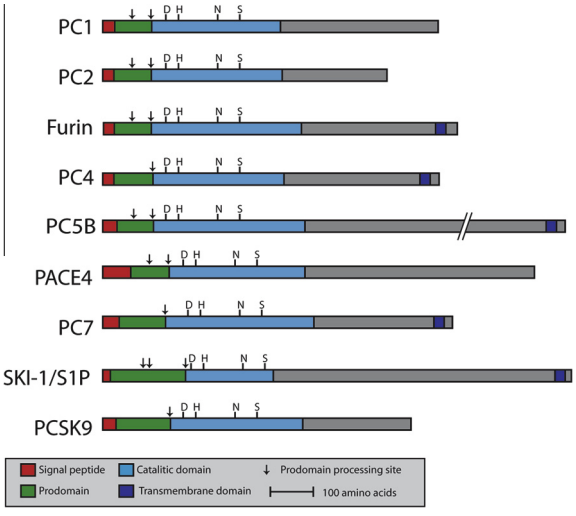

Fig. 1. Schematic representation of the domain arrangement of human proprotein convertases. Residues belonging to the catalytic triad and oxyanion hole are indicated.

All PCs have a well-conserved modular structure comprised of an N-terminal prodomain, followed by a structurally conserved catalytic domain, and variable C-terminal domains (Fig. 1). Four PCs (furin, PC5/6 isoform B, PC7, and SKI-1/S1P) are anchored to cellular membranes while the remaining enzymes are either secreted (PC4, PC5/6 isoform A, PACE4, and PCSK9) or retained in dense core granules (PC1/3, PC2) (Seidah and Prat, 2012) Despite considerable variation in PC sequences, the catalytic subunit cores of these 7 basic aa-specific PCs share $\geqslant$50% sequence identity (Henrich et al., 2005) with similar but not identical recognition of clusters of amino acids (K/RXnK/R↓, *consensus* motif) which interact with the catalytic pocket and define the boundaries of the newly generated fragments. The overall similarity of *consensus* sequences, and thus apparent redundancy of basic aa-specific PCs, suggests overlapping patterns of substrate cleavage, found e.g. in *in vitro* and in overexpression systems. However, there is evidence that subtle differences in amino acid residues in the vicinity of the *consensus* sequence crucial for enzyme/substrate recognition may modulate the preference of a given basic aa-specific PC for specific substrate proteins (Essalmani et al., 2008; Remacle et al., 2006; Zhang et al., 2012). In contrast to basic aa-specific PCs, SKI-1/S1P and PCSK9, the last discovered members of the family, cleave after hydrophobic/small residues, BX(hydrophobic)X↓ (Pasquato et al., 2006) and VFAQ↓, respectively (Benjannet et al., 2004).

A common feature of all PCs is their maturation involving autoproteolytic processing in order to exit the endoplasmic reticulum (ER) and to acquire their specific activity. This step-wise zymogen activation is an essential step in the biosynthesis of basic aa-specific PCs and includes two subsequent cleavages at the N-terminus (Basak et al., 1999; Rousselet et al., 2011). Following removal of the signal peptide in the ER, basic aa-specific PCs auto-process the N-terminal pro-segment, which in turn helps the enzyme polypeptide chain to fold and adopt the correct conformation of the catalytic pocket. The activation of basic aa-specific PCs is regulated in an organelle-specific manner by their pro-segments which undergo pH-dependent auto-cleavage following exit from the ER (Dillon et al., 2012).

The dual function of the pro-segment as chaperone/inhibitor has been well characterized for furin, the prototypic member of PCs. After the first step of prodomain processing, the enzyme is retained in a latent form. The full enzymatic activity is acquired by a second cleavage within the pro-region in a spatially and temporally controlled fashion (Benjannet et al., 2004). Notably, PCs vary in the sub-cellular site of their activation. Furin and PC7 become fully active in the *trans* Golgi network (TGN), PC1/3 and PC2 in the dense core secretory granules, and PC5/6 and PACE4 at the cell surface. In case of SKI-1/S1P (Toure et al., 2000) and differently from basic aa-specific PCs, the two cleavage steps required for activation occur first in the middle of the pro-segment and then at its C-terminus, releasing a fully active enzyme already in the ER/cis Golgi. In contrast to the other PC family members, PCSK9 is kept in a latent form due to the formation of a stable complex with its pro-segment (Piper et al., 2007). With the exception of SKI-1/S1P and PC2, all PC pro-segments are inhibitors of their cognate enzymes (Benjannet et al., 2001; Nour et al., 2003; Zhong et al., 1999).

The proprotein convertases follow distinct sorting routes to reach specific sub-cellular compartments. PC1/3, PC2 (Tanaka et al., 1996) and rarely PC5/6A (De Bie et al., 1996) are found mainly within the dense core vesicles of the regulated secretory pathway. PACE4, PC5/6B, PC7, and furin contain sorting signals in their cytosolic tails allowing them to cycle from the cell surface back to the TGN through endosomes. PC4 exclusively localizes at the plasma membrane (Gyamera-Acheampong et al., 2006), PCSK9 is secreted (Seidah et al., 2003), whereas SKI-1/S1P shows a wide pattern of subcellular distribution being detectable in the ER, Golgi, in endosomes and in lysosomes (Pullikotil et al., 2004). SKI-1/S1P and furin further undergo shedding to release a soluble enzymatic form in the extracellular *milieu* whose function is still not adequately defined.

Due to its dynamic trafficking and highly regulated subcellular localization, furin-mediated processing can occur in the TGN, in endosomes, and at the plasma membrane (Rehemtulla and Kaufman, 1992) where substrates normally reside. There is currently no evidence that endogenous furin is active on biological substrates in the early secretory pathway, although overexpressed mutated (Salvas et al., 2005) or misfolded (Bass et al., 2000) proteins may be cleaved in the ER/*cis* Golgi compartments.

A different scenario has been described for the two non-basic aa-specific PCs, SKI-1/S1P and PCSK9. Both are linked to lipid metabolism: SKI-1/S1P is involved in cholesterol and fatty acid homeostasis whereas PCSK9 is a major regulator of the low density lipoprotein receptor (LDLR) on the plasma membrane. Following cholesterol deprivation or ER stress, the SKI-1/S1P substrates Sterol Regulatory Element-Binding Proteins (SREBP) and activating transcription factor 6 respectively move to the *cis* Golgi where sequential processing by SKI-1/S1P and Site-2 Protease occurs. Proteolytic processing liberates the active form of the transcription factors from the cytosolic face, which then enter the nucleus resulting in the active transcription of specific genes. PCSK9, which does not possess any catalytic activity other than its own auto-processing in the ER, exerts its biological activity *via* escorting target proteins to lysosomes for degradation.

In vivo knockouts (KO) of PCs highlight the physiological importance of these enzymes. Furin, PC5/6, and S1P/SKI-1 KO result in a lethal phenotype in mice; PACE4 KO mice exhibit partial lethality. In a mixed genetic background, PC1/3 and PC2 KO animals show multiple neuroendocrine peptide processing defects, whereas PC7 mice exhibit an anxiolytic phenotype (Seidah and Prat, 2012). Finally, PCSK9 deficiency lowers plasma cholesterol and inactivation of the PC4 gene in mouse causes male infertility (Creemers and Khatib, 2008).

In addition to their crucial role in normal host cell physiology, PCs are hijacked by a variety of pathogens, including several major human pathogenic enveloped viruses. The common feature that links PCs to most of these viruses is the strict requirement of their viral envelope glycoprotein precursors for proteolytic processing by PCs to acquire fusogenic properties required for productive infection and viral spread. The fusion peptide (FP) is a hydrophobic stretch of amino acids contained in the envelope glycoprotein that contacts cellular membranes to allow virus-cell fusion. In the fusion-inactive form of the precursor, the FP is flanked by residues within the envelope glycoprotein. Proprotein convertases recognize and process the viral envelope glycoprotein precursors in the proximity of the FP during their trafficking through the secretory pathway. Upon cleavage, the FP is "liberated" rendering the glycoproteins fusion competent.

In the following sections, we will discuss the role of PCs in maturation of envelope glycoproteins of selected pathogenic viruses of medical importance. We will describe the basic molecular mechanisms underlying the recognition of viral glycoproteins by PCs, including recent evidence for differential recognition of viral and cellular substrates by PCs. In specific cases, we will show how viruses evolved their glycoproteins to hijack the activity of cellular PCs to their own benefit during virus-host co-evolution and discuss the consequences for virus–host interaction and pathogenesis. Particular attention will be given to past and current efforts to evaluate cellular PCs as targets for antiviral therapeutic intervention, in particular in the case of emerging highly pathogenic viruses for which no effective drugs or vaccines are currently available.

3. Viruses depending on basic aa-specific PCs for glycoprotein processing

3.1. Influenza A virus

Influenza A virus belongs to the family of *Orthomyxoviridae*, a large family of enveloped viruses containing a negative-sense, single-stranded segmented RNA genomes with six to eight fragments. The orthomyxoviruses include three major genera, *Influenzavirus*, *Isavirus*, and *Thogotovirus* (Cox et al., 2000). Influenza A virus represents a significant source of global morbidity and mortality

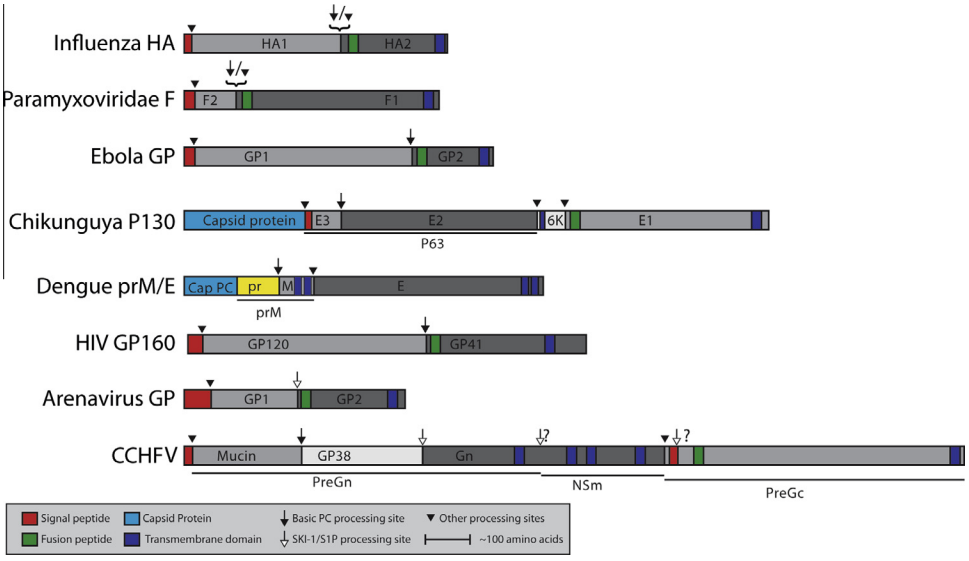

Fig. 2. Schematic representation of the domain arrangement of several viral glycoproteins. Maturation sites are indicated by black arrows for basic PCs; by white arrows for SKI-1/S1P and by arrowheads for non-PCs. See main text for more information.

(Morse et al., 2012; Neumann et al., 2009). This virus is characterized by its ability to undergo rapid changes *via* either antigenic drift, causing the recurring seasonal influenza epidemics, or antigenic shift, involving the exchange of entire genomic segments in re-assortants upon co-infection of a host cell, with the risk of global pandemics (Zambon, 1999). The continuing circulation of highly pathogenic H5N1 influenza viruses in Eurasia and the recent emergence and global spread of pandemic H1N1 2009 are examples of influenza evolution.

Pioneering studies on the influenza hemagglutinin (HA) (Wilson et al., 1981) provided the first insights into the molecular structure of a viral envelope glycoprotein. Since then, influenza HA has served as a classical paradigm for viral fusion active proteins and the determination of its structure provided important concepts on the role of host proteases in viral glycoprotein maturation with important implication for viral pathogenesis. Initially synthesized as a single polypeptide chain, the precursor, HA0 undergoes proteolytic processing into a cell surface exposed globular HA1 and a transmembrane HA2 (Fig. 2). The HA1 subunit is responsible for attachment to the host cell *via* α3-6- or α2-3-linked sialic acid, while HA2 mediates virus–host membrane fusion, triggered by low pH (Skehel and Wiley, 2000). The ability of influenza virus to infect target cells strictly depends on the liberation of the HA2 N-terminal fusion peptide upon cleavage by a host cell protease(s). Initial processing of HA0 is followed by trimming of the C-terminal Arg from HA1 by a virion-associated carboxypeptidase (Garten and Klenk, 1983), resulting in a structural rearrangement of the fusion peptide (Chen et al., 1998). High resolution structures for pre-fusion conformations of HA prior and after cleavage revealed that the overall conformation before and after proteolytic processing are largely identical, with exception of polypeptide structures surrounding the cleavage site (Chen et al., 1998; Wilson et al., 1981).

In addition to its critical role in HA maturation, the amino acid sequence encompassing the HA0 cleavage site has been recognized as a determinant of virulence in some avian strains of influenza. Single basic amino acids are found at the HA0 processing site of low pathogenic avain influenza (LPAI) strains that are typically cleaved by trypsin-like endoproteases. In contrast, HA0 cleavage sites in highly pathogenic avian influenza (HPAI) isolates contain multibasic residues and are processed by PCs (Stieneke-Grober et al., 1992). Differently from trypsin-like enzymes whose tissue expression is restricted to the respiratory and/or gastrointestinal tracts, PCs are widely distributed, likely contributing to the ability of HPAI strains to establish systemic infections. In addition, the subcellular processing sites of HA0 derived from LPAI and HPAI strains are different. LPAI HA0 processing at monobasic sites occurs at the cell surface, e.g. involving human airway trypsin-like type II transmembrane serine proteases TTSPs (Bottcher-Friebertshauser et al., 2010; Okumura et al., 2010), or on released viruses by trypsin (Klenk et al., 1975).

In contrast, HPAI HA0 maturation by PCs (Hamilton et al., 2012; Horimoto et al., 1994) occurs in the TGN, which is consistent with the subcellular localization of furin and furin-like enzymes. It is conceivable that the higher efficiency of intracellular PC-mediated processing of HA0 compared to extracellular cleavage, combined with the wide tissue expression of the basic aa-specific PCs contributes to the widespread systemic infections observed with HPAI strains (Kido et al., 2012; Steinhauer, 1999). However, there is evidence that TMPRSS2, most-likely the more important trypsin-like protease at least in some cell lines, is nearly inactive at the cell surface suggesting that it cleaves HA0 also in a subcellular compartment, similar to PCs (Bottcher-Friebertshauser et al., 2011). In addition, specific amino acids at and surrounding the processing site may modulate the ability of PCs to cleave HA0, as e.g. Gly at position P1′ is highly preferred over other residues (Horimoto and Kawaoka, 1995). Single amino acid mutation/deletion in the proximity of the cleavage site observed in the context of large scale poultry vaccination campaigns were also found to affect furin-mediated processing of HA0 (Pasquato and Seidah, 2008).

Insertion of polybasic motifs upstream the actual *consensus* motif is typically found in HPAI HA0s and correlates with increased HA0 cleavage due to better docking of the substrate to the catalytic pocket of furin (Decha et al., 2008). The ability of HA0 to acquire residues highly favorable for furin recognition has been recently highlighted by Izidoro et al. who reported a major and selective cleavage by furin of substrates sequences derived from virus envelope glycoproteins as compared to other eukaryotic proproteins (Izidoro et al., 2010).

3.2. Paramyxoviruses

The *Paramyxoviridae* are a family of enveloped, negative-strand RNA viruses that include major human pathogens such as measles virus (MV), respiratory syncytial virus (RSV), the parainfluenza viruses, and mumps virus (Lamb and Kolakofsky, 2001). Two envelope glycoproteins are encoded by the paramyxoviruses: the fusion protein (F) and a second glycoprotein responsible for the attachment to the host cell, whose nomenclature varies depending on the particular virus, and has been designated as either hemagglutinin-neuraminidase protein (HN), hemagglutinin protein (H), or glycoprotein (G). The attachment proteins of paramyxoviruses are type II integral membrane proteins, and mostly do not require any post-translational protease processing.

The F protein of paramyxoviruses is responsible for the frequently pH-independent membrane fusion (Morrison, 2003), which can occur between viral and cellular membranes, but also between membranes of infected neighboring cells, resulting in the formation of syncytia (Horvath et al., 1992). The F glycoproteins are type I proteins that form homotrimeric structures, initially synthesized as inactive precursors (F0) to undergo proteolytic cleavage by host proteases into F1 and F2 subunits (Fig. 2). Activation of F0 involves the sequential action of two host enzymes, an endoprotease that cleaves at the carboxyl terminus of a basic residue, and a carboxypeptidase that trims out the newly formed C-terminal basic residue (Homma and Ouchi, 1973; Scheid and Choppin, 1974). Cleavage of F0 occurs at either single or multiple basic residues. Processing of F proteins containing multibasic sequences, e.g. Arg-X-Arg/Lys-Arg↓, occurs in the TGN and seems to involve furin (Gotoh et al., 1992; Klenk and Garten, 1994). F0 proteins that do not contain a *consensus* sequence recognized by furin, but rather a single basic residue, typically Arg, are either cleaved extracellularly by exogenous trypsin-like proteases or undergo re-internalization, followed by processing by cathepsins in endosomal compartments (Diederich et al., 2008; Pager et al., 2006; Pager and Dutch, 2005).

Human respiratory syncytial virus (RSV) causes acute respiratory tract infections frequently associated with severe lung disease, accounting for nearly 50% of all viral pneumonias (Simoes, 1999). Unlike most other paramyxoviruses, the RSV F protein alone, without contribution of the glycoprotein (G), is sufficient to mediate membrane fusion and virus infection (Karron et al., 1997). The RSV F0 undergoes priming in the TGN by processing by basic aa-specific PCs (Basak et al., 2001) leading to the mature and active form of the fusion protein (F2/F1). Interestingly, two conserved cleavage sites have been observed (site I, $RARR^{109}$ and site II, $KKRKRR^{136}$), separated by a stretch of 27 amino acids (pep27) (Gonzalez-Reyes et al., 2001). The presence of two processing sites is rather unusual and their exact role is not yet fully understood. Interestingly, the insertion of the RSV region encompassing the two cleavage sites into the Sendai virus (SeV) glycoprotein, results in SeV chimera that mimic RSV's ability to infect cells in absence of

the attachment protein. Thus, the double processing sites may represent an alternative mechanism to regulate fusion (Rawling et al., 2011).

Measles virus is one of the most contagious human pathogens and represents still a major cause of infant mortality and severe disease at the global scale (Moss and Griffin, 2012). MV displays two envelope glycoproteins, hemagglutinin (H) and the F1/F2 complex. The non-fusogenic precursor F0 is processed in the TGN into F1 and F2 subunits by furin, but not PC5/6B or PC7, (Bolt and Pedersen, 1998). Point mutation of F0 Arg_{112}, critical for furin recognition, leads to aberrantly cleaved F proteins, unable to generate fusion competent viral particles (Alkhatib et al., 1994). Thus, maturation of F0 by furin is a pre-requisite for syncytium formation, hemolysis, and virus entry (Scheid and Choppin, 1977). While MV particles containing uncleaved F0 proteins are still released, they are not infectious unless processing takes place, similar to influenza virus (Fujinami and Oldstone, 1981).

3.3. Alphaviruses

The alphaviruses are an important family of emerging and re-emerging viruses that are transmitted by arthropod vectors to humans and a wide range of mammals (Gould et al., 2010). With several million infections annually, alphaviruses cause diseases such as arthritis and encephalitis. Among the alphaviruses, chikungunya virus (CHIKV) and the related Venezuelan equine encephalitis virus (VEEV) pose serious public health problems and are defined as Category C and B Select Agents, respectively (Weaver and Reisen, 2010).

CHIKV is a mosquito-transmitted *Alphavirus* that causes in humans an acute infection characterized by polyarthralgia, myalgia, headache, and fever (Staples et al., 2009). CHIKV envelope proteins consist of two type I glycoproteins p62, also known as E3E2, and E1 (Fig. 2). The p62/E1 interaction is essential for proper folding. P62 is initially synthesized as precursor and during virion maturation cleaved at multibasic motifs by basic aa-specific PCs in the (post-)TGN compartments (Barr, 1991; Zhang et al., 2012). Viral production of chimeric viruses carrying glycoprotein defective in E3E2 processing was found to be compromised (Heidner et al., 1994). Interestingly, the accessibility of the E3E2 protein to various basic aa-specific PCs may be modulated by the nature of the residues surrounding the four amino acid *consensus* sequence at the cleavage site: E3E2 from African CHIKV strains undergoes efficient cleavage at the HRQRR↓ site only by membrane-anchored furin whereas a CHIKV strain of Asian origin is processed by a larger spectrum of PCs at a RRQRR↓ site. The latter shows further a lower susceptibility to E3E2 cleavage inhibition by chloroquine (Ozden et al., 2008). Considering the crucial role of PC-mediated processing of viral envelope GPs for viral multiplication, it is conceivable that this one amino acid difference can critically influence virus spread and tropism, similarly to the situation found with some avian influenza isolates described above.

3.4. Dengue virus

The *Flaviviridae* family includes several important human pathogens, including West Nile virus (WNV), tick-borne encephalitis virus (TBEV), and dengue virus (DENV) (Lindenbach et al., 2007). DENV is the most prevalent mosquito-borne viral pathogen and threatens today almost half of the world's population with an estimated 50–100 million new infections and up to 500,000 hospitalized cases occurring annually (Gould and Solomon, 2008). Dengue is found in tropical and sub-tropical regions around the world, predominantly in urban and semi-urban areas and thereby affecting mostly developing countries. While the majority of primary DENV infections results in classical dengue fever, secondary infection with different DENV serotypes is frequently associated with severe Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Halstead, 2007).

There is currently no licensed vaccine available against DENV, and the lack of antiviral drugs severely restricts therapeutic options, resulting in high mortality of hospitalized patients. In addition to the extent of human suffering inflicted by DENV in large regions of the developing world, epidemiological data predict a significant geographic expansion of this pathogen with an increasing risk of secondary infections associated with severe DHF/DSS. Elegant structural and biochemical studies shed light on the molecular mechanisms of DENV maturation, revealing a key role of PC-mediated processing of DENV envelope (E) protein, which is responsible for the host cell attachment and subsequent fusion (Li et al., 2008; Yu et al., 2008). Assembly and budding of DENV take place at the ER membrane, resulting in the release of immature virions of non-infectious nature into the ER lumen. These immature particles are decorated with heterodimers of the precursor membrane protein (prM) and E protein. During transport through the secretory pathway, the luminal pH drops, triggering the exposure of a furin *consensus* sequence previously hidden within the prM (Fig. 2).

Cleavage of prM by furin-like proteases in the TGN is crucial for maturation of the E protein involving rearrangement and exposure of the receptor-binding domain conferring infectivity (Yu et al., 2008). However, after cleavage, the pr stays associated with the fusion loops and dissociation of pr requires a change in pH above the mildly acidic conditions of the TGN. The data at hand suggest that exposure to neutral pH after exit from the secretory pathway is required for DENV virion to acquire full fusion competence and illustrates how viruses hijack PCs within particular subcellular compartment to orchestrate glycoprotein maturation with viral egress to avoid premature fusion. Interestingly, the charged residues around the processing site of a large number of DENV isolates show little variation, suggesting a role of the conserved sequence recognition sequence at the prM junction. Accordingly, substitution of the prM cleavage site with those found in other flaviviruses greatly enhanced prM cleavability, but impaired DNEV export (Keelapang et al., 2004), suggesting specific adaptation of the prM cleavage sites during virus-host co-evolution.

3.5. Human immunodeficiency virus

The lentivirus human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS). The HIV-1 envelope glycoprotein precursor gp160 (*Env*) is processed into the mature gp41/gp120 heterodimers by furin-like proteases (Fig. 2) (Hallenberger et al., 1992). Gp120 is responsible for initial target cell attachement *via* the CD4 receptor and the co-receptors CCR5/CXCR4, while gp41 has structural features of class I viral fusion proteins and is responsible for membrane fusion. So far, the structure of the gp120 core in complex with CD4 (Kwong et al., 1998) and that of a post-fusion form of gp41 have been solved (Chan et al., 1997), but little information is available on the entire gp160 precursor. Two monoclonal antibodies directed against the V3 loop do recognize immature gp160 but not gp120 (Pinter et al., 1993), suggesting that cleaved and uncleaved HIV-1 envelope glycoproteins have different conformations. The gp160 is cleaved at an REKR↓ motif (site-1) by basic proprotein convertases (Decroly et al., 1994). A second potential PC *consensus* sequence, KAKR (site-2), is present upstream of the physiological processing site and cleaved only in ca. 15% of total gp160 (Fenouillet and Gluckman, 1992).

Conformational studies revealed that site-2 is embedded in a helical segment, whereas site-1 resides in an exposed loop (Oli-

va et al., 2002). Although not usually cleaved, site-2 plays a crucial role in the maturation of the envelope glycoprotein at site-1 because point mutations in the KAKR sequence abolish cleavage at site-1 (Bosch and Pawlita, 1990). In short peptides mimicking the gp160 fragments, the presence of a helical segment upstream the cleavage site favors enzyme/substrate recognition (Falcigno et al., 2004). Moreover, recent investigations on synthetic peptides encompassing the gp160 cleavage site suggest that these multibasic regions provide inverted glycosaminoglycans (GAGs) *consensus* motifs whose binding to GAGs favors exposure of the physiological processing site (Pasquato et al., 2007). Nonetheless, the exact role of site-2 remains to be defined.

Processing at site-1 is necessary for infectious particle production and required for efficient incorporation of the envelope glycoprotein into virions (Dubay et al., 1995). Using co-expression of recombinant proteins, the preference of basic aa-specific PCs for gp160 processing has been studied, revealing an efficiency of cleavage of furin >PACE4 > PC7 > PC5/6 > PC1, and no significant activity of PC2 (Decroly et al., 1997). The absence or weak gp160 processing by PC1 and PC2 is likely related to their normal specific subcellular localization in dense core granules and optimal activity at low pH. Intact gp160 appears to be processed mainly by exogenous soluble furin at the cell surface (Moulard et al., 1999). Inhibitors of basic aa-specific PCs (Moulard et al., 1994) or mutations at the highly conserved REKR↓ sequence of gp160 (Willey et al., 1991) results in the incorporation of uncleaved gp160 into budding particles and subsequent production of non-infectious HIV-1 virus that are incapable of fusion.

4. Viruses depending on SKI-1/S1P for glycoprotein processing

In contrast to basic aa-specific PCs that are used by a wide variety of viruses to process their envelope glycoproteins, the eight member of the PC family, SKI-1/S1P is hijacked by only two families of emerging viruses, the Arenaviruses and some members of the Bunyavirus family. Research in the past years on the use of SKI-1/S1P by Arena- and Bunyaviruses revealed some interesting aspects of the interaction of emerging viruses with host cell components that are of relevance for the design of novel antiviral therapeutic against these pathogens.

4.1. Arenaviridae

The arenaviruses are a large and diverse family of emerging enveloped negative strand viruses that includes several severe pathogens associated with hemorrhagic fevers with high mortality in humans. The prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) represents a powerful model for experimental virology (Buchmeier et al., 2007; Oldstone, 2002) and is a re-emerging human pathogen of relevance in pediatrics and transplantation medicine (Bonthius et al., 2007; Bonthius and Perlman, 2007; Fischer et al., 2006). The *Arenaviridae* family is currently classified into more than twenty species divided in Old (OW) and New World (NW) virus groups (Charrel et al., 2008). The OW arenavirus Lassa virus (LASV) and Lujo virus as well as the NW arenaviruses Junin, Machupo, Guanarito, Sabia, and Chapare are the causative agents of severe viral hemorrhagic fevers with high mortality and represent serious public health problems (Briese et al., 2009; Delgado et al., 2008; Geisbert and Jahrling, 2004). Arenaviruses have non-lytic life cycle and are able to establish persistent infections in mammalian cells *in vitro* and in their natural rodent reservoirs *in vivo*.

A crucial step in the arenavirus virus life cycle is the maturation of the viral envelope glycoprotein precursor (GPC) (Fig. 2). The arenavirus GPC is synthesized initially as a single polypeptide that is sequentially cleaved by cellular signal peptidases and then by SKI-1/S1P (Beyer et al., 2003; Kunz et al., 2003; Lenz et al., 2001; Rojek et al., 2008). Processing of GPC by SKI-1/S1P yields the N-terminal GP1 (44 kDa), which is implicated in binding to cellular receptors (Borrow and Oldstone, 1992), and the transmembrane GP2 (35 kDa) that mediates fusion and resembles class I viral fusion proteins (Eschli et al., 2006; Igonet et al., 2011).

Arenavirus GPs contain a remarkably stable signal peptide (SSP) of unusual length, which forms part of a mature tripartite complex SSP/GP1/GP2, representing the functional unit of receptor binding and fusion (Eichler et al., 2003a,b; Froeschke et al., 2003; York et al., 2004). Due to the unusual residue pattern at the GP1/GP2 cleavage site, the protease responsible was found long after the identification of the typical *consensus* motifs of arenavirus GPCs. SKI-1/S1P, the eight member of PCs, cleaves OW LASV (Lenz et al., 2001), LCMV (Beyer et al., 2003) and NW arenavirus (Rojek et al., 2008) GPCs. Maturation of GPC by SKI-1/S1P is strictly required for the production of infectious particles and viral spread, and inhibition of SKI-1/S1P results in the formation of non-infectious "naked" particles lacking GP (Beyer et al., 2003; Kunz et al., 2003; Lenz et al., 2001; Rojek et al., 2008). Proof-of-concept studies with protein- and peptide-based SKI-1/S1P inhibitors revealed that targeting of GPC maturation represents a novel and promising antiviral strategy (Maisa et al., 2009; Rojek et al., 2010), an aspect that will be further developed below.

The processing sites of arenavirus GPCs are unique and differ from cellular substrates because of their mimicry of SKI-1/S1P B/B' or C autoprocessing motifs. Junin GPC contains the RSLK↓ (B/B′ site) sequence while LASV and LCMV GPCs are cleaved at RRLL↓ and RRLA↓ motifs (C site), respectively (Pasquato et al., 2011). Lassa GPC undergoes SKI-1/S1P processing early in the secretory pathway while LCMV GPC was shown to be processed in a late Golgi or post-Golgi compartment. However, membrane-associated SKI-1/S1P is found predominantly in the early Golgi where cellular SKI-1/S1P substrates are cleaved (Pullikotil et al., 2007). The non-overlapping sub-cellular localization of viral *vs* cellular substrates has been further supported by the recent finding that SKI-1/S1P mediated processing of the host cell's activating transcription factor-6 is unaffected by arenavirus infection (Pasqual et al., 2011).

How arenaviruses are able to select a specific sub-cellular compartment for SKI-1/S1P-mediated maturation is still matter of investigation. Subtle changes of the sequence at the cleavage site have drastic effects on GPC maturation, despite maintaining the RXLX↓ *consensus* cleavage motif of SKI-1/S1P. Processing of a LCMV GPC chimera carrying the LASV cleavage site (RRLL) is re-directed from the Golgi to the ER, whereas introduction of the LCMV GPC cleavage motif (RRLA) into the LASV GPC backbone results in an uncleavable protein (Burri et al., 2012). Similar to basic aa-specific PCs, residues surrounding the SKI-1/S1P processing site may affect substrate-enzyme recognition. In particular, an aromatic amino acid at P7 highly favors cleavage of both LASV (Pasquato et al., 2006) and LCMV GPC (Beyer et al., 2003), becoming a likely attractive viral drug target. Interference with P7–S7 interaction by a small molecule may not alter the SKI-1/S1P catalytic triad and thus its activity towards cellular substrates that generally lack aromatic P7 amino acids. Modulation of SKI-1/S1P activity is feasible and can be achieved by specific point mutations within its prosegment. Replacement of R130, R134 with E at the B/B′ site results in impairment of SKI-1/S1P maturation and selective inhibition of viral GPC cleavage without affecting processing of cellular substrates (Burri et al., 2012; Popkin et al., 2011). The different extent of viral *vs* cellular protein processing provided the first evidence for differential recognition of arenaviral and cellular substrates by SKI-1/S1P.

These data suggest a strategy developed by viruses permitting viral persistence in their natural rodent host species without affecting host viability.

4.2. Bunyaviridae

The bunyaviruses are a complex and diverse family of emerging enveloped negative strand viruses that contains a number of arthropod-born human pathogens that merit significant attention as public health threats. Crimean–Congo hemorrhagic fever (CCHF) is a widespread tick-borne viral disease that may affect humans who, upon infection, commonly show severe symptoms, with a 30% mortality rate. The processing of CCHF viral proteins is more complicated compared to other Bunyaviruses and not yet fully understood. A crucial step in the life cycle of CCHF virus is the biosynthesis of the envelope GN and GC glycoproteins which are responsible for the virus attachment to the target cell through a presently unknown receptor (Ergonul, 2006). The M segment of CCHF virus has one open reading frame which encodes a precursor polypeptide, with a highly variable amino-terminal domain and a fairly conserved carboxyl-terminal region (Seregin et al., 2004). The precursor is processed by co-translational cleavage into two primary precursor proteins, PreGN and PreGC (Fig. 2), which give rise to the two mature structural proteins, GN and GC, respectively, through further posttranslational cleavages.

The PreGN glycoprotein is cleaved early in the secretory pathway by SKI-1/S1P at its N-terminus following the *consensus* motif $RRLL_{519}$, resulting in mature GN and the GP85/GP160 protein (Sanchez et al., 2002; Vincent et al., 2003). The latter includes the mucin-like highly variable domain and a second region, namely GP38. The two units are cleaved at the furin-like motif $RSKR_{247}$ (Vincent et al., 2003). Inhibition of the furin-like mediated processing does not interfere with the SKI-1/S1P processing (Sanchez et al., 2006). In addition, C-terminal cleavage of preGN leads to the generation of a NSM non structural protein. The exact processing site has not been identified and might occur following the $QSAP_{841}$ sequence, whereas the associated enzyme has been proposed to be a member of the intramembrane cleaving proteases family (Altamura et al., 2007). The functional roles of mucin-like, GP38, and NSM regions are not known.

PreGC maturation occurs upon processing at the N-terminal $RKPL_{1040}$, which does not appear to be recognized by SKI-1/S1P but is likely cleaved by a related protease (Vincent et al., 2003). This hypothesis is supported by the evidence that mature GC is predominantly localized in the ER (Haferkamp et al., 2005), while the membrane bound SKI-1/S1P is mostly found in the Golgi and endosomal compartments (Pullikotil et al., 2007). Interestingly, absence of SKI-1/S1P induces the release of non-infectious particles deficient in either cleaved or uncleaved glycoproteins (Bergeron et al., 2007), similarly to the SKI-1/S1P dependent arenaviruses and differently from other furin-like dependent viruses (Elshuber et al., 2003; Zhang et al., 2003).

5. PCs as targets for antiviral therapy

The history of drugs designed to block enzymatic activity dates back to the 1950s. Since then, many new inhibitors have been discovered against more than 50 different proteases, with several compounds currently being in advanced clinical trials or used clinically (Turk, 2006). Protease genes represent ~2% of the mouse and human genome (Puente et al., 2003) and are involved in many physiological and pathological processes, becoming important drug targets in the control of a large number of disease states, e.g. cardiovascular diseases (targeting angiotensin-converting enzyme, ACE and renin), and diabetes type II. In addition, viral proteases are targeted by several clinically approved drugs against HIV-1 and hepatitis C virus.

Direct inhibition of the mature protease is the most widely used approach to interfere with the normal enzymatic activities. Although this technique often gives excellent results, a catalytically dead enzyme results in a general loss of activity towards all substrates, cellular and viral alike. Thus, unwanted side effects must be carefully taken into consideration when direct inhibition of a given protease is envisioned as antiviral therapeutic approach. Given the crucial role of PCs in the maturation of several viral glycoprotein precursors and thus infection, efforts have been made to develop inhibitors mainly against furin, representative of the basic aa-specific PCs, and SKI-1/S1P. Here, we divide the inhibitors into two classes, large and small molecules for each group of proteases.

5.1. Basic aa-specific PC inhibitors

5.1.1. Protein-based strategies

Three major classes of protein-based inhibitors have been developed as antiviral agents targeting PC-mediated processing: α_1-Antitrypsin and α_2-macroglobulins variants, and the prosegments of PCs.

α_1-Antitrypsin is a 494-aa inhibitor of neutrophil elastase. Consistent with the mechanism of action of other serpins, α_1-Antitrypsin acts as a suicide inhibitor, entrapping the enzyme in a stable complex upon cleavage at the reactive site loop (RSL) $AIPM_{358}\downarrow$ which allows the substrate to rearrange into a new conformation. A naturally occurring mutation of M_{358} to R within the RSL changes the serpin specificity from neutrophil elastase to thrombin inhibitor and is called α_1-AT-Pittsburgh (α_1-PIT). Further mutations have been designed in the RSL of α_1-PIT to introduce the PCs $B(X)_nB\downarrow$ motif (B, basic aa; X any aa except Cys; $n = 0, 2, 4, 6$), resulting in a furin-like enzyme inhibitor (α_1-PDX) with a potency 3000-fold higher than α_1-PIT (Anderson et al., 1993).

Recently, a novel α1-antitrypsin variant AVNR ($AVPM_{352}$ into $RVNR_{352}$) was identified as a highly selective inhibitor of furin *ex vivo* and *in vitro* whereas other PCs members including PACE4, PC5/6 and PC7 were not affected (Hada et al., 2012). Alpha1-antitrypsin variants were found to block processing of HIV-1 gp160 (Anderson et al., 1993), MV F0 (Watanabe et al., 1995), and Zaire Ebola and Marburg viruses GP (Stroher et al., 2007). Interference with HIV-1 gp160 maturation by α_1-PDX does correlate with significant inhibition of viral replication (Bahbouhi et al., 2000). However, extensive processing of the inhibitor was observed during infection thus preventing its use to stably block HIV-1 replication (Bahbouhi et al., 2001). Glioma cells stably expressing α_1-PDX and infected with MV did not show syncytia formation with consequent infectious-virus titer reduction by 3–4 orders of magnitude (Watanabe et al., 1995). On the contrary, filoviruses are weakly affected by this class of inhibitors, according to the evidence that furin-mediated envelope glycoprotein cleavage is not required for efficient virus cell-to-cell propagation (Stroher et al., 2007).

$Alpha_2$-macroglobulin (α_2-M) is a 1,474 aa homotetrameric glycoprotein, containing an internal unusual S-ester bond which confers inhibitory properties against all classes of proteases (Kan et al., 1985). $Alpha_2$-M is characterized by a flexible peptide stretch called bait which includes cleavage sites specific to different enzymes. Introduction of a multibasic motif lead to the discovery of a potent furin inhibitor (FUR-α_2-M). Interestingly, although the cleavage of several cellular furin substrates is totally abrogated by FUR-α_2-M overexpression, only soluble HIV-1 gp60 maturation is impaired whereas the full length form is efficiently processed (Van Rompaey et al., 1997).

Another class of protein-based inhibitor is based on the property of intra-cellularly expressed prepro-segments of PCs (ppPC) to act as cellular inhibitors of the cognate enzyme. HIV-1 gp160

processing is inhibited by ppfurin as well as ppPC7, although the latter is less efficient (Zhong et al., 1999).

5.1.2. Peptide-based compounds and small molecules

Decanoylated chloromethylketone (cmk) – peptides represent very well characterized basic aa-specific PC inhibitors developed by Garten's research group in the early 1990s (Hallenberger et al., 1992). Cmks contain a peptidic core that mimics the $B(X)_nB\downarrow$ recognition motif of furin-like enzymes linked to a chloromethylketone moiety at the C-terminus. When cleavage takes place, cmks act as irreversible inhibitors by alkylation of the active site histidine. The long hydrophobic tail at the N-terminus renders the small molecule cell permeable. Thus, *in vitro* dec-RVKR-cmk was shown to potently block the cleavage of viral envelope precursors including HPAIV HA0 (Garten et al., 1994; Stieneke-Grober et al., 1992), HIV-1 gp160 (Garten et al., 1994; Hallenberger et al., 1992), CCHFV preGN (Sanchez et al., 2002), CHIKV p62 (Ozden et al., 2008), and parainfluenza virus F protein (Garten et al., 1994).

Blocking basic aa-specific PCs by cmks induces a stronger inhibition of CHIKV infection than the lysosomotropic agent chloroquine that blocks fusion (Ozden et al., 2008). Despite an IC_{50} of dec-RVKR-cmk in the low micromolar range, the molecule cannot be used as therapeutic agent due to its chemical instability, relative cytotoxicity, and lack of specificity. New generations of peptide-based small inhibitors with improved stability and specificity were recently published. Phenylacetyl-Arg-Val-Arg-amidinobenzylamide and N-terminal 4-/3-(guanidinomethyl)phenylacetyl-Arg-Val-Arg-amidinobenzylamide are newly designed furin inhibitors containing decarboxylated P1 arginine mimetics. Side-by-side comparison showed that they block influenza virus HA0 processing similarly to dec-RVKR-cmk, significantly reducing Influenza A H7N1 virus propagation in a long-term infection test (Becker et al., 2012, 2010). Also polyarginines are potent inhibitors of furin-like proteases with an IC_{50} in the low nanomolar range (Cameron et al., 2000). Made of six or more residues of arginine, this class of inhibitors contains several PCs *consensus* sites which compete with natural substrates for cleavage. Their efficiency in blocking gp160 processing was shown to suppress productive HIV-1 infection *in vitro* (Kibler et al., 2004), thus opening the possibility of therapeutic applications.

Other peptides, mimicking the cleavage site of different enveloped viruses and derivatized with a cell-permeable N-terminal decanoyl group, were found to potently inhibit gp160 processing but significant cytotoxicity limits their use as antiviral drugs (Decroly et al., 1994). More recently, peptides encompassing the cleavage motif of avian influenza virus HA0 were found to inhibit H5N1 envelope glycoprotein processing (Shiryaev et al., 2007). Finally, multibranched peptides consisting of the gp160 derived KIEPLGVAPTKAKRRVVNREKR linked to a lysine core (CLV) are potent inhibitors of HIV-1 infection without important toxicity. The mechanism of action has not been fully understood, although a delay in gp160 processing has been detected (Barbouche et al., 2000, 1998).

5.2. SKI-1/S1P inhibitors

So far, two classes of viruses were found to be SKI-1/S1P dependent, the Bunyavirus CCHFV and Arenaviruses. As peculiar characteristic of these pathogens, infection of SKI-1/S1P null cells gives rise to noninfectious naked particles devoid of envelope glycoproteins, based on a yet unknown mechanism of selective incorporation of cleaved glycoproteins (Kunz et al., 2003). In addition, the lack of active SKI-1/S1P does not induce the appearance of arenavirus escape variants, commonly observed for viruses dependent on basic aa-specific PCs. Thus, increasing pieces of evidence suggest that inhibition of SKI-1/S1P is a promising therapeutic approach against SKI-1/S1P dependent infection.

5.2.1. Protein-based strategies

α_1-PDX is a potent furin-like enzyme inhibitor engineered from the naturally occurring α_1-AT protein. Based on the strict homology of the catalytic sites of basic and non-basic aa-specific PCs and the prediction that this serpin would react equally with all members of active proprotein convertases, the reactive site loop of α_1-AT was further mutated to introduce the SKI-1/S1P BX(hydrophobic)X$\downarrow$ motif. Overexpression of the α1-AT RRVL variant was found to inhibit both CCHFV preGC (Pullikotil et al., 2004) and LASV GPC maturation (Maisa et al., 2009). Moreover, blocking arenavirus GPC processing has a strong antiviral effect, highly containing cell-to-cell spread and *de novo* viral particles production (Maisa et al., 2009).

5.2.2. Peptide-based compounds and small molecules

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

PF-429242 is a reversible, competitive aminopyrrolidineamide inhibitor of SKI-1/S1P, recently discovered by Pfizer Inc. and able to efficiently block processing of endogenous cellular substrates (Hay et al., 2007). Following PF-429242 administration *in vivo*, SREBP-2 activation in mice is dramatically reduced and this translates in a marked drop of plasma cholesterol (Hawkins et al., 2008). Pharmacological inhibition of SKI-1/S1P activity by PF-429242 also blocks LASV and LCMV GPC maturation with a potent containment of virus cell-to-cell propagation and no off-target effects (Pasquato et al., 2012; Urata et al., 2011). Of particular interest is the inability of arenavirus to overcome the inhibition of the enzyme since no escape variants have ever been detected during/after treatment of persistent infection over several weeks. On the contrary, chronically LCMV infected cells are rapidly and efficiently cleared by low concentrations of PF-429242 (Pasquato et al., 2012). Lack of reported cytotoxicity together with the excellent *in vitro* IC_{50} make this small organic compound an attractive antiviral drug for further development in the context of treatment of human arenavirus infections.

6. Conclusions

Processing of viral envelope glycoprotein precursors is a crucial step common to several enveloped viruses to achieve a fully mature infectious status. This crucial step in the virus life cycle is frequently dependent on host proteases, in particular PCs employed by several important human pathogens, including HIV-1, highly pathogenic influenza viruses, paramyxoviruses, arenaviruses, and bunyaviruses. Given the PCs key role in virus pathogenesis and the experimental evidence that *in vitro* enzymatic inhibition has drastic consequences on viral spread and production, several attempts have been made to develop specific PCs inhibitors. Although proprotein convertases are essential for normal cell functions in adults, several studies have nevertheless shown that specific inhibition of a particular PC is not detrimental in case of *in vivo* short-term treatment (Hay et al., 2007; Shiryaev et al., 2007).

Targeting viral glycoprotein cleavage therefore appears to be a novel promising therapeutic approach, especially to combat pathogenic emerging viruses that cause acute infection with high mortality in man. 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. Therefore, the development of novel broad antivirals is critical to meet these unmatched medical problems. Inhibition of proprotein convertases is emerging as a possible answer to this need.

Acknowledgements

This work was supported by the Swiss National Science Foundation grant FN 31003A-135536 (S.K.), funds from the University of Lausanne (S.K.). This work was partially supported by a Canada Research Grant to NGS (# 216684).

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