Review Articles

Subtilisin-like proprotein convertases (SPCs); host enzymes controlled viral protein processing and maturation

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Abstract

The cellular subtilisin-like proprotein convertases (SPCs) are responsible for virion maturation process which occurs in secretory vesicles, primes virion maturation and viral infectivity. Eight SPCs, SPC1 (furin/PACE), SPC2 (PC2), SPC3 (PC1/PC3), SPC4 (PACE4), SPC5 (PC4), SPC6 (PC5/PC6A) and SPC7 (LPC/PC7/PC8) and PCSK9, were identified. The consensus substrate sequence is -RX(K/R)R▼X- (X can be any amino acid, ▼ represents the cleavage site). The conformational change of viral proteins can be triggered by a low pH in the endosomes, as in the case of influenza virus, or by the interaction with a secondary receptor protein at the cell surface, as the case of HIV. In flaviviruses, the functional roles of charged residues locate to the SPC consensus sequence in cleavage site of prM protein and provide cleavability affect to virus replication. Changes in the prM-cleavage level were associated with altered proportions of extracellular virions and subviral particles. The hemagglutinin (HA) protein is a critical determinant of the pathogenicity of avian influenza viruses, with a clear link between HA cleavability and virulence. The highly pathogenic avian influenza virus, in which contain high numbers of basic amino acid sequence at the HA cleavage site, can be converted to low numbers of basic amino acid sequence of a typical avirulent virus. The processing by SPCs is an important control mechanism for the biological activity of viral surface proteins. The molecular mechanisms underlying the recognition of SPCs by viral glycoproteins were described, including recent findings demonstrating differential SPC-recognition of viral and cellular substrates. Proteolytic activation of envelope glycoproteins is necessary for entry of viruses into the host cell and, hence, for their ability to undergo multiple replication cycles. Proteolytic cleavage is the first step in the activation of virus fusion proteins and is followed by a conformational change resulting in the exposure of the fusion domain. The conformational change can be triggered by a low pH in the endosomes, or by the interaction with a secondary receptor protein at the cell surface.

Keywords: subtilisin-like proprotein convertases, proteolytic cleavage, viral protein, virus maturation

กลุ่มเอนไซม์ Subtilisin-like proprotein convertases (SPCs); เอนไซม์ของเซลล์โฮสต์ที่ควบคุมการตัดโปรตีนและการสมบูรณ์แบบของอนุภาคเชื้อไวรัส

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บทคัดย่อ

กลุ่มเอนไซม์ subtilisin-like proprotein convertases (SPCs) ของเซลล์ มีส่วนสำคัญสำหรับขบวนการเปลี่ยนแปลงอนุภาคของเชื้อไวรัสจากรูปแบบของอนุภาคไม่สมบูรณ์สู่รูปแบบของอนุภาคสมบูรณ์และให้มีความสามารถในการติดต่อเข้าเซลล์ ซึ่งเอนไซม์กลุ่มนี้พบแล้ว 8 ชนิด คือ SPC1 (furin/PACE) SPC2 (PC2) SPC3 (PC1/PC3) SPC4 (PACE4) SPC5 (PC4) SPC6 (PC5/PC6A) SPC7 (LPC/PC7/PC8) และ PCSK9 ลำดับกรดอะมิโนที่เอนไซม์กลุ่มนี้จดจำและเข้าไปตัดที่ตำแน่งนั้น คือ -RX(K/R)R▼X- (X แทนกรดอะมิโนชนิดใดๆ และเครื่องหมาย ▼ แทนตำแหน่งกรดอะมิโนที่โปรตีนถูกตัด) หลังจากโปรตีนของไวรัสถูกตัดแล้วจะมีการเปลี่ยนแปลงรูปลักษณะของโปรตีนซึ่งถูกเหนี่ยวนำโดยสภาวะความเป็นกรดระดับต่ำๆ ภายในเอนโดโซมของเซลล์ พบได้ในกรณีขบวนการเพิ่มจำนวนของไวรัสไข้หวัดใหญ่ (influenza virus) และไวรัสเอดส์ (HIV) ในทำนองเดียวกันเชื้อไวรัสในกลุ่ม flaviviruses การตัดที่โปรตีน prM ของไวรัสมีบทบาทต่อความสามารถในการเพิ่มจำนวนของเชื้อภายในเซลล์โฮสต์ และขึ้นอยู่กับชนิดกรดอะมิโนที่มีประจุรอบบริเวณตำแหน่งที่ถูกตัดโดยเอนไซม์ SPCs การถูกตัดโปรตีนฮีแมกกลูตินิน (hemagglutinin; HA) ของไวรัสไข้หวัดใหญ่มีความสำคัญอย่างมากต่อการก่อความรุนแรงของเชื้อไวรัสสายพันธุ์นั้น เชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ที่มีความรุนแรงสูงจะมีกรดอะมิโนที่มีประจุบวก (basic amino acid) จำนวนมากที่ตำแหน่งที่ถูกตัดของโปรตีน HA ในทางตรงข้ามกันเชื้อไข้หวัดใหญ่สายพันธุ์ที่มีความรุนแรงต่ำจะมีกรดอะมิโนที่มีประจุบวกจำนวนน้อยที่ตำแหน่งดังกล่าว กระบวนการตัดโปรตีนโดยเอนไซม์ SPCs จึงเป็นขั้นตอนที่สำคัญที่ควบคุมการสมบูรณ์แบบ (maturity) ของไวรัส ซึ่งกระบวนการในระดับโมเลกุลในไวรัสหลายชนิดนอกเหนือจากที่กล่าวได้ถูกค้นพบและอธิบายมาแล้วและได้รวบรวมมาในบทความนี้ กระบวนการ proteolytic activation เหล่านี้ เป็นสิ่งจำเป็นสำหรับไวรัสในการเข้าสู่เซลล์ และเกี่ยวข้องกับความสามารถในการเพิ่มจำนวนของไวรัสในเซลล์โฮสต์ และเป็นขั้นตอนแรกๆ ที่กระตุ้น fusion proteins ของอนุภาคไวรัสให้มีการเปลี่ยนแปลงโครงสร้างอันเป็นผลให้แสดง fusion domain ออกมา และทำให้เกิดขบวนการเชื่อมรวม (fusion) กับเซลล์โฮสต์ ซึ่งมักจะเกิดในสภาวะที่สิ่งแวดล้อมโดยรอบเซลล์มีสภาวะเป็นกรดระดับต่ำ

คำสำคัญ : เอนไซม์ SPCs, การตัดโปรตีนของเอนไซม์, โปรตีนของเชื้อไวรัส, การเป็นอนุภาคที่สมบูรณ์ของไวรัส

Introduction

There are many mechanisms of viruses using host machinery for their replication, viral protein processing and virion maturation. The virion maturation process is one of the most challenging events to be completely understood. Virion maturation usually involves structural changes in the virus particle, which may result from specific cleavage of envelop or capsid proteins to form the mature particle or conformational changes in protein during assembly. It completely changes the morphology and conditions within the virion and primes viral infectivity by properly arranging the viral envelop or capsid protein, which contains the viral genome and enzymes. In many viruses such as *flaviviruses, orthomyxoviruses, paramyxoviruses, retroviruses;* cellular subtilisin-like proprotein convertases (SPCs) or trypsin-like molecules are responsible for virion maturation process which occurs in secretory vesicles as the virus buds into them prior to release at the cell surface.

The processing of precursor proteins via limited proteolysis is an important and widely used cellular mechanism for the generation of biologically active proteins and peptides in appropriate cellular compartments. The major endoproteolytic processing enzymes of the secretory pathways are the homologous endoproteases with bacterial subtilisin or the yeast processing protease, Kexin (Kex2p) (Nakayana, 1997; Steiner, 1998; Zhou *et al,* 1999), which is encoded by the *fur* gene on chromosome 15. Subsequently, eight mammalian Kexin-homologue enzymes were identified. Although each enzyme had been independently named by it discoverers, a simplified nomenclature for the group of mammalian processing proteases has been proposed (Chan *et al,* 1992; Bergeron *et al,* 2000), using the term subtilisin-like proprotein convertases (SPCs); SPC1 (furin, or pair amino acid convertase enzyme: PACE), SPC2 (Prohormone convertase 2: PC2), SPC3 (Prohormone convertase 1: PC1, or Prohormone convertase 3: PC3), SPC4 (Pair amino acid convertase enzyme 4: PACE4), SPC5 (Prohormone convertase 4: PC4), SPC6 (Prohormone convertase 5: PC5, or Prohormone convertase 6A: PC6A) and SPC7 (Lymphoma prohormone convertase: LPC, or Prohormone convertase 7: PC7, or Prohormone convertase 8: PC8). Recently, Proprotein convertase subtilisin/kexin type 9 (PCSK9), new member of SPC family was found. PCSK9 cleave amino acid residues and modulate the activity of precursor proteins. Evidence from patients and animal models carrying genetic alterations in PCSK9 members show that PCSK9 members are involved in various metabolic processes (Choi and Korstanje, 2013)

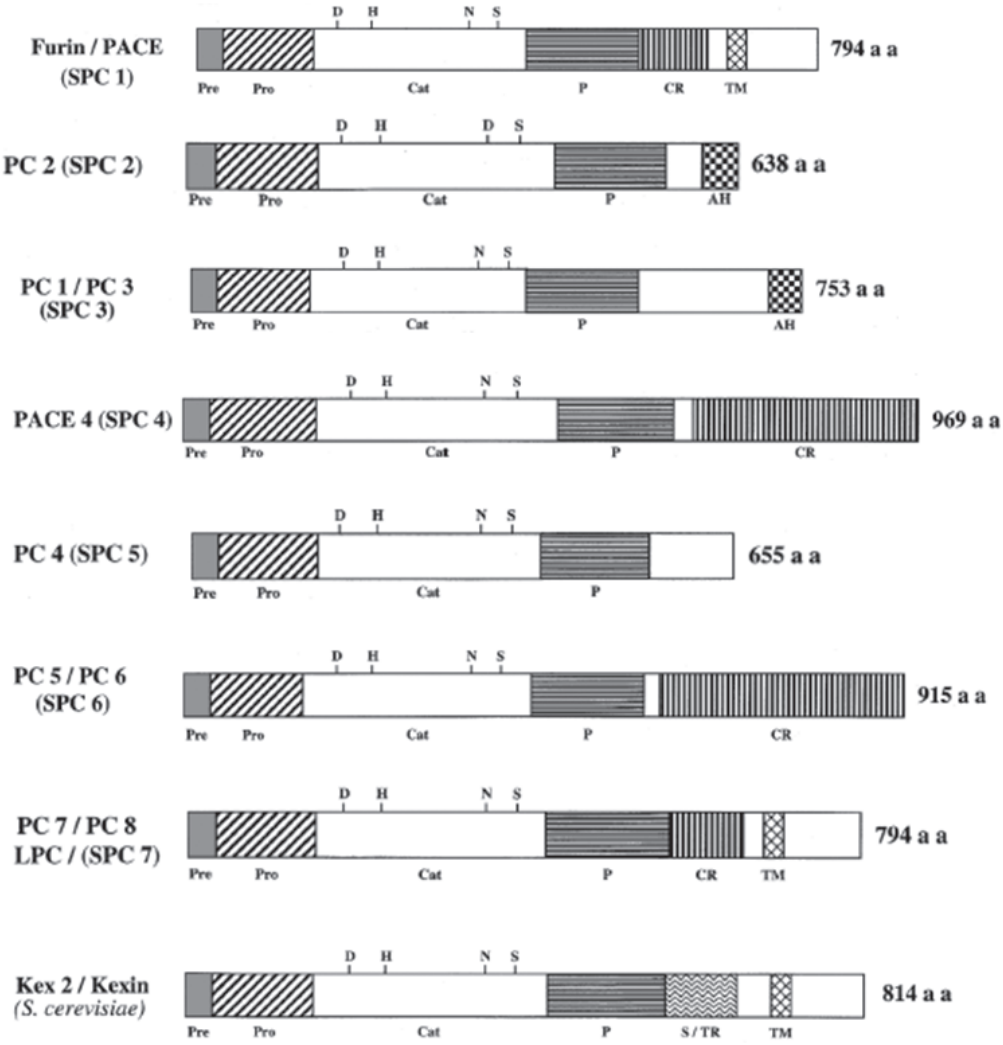
Structure and function of SPCs

SPCs, the enzymes mediating this endoproteolysis, most of them are calcium dependent serine proteases of the subtilase subfamily, collectively designated as proprotein convertases. These endoproteinases travel through, reside within, or cycle between the various compartments of the secretory pathway. The SPCs all have a characteristic amino-terminal propeptide (Pro). The Pro is followed by a well conserved, but modified (from that of subtilisin); catalytic module (Cat) and a conserved down-stream domain of 150 amino acids called the "P domain" or "homo B-domain". A three-dimensional structure has not yet been obtained for any of these enzymes, although their catalytic modules have been modeled on the basis of the known structure of subtilisin (Lipkind *et al*, 1998). The catalytic domain contains the active site of the enzyme, with the typical catalytic triad of subtilisin-related serine proteases, including the asparagine, histidine and serine active site residues. The role of the P domain appears to be regulatory and it may influence the marked calcium dependency and increased acidic pH optima of some of the SPCs. In addition, the P domain also appears to stabilize the catalytic domain structurally. It may do this by helping to balance the surface charge asymmetry in the substrate-binding region of the catalytic domain. This is caused by the characteristic multibasic residue

specificity of these enzymes. The roles of the substrate recognition of SPCs have been identified by mutational analysis confirming that negative charged residues of a catalytic pocket interact with positive charged residues of the substrate (Creemers *et al,* 1993). The consensus substrate sequence is -$R_{(P4)}X_{(P3)}(K/R)_{(P2)}R_{(P1)}$ ▼ $X_{(P1')}$-, where X can be almost any amino acid and ▼ represents the cleavage site where the peptide bone is hydrolyzed.

SPC1 (furin/PACE) is a secretory pathway endoprotease that catalyses the maturation of a strikingly diverse group of proprotein substrates, ranging from growth factors and receptors to pathogen proteins in multiple compartments within the *trans*-Golgi network/endosomal system. Human furin is initially synthesized as 100 kDa core-glycosylated pro-furin, which is converted into 94 kDa forms by a cleavage of propeptide at the -RAKR▼X- site, at residue 104-107 (Denault and Luduc, 1995; Nakayama, 1997). SPC2 is more complex in transport and activation. This convertase, which is unique, requires the acidic conditions of a late post-Golgi compartment for activation. In the endoplasmic reticulum (ER), proSPC2 interacts with 7B2: a 27-kDa neuroendocrine secretory protein that is coexpressed with SPC2 in many neuroendocrine tissues. The 7B2 is required for the production of active SPC2, which might function as a chaperone protein to assist in pro SPC2 folding. The 7B2 is cleaved at a polybasic site toward the C-terminus, similar to the cleavage of furin or related TGN protease, which results in the release of an N-terminal 21-kDa form and inhibitory C-terminal fragment (Steiner, 1998; Bergeron *et al*, 2000). The other SPCs: SPC3, SPC4, SPC5 and SPC7, have sequence specificity similar to that of SPC1 (furin). The mammalian SPCs can be classified into three groups based on their tissue distribution. The SPC1, SPC4, SPC6 and SPC7 are expressed in a broad range of tissues and cell lines. In contrast, the expression of SPC2 and SPC3 are limited to neuroendocrine tissues, such as pancreatic islets, pituitary, adrenal medulla and many brain areas. The expression of SPC5 is highly restricted to testicular spermatogenic cells. Within cells, SPC1 and SPC7, both of which have a transmembrane domain, are localized in the *trans*-Golgi network (TGN). Another SPC with a transmembrane domain, SPC6B, is also localized in the Golgi area, although it appears not to concentrate in the *trans*-Golgi network. The neuroendocrine-specific convertases (SPC2 and SPC3), are mainly localized in secretory granules. The SPC6A has been reported to be localized to secretory granules (Nakayama, 1997; Seidah and Chretien, 1997).

The autoactivation of SPC1 (furin) serves as a model for the other subtilisin-like proprotein convertases, with the exception of SPC2. Intramolecular cleavage of the propeptide (Pro) allows furin to exit the endoplasmic reticulum. However, the propeptide remains attached noncovalently until the cleaved inactive proenzyme reaches the *trans*-Golgi network, where an increased acidic (pH~6.5) and calcium-enriched environment facilitates dissociation from the propeptide. A second cleavage within the propeptide then precludes further inhibitory interactions, which result in full activation. A similar mechanism of activation has been demonstrated for SPC3, SPC5 and SPC7. Propeptide of SPC4 is autocleaved slowly, but it can probably also occur prior to its exit from the endoplasmic reticulum (Zhou *et al,* 1999).



Letters above the open boxes represent the catalytic triad (D, H, S) and the oxyanion residue (N; D only in PC2). Other abbreviations are as follows: Pre, signal sequence; Pro, proregion; Cat, catalytic domain; P, P or homo B domain; AH, predicted amphipathic helix; CR, cysteine-rich region; TM, transmembrane domain; S/TR, serine/threonine-rich region.

Figure 1 Members of the mammalian family of SPCs compare with Kex2 (Kexin). Figure modified from Steiner (1998).

Creemers *et al* (1993) studied the binding of furin and a precursor substrate, von-Willebrand factor, by site-directed mutagenesis of several amino acids in the substrate-binding region of furin. They found that mutation of two negatively charged amino acids, Asp199 and Asp47, strongly inhibited enzyme activity. In a three-dimensional structure model of the catalytic domain of human furin constructed on the basis of the crystal structure of subtilisin BPN' (bacterial protease Nagase) and termitase, it was predicted that these two acidic amino acids provided critical negatively-charged side chain within the S1 and S2 subsites of the substrate-binding region required for charge-charge interaction with the positively charged amino acids at the substrate P1 and P2 positions (Creemers *et al*, 1993). In this model, mutation of these critical negatively charged residues alters the specificity of furin for multiple basic amino acid residues of the target substrate (Figure 2).

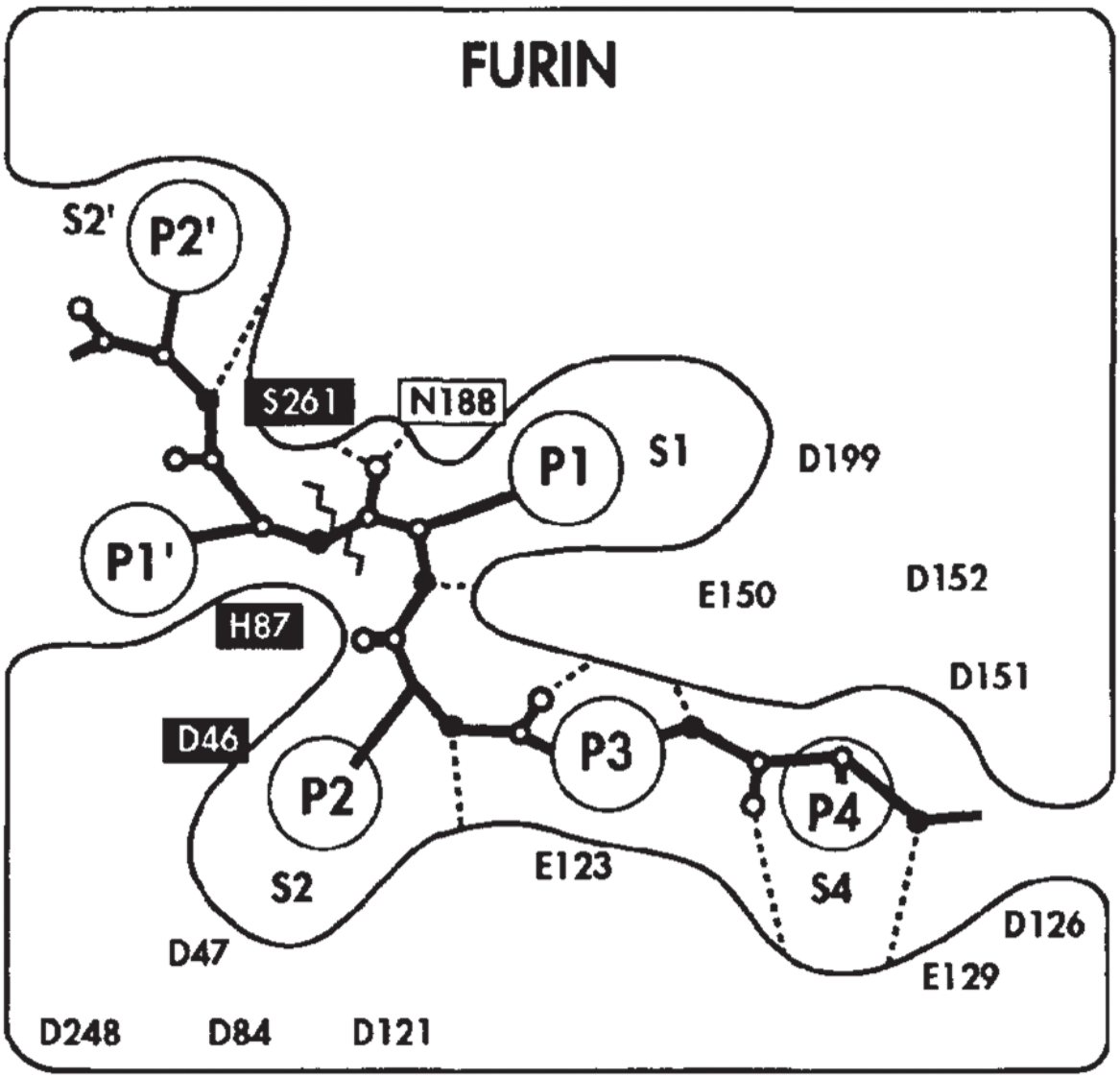


Figure 2 Schematic representation of the substrate binding region of the enzyme furin (Creemers *et al,* 1993). The positions of negatively charged residues, which were predicted to be in or near the substrate-binding region of furin, are indicated around the binding pockets S1, S2 and S4. The position of the residues of the catalytic triad, Asp46, His87 and Ser261, and the oxyanion-binding site Asn188 are boxed. The cleavage site of hexapeptide substrate (-P4-P3-P2-P1▼-P1'-P2'-), carboxyl-terminal of P1, is indicated by a zig-zag line.

Target of SPCs in the surface proteins of flaviviruses, influenza viruses and other viruses

As listed in Table 1, the cleavage sites of these flavivirus prM precursors and other viral coated glycoproteins fully fit the -RX(K/R)R▼X- consensus sequence. These viral precursors were delineated the following sequence rules that govern the cleavage by SPCs, as follows: 1) An Arginine residue is essential at the P1 position 2) In addition to the Arg(P1), basic residues at the P2 and P4 are required for efficient cleavage 3) At P1' position, an amino acid with a hydrophobic aliphatic side chain is not suitable.

Table 1 Sequences around the cleavage site of precursor proteins

Precursors	GenBank no.	Cleavage site ($...P_6P_5P_4P_3P_2P_1{\blacktriangledown}P_{1'}...$)	References
Flaviviruses prM proteins			
Dengue virus group			
Den 1 (West Pacific)	GI: 1854036	$HRRDKR{\blacktriangledown}S_{206}$	Puri *et al,* 1997
Den 2 (16681)	GI: 2155257	$HRREKR{\blacktriangledown}S_{206}$	Kinney *et al,* 1997
Den 3 (H87)	GI: 323468	$HRRDKR{\blacktriangledown}S_{206}$	Osatomi and Sumiyoshi, 1990
Den 4	GI: 6978317	$RRREKR{\blacktriangledown}S_{205}$	Zhao *et al,* 1986
Japanese encephalitis virus group			
JEV (JaOArS982)	GI: 9626460	$SKRSRR{\blacktriangledown}S_{220}$	Sumiyoshi *et al,* 1987
Murray Valley encephalitis virus	GI: 9633622	$SKRSRR{\blacktriangledown}S_{218}$	Hurrelbrink *et al,* 1999
St. Louis encephalitis virus (MSI.7)	GI: 334865	$SRRSRR{\blacktriangledown}S_{214}$	Trent *et al,* 1987
West Nile virus	GI: 11528013	$SRRSRR{\blacktriangledown}S_{216}$	Castle *et al,* 1985
Kunjin virus (MRM61C)	GI: 221966	$SRRSRR{\blacktriangledown}S_{216}$	Coia *et al,* 1988
Rio Bravo virus group			
Apoi virus (ApMAR)	GI: 7939633	$NTRTRR{\blacktriangledown}D_{197}$	Billoir *et al,* 2000
Rio Bravo virus (RiMAR)	GI: 7144649	$GHRLKR{\blacktriangledown}S_{193}$	Billoir *et al,* 2000
Tick-borne encephalitis virus group			
Langat virus (TP21)	GI: 8453150	$GSRSRR{\blacktriangledown}S_{206}$	Campbell and Pletnev, 2000
Louping ill virus (369/T2)	GI: 9629456	$GSRTRR{\blacktriangledown}S_{206}$	Gritsun *et al,* 1997
Powassan virus (LB)	GI: 309916	$GSRGKR{\blacktriangledown}S_{204}$	Mandl *et al,* 1993
TBE virus (Neudoerfl)	GI: 9628431	$GSRTRR{\blacktriangledown}S_{206}$	Mandl *et al,* 1989

The P6-P1' cleavage site sequences for a selected list of proposed SPC substrates are shown above. The cleavage sites of these enveloped viruses consist of the highly conserved sequence, $-R_{(P4)}X_{(P3)}[K/R]_{(P2)}R_{(P1)}{\blacktriangledown}X_{(P1')}-$, with basic amino acid motif. The bole capital letter represented as positive charge amino acids at cleavage sites.

Table 1 (Continued)

Precursors	GenBank no.	Cleavage site ($...P_6P_5P_4P_3P_2P_1{\blacktriangledown}P_{1'}...$)	References
Flaviviruses prM proteins			
Yellow fever virus group			
Yellow fever virus (17D)	GI: 59338	SRRSRR$\blacktriangledown$A$_{211}$	Rice *et al,* 1985
Unclassified Flavivirus			
Cell fusing agent	GI: 336190	KKREKR$\blacktriangledown$S$_{220}$	Cammisa-Parks *et al,* 1992
Avian influenza virus - HA0 precursor			
A/ck/Mexico/31381/94 (H5N2), LPAI	GI: 1125704	PQ----RETR$\blacktriangledown$G$_{327}$	Garcia *et al*, 1996
A/ck/Hong Kong/990/97 (H5N1), HPAI	GI: 4240441	PQRERRRKKR$\blacktriangledown$G$_{347}$	Matrosovich *et al*, 1999
A/ck/Thailand/ICRC-213/07(H5N1), HPAI	GI:193795159	PQRERRRKKR$\blacktriangledown$G$_{347}$	Chaichoune *et al*, 2009
A/tern/Potsdam/79 (H7N7), LPAI	GI:902774	PE----IPKGR$\blacktriangledown$G$_{340}$	Rohm *et al*, 1995
A/Netherlands/219/03(H7N7), HPAI	GI:37786137	PE--IPKRRRR$\blacktriangledown$G$_{349}$	Fouchier *et al*, 2004
Other viral coat proteins			
Borna disease virus	GI: 15718111	LVRRRR$\blacktriangledown$D$_{250}$	Pleschka *et al,* 2001
Cytomegalovirus glycoprotein B	GI: 138193	HNRTKR$\blacktriangledown$S$_{461}$	Spaete *et al,* 1988
Eastern equine encephalomyelitis virus	GI: 2120048	NARTRR$\blacktriangledown$D$_{324}$	Volchkov *et al,* 1991
Ebola virus (Zaire) Glycoprotein	GI: 465411	GRRTRR$\blacktriangledown$E$_{502}$	Volchkov *et al,* 1998
HIV gp160	GI: 119437	VQREKR$\blacktriangledown$A$_{507}$	York-Higgins *et al,* 1990
Measles virus F_0	GI: 9181897	SRRHKR$\blacktriangledown$F$_{116}$	Parks *et al,* 2001
Sindbis virus (HRSP) gpE2	GI: 74511	SGRSKR$\blacktriangledown$S$_{329}$	Strauss *et al,* 1984

The P6-P1' cleavage site sequences for a selected list of proposed SPC substrates are shown above. The cleavage sites of these enveloped viruses consist of the highly conserved sequence, -$R_{(P4)}X_{(P3)}[K/R]_{(P2)}R_{(P1)}$ $\blacktriangledown$$X_{(P1')}$-, with basic amino acid motif. The bole capital letter represented as positive charge amino acids at cleavage sites.

The processing by SPCs is an important control mechanism for the biological activity of viral surface proteins. Proteolytic activation of envelope glycoproteins is necessary for entry of viruses into the host cell and, hence, for their ability to undergo multiple replication cycles. In some cases, it has also been shown that the cleavability of the envelope glycoproteins is an important determinant for pathogenicity. Proteolytic cleavage is the first step in the activation of these fusion proteins and is followed by a conformational change resulting in the exposure of the fusion domain (Miranda *et al,* 1996; Stadler *et al,* 1997; Sabbarao *et al,* 1998; Volchkov *et al,* 1998). The conformational change may be triggered by a low pH in the endosomes, as in the case of influenza virus (Sabbarao *et al,* 1998), or by the interaction with a secondary receptor protein at the cell surface, e.g. in the case of HIV (Miranda *et al,* 1996).

SPCs and cleavage of Flavivirus prM protein

Among flaviviruses with known insect vectors, the presence of an acidic residue at the P3 cleavage position appears to be unique to all four dengue virus serotypes. The P3 acidic residue is highly conserved among dengue viruses, and the only other known example of such a residue is found in the cell fusing agent virus, which also exhibits minimal prM cleavage (Nakayama, 1997). The structural basis for the requirement of arginine at the P1 position and Arg(R) or Lys(K) at the P2 position of the dengue prM cleavage junction can be extrapolated from studies of other proteins. In SPCs, there are subsites in the substrate-binding region containing negatively charged amino acids, which may interact with the positively charged residue of substrates (Nakayama, 1997). It was suggested that interactions between the positive and negative charges in these subsites determined the affinity of the substrate for SPCs.

In study of Junihon *et al,* (2008), examined the influence of the Glu(P3) residue and other nonconsensus charged residues on the efficiency of dengue virus prM cleavage. The mutant viruses harboring the alanine-scanning and other multiple-point mutations of the prM junction were generated, employing a dengue virus background that exhibited 60 to 70% prM cleavage and a preponderance of virion-sized extracellular particles. Analysis of prM and its cleavage products in viable mutants revealed a cleavage-suppressive effect at the conserved Glu(P3) residue, as well as the cleavage-augmenting effects at the Arg(P5) and His(P6) residues, indicating an interplay between opposing modulatory influences mediated by these residues on the cleavage of the prM junction. Changes in the prM-cleavage level were associated with altered proportions of extracellular virions and subviral particles; mutants with reduced cleavage were enriched with subviral particles and prM-containing virions, whereas the mutant with enhanced cleavage was deprived of these particles. Alterations of virus multiplication were detected in mutants with reduced prM cleavage and were correlated with their low specific infectivities. These findings define the functional roles of charged residues located adjacent to the furin consensus sequence in the cleavage of dengue virus prM and provide plausible mechanisms by which the reduction in the prM junction cleavability may affect virus replication.

In the C6/36 mosquito cell-line that usually used to be host cells for isolation or propagation of any flaviviruses, the pro-vitellogenin convertase deduced amino acid sequence has a high similarity to a domain structure characteristic of subtilisin-like convertases. The SPC-like protein in C6/36 cell-line was identified and characterize as a 115-kDa pro-vitellogenins (pro-Vg) processing enzyme or pro-vitellogenins convertase (VC), from a vitellogenic female fat body cDNA library of *Aedes aegypti* mosquito. The amino acid sequence of vitellogenins convertase reveals a high structural similality to furin-like convertase (SPC1). It has a typical structure of furin-like convertases which comprised of pre-propeptide, catalytic, cysteine-rich, C-terminal transmembrane, and cytoplasmic domains and recognizes the same cleavage sites that contain paired basic amino

acid motif, -RX(K/R)R▼X- (Chen and Raikhel, 1996).

Lack of prM-M cleavage may affect dengue virus replication in many ways. Some studies indicated that the processing of prM to M protein is a mechanism used for activation of the flavivirus fusion protein, E glycoprotein. A study by Guirakhoo *et al,* (1991) showed fusion inhibition of tick-borne encephalitis virus and Japanese encephalitis virus based on the "fusion from without assay" in C6/36 mosquito cells. They experimented with prM-containing (immature) virions which were grown in the presence of ammonium chloride. These particles did not cause fusion under the fusion-from without-assay conditions. The acquisition of fusion activity, therefore, also depends on the proteolytic cleavage of the prM protein. In similar study in the West Nile virus, the prM processing was found to be required for full infectivity and the rearrangement of oligomeric structures on the surface of virions (Wengler and Wengler, 1989).

Recent cryo-electron microscopy studies of a virus-like particle have provided evidence that the E protein dimers on its surface form an extensive network of specific lateral interactions, in which domain II of one E dimer contacts domains I and III of a neighboring dimer, resulting in a highly ordered outer shell with icosahedral symmetry (Ferlenghi *et al,* 2001). These capsidless recombinant subviral particles are generated by co-expression of the prM and E protein in COS1 cells. Their structure was determined to a resolution of 19Å by image reconstruction of electron micrographs and this allowed fitting of the atomic structure of the E protein in to the lower-resolution structure obtained by the cryo-electron microscope.

At the pH of fusion, the E proteins on the surface of virus particles and recombinant subviral particles undergo dramatic irreversible structure change that involve not only the conformation of the individual protein subunits, but also the lateral intersubunit interactions that make up the icosahedral lattice structure (Ferlenghi *et al,* 2001; Heinz and Allison, 2001). The information provided by cryo-electron microscope reveals that the uncleaved prM protein would restrict the lateral freedom of E, and prevent the loosening of the intermolecular E contact for conformational change. Based on these studies, it is quite likely that the underlying basis for non-viability of the eight prM junction mutants lays in the failure of E protein to undergo conformational change required for fusion activity of flaviviruses (Figure 3).

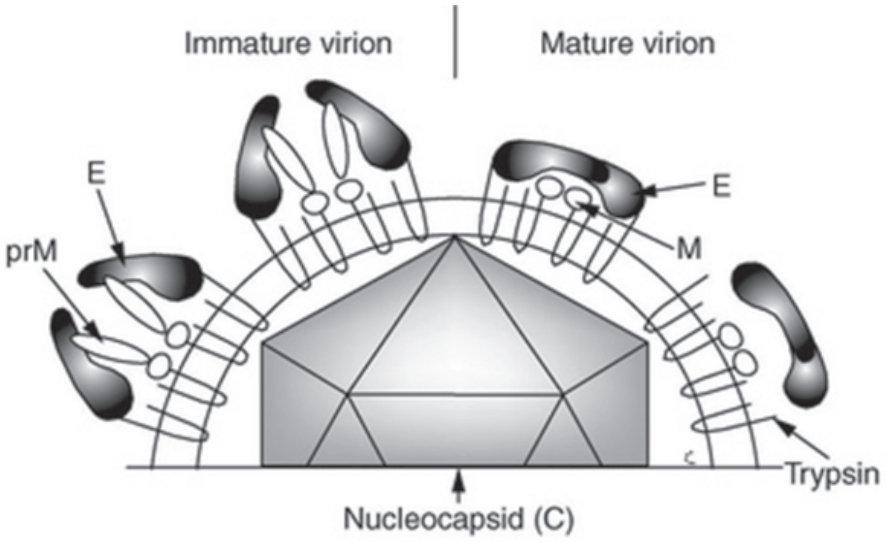


Figure 3 Schematic diagram of the composition of immature and mature flaviviruses (Figure reproduced from Heinz and Allison, 2001.)

SPCs and cleavage of influenza virus HA0 precursor

In the case of influenza virus, the hemagglutinin (HA) is synthesized as a precursor (HA0) that associates noncovalently as homotrimers. The precursor polypeptides are post-translationally cleaved at a conserved arginine residue into two subunits, HA1 and HA2, which linked by a single disulfide bond (Figure 4). This cleavage step is necessary for virus infectivity because it activates the membrane fusion potential of hemagglutinin (Steinhauer, 1999).

In 1997, the avian influenza A virus outbreak in Hong Kong, which inflicted a fatal respiratory illness (30% lethality), was traced to an H5N1 strain. Sequence analysis of genes encoding hemagglutinin (HA) from 16 isolates revealed a consistent alteration in the viral genomes to generate a second consensus furin site at the HA1-HA2 junction (-RKKR▼G- to -RGRRRKKR▼G-). Exactly how the addition of a tandem consensus site results in enhanced virulence remains to be determined (Suarez *et al,* 1998; Sabbarao *et al,* 1998). The mutant avian influenza A virus, in which the amino acid sequence at the HA cleavage site (-PGRERRRKKR▼G-), was converted to sequence of a typical avirulent virus (-PE---RETR▼G-, where dash indicates a deletion). When tested in mice, this HA mutant was highly attenuated (virus dose lethal to 50% of mice, MLD50, $>10^5$ pfu), and none of the infected mice showed signs of disease. The virulence of H5N1 virus in mice appears to involve HA cleavability (Hatta *et al,* 2001).

The HA is a critical determinant of the pathogenicity of avian influenza viruses, with a clear link between HA cleavability and virulence (Horimoto and Kawaoka, 2001; Klenk and Rott, 1988). The HA0 proteins of highly pathogenic H5 and H7 viruses contain multiple basic amino acids at the cleavage site, which are recognized by ubiquitous proteases, furin and PC6. For this reason, these viruses can cause systemic infections in poultry. In cell culture, the HAs of these viruses do not need exogenous proteases to form plaques. In contrast, the HA0 proteins of low pathogenic avian and non-avian influenza A viruses, with the exception of H7N7 equine influenza viruses (Kawaoka, 1991), contain a single arginine residue at the HA cleavage site (Bosch *et al,* 1979) and are cleaved in only a few organs. These viruses, therefore, produce localized infection of the respiratory and/or intestinal tract that is usually asymptomatic or mild. The tissue tropism of viruses is thus partly determined by the availability of host proteases to recognize and cleave the two types of amino acid sequences found at the HA0 cleavage site (Figure 4).

Two groups of proteases are responsible for HA cleavage. The first group recognizes a single arginine and cleaves all HAs. Members of this group include plasmin (Lazarowitz *et al,* 1973), blood-clotting factor X-like proteases (Gotoh *et al,* 1990), tryptase Clara, miniplasmin (Kido *et al,* 1999), and bacterial proteases (Kido *et al,* 1992). In ovo, a protease similar to the blood-clotting factor Xa that is present in the allantoic fluid cleaves HA, which explains why influenza viruses grow efficiently in eggs (Kawaoka and Webster, 1988). Tryptase Clara is secreted from specialized respiratory epithelial cells in rats and mice (Goto and Kawaoka, 1998); whether similar proteases are responsible for HA cleavage in humans and birds remains unknown. Miniplasmin is a trypsin-type serine protease in the epithelial cells of the bronchia that cleaves HA downstream of the consensus motif -(Q/E) XR▼X- (Kido et al, 1992). Cleavage of HA0 by plasmin can be augmented by the ability of the A/WSN/33(H1N1) neuraminidase (NA) proteins to sequester its protease precursor, plasminogen (Tashiro *et al,* 1987). This NA function depends on a carboxy-terminal lysine residue and the absence of an oligosaccharide side chain at position 146 (N2 numbering). Bacterial proteases can also activate HA, either directly or indirectly by activating plasminogen, a property that may explain the development of pneumonia after dual infections with viruses and bacteria (Horimoto and Kawaoka, 1994).

The second group of proteases that cleaves HA proteins (Stieneke-Gröber *et al,* 1992; Klenk *et al,* 1984) comprises the ubiquitous intracellular subtilisin-related endoproteases furin and PC6 (Klenk *et al,* 1984). These enzymes are calcium dependent, have an acidic pH optimum, and are located in the Golgi and/or *trans*-Golgi network (Kawaoka *et al,* 1984). The cleavage efficiency of these ubiquitous proteases is determined by the sequence at the cleavage site and the absence or presence of a nearby carbohydrate chain on the HA molecule (Stieneke-Gröber *et al,* 1992; Kido *et al,* 1999; Vey *et al,* 1992; Perdue *et al,* 1997; Horimoto and Kawaoka, 1995). The proposed sequence required for HA cleavage is-Q(R/K)X(R/K)R▼X- (X, nonbasic amino acid) in the absence of a nearby carbohydrate chain. The presence of a nearby carbohydrate chain requires insertion of two additional residues, -QXXRX(R/K)R▼X-, or alteration of the conserved glutamine at position -5 or the proline at position -6, -BB(R/K)X(R/K)R▼X- (B, basic residue; X, nonbasic amino acid). The presence of direct repeats of basic amino acid insertions of various lengths in the HA proteins of several H5 and H7 viruses suggests that these sequences arose from polymerase stuttering (Chen *et al,* 1998) likely caused by secondary structure in the template RNA. HA cleavage efficiency can also be affected by the nature of the amino acid immediately downstream of the cleavage site, that is, the N-terminal amino acid of HA_2 (Feldmann *et al,* 2000).

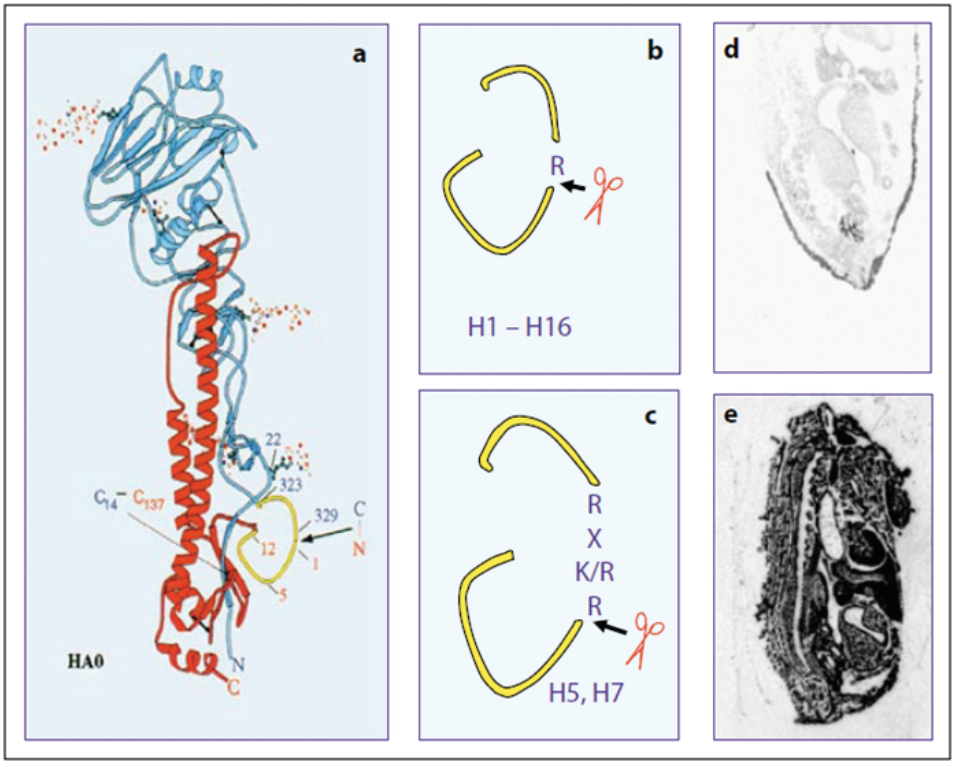


Figure 4 The cleavage site of HA determines the pathogenicity of avian influenza viruses. HA0 is cleaved into subunits HA1 and HA2. The cleavage site is located in a loop projecting from the surface of the molecule (Schauer and Kamerling, 1997) (a). LPAI viruses have a single arginine at the cleavage site that is recognized by trypsin-like proteases that are present only in specific tissues, such as intestinal epithelia (b). HPAI viruses (serotypes H5 and H7) are activated at a multibasic cleavage site -RX(K/R)R▼X- by the ubiquitous protease furin and furin-like proteases (c). Spread of the LPAI virus A/Chicken/Germany/N/49(H10N9) (d) and of the HPAI virus A/FPV/Rostock/34(H7N1) (e) in chicken embryos. Thin sections were subjected to in situ hybridization with ^{35}UTP-labeled riboprobes specific for viral mRNA (Schauer, 2004).

SPCs and cleavage of Paramyxovirus glycoprotein

For many viruses in the *Paramyxoviridae* family, cleavage of viral glycoprotein by furin cleavage and other proprotein convertases is absolutely required for their activity and thus determines the extent of virus pathoginicity. Measles virus is the prototype member of the morbillivirus genus in the *Paramyxoviridae* family of negative-stranded RNA viruses. The virions contain envelope with two virus-encoded integral membrane glycoproteins, the viral attachment protein hemagglutinin (H) and the fusion (F) protein, which form spike-like projections on the outer surface. The F protein is synthesized as an inactive precursor molecule F0, which is cleaved intracellularly by SPCs to generate two polypeptide subunits, F_1 and F_2, held together by disulfide bonds. Infected cells exposing cleaved F protein on the surface fuse with adjacent cells at neutral pH, thereby causing syncytium formation. The multibasic cleavage site at which the protein of measles virus is activated consists of five basic amino acids, -RRHKR$^{\blacktriangledown}$X-, at the positions 108-112. Correct proteolytic cleavage after Arg112 is essential, because changing this residue to leucine (clevage site: -$R_{(P4)}H_{(P3)}K_{(P2)}L_{(P1)}{}^{\blacktriangledown}X_{(P1')}$) was shown to result in aberrant cleavage and loss of fusion ability (Alkathib *et al,* 1994). Maisner *et al* (2000) generated mutant F protein of measles virus of which the sequence -RRHKR$^{\blacktriangledown}$X- was changed by site-directed mutagenesis to $R_{(P5)}$-$N_{(P4)}$-$H_{(P3)}$-$N_{(P2)}$-

SPCs and activation of HIV-1 gp120

There is currently a debate on SPCs that are physiologically involved in cleavage and activation of HIV-1 gp160. The production of infectious HIV-1 virions is dependent on the processing of envelope glycoprotein gp160 by the host cell proteinase. The furin and the other subtilisin-like proteases can cleave and activate HIV-1 gp160 to yield gp120 and gp41. This, taken together with furinís expression in $CD4^+$ cell lines, has led to propose that furin is the proteinase activating gp160. However, other proteinases that are subtilisin-like convertase, are also involved physiological in gp160 cleavage (Miranda *et al,* 1996). Seidah and Chretien (1997) have suggested that furin, SPC6 and SPC7, are the major gp160-convertase enzymes in T lymphocytes.

SPCs and activation of Filoviruses

The processing of the Ebola Filovirus glycoprotein (GP) by furin was determined by Volchkov *et al* (1998). This was indicated by the observation that cleavage did not occur when GP was expressed in furin-defective LoVo cells, but it was restored in these cells by vector-expressed furin. The Reston subtype, which differs from all other Ebola viruses in its low human pathogenicity, has reduced cleavability due to a mutation at the cleavage site. As a result of these observations, it should now be considered that proteolytic processing of GP might be an important determinant for the pathogenicity of the Ebola virus. The studies of glycoprotein processing of the Ebola virus (subtype Zaire) by furin and other SPCs, correlated with other virus glycoprotein processing. The glycoprotein (GP) of Ebola virus is the only surface protein of virions with suggested function in receptor binding and fusion with cellular membranes. As seen in other viruses, the fusogenic property of Ebola virus glycoprotein requires posttranslational proteolytic processing. The maturation involves posttranslational cleavage of a precursor at the C-terminal end of the sequence -RTRR$^{\blacktriangledown}$X- (at positions 498-501) into the disulfide-linked fragments GP_1 and GP_2. The proprotein convertase furin has been identified as a cleavage enzyme. Volchkov *et al* (1998) modified the internal cleavage sequence by site-directed mutagenesis. In the first mutation at Arg501 was substituted by lysine (changed from -RTRR$^{\blacktriangledown}$X- to -RTRK$^{\blacktriangledown}$X-). Besides this, in the second mutant the arginine residue at positions 500 and 501 were changed to asparagine and methionine, respectively (changed from -RTRR$^{\blacktriangledown}$X-to-RTNM$^{\blacktriangledown}$X-). Both mutants were evaluated for proteolytic processing by transient expression in HeLa cell. Unlike wild-type glycoprotein, which was processed into subunit GP_1 and GP_2, both mutants expressed only the

uncleaved glycoprotein. This result indicates that glycoprotein is cleaved at the C-terminal side of Arg501 and that the cleavage site has the classical consensus sequence -RXRR▼X- recognized by SPCs. However, mutant Ebola viruses containing mutated furin cleavage sequence were viable. Ebola virus with uncleaved glycoprotein was able to mediate infection in various cell lines as efficiently as the wild type virus (Wool-Levis and Bates, 1999).

Conclusion

The SPCs 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 SPCs. Based on their crucial role in virus-host interaction, SPCs can be important determinants for viral pathogenesis and represent promising targets of therapeutic antiviral intervention. In the present review, we covered basic aspects and recent developments of SPC-mediated maturation of viral envelope glycoproteins of selected medically important viruses. The molecular mechanisms underlying the recognition of SPCs by viral glycoproteins were described, including recent findings demonstrating differential SPC-recognition of viral and cellular substrates. Particular attention have given to past and current efforts to evaluate cellular SPCs as targets for antiviral therapeutic intervention, with emphasis on emerging highly pathogenic viruses for which no efficacious drugs or vaccines are currently available.

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