Implication of the proprotein convertases furin, PC5 and PC7 in the cleavage of surface glycoproteins of Hong Kong, Ebola and respiratory syncytial viruses: a comparative analysis with fluorogenic peptides

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Fluorogenic peptides encompassing the processing sites of envelope glycoproteins of the infectious influenza A Hong Kong virus (HKV), Ebola virus (EBOV) and respiratory syncytial virus (RSV) were tested for cleavage by soluble recombinants of the proprotein convertases furin, PC5 and PC7. Kinetic studies with these intramolecularly quenched fluorogenic peptides revealed selective cleavages at the physiological dibasic sites. The HKV peptide is cleaved by both furin and PC5 with similar efficacy; in comparison, PC7 cleaves this substrate poorly. In contrast with the basic tetrapeptide insertion within the haemagglutinin sequence of HKV, two other dipeptide insertions revealed a poorer cleavage with a similar rank order of potency. These results demonstrate that the N-terminal RERR insertion to the wild-

type avian RKKR↓ sequence is functionally significant, and suggest that the approx. 5-fold increase in cleavage efficacy contributes to the high infectivity of the H5N1 virus subtype. With regard to RSV peptide processing, PC7 is twice as effective as PC5 and furin. The EBOV peptide was processed with similar efficiency by the three enzymes. Our observations that all of these cleavages can be effectively inhibited by a plant andrographolide derivative at 250 μM or less might aid in the design of potent convertase inhibitors as alternative antiviral therapies.

Key words: cleavage specificity, dibasic recognition, kinetic studies, precursor processing, viral glycoproteins.

INTRODUCTION

Viral outbreaks are responsible for some of the worst pandemic infections in human history. Of these, influenza viruses have contributed to the spread of deadly influenza infections such as those caused by the Spanish (H1N1 swine flu), Asian (H2N2), Hong Kong (H3N2) and Russian (H1N1) subtypes [1,2]. The proteolytic maturation of surface envelope glycoproteins is a common virulence-activating step of many pathogenic viruses, leading to the formation of 'activated' proteins, which allow the fusion of the virions with host cell membranes. Therefore proteolytic processing of envelope glycoproteins is an important factor in pathogenicity [3,4]. This limited proteolysis of proproteins is responsible for the generation not only of fusogenic viral glycoproteins but also of diverse bioactive polypeptides [5–7]. It is noted that for all viral glycoproteins, cleavage occurs at the C-terminus to a single or pairs of basic amino acids within the consensus sequence (R/K/H)-(X)_n-(K/R)↓ (where *n* = 0, 2, 4, or 6 and X is any amino acid except Cys), highly compatible with the specificity of proprotein convertases (PCs) [6–9].

PCs are Ca²⁺-dependent subtilisin-like serine proteinases related to the yeast enzyme kexin; so far seven convertases are known: furin ('PACE'), PC1 (PC3), PC2, PC4, PACE4, PC5 (PC6) and PC7 (LPC, PC8) (reviewed in [6–9]). Accumulated research suggests a role for PCs in several human patho-

physiological conditions including viral infections, arterial stenosis and cancer [10–13]. Thus it has been demonstrated that conditions favouring viral replication cause a significant up-regulation of candidate processing enzymes such as furin and PC7 [14–18]. These and other studies have indicated that furin and PC7 are the major candidate surface glycoprotein (including HIV gp160) convertases in lymphocytes [15–18]. This finding inspired various groups to examine the possible participation of PCs in other viral and opportunistic infections [19–22].

Avian viruses, particularly the Hong Kong virus (HKV) poultry variant H5N1, as well as Ebola virus (EBOV) and respiratory syncytial virus (RSV), have been studied particularly closely because of their rapid infectivity, morbidity and mortality. The H5N1 avian influenza virus, which is extremely pathogenic for chickens, was transmitted from birds to humans in 1997 in Hong Kong, infecting 18 humans, 6 of whom died [22,23]. The wild-type virus (pathogenic to birds) does not cause disease in humans but the H5N1 variant is lethal to both humans and chickens [24]. The variant H5N1 gene encodes a basic tetrapeptide insertion (RERR) upstream of the cleavage site [22]. RSV, a single-stranded RNA virus, belongs to the family Paramyxoviridae, subfamily Pneumovirinae, genus Pneumovirus. It is described as the single most important virus causing acute respiratory-tract infection and severe lung disease in children and adults, especially in the elderly [25,26]. It also compromises immunity [27] and

Abbreviations used: Abz, 2-aminobenzoic acid; AMC, 7-amino-4-methylcoumarin; EBOV, Ebola virus; F, fusion protein; Fmoc, fluorenyl-methoxycarbonyl; GP, glycoprotein; HA, haemagglutinin; HKV, Hong Kong virus; IQF, intramolecularly quenched fluorogenic; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; MCA, 4-methyl-7-coumarinamide, PC, proprotein convertase; RP-HPLC, reverse-phase HPLC; RSV, respiratory syncytial virus; SEA, succinoyl ester of andrographolide; Tys, 3-nitrotyrosine; TFA, trifluoroacetic acid.

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Table 1 Proteolytic activation sites of selected viral envelope glycoproteins

Sequences derived from viruses in bold are investigated in this work. The important basic residues at P1, P2, P4, P6 and P8 are also shown in bold. The underlined sequence in the Hong Kong virus emphasizes the RERR insertion. Symbol: ↓, physiological cleavage site.

Virus	Glycoprotein	Processing site	Reference
Human cytomegalovirus	gB	H N R T K R ↓ S T	[6]
Infectious bronchitis	E2	T R R F R R ↓ S I	[21]
Borna disease	p57/gp94	L K R R R R ↓ D T	[21]
Epstein-Barr	Gb	L R R R R R ↓ D A	[21]
Japan B encephalitis	M	S K R R R R ↓ S V	[21]
Mumps	F	S R R H K R ↓ F A	[6]
Measles	F	S R R H K R ↓ F A	[21]
Yellow fever	prM	S R R S R R ↓ A I	[21]
Varicella zoster	gpII	N T R S R R ↓ S V	[6]
HIV-1	gp160	V Q R E K R ↓ A V	[6]
Bovine leukaemia	gp72	P T R V R R ↓ S P	[6]
Rous sarcoma	env	G I R R K R ↓ S V	[6]
Marburg	GP	V Y R R K R ↓ S I	[55]
Respiratory syncytial	F	K K R R R R ↓ F L	[21]
Ebola (Zaire)	GP	G R R T R R ↓ E A	[6]
Ebola (Sudan)	GP	R K R S R R ↓ Q V	[6]
Ebola (Ivory Coast)	GP	S R K R R R ↓ D V	[6]
Ebola (Reston)	GP	T R K Q K R ↓ S V	[6]
Avian influenza WT	HA	T P R K K R ↓ G L	[6]
Avian Hong Kong (H5N1)	HA	T P R E R R R K K R ↓ G L	[22]

invades the middle ear during the acute otitis phase [28]. RSV accounts for nearly 50 % of all pneumonia and up to 90 % of the reported cases of bronchiolitis in infancy [26]. RSV infections are abrupt in onset and the symptoms can last for up to 5 months. Nearly 60 % of infants are infected with RSV in their first year of life, and 20 % develop lower-respiratory-tract disease resulting in up to 0.6 % mortality [26]. EBOV, a member of the Filoviridae family, is also virulent to primates. It induces acute, rapidly progressing, frequently fatal pathologies typically characterized by haemorrhages, and shock affecting a number of organs [29]. This virus primarily targets macrophages, which have an important role in the aetiology of the disease. Phylogenetic analysis reveals four distinct EBOV subtypes with different virulence properties: Zaire (including Gabon strains), Sudan, Reston and Côte d'Ivoire [29]. Currently, immunization is the most effective preventative measure against viral diseases but the formulations cannot cover every single strain known. Therefore alternative approaches are needed.

On the basis of the sequence motifs at the processing sites of infectious viruses (Table 1), the nature of their target host cells and the subcellular site of processing of viral glycoproteins [30,31], it is thought that PCs are the best candidate cellular convertases of viral envelope glycoproteins. In the present study we designed a number of intramolecularly quenched fluorogenic (IQF) peptide substrates [32–35] mimicking the activation sites of envelope coat glycoproteins of HKV and three of its mutants, as well as EBOV and RSV. These substrates are good fluorescence quenching release systems in which the fluorescent (donor) and quencher (acceptor) groups respectively are positioned at the termini of the peptide chain. Kinetic analysis of the cleavage of these model peptides was performed with the convertases furin, PC5 and PC7, which are likely to be involved in the processing of the selected HKV, EBOV and RSV surface glycoproteins. We also examined the inhibitory potency of a succinyl ester of andrographolide (SEA), derived from plant extracts [36], on PC-mediated cleavages *in vitro*.

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EXPERIMENTAL

Materials

For enzyme assay, the pentapeptide pGlu-Arg-Thr-Lys-Arg-MCA (in which pGlu stands for pyroglutamic acid and MCA for 4-methyl-7-coumarinamide) was obtained from Peptides International (Louisville, KY, U.S.A.). Fluoren-9-ylmethoxycarbonyl (Fmoc)-protected amino acid derivatives (L-configuration) including the two acceptor and donor compounds Fmoc-2-Abz (in which Abz stands for 2-aminobenzoic acid) and Fmoc-3-nitrotyrosine (Fmoc-Tyx), the coupling reagents and the solvents were purchased from PE Biosystems (Framingham, MA, U.S.A.), Calbiochem (San Diego, CA, U.S.A.) and Chem-Impex International (Wood Dale, IL, U.S.A.). Amino acid analyses were performed after hydrolysis for 18–24 h in 5.7 M HCl at 110 °C *in vacuo* with a modified Beckman 120C autoanalyser equipped with a Varian DS604 integrator/plotter [32]. SEA was prepared by following the procedure described previously [36].

All reverse-phase (RP) HPLC runs were performed on a Rainin Dynamax instrument equipped with an SD-1 pump, with the use of a C₁₈ column [Jupiter, Phenomenex; column size 0.46 cm × 25 cm (analytical) or 1 cm × 25 cm (semipreparative); particle size 10 µm; pore size 300 Å]. The buffer system comprised an aqueous 0.1 % (v/v) trifluoroacetic acid (TFA) solution and an organic phase of acetonitrile containing 0.1 % (v/v) TFA. Peptides were eluted with a 1 %/min linear gradient (5–60 %) of 0.1 % (v/v) aqueous TFA/acetonitrile at a flow rate adjusted to 1 ml/min (analytical run) or 2 ml/min (semipreparative run), after a 5 min isocratic elution at 5 % (v/v) (0.1 % TFA/acetonitrile) in water. The elution of the peptides was monitored on-line by measuring UV absorbance with wavelength (λ) set at 210 nm and the fluorescence (Rainin, Dynamax fluorescence detector FL-2 model) λ_{ex} and λ_{em} fixed at 320 ± 5 and 420 ± 3 nm respectively. The fluorimetric enzyme assay was performed with an LS 50B (Perkin–Elmer, Gaithersburg, MD, U.S.A.) spectrofluorimeter.

Sources of recombinant PCs

The endoproteases used in this study, human shed-furin, mouse PC5 and before transmembrane domain ('BTMD') rat PC7 were all obtained from the conditioned medium of BSC40 cells after infection with the respective recombinant vaccinia virus bearing the full-length enzymes as described [37–39]. For furin, the final enzyme preparation was further diluted 1:4 to obtain an enzymic activity comparable to those of the other two PCs, as measured with the fluorogenic substrate pERTKR-MCA [37–39].

Design of IQF peptide substrates

We designed a number of IQF peptides with a donor Abz group and an acceptor Tyx group attached respectively to the N-terminus and C-terminus of the peptide chain. An alanine residue was incorporated at the C-terminus to facilitate the solid-phase peptide synthesis starting from the C-terminus.

Table 2 lists the various IQF peptides prepared for the present study. In addition, another peptide called FL-HA_{HKV} (in which HA stands for haemagglutinin), Abz-Arg-Asn-Thr-Pro-Arg-Glu-Arg-Arg-Arg-Lys-Lys-Arg representing the fluorescent N-terminal portion preceding the cleavage site in Q-HA_{HKV}, was also synthesized for the purpose of generating standard curves and for quenching corrections (see below). All peptides except FL-

Table 2 Intramolecularly quenched fluorogenic peptides

Abbreviations: EBOV (Z), Ebola virus (Zaire); Q, quench; mut, mutant; F, F protein; WT, wild-type. The inserted mutant variant amino acid residues are underlined. Symbol: ↓, physiological cleavage site.

Virus	Name	Amino acid sequence	Mass (Da)
Avian wild-type	Q-HA _{WT}	Abz-R-N-T-P-R-K-K-R ↓ G-L-Tyx-A	1622
Mutant1	Q-HA _{mut1}	Abz-R-N-T-P- <u>R</u> -R-K-K-R ↓ G-L-Tyx-A	1934
Mutant2	Q-HA _{mut2}	Abz-R-N-T-P- <u>R</u> - <u>E</u> -R-K-K-R ↓ G-L-Tyx-A	1907
HKV	Q-HA _{HKV}	Abz-R-N-T-P- <u>R</u> - <u>E</u> -R-K-K-R ↓ G-L-Tyx-A	2219
RSV	Q-F _{RSV}	Abz-S-K-K-R-K-R-R ↓ F-L-G-Tyx-A	1672
EBOV (Z)	Q-GP _{EBOV}	Abz-G-G-R-R-T-R-R ↓ E-A-I-Tyx-A	1568

HA_{HKV} were prepared with the C-terminus in the amide (CONH₂) form.

Peptide synthesis

All peptides were synthesized on a solid-phase automated peptide synthesizer instrument (Pioneer; PE-PerSeptive Biosystems, Framingham, MA, U.S.A.), by following the *O*-hexafluorophospho-[7-azabenzotriazol-1-yl]-*N,N,N',N'*-tetramethyluronium ('HATU')/di-isopropylethylamine ('DIEA')-mediated Fmoc chemistry [39,40]. For the incorporation of two unnatural amino acids, 2-Abz and Tyx, into the growing peptide chain an extended coupling cycle (45 min) was used instead of normal standard (20 min) or fast (10 min) cycle. The crude peptides were recovered as described [39,40].

Peptide purification and MS

The crude peptides were purified by RP-HPLC, first with a semipreparative and then an analytical C₁₈ column with the conditions as described above in the Materials section. Matrix-assisted laser desorption ionization–time-of-flight MS (MALDI–TOF–MS) was performed on a Voyager DE-Pro instrument (PE PerSeptive Biosystems) as described [39,40]. For fractions obtained from RP-HPLC, MS was performed directly on 1 µl of the eluates.

Enzymic assay

For proteolytic activity measurements, the enzyme samples were assayed by cleavage of the fluorogenic substrate pERTKR-MCA [38,39]. For each assay, 5 µl of the enzyme sample was added to a solution containing 50 mM Tris/HCl, pH 7.0, 2 mM CaCl₂ and 100 µM pERTKR-MCA in a total volume of 100 µl.

Correction for fluorescence quenching

Consistent with earlier observations [32,33,35] was our finding that the use of an extended sequence between the acceptor/donor pair led to a significant increase in the level of background fluorescence as well as a decrease in the intramolecular quenching effect. This problem was overcome either by coupling RP-HPLC analysis to each time point or by the use of initial velocity measurement with increasing concentrations of substrate, to estimate the correct amount of product released. In agreement with a previous study [32], we noted a significant intermolecular quenching of the released fluorescent material by the undigested substrate molecule. To correct for this, increasing amounts of the fluorescent N-terminal cleavage product FL-HA_{HKV} were incubated in the presence and the absence of a single representative

substrate, namely Q-HA_{HKV}, up to a concentration of 30 µM; a correction factor was established [32].

Determination of kinetic parameters $V_{\max}$ (app) and K_m (app) and site of cleavage

For the determination of the kinetic parameters $V_{\max}$ (app) and K_m (app), enzyme samples, typically 5 µl, equivalent to an activity leading to the release of 4.1 µmol/h (furin), 3.9 µmol/h (PC5) and 3.6 µmol/h (PC7) of 7-amino-4-methylcoumarin (AMC) from pERTKR-MCA (100 µM) were used. To these were added increasing concentrations of each intramolecularly quenched peptide ranging from 1 to 20 µM in a total volume of 100 µl of buffer described above, in a 96-well microtitre plate; the assay was conducted for 30 min at 37 °C. The rate of substrate hydrolysis was obtained from the changes in fluorescence readings and the values were transformed into amounts of µM/h cleaved by using the standard curve and quenching corrections as described above with FL-HA_{HKV}. $V_{\max}$ (app) and K_m (app) were calculated from a non-linear regression analysis (GrafFit v4.0 from Erthacus Software; distributed by Sigma Chemical Co.) of plots of the hydrolysis rate against the substrate concentration. For recording the scanning fluorescence spectrum, the peptide, e.g. Q-HA_{HKV} (10 µM) in the above buffer mixture (100 µl) was incubated with the enzyme (5 µl) and scanned for the emission spectrum with λ_{ex} fixed at 320 nm at various time points.

RESULTS

Processing of IQF peptides by furin, PC5 and PC7

The identities of all synthetic peptides reported in this study were confirmed by both amino acid analysis (results not shown) and by MALDI–TOF–MS (Table 2). We selected furin, PC5 and PC7 as the candidate enzymes for the processing of the chosen viruses (HKV, EBOV and RSV) because primary replication takes place in the respiratory tract for HKV and RSV and in monocytes/macrophages for EBOV and because expression of these PCs is elevated in the appropriate cells [7,41,42]. All IQF peptides are appropriately cleaved *in vitro* by furin, PC5 and PC7 with different kinetic efficiencies (Table 3). In each case the major cleavage occurs at the physiological site, as confirmed by RP-HPLC and MALDI–TOF–MS analyses of the digestion products. It is further noted that despite the presence of potential multiple PC-like cleavage sites in some of these model peptides such as Q-HA_{mut1}, Q-HA_{HKV}, Q-F_{RSV} (in which F stands for fusion protein) and Q-GP_{EBOV}, we noticed only a single cleavage consistent with the physiological site (Table 2). Figure 1 shows representative RP-HPLC chromatograms of the products of 10.2 µM Q-HA_{HKV} digested by furin for 1 h and 16 h. The peptide was fully cleaved by furin in 1 h, as confirmed by the complete disappearance of the parent peptide (Figure 1; retention time 24.4 min) and the emergence of two additional peaks that were eluted at 23.7 and 16.2 min. The last two peaks are attributed to the cleaved fragments Gly-Leu-Tyx-Ala-CONH₂ [observed m/z 466.9 ($M+H$)⁺ by MALDI-MS, calculated 465.5] and Abz-Arg-Asn-Thr-Pro-Arg-Glu-Arg-Arg-Arg-Lys-Lys-Arg-OH [observed m/z 1771.8 ($M+H$)⁺ by MALDI-MS, calculated 1770.1] respectively. As expected, the early-eluting peak was detectable by both UV and fluorescence absorbance owing to the highly fluorescent Abz-containing N-terminal fragment. In contrast, the late-eluting peak was the non-fluorescent, UV-absorbing C-terminal fragment. Figure 1 also reveals that after prolonged incubation with furin (approx. 16 h), Q-HA_{HKV} exhibited less than 20% additional cleavage (Pro-Arg-Glu-Arg-Arg↓Arg). Similarly, prolonged incubation with PC5 led to two minor additional

Table 3 Kinetic constants for the cleavage of intramolecularly quenched fluorogenic peptide substrates derived from viral glycoproteins with furin, PC5 and PC7

Results are means $\pm$ S.D. for independent experiments in triplicate. The underlined values are the most significant in each enzyme category.

Convertase	Substrate	$V_{\max}$ (app) ($\mu\text{mol s}^{-1}$)	K_m (app) (μM)	$V_{\max}$ (app)/ K_m (app) ($\text{litre}^{-1} \cdot \text{s}^{-1}$)	$V_{\max}$ (app)/ K_m (app) (relative)
Furin	Q-HA _{WT}	116 $\pm$ 4.8	6.3 $\pm$ 1.4	18.3	0.2
	Q-HA _{mut1}	77.2 $\pm$ 3.3	1.7 $\pm$ 0.8	45.4	0.5
	Q-HA _{mut2}	73.8 $\pm$ 8.3	5.2 $\pm$ 1.5	14.2	0.2
	Q-HA _{HKV}	513 $\pm$ 10.1	5.7 $\pm$ 0.5	<u>90.0</u>	<u>1.0</u>
	Q-F _{RSV}	64.6 $\pm$ 3.4	3.6 $\pm$ 0.9	17.9	0.2
	Q-GP _{EBOV}	157 $\pm$ 4.7	3.4 $\pm$ 0.5	46.1	0.5
PC5	Q-HA _{WT}	96.5 $\pm$ 2.7	4.2 $\pm$ 0.8	22.9	0.3
	Q-HA _{mut1}	68.5 $\pm$ 1.7	3.3 $\pm$ 0.3	20.6	0.2
	Q-HA _{mut2}	52.8 $\pm$ 1.3	2.1 $\pm$ 0.3	25.1	0.3
	Q-HA _{HKV}	470 $\pm$ 15.1	5.1 $\pm$ 0.6	<u>92.2</u>	<u>1.0</u>
	Q-F _{RSV}	51.5 $\pm$ 1.4	3.1 $\pm$ 0.4	16.6	0.2
	Q-GP _{EBOV}	127 $\pm$ 4.5	3.1 $\pm$ 0.7	40.8	0.4
PC7	Q-HA _{WT}	78.7 $\pm$ 2.1	5.0 $\pm$ 0.7	15.9	0.3
	Q-HA _{mut1}	75.6 $\pm$ 2.8	1.5 $\pm$ 0.6	<u>50.4</u>	<u>1.0</u>
	Q-HA _{mut2}	57.9 $\pm$ 2.4	2.9 $\pm$ 0.7	19.9	0.4
	Q-HA _{HKV}	12.2 $\pm$ 1.1	10.1 $\pm$ 0.9	1.2	0.02
	Q-F _{RSV}	33.1 $\pm$ 0.8	0.9 $\pm$ 0.2	36.8	0.7
	Q-GP _{EBOV}	71.6 $\pm$ 1.3	1.4 $\pm$ 0.2	<u>50.4</u>	<u>1.0</u>

Figure 1 RP-HPLC chromatograms showing the separation of digestion products of Q-HA_{HKV} by furin after incubation for 1 h (upper panel) and 16 h (lower panel)

Each chromatogram represents a digestion of 10.2 μM of peptide with furin having an activity of 4.1 μmol of AMC/h released from pERTKR-MCA. The separation was monitored on-line by both UV absorbance at 210 nm and fluorescence ($\lambda_{\text{ex}} = 320 \text{ nm}$ and $\lambda_{\text{em}} = 420 \text{ nm}$), measured in arbitrary units. The various peaks with retention times shown in parenthesis were analysed by amino acid composition and MALDI-TOF-MS. Abbreviations: NT₁, fluorescent N-terminal fragment (1–13) generated by cleavage at the physiological site (heavy arrow); NT₂, fragment (1–9) generated by cleavage at an alternate site (thin arrow); CT₁ and CT₂, non-fluorescent C-terminal fragments (14–17) and (10–17) respectively.

cleavages. This is illustrated in Figure 2, where major and minor cleavages as indicated respectively by heavy and light arrows in Abz-Arg-Asn-Thr-Pro-Arg-Glu-Arg-Arg-Arg-Lys-Lys-Arg-Gly-Leu-Tyr-Ala. Interestingly, PC7 did not exhibit any additional cleavage of Q-HA_{HKV} even when the peptide was fully digested (Figure 3). Although kinetically slow, PC7 seems to be more selective than furin and PC5 in cleaving Q-HA_{HKV} (Figures 1–3). The cleavage efficiencies of various IQF peptides

for each convertase were further investigated by detailed kinetic analyses (see below).

Kinetic parameters of PC-mediated cleavage of IQF peptides

An analysis of the rate of cleavage of each IQF peptide was performed with furin, PC5 and PC7. The study was conducted with a fixed amount of peptide and enzyme activity as measured

Figure 2 RP-HPLC chromatograms showing the separation of the digestion products of Q-HA_{HKV} by PC5 after incubation for 1 h (upper panel) and 16 h (lower panel)

The undigested peptide was eluted at 24.4 min. Conditions were the same as for furin (Figure 1) except that the sample of PC5 used had an activity of 3.9 μmol of AMC/h released. The peptides NT₁, NT₂, CT₁ and CT₂ are identical with those in Figure 1. In addition, prolonged incubation (16 h) led to the formation of another minor peptide, NT₃ (1–10). The corresponding CT₃ peptide was not detected as a separate peak. Absorbance and fluorescence are measured in arbitrary units.

Figure 3 RP-HPLC chromatograms showing the separation of the digestion products of Q-HA_{HKV} by PC7 after incubation for 1 h (upper panel) and 16 h (lower panel)

Conditions were the same as for furin (Figure 1) except that PC7 had an activity equivalent to 3.6 μmol of AMC/h. NT₁ and CT₁ are the same as in Figure 1. Absorbance and fluorescence are measured in arbitrary units.

with pERTKR-MCA. With the exception of Q-F_{RSV} and Q-GP_{EBOV}, all peptides were fully processed by furin and PC5 within 2 h. For example, in 1 h the Q-HA_{HKV} peptide was cleaved

more than 90 % by furin, compared with 65 % and 10 % by PC5 and PC7 respectively.

After incubations of each IQF peptide (Table 2) with furin,

Figure 4 Representative plot of the initial rate of cleavage of Q-GP_{EBOV} by furin (■, □), PC5 (▼, ▽) and PC7 (●, ○)

The enzymic activities used were the same as in Figures 1–3 and were monitored on-line by the change in fluorescence intensity as a function of substrate concentration. Symbols: □, ▽, ○, raw data; ■, ▼, ●, quench-corrected data [32].

PC5 or PC7, we determined their kinetic constants $V_{\max}$ (app) and K_m (app) by monitoring the changes in fluorescence readings [32–34]. Each incubation was performed for no more than 1 h, such that only the single physiologically relevant cleavage occurred. The determination of $V_{\max}$ (app) and K_m (app) from Michaelis–Menten plots, including corrections for fluorescence quenching by the undigested peptide on the fluorescence derived from the processed N-terminal fragment, was performed as published [32]. Figure 4 is a representative example showing the

importance of quench correction, especially at high substrate concentration, for the analysis of the kinetic data of the processing of Q-GP_{EBOV} by each convertase.

Furin parameters

Our results revealed that most IQF peptides are efficiently cleaved by furin with $V_{\max}$ (app)/ K_m (app) ranging from a low of 14.2 to a high of 90.0 litre⁻¹·s⁻¹. The most efficient processing was observed with the HKV-derived Q-HA_{HKV} peptide. This value was 5–6-fold higher than those obtained with Q-F_{RSV}, Q-HA_{wt} and Q-HA_{mut2} (Table 3). The next best peptides cleaved by furin were Q-GP_{EBOV} and Q-HA_{mut1}, which were cleaved approximately half as efficiently as Q-HA_{HKV}.

PC5 parameters

Like furin, PC5 cleaved most IQF peptides fairly well (Table 3). The peptide most effectively processed was again Q-HA_{HKV}, with a $V_{\max}$ (app)/ K_m (app) of 92.2 litre⁻¹·s⁻¹; the next best cleaved peptide was Q-GP_{EBOV}. However, in contrast with furin, Q-HA_{mut1} was also one of the worst substrates, together with Q-F_{RSV}, Q-HA_{wt} and Q-HA_{mut2} (Table 3).

PC7 parameters

PC7 cleaved the EBOV Q-GP_{EBOV} and avian RR insertion mutant Q-HA_{mut1} with the highest efficiency and exhibited an approx. 1.3-fold lower $V_{\max}$ (app)/ K_m (app) for Q-F_{RSV} (Table 3). Interestingly, unlike furin and PC5, the HKV-derived peptide Q-HA_{HKV} was processed very poorly by PC7, exhibiting an approx. 75-fold lower $V_{\max}$ (app)/ K_m (app) (Table 3). The remaining peptides, namely Q-HA_{wt} and Q-HA_{mut2}, were cleaved with intermediate efficiencies.

When the three sets of kinetic data are compared, it is revealed that both furin and PC5 cleaved Q-HA_{HKV} with the highest

Figure 5 RP-HPLC showing the effect of various SEA concentrations on the 16 h furin-mediated cleavage of Q-HA_{HKV}

For the sake of clarity the chromatograms have been overlaid with a 1 min offset along the time axis, starting from the uppermost panel. Absorbance and fluorescence are measured in arbitrary units.

degree of efficiency. In contrast, Q-GP_{EBOV} seemed to be processed with comparable ease by the three convertases, whereas Q-F_{RSV} was cleaved best by PC7.

Processing of IQF peptides is blocked by SEA

The plant-derived inhibitor SEA, also called 14-dehydroandrographolide succinoyl monoester ('DASM') [36], was next tested for its ability to inhibit the PC-generated cleavage of Q-HA_{HKV}. As a representative example, the furin-dependent cleavage of Q-HA_{HKV} was inhibited by SEA in a dose-dependent manner; maximal inhibition was reached at levels below 75 μ M (Figure 5). Comparison with the other PCs revealed that the ED₅₀ values for furin, PC5 and PC7 were approx. 20, 120 and 200 μ M respectively. This result suggested that the non-peptide convertase inhibitor SEA is 6-fold and 10-fold more potent in inhibiting the cleavage of Q-HA_{HKV} *in vitro* by furin than that by PC5 and PC7 respectively.

DISCUSSION

The kinetic parameters $V_{\max}$ (app)/ K_m (app) *in vitro* presented above (Table 3) using model synthetic peptides derived from viral glycoproteins suggested that the host cellular enzymes furin and PC5 represent the best-candidate Hong Kong viral glycoprotein convertases, whereas PC7 seems to be the optimal convertase for the activation of RSV F-protein. For the proteolytic maturation of EBOV glycoprotein, our results with synthetic peptides indicate that all three convertases are almost equally effective. These observations obtained *in vitro* with synthetic peptides can be extended to more physiological models, as has been done for the proteolytic maturation of the glycoprotein gp160 of HIV in which a synthetic peptide P⁵⁰⁰TKAKRVVQREKR↓AVGI⁵¹⁶ spanning the expected physiological site was used as a substrate for various PCs [15]. This and a parallel *ex vivo* study [43] concluded that gp160 is processed intracellularly best by furin, followed by PACE4, PC5-B and PC7.

The comparative kinetic data on the model peptides derived from the avian wild-type HA and its virulent insertion mutants indicate a potential relationship between the proteolytic processing rate and viral infectivity. Out of 15 HA and 9 neuraminidase ('NA') subtypes of influenza virus so far identified in avian species, HKV H5N1 is the most infectious variant [24], correlating with the basic tetrapeptide RERR insertion within the HA wild-type sequence. This observation, in conjunction with our results showing that RERR incorporation enhances the proteolytic processing by furin and PC5 at least 4-fold over the wild type, suggests the participation of these convertases in the pathogenicity of HKV. In contrast, PC7 poorly cleaves the extended processing site (7-fold decrease). Moreover, the dipeptide insertions RR and RE did not have any significant effect on PC5-mediated cleavages. This suggests that the incorporation of an additional P6 Arg residue at the N-terminus of Arg-Xaa-Lys-Arg↓ did not produce any significant effect on processing, whereas the combination of P8 and P6 Arg residues (e.g. in the RERR insertion) led to an enhanced processing rate by PC5. For furin processing, the RR insertion led to an approx. 3-fold increase in cleavage efficiency over the wild-type sequence and the RE insertion, suggesting that furin prefers a positively charged P5 Arg residue rather than the negatively charged Glu residue [43]. Moreover, the results revealed that furin, PC5 and PC7 did not cleave Q-HA_{HKV} at either the Arg-Lys↓ or the Lys-Lys↓ site despite the presence of an Arg residue at P4 or P6 or at both positions.

It was demonstrated previously that interactions involving amino acid residues occupying positions from P1 to P4 of a substrate are more critical determinants for cleavage efficiency by furin [43,44]. Molecular modelling and substrate specificity studies have identified important residues in the catalytic subsites, S4 to S1, of a number of PCs including furin that are implicated in substrate recognition and binding [45]. This study now shows that, for furin and PC5, residues distant from the cleavage site, such as those at P5 to P10, can make important contributions towards substrate recognition. In contrast, PC7 does not require such an extended sequence for maximum cleavage. It should be noted that, similarly to the H5N1 variant, subtle modifications of the envelope protein such as those occurring in a Mexico variant of H5N2 of influenza also result in increased pathogenicity due either to a tetrabasic RKRK insertion at the PC-like cleavage site of HA [46] or to the loss of an N-glycosylation site [47], demonstrating that masking of the cleavage site by an oligosaccharide can be overcome by an enhancement of the basic charge at the processing site. Finally, besides the envelope glycoprotein, other proteins such as nucleoproteins might also have a role in the pathogenicity of influenza viruses [48].

SEA was previously shown to be a reversible inhibitor of the processing of pERTKR-MCA by furin, PC1 and PC7, with K_i values in the low micromolar range [36]. The present study revealed that SEA inhibits the cleavage of Q-HA_{HKV} by furin (Figure 5) and also by PC5 and PC7 (results not shown). Furthermore, SEA exhibits antiviral properties against HIV-1 and HIV-2 in H9 cells at concentrations in the low micromolar range [49], which is the range observed from the *in vitro* processing of Q-HA_{HKV} by furin. Thus future studies aimed at improving the inhibitory and cell-permeability properties of SEA might be very beneficial. The present study suggests that the development of convertase-based inhibitors might be considered as an alternative approach to broad-based anti-pathogens of infectious viruses. Current antiviral agents that target viral gene products such as DNA polymerase and viral proteases invariably lead to the generation of drug-resistant strains [50]. Moreover, many retroviruses mutate at such a rate that viral proteins might not always be an optimal pharmacological target [51]. In fact, because PCs are host cell proteinases, resistance through viral mutation is less likely. Therefore adjunct therapies could also involve the strategic regulation of the activity or levels of furin, PC5 and PC7 (and possibly PACE4) [52], which allow the management of retroviral infections as well as a variety of acute pathogenic infections [19,20,22,31,53]. Indeed, a strategy with α 1-PDX, a potent furin inhibitor, provided a protein-based therapeutic for human cytomegalovirus infections [20]. Potentially, other PC-inhibitors might also find future applications in the field of viral infectious diseases [33,39,54].

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