A rapid fluorometric assay for the proteolytic activity of SKI-1/S1P based on the surface glycoprotein of the hemorrhagic fever Lassa virus

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Abstract The subtilase subtilisin kexin isozyme-1 (SKI-1)/site 1 protease (S1P), has been implicated in the processing of Lassa virus glycoprotein C (GP-C) precursor into GP1 and GP2 that are responsible for viral fusion with the host cell membrane. Here, we studied in vitro the kinetics of this cleavage by hSKI-1 using an intramolecularly quenched fluorogenic (IQF) peptide, Q-GPC$^{251-263}$ [Abz-^{251}Asp-Ile-Tyr-Ile-Ser-Arg-Arg-Leu-Leu↓Gly-Thr-Phe-Thr^{263}-3-NitroTyr-Ala-CONH$_2$], containing the identified site. The measured $V_{max\ (app)}/K_{m\ (app)}$ was compared to those for other IQF SKI-substrates. Q-GPC$^{251-263}$ is cleaved 10-fold more efficiently than the previously known best SKI-substrate, Q-hproSKI$^{134-142}$. This study confirmed the role of SKI-1 in GP-C processing and provides a novel, rapid and efficient enzymatic assay of SKI-1. © **2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Subtilisin kexin isozyme 1; Site 1 protease; Lassa virus glycoprotein; Intramolecularly quenched fluorogenic substrate; Enzyme assay; Kinetic study; $V_{max\ (app)}/K_{m\ (app)}$

1. Introduction

Viral infections are often mediated by the cleavage of associated precursor surface glycoproteins into their functionally active forms [1–3]. Following proteolysis, the smaller 'activated' polypeptide fragments become highly virulent and fusogenic to the host cell membrane leading to the onset or progression of infection [4,5]. It is now recognized that besides viral protease(s), single or multiple host enzymes also play a crucial role in viral pathogenesis by providing necessary assistance to the activation of surface glycoprotein of the invading virus particle. Hence, identifying the host protease/s responsible for maturation of precursor glycoproteins and silencing their activity via specific inhibitors may provide an effective alternative to viral therapeutics [5–7]. Several studies indicated that the cellular proteases 'proprotein convertases' (PCs) (reviewed in [8]) are responsible for proteolytic maturation of envelope glycoproteins of HIV [4,5], Ebola [9], and other viruses [2,7,10]. It was further demonstrated that PC-specific inhibitors are capable of blocking these cleavages and display antiviral properties [5,7,11]. The primary description of such inhibitors (against furin) capable of blocking the cleavage of HIV-1 glycoprotein gp160 was reported by Hallenberger et al. [5]. PCs are Ca^{2+}-dependent serine proteases related to the family of subtilases that include bacterial subtilisin and yeast kexin. These cleave proproteins at selected single or pairs of basic residues characterized by the motif R/H/KXK/RR↓ or R/H/KXXR↓ [8], where X = any amino acid except Cys. Recently a paralog of PCs, called 'subtilisin kexin isozyme-1' (SKI-1) or site 1 protease (S1P) has been discovered. The latter cleaves at the carboxy-terminus of non-basic amino acids within the consensus sequence R/KX-hydrophobic-L/I/S/T↓ [12–15]. So far five physiological substrates of SKI-1 have been identified. They are proBDNF [13,14], SREBPs [12,15], the transcription factor ATF-6 [16], pro-somatostatin [17] and the prodomain of SKI-1 itself [14,18]. Recently SKI-1 has been identified by Lenz et al. as the cleavage enzyme of 76 kDa Lassa virus precursor glycoprotein C (GP-C) into two active subunits, the N-terminal GP-1 and the C-terminal membrane-bound GP-2 [19]. Earlier, the site of this cleavage had been indicated by the same group as RRLL^{259}↓GTF [20]. Lassa virus, a member of the *arenavirus* family, mostly found in West Africa, causes hemorrhagic fever with flu-like symptoms, replication in the cytoplasm, and bud-ding at the plasma membrane [21,22]. Between 100 000 and 500 000 cases have been reported annually, with a mortality rate of ~15%, particularly among the pregnant women, while their fetuses have the highest rate of mortality (92–100%). Infection is generally caused by exposure to the virus via infected feces, saliva, etc., through broken skin and mucous membranes [19,21,22]. To further confirm the role of SKI-1 in the proteolytic activation of GP-C and to develop a rapid bioassay for its activity, we prepared an internally quenched fluorogenic (IQF) peptide [7,23,24] encompassing the GP-C cleavage site. The measured kinetic parameter $V_{max\ (app)}/K_{m\ (app)}$ was compared with those for other IQF peptides derived from proregion of hSKI-1, hproBDNF and potential SKI-1 substrates. The latter include the proposed 19 kDa cleavage site of malaria parasite *Plasmodium falciparum* merozite surface protein 1 (PfMSP-1) [25]. The Cys-rich 19 kDa fragment is

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Abbreviations: Abz, 2-amino benzoic acid; Tyx, 3-nitro tyrosine [Tyr(3-NO$_2$)]; SKI-1, subtilisin kexin isozyme-1; S1P, site 1 protease; PC, proprotein convertase; Fmoc, fluorenylmethyloxycarbonyl; RP-HPLC, reverse-phase high performance liquid chromatography; GP-C, glycoprotein C of Lassa virus; MALDI-tof MS, matrix-assisted laser desorption ionization time of flight mass spectroscopy; R_t, retention time; *m/z*, mass/charge; TFA, trifluoroacetic acid

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retained by the merozite during erythrocyte invasion and the processing leading to its formation plays an essential role in malarial infection [26].

2. Materials and methods

All fluorenylmethyloxycarbonyl (Fmoc)-amino acids (L-configuration), the coupling agents and solvents were purchased from PE Biosystems Inc. (Framingham, MA, USA), Calbiochem Novabiochem, (San Diego, CA, USA), Chem-Impex International Inc., (Wood Dale, IL, USA) and Aldrich Chemical Company (Milwakee, WI, USA). Amino acid analyses were performed following 24 h hydrolysis in 6 N HCl at 110°C in vacuo using a Dionnex autoanalyzer [7].

Reverse-phase high performance liquid chromatography (RP-HPLC) was performed on a Rainin (Dynamax) instrument, using a 10 μ, 300 Å C_{18} column [Jupiter, Phenomenex, 0.46×25 cm (analytical) or 1×25 cm (semipreparative)]. The buffers comprised an aqueous 0.1% (v/v) trifluoroacetic acid (TFA) solution and an organic phase of CH_3CN containing 0.1% (v/v) TFA. Peptides were eluted with a 1%/min linear gradient (5–60%) of 0.1% aqueous TFA/CH_3CN at a flow rate of 1 ml/min (analytical run) or 2 ml/min (semipreparative run), following a 5 min isocratic at 5% of 0.1% TFA/CH_3CN. The elution of the peptides was monitored by UV absorbancy ($\lambda = 210$ or 230 nm) and by fluorescence measurement (Dynamax fluorescence detector, model FL-2) at λ_{ex} and λ_{em} fixed at 320 and 420 nm respectively. All fluorometric assays were performed with a spectofluorometer instrument (Gemini, Molecular Dynamics, Sunnydale, CA, USA) as described [7].

2.1. Source of soluble recombinant hSKI-1

The soluble hSKI-1 was obtained as described using a BTMD hSKI-1 construct ending at PGRYNQE^{997} [14]. Following a 6 h incubation of vaccinia virus infected BTMD SKI-1 recombinant in HK-293 cells, the conditioned medium was concentrated (~15-fold) using centricon-30 (Amicon, USA), dialyzed and stored with 40% glycerol at −20°C before use.

2.2. Design of IQF peptide substrates

Several IQF-peptides with an electron donor 2-amino benzoic acid (2-Abz) and an acceptor 3-nitro tyrosine (Tyx) group attached respectively to the amino and carboxy-terminals of the peptide were designed. An additional Ala residue was incorporated at the carboxy-terminus to facilitate synthesis. Table 1 lists all nine IQF peptides. Among these, Q-hSKI$^{134-142}$ and Q-hSKI$^{132-142}$ were previously reported by us [14]. Another peptide, FL-GPC$^{251-259}$, representing the NH_2-terminal portion preceeding the GP-C cleavage site was also synthesized for generating a standard curve needed for quenching corrections (see below). All peptides, except FL-GPC$^{251-259}$, were prepared with the carboxy-terminal in the amide ($CONH_2$) form.

2.3. Peptide synthesis

All peptides were synthesized on a solid phase automated peptide synthesizer (Pioneer model, PE-PerSeptive Biosystems, Framingham, MA, USA), using *O*-hexafluorophospho-[7-azabenzotriazol-1-yl]-*N*,*N*,*N*′,*N*′-tetramethyluronium/diisopropyl ethyl amine-mediated Fmoc chemistry [7,14] and polyamino linker polyethylene glycol resin. For incorporation of unnatural amino acids, 2-Abz and Tyx, a double coupling each with extended cycle was used.

2.4. Peptide purification and mass spectrometry (MS)

Peptides were purified by RP-HPLC using C_{18} semipreparative and analytical columns. They were characterized by matrix-assisted laser desorption ionization time of flight MS (MALDI-tof; Voyageur DE-Pro, PerSeptive Biosystems) using CHCA matrix [7,14].

2.5. Enzyme assay

For measurement of SKI-1 activity, the enzyme sample was incubated at 37°C with varying concentrations of IQF peptides (Table 1). For each assay, 10 μl of the enzyme sample was added to a solution containing the peptide (100 μM) in buffer consisting of 25 mM Tris–HCl+25 mM 4-morpholine ethane sulphonic acid monohydrate, pH 7.4, 2.5 mM $CaCl_2$, in a total volume of 100 μl. Both on-line and stop time assays were performed with λ_{ex} and λ_{em} at 320 and 420 nm respectively.

2.6. Correction for fluorescence quenching

Consistent with earlier studies [7,23], we noted a significant intermolecular quenching of the released fluorescent material by the undigested substrate, especially at high concentrations of the substrate. This is corrected by using a standard quenching curve derived from each IQF peptide and FL-GPC$^{251-259}$ [23].

2.7. Determination of kinetic parameters $V_{max\,(app)}$ and $K_{m\,(app)}$ and sites of cleavages

For measurements of $V_{max\,(app)}$ and $K_{m\,(app)}$, hSKI-1 (10 μl) was incubated (4–8 h) with increasing concentrations (1–20 μM or more) of each IQF peptide in 100 μl of buffer in a 96-well microtiter plate at 37°C. The rate of hydrolysis was obtained from the changes in fluorescence readings and the values transformed into μmol/h of peptide cleaved by using standard curve and quenching corrections [7,14,23]. Data were collected in duplicates and each value is the mean of three independent experiments. $V_{max\,(app)}$ and $K_{m\,(app)}$ were calculated as described [7,14,23]. For determination of site of cleavage, each digest was analyzed by RP-HPLC using the C_{18} analytical column followed by MALDI-tof MS of each isolated peak.

3. Result and discussion

3.1. Design of IQF peptide substrates for SKI-1

IQF peptides (Table 1) were designed from the amino acid sequences around the processing sites of substrates and potential substrates of SKI-1. These include the GP-C cleavage site RRLL^{259}↓GT, the hproSKI-1 primary (RSLK^{137}↓YA) and secondary (RRLL^{186}↓RA) activation sites, the proposed malarial PfMSP-1 processing site (KFQDML^{1605}↓NIS) of the 19 kDa form and the hproBDNF cleavage site (RGLT^{57}↓SL) resulting in the 28 kDa form. We have also included two additional IQF peptides derived from the PC-processing site of Hong Kong virus glycoprotein [27] and NRD convertase processing site of prodynorphin [28], since they also contain a potential site of cleavage for SKI-1 (RKKRGL^{332}↓Tyx-A and RRIQ^{9}↓Tyx respectively).

3.2. Digestion of Q-GPC$^{251-263}$ by recombinant SKI-1

Among the nine peptides tested, only Q-GPC$^{251-263}$, Q-SKI-1$^{134-142}$, Q-SKI-1$^{132-142}$, Q-SKI-1$^{181-187}$+LE, Q-SKI-1$^{178-188}$ and Q-HKV$^{318-332}$ were cleaved in vitro by SKI-1 with varying kinetic efficiencies. In all cases, the cleavage occurs mostly at the physiological site (Table 1). The exact cleavage site was confirmed by RP-HPLC of the digest and MALDI-tof MS analysis of the materials under the collected peaks (Table 2). Fig. 1A, shows that Q-GPC$^{251-263}$ is almost fully cleaved by SKI-1 in 12 h. This is confirmed by near disappearance of the parent peptide peak [retention time (R_t) ~45.3 min, mass/charge (m/z) observed = 1949.8 $(M+H)^+$, calculated = 1951] and the emergence of two additional peaks at 27.1 and 39.1 min, identified respectively as the cleaved fragments C-terminal GTFT-Tyx-A-NH_2 [m/z observed = 702.06 $(M+H)^+$, calculated = 701.2] and N-terminal Abz-DIYISRRLL-OH [m/z observed = 1271.9 $(M+H)^+$, calculated = 1270.2]. The result confirmed the previously observed in vivo cleavage by SKI-1 of GP-C at RRLL^{259}↓GTF [19,20]. Fig. 1 also reveals that even upon prolonged incubation, no cleavage at the potential R-R-L-L-G^{260}↓T-F site is observed. This suggests that despite the presence of P4 Arg and P2 Leu, SKI-1 does not accept a Gly at P1 position, an alkyl hydrophobic residue at P3 position or an aromatic hydrophobic residue at P2′ position or a combination thereof. As expected, treatment of Q-GPC$^{251-263}$ with control wild-type (WT) medium concentrate produced no peptide hydro-

Table 1
List of various IQF peptides

Peptides	P_{16} P_{15} P_{14} P_{13} P_{12} P_{11} P_{10} P_9 P_8 P_7 P_6 P_5 P_4 P_3 P_2 P_1	P_1' P_2' P_3 P_4 P_5 P_6 P_7'
1. Q-GPC$^{251-263}$	**Abz**-Asp-Ile-Tyr-Ile-Ser-Arg-Arg-Leu-Leu ↓	Gly-Thr-Phe-Thr-**Tyx**-Ala
2. Q-hSKI$^{134-142}$	**Abz**-Arg-Ser-Leu-Lys	Tyr-Ala-Glu-Ser-Asp-**Tyx**-Ala
3. Q-hSKI$^{132-142}$	**Abz**-Val-Phe-Arg-Ser-Leu-Lys	Tyr-Ala-Glu-Ser-Asp-**Tyx**-Ala
4. Q-hSKI$^{181-187}$+LE	**Abz**-Ser-Arg-Arg-Leu-Leu	Arg-Ala-Leu-Glu-**Tyx**-Ala
5. Q-hSKI$^{178-188}$	**Abz**-Arg-His-Ser-Ser-Arg-Arg-Leu-Leu	Arg-Ala-Ile-**Tyx**-Ala
6. Q-HKV$^{318-332}$	**Abz**-Arg-Asn-Thr-Pro-Arg-Arg-Glu-Arg-Arg-Arg-Lys-Lys-Arg-Gly-Leu	**Tyx**-Ala
7. Q-Dyn$^{2-9}$	**Abz**-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Gln	**Tyx**-Ala
8. Q-PfMSP-1$^{1600-1608}$	**Abz**-Lys-Phe-Gln-Asp-Met-Leu	Asn-Ile-Ser-**Tyx**-Ala
9. Q-BDNF$^{47-58}$	**Abz**-Asn-Gly-Pro-Lys-Ala-Gly-Ser-Arg-Gly-Leu-Thr	Ser-**Tyx**-Ala
10. FL-GPC$^{251-259}$	**Abz**-Asp-Ile-Tyr-Ile-Ser-Arg-Arg-Leu-Leu	

Important basic (Arg/Lys) and hydrophobic (Leu/Ile) residues are indicated by underline. The arrow indicates the potential cleavage site for SKI-1.

lysis (Fig. 1C). Moreover RP-HPLC of 4 h digest of Q-GPC$^{251-263}$ with varying amounts of SKI-1 (2.5, 5, 7.5 and 10 μl) showed a gradual diminution of peak intensity for the parent peptide ($R_t \sim 45.4$ min) (data not shown). A similar digestion profile was also noted with time of incubation for a fixed amount of the enzyme.

3.3. Digestion of other IQF peptides by recombinant SKI-1

Both Q-hSKI$^{134-142}$ and Q-hSKI$^{132-142}$ were cleaved by SKI-1 at the RSLK^{137}↓Y site (major), as observed earlier [14,15], and at the RSLKYA^{139}↓ site (minor). This is confirmed by RP-HPLC analysis of each peptide digest showing the presence of peaks for the parent peptide and cleaved prod-

Table 2
MALDI-tof MS data of various fractions obtained from RP-HPLC chromatograms of SKI-1 digests of Q-GPC$^{251-264}$ and Q-hSKI$^{134-142}$

Parent peptide	RP-HPLC Peak position (R_t, min)	Observed Mass $(M+H)^+$	Calculated Mass $(M+H)^+$	Peptide identified
Q-GPC$^{251-263}$	45.4	1949.8	1951.0	Abz-D-I-Y-I-S-R-R-L-L-G-T-F-T-Tyx-A-NH_2
	39.1	1271.9	1270.2	Abz-D-I-Y-I-S-R-R-L-L-OH
	27.1	702.1	701.2	G-T-F-T-Tyx-A-NH_2
Q-hSKI$^{134-142}$	32.8	1465.7	1463.3	Abz-R-S-L-K-Y-A-E-S-D-Tyx-A-NH_2
	23.4	622.3	621.1	Abz-R-S-L-K-OH
	24.4	861.6	860.1	Y-A-E-S-D-Tyx-A-NH_2
	26.2	855.6	854.1	Abz-R-S-L-K-Y-A-OH
	19.8	627.5	626.5	E-S-D-Tyx-A-NH_2

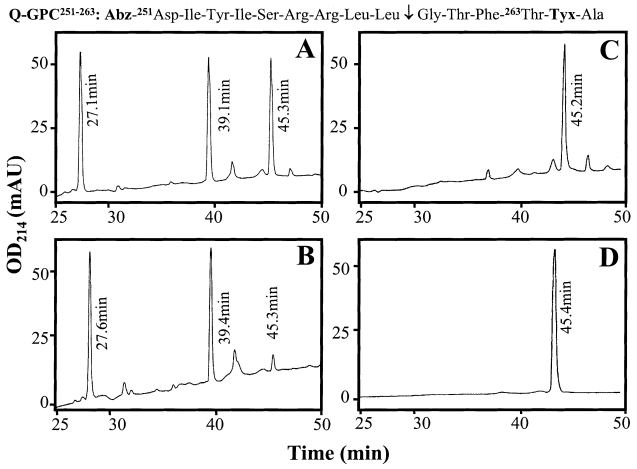

Fig. 1. RP-HPLC chromatograms showing the separation of digestion products of Q-GPC$^{251-263}$ (10 μg) by hSKI-1 (10 μl) after (A) 4 h, (B) 12 h incubation and (C) by HK293 control medium concentrate (10 μl) following 12 h incubation. D: Peptide alone. The separation was monitored on-line by both UV absorbance (210 nm) and fluorescence (λ_{ex} = 320 nm and λ_{em} = 420 nm) (data not shown). The peaks were identified by amino acid composition and MALDI-tof MS (Table 2). Peak 39.1 min = fluorescent N-terminal fragment (Abz-DIYISRRLL-OH), peak 27.1 min = non-fluorescent C-terminal fragment (GTFT-Tyx-A-NH_2), and peak 45.2 min = undigested Q-GPC$^{251-263}$.

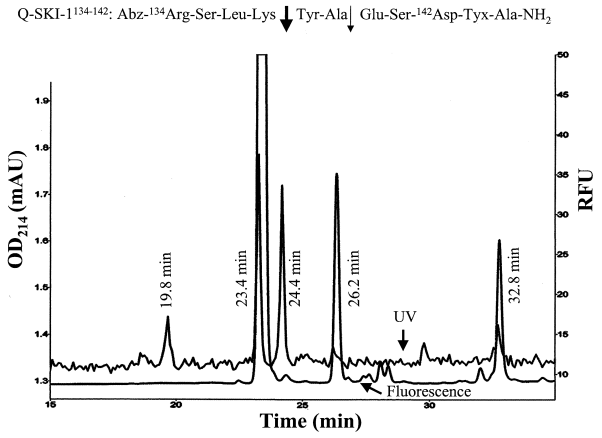

Fig. 2. RP-HPLC chromatograms on digestion products of Q-hSKI$^{134-142}$ by hSKI-1 (10 μl) following 14h incubation. The chromatograms were monitored on line by both UV (top, λ = 214 nm) and fluorescence (bottom, λ_{ex} = 320 nm, λ_{em} = 420 nm) detectors. The peaks were identified by amino acid composition and MALDI-tof MS (Table 2). Peak 23.4 min = fluorescent N-terminal fragment (Abz-RSLK-OH), peak 24.4 min = corresponding non-fluorescent C-terminal fragment (YAESD-Tyx-A-NH_2), peak 26.2 min (minor) = fluorescent N-terminal fragment (Abz-RSLKYA-OH) generated by cleavage at alternate site (thin arrow), peak 19.8 min = corresponding non-fluorescent C-terminal fragment (ESD-Tyx-A-NH_2) and peak 32.8 min = undigested Q-SKI$^{134-142}$.

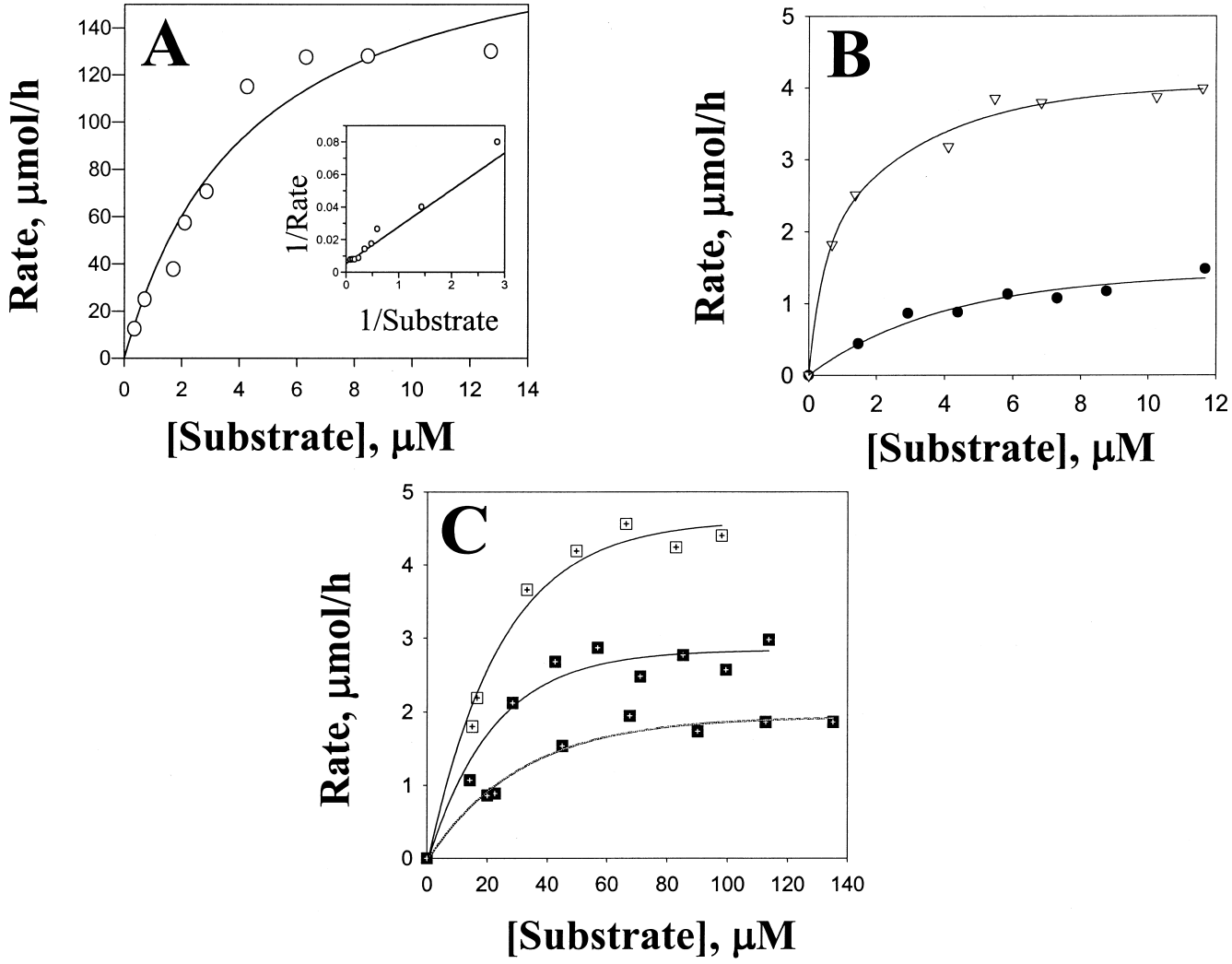

Fig. 3. Michaelis–Menten curves for the cleavages of various IQF peptides by hSKI-1 following 4–8 h incubation. A: Q-GPC$^{251-263}$, B: Q-hSKI$^{134-142}$ (open triangle), Q-hSKI$^{132-142}$ (solid circle), and C: hSKI$^{181-187}$+LE (top), Q-hSKI$^{178-188}$ (middle) and Q-HKV$^{318-332}$ (bottom).

Table 3
Measured kinetic parameters of cleavages of various IQF peptides by soluble recombinant hSKI-1

Peptides	V_{max} (μmol/h)	$K_{m\ (app)}$ μM	$V_{max}/K_{m\ (app)}$ (h^{-1} l^{-1})	Fold
Q-GPC$^{251-263}$	192.7	4.4	43.8	1.0
Q-hSKI$^{134-142}$	4.27	0.96	4.45[a]	0.102
Q-hSKI$^{132-142}$	1.86	4.28	0.44[a]	0.01
Q-hSKI-1$^{181-187}$+LE	5.87	23.08	0.25	0.0057
Q-hSKI$^{178-188}$	3.44	20.02	0.17	0.00388
Q-HKV$^{318-332}$	2.36	27.87	0.085	0.00194
Q-Dyn$^{2-9}$		No cleavage		
Q-PfMSP-1$^{1600-1608}$		No cleavage		
Q-BDNF$^{47-58}$		No cleavage		

[a]Data were taken from our earlier study [14].

ucts. Fig. 2 shows the RP-HPLC chromatogram of a 12 h digest of Q-hSKI$^{134-142}$. The peptides eluting at R_t 32.8, 24.4 and 23.4 min were respectively characterized as undigested peptide (*m/z* observed = 1465.7 $(M+H)^+$, calculated = 1463.3], the C-terminal YAESD-Tyx-A-NH_2 [*m/z* observed = 861.6 $(M+H)^+$, calculated = 860.1] and the highly fluorescent N-terminal Abz-RSLK-OH [*m/z* observed = 622.3 $(M+H)^+$, calculated = 621.l], confirming a cleavage at RSLK$^{137}\downarrow$Y. The peptide eluting at R_t = 26.2 min, visible with the fluorescence detector, was identified as the N-terminal Abz-RSLKYA-OH, [*m/z* observed = 855.6 $(M+H)^+$, calculated = 854.1], indicating an additional relatively minor cleavage at RSLKYA$^{139}\downarrow$E. The corresponding C-terminal fragment of this cleavage [ESD-Tyx-A-NH_2] was attributed to the peak eluting at R_t = 19.8 min (*m/z* observed = 627.6, calculated = 626.5). Q-hSKI$^{181-187}$+LE, Q-hSKI$^{178-188}$ and Q-HKV$^{318-332}$ were all poorly cleaved by SKI-1 (Table 1), as confirmed by MS and RP-HPLC analyses (not shown). These data suggest that although P4 Arg is crucial for recognition by SKI-1, a P6 Arg can compensate for the absence of a P4 Arg.

3.4. Kinetic parameters, $V_{max\ (app)}$ and $K_{m\ (app)}$

Graphic presentations used for the determination of $V_{max\ (app)}$ and $K_{m\ (app)}$ using Michaelis–Menten plots of the digestion of various IQF peptides by SKI-1 are shown in Fig. 3A–C. The data (Table 3) indicate that Q-GPC$^{251-263}$ is the most potent in vitro substrate of SKI-1. It is cleaved ~10-fold more efficiently than the previously known best substrate Q-SKI-1$^{134-142}$ [14]. Q-GPC$^{251-263}$ exhibited $V_{max\ (app)}$ and $K_{m\ (app)}$ 45- and 4.6-fold higher than that for Q-SKI-1$^{134-142}$. $V_{max\ (app)}/K_{m\ (app)}$ ratio for SKI-1-mediated cleavage of Q-GPC$^{251-263}$ is respectively ~100-, 175-, 258- and 515-folds higher than those of Q-SKI-1$^{132-142}$, Q-SKI-1$^{181-187}$+LE, Q-SKI-l$^{178-188}$ and Q-HKV$^{318-332}$, respectively. Thus, P4 Arg, alkyl hydrophobic groups at both P1 and P2

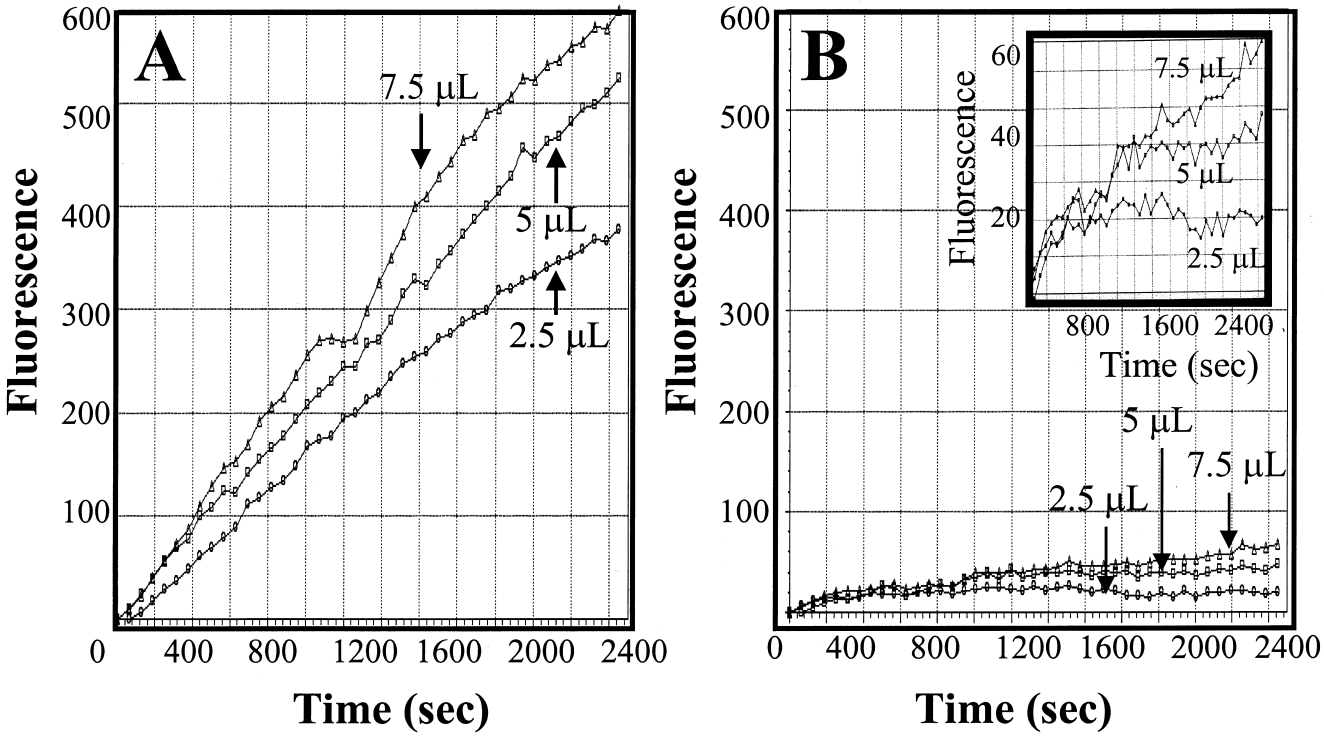

Fig. 4. Progress curves for the release of fluorescence upon incubation of Q-GPC$^{251-263}$, [Abz-DIYISRRLL↓GTFTY(NO_2)A-NH_2], with (A) various amounts of recombinant hSKI-1 (2.5, 5 and 7.5 μl from bottom to top), and (B) WT control HK293 medium concentrate (expanded in the enclosed inset). The fluorescence is monitored over 40 min at λ_{ex} = 320nm, λ_{em} = 420 nm.

and a small amino acid at P1′ are favorable for SKI-1. Moreover, lack of processings of Q-Dyn$^{2-9}$ and Q-PfMSP-1$^{1600-1608}$ and a poor cleavage of Q-HKV$^{318-332}$ suggest that the presence of highly hydrophobic and bulky unnatural aromatic residue Tyx at P1′ position is not accepted by SKI-1 nor a P6 Lys residue for P4 Arg. Moreover, unlike the PCs, presence of consecutive basic residues at P4–P8 may not be well accepted by SKI-1. Inability of SKI-1 to cleave Q-BDNF$^{47-58}$ (Abz-^{47}NG**P**KAGSRGLT↓S^{58}**Tyx**-A) in contrast to hproBDNF$^{50-63}$ (^{50}KAGSRGLT↓SLADTF^{63}), which is readily processed by SKI-1 at the site indicated [14], suggests that the unnatural aromatic Tyx residue at P2′ position and/or a Pro at P9 position (shown in bold) are not suitable for recognition by SKI-1. Thus, a Tyx residue at P1′, P2′ or P4′ is not accepted by SKI-1, even in the context of a sequence with P4 Arg and P2 Leu.

3.5. Progress curve for the cleavage of Q-GPC$^{251-263}$ by SKI-1

Fig. 4A depicts progress curves for the cleavage of Q-GPC$^{251-263}$ (100 μM) with variable amounts of hSKI-1 (2.5, 5 and 7.5 μl). The fluorescence release following cleavage increases gradually with enzyme used. Using Q-GPC$^{251-263}$ as substrate, measured SKI activity in 7.5 μl was 1.3- and 1.8-fold higher than in 5 and 2.5 μl respectively. Similarly, RP-HPLC of respective digests confirm a systematic increase in cleavage with enzyme levels. In contrast, WT medium did not exhibit significant dose-dependent release of fluorescence (Fig. 4B). Furthermore, incubation of Q-GPC$^{251-263}$ with various PCs, such as PC1, furin, PACE4, PC5, PC7 and kexin [8] did not yield any significant release of fluorescence (data not shown), suggesting its specificity towards SKI-1.

4. Conclusion

In summary, Q-GPC$^{251-263}$ is a highly potent and selective fluorogenic substrate for SKI-1. It provides a very efficient, specific and a rapid on-line assay for proteolytic activity of SKI-1. The assay works best with 50–100 μM concentration of the substrate. At higher concentration, the released fluorescence is significantly suppressed by the undigested peptide while at lower concentration, the fluorescence release may not be sufficient for precise measurement. The present study provided in vitro evidence for the role of SKI-1 in the maturation of Lassa virus GP-C and confirmed the previously identified in vivo cleavage site RRLL^{259}↓GT [19,20]. This highly sensitive bioassay of SKI-1 will find useful applications to monitor SKI-1 activity in both tissue and animal models. This is particularly significant in view of recent observation that the selective disruption of SKI-1/S1P gene expression in the liver of mice leads to a significant reduction in lipid and cholesterol biosynthesis [29]. The assay will also help in the development of specific SKI-1 inhibitors that may function as potential lipid-lowering agents for which a close to complete enzyme inhibition is required in order to achieve significant biological effects.

Our study combined with previous ones revealed that (i) SKI-1 recognizes best the consensus motif Arg-X-[Leu/Ile]-[Lys/Ser/Thr/Leu]↓, (ii) Arg but not Lys at P6 may compensate partly for P4 Arg, (iii) the aromatic hydrophobic residue Tyx at P1′, P2′ or P4′ positions is not accepted. The data also suggests that possibly SKI-1 is not a candidate protease for the maturation of merozite surface protein PfMSP-1 [26].

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References

[1] Snacken, R., Kendal, A.P., Haaheim, L.R. and Wood, J.M. (1999) Emerg. Infect. Dis. 5, 1–9.
[2] Klenk, H.D. and Garten, W. (1994) in: Cellular Receptors for Animal Viruses. Monograph 28 (Wimmer, E. Ed.), pp. 241–280, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[3] Moulard, M., Challoin, L., Canarelli, S., Mabrouk, K. and Darbon, H. (1998) Biochemistry 37, 4510–4517.
[4] Decroly, E., Wouters, S., Dibello, C., Lazure, C., Ruysschaert, J.M. and Seidah, N.G. (1996) J. Biol. Chem. 271, 30442–30450.
[5] Hallenberger, S., Bosch, V., Angliker, H., Shaw, E., Klenk, H.D. and Garten, W. (1992) Nature 360, 358–361.
[6] Hallenberger, S., Moulard, M., Sordel, M., Klenk, H.D. and Garten, W. (1997) J. Virol. 71, 1036–1045.
[7] Basak, A., Zhong, M., Munzer, J.S., Chrétien, M. and Seidah, N.G. (2001) Biochem. J. 353, 537–545.
[8] Seidah, N.G. and Chrétien, M. (1999) Brain Res. 848, 45–62.
[9] Volchkov, V.E., Feldmann, H., Volchkova, V.A. and Klenk, H.-D. (1998) Proc. Natl. Acad. Sci. USA 95, 5762–5767.
[10] Bolt, G., Pedersen, L.O. and Birkeslund, H.H. (2000) Virus Res. 68, 25–33.
[11] Anderson, E.D., Thomas, L., Hayflick, J.S. and Thomas, G. (1993) J. Biol. Chem. 268, 24887–24891.
[12] Espenshade, P.J., Cheng, D., Goldstein, J.L. and Brown, M.S. (1999) J. Biol. Chem. 274, 22795–22804.
[13] Seidah, N.G., Mowla, S.J., Hamelin, J., Mamarbachi, A.M., Benjannet, S., Touré, B.B., Basak, A., Munzer, J.S., Marcinkiewicz, J., Zhong, M., Barale, J.C., Lazure, C., Nomura, N., Murphy, R.A., Chrétien, M. and Marcinkiewicz, M. (1999) Proc. Natl. Acad. Sci. USA 96, 1321–1326.
[14] Touré, B.B., Munzer, J.S., Basak, A., Benjannet, S., Rochemont, J., Lazure, C., Chrétien, M. and Seidah, N.G. (2000) J. Biol. Chem. 275, 2349–2358.
[15] Sakai, J., Rawson, R.B., Espenshade, P.J., Cheng, D., Seegmiller, A.C., Goldstein, J.L. and Brown, M.S. (1998) Mol. Cell 2, 505–514.
[16] Ye, J., Rawson, R.B., Komuro, R., Chen, X., Dave, U.P., Prywes, R., Brown, M.S. and Goldstein, J.L. (2000) Mol. Cell 6, 1355–1364.
[17] Mouchantaf, R., Seidah, N.G. and Patel, Y.C. (2000) 'Processing of Prosomatostatin (PSST) to PSST$_{1-10}$ by the Mammalian Proteinase SKI-1/SIP'. Society for Neuroscience 30th Annual Meeting, New Orleans, LA, Nov. 4–9. Abstract # 18.7, p. 27.
[18] Elagoz, A., Benjannet, S., Mammarbassi, A., Wickham, L. and Seidah, N.G. (2001) J. Biol. Chem. M109011200 (published online on Dec 26).
[19] Lenz, O., ter Meulen, J., Klenk, H.D., Seidah, N.G. and Garten, W. (2001) Proc. Natl. Acad. Sci. USA 98, 12701–12705.
[20] Lenz, O., ter Meulen, J., Feldmann, H., Klenk, H.D. and Garten, W. (2000) J. Virol. 74, 11418–11421.
[21] McCormick, J.B., Webb, P.A., Krebs, J.W., Johnson, K.M. and Smith, E.S. (1987) J. Infect. Dis. 155, 437–444.
[22] McCormick, J.B., King, I.J., Webb, P.A., Johnson, K.M., O'Sullivan, R., Smith, E.S., Trippel, S. and Tong, T.C. (1987) J. Infect. Dis. 155, 445–455.
[23] Lazure, C., Gauthier, D., Jean, F., Boudreault, A., Seidah, N.G., Bennett, H.P.J. and Hendy, G.N. (1998) J. Biol. Chem. 273, 8572–8580.
[24] Berman, Y., Juliano, L. and Devi, L.A. (1999) J. Neurochem. 72, 2120–2126.
[25] Barale, J.-C., Blisnick, T., Fujioka, H., Alzari, P.M., Aikawa, M., Braun-Breton, C. and Langsley, G. (1999) Proc. Natl. Acad. Sci. USA 96, 6445–6450.
[26] Longacre, S., Mendis, K.N. and David, P.H. (1994) Mol. Biochem. Parasitol. 64, 191–205.

[27] Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., Hemphill, M., Rowe, T., Shaw, M., Xu, X., Fukuda, K. and Cox, N. (1998) Science 279, 393–396.
[28] Song, E.-S., Mukherjee, A., Juliano, M.A., Pyrek, J.S., Goodman Jr., J.P., Juliano, L. and Hersh, L.B. (2001) J. Biol. Chem. 276, 1152–1155.
[29] Yang, J., Goldstein, J.L., Hammer, R.E., Moon, Y.-A., Brown, M.S. and Horton, J.D. (2001) Proc. Natl. Acad. Sci. USA 98, 13607–13612.