THE PROPROTEIN CONVERTASE SKI-1/S1P: *IN VITRO* ANALYSIS OF LASSA VIRUS GLYCOPROTEIN-DERIVED SUBSTRATES AND *EX VIVO* VALIDATION OF IRREVERSIBLE PEPTIDE INHIBITORS

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Running title: SKI-1/S1P fluorogenic substrates and inhibitors

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Herein we designed, synthesized, tested, and validated fluorogenic methyl coumarinamide (MCA) and chloromethylketone-peptides spanning the Lassa virus GPC cleavage site as substrates and inhibitors for the proprotein convertase SKI-1/S1P. The 7mer-MCA (YISRRLL-MCA) and 8mer-MCA (IYISRRLL-MCA) are very efficiently cleaved with respect to both the 6mer-MCA (ISRRLL-MCA) and point mutated fluorogenic analogues, except for the 7mer mutant Y253F. The importance of the P7 phenyllic residue was confirmed by digestions of two 16mer non fluorogenic peptidyl substrates which differ by a single point mutation (Y253A). Since NMR analysis of these 16mer peptides did not reveal significant structural differences at the recognition motif RRLL, the P7 Tyr residue is likely important in establishing key interactions within the catalytic pocket of SKI-1. Based on these data, we established through analysis of proATF6 and proSREBP-2 cellular processing that decanoylated chloromethylketone 7mer, 6mer and 4mer peptides containing the core RRLL sequence are irreversible and potent ex vivo SKI-1 inhibitors. Although caution must be exercised in using these inhibitors in in vitro reactions, as they can also inhibit the basic amino acid specific convertase furin, within cells and when used at concentrations ≤100 μM these inhibitors are relatively specific for inhibition of SKI-1 processing events, as opposed to those performed by furin-like convertases.

INTRODUCTION

With the advent of the genome sequencing, it became apparent that hydrolases represent ~4% of the total human/mouse genome, with an estimated 500-550 members comprised within the 5 classes of proteases (1). While these proteases cleave their substrates either intra- or extra-cellularly, some of them are implicated in the limited proteolysis of secretory precursors. Among those, the 9 known proprotein convertases (PCs) are members of a unique family of mammalian serine proteinases related to bacterial subtilisin (2-4). The PCs are responsible for the tissue-specific limited proteolysis of multiple polypeptide precursors, generating a large diversity of bioactive molecules in an exquisitely regulated manner. While seven PCs (PC1/3, PC2, furin, PC4, PC5/6, PACE4 and PC7) cleave secretory precursors within the motif R-Xn-R↓, where n=0, 2, 4, or 6, and X is a variable amino acid (aa), except Cys, the other two convertases (SKI-1/S1P and NARC-1/PCSK9) cleave within the motifs R-X-(hydrophobic)-X↓ (3,5-8) and VFAQ↓ (9), respectively.

Subtilisin-kexin-isozyme-1, known as SKI-1 (6,10), is synthesized as an inactive precursor that is autocatalytically cleaved in the endoplasmic reticulum (ER) at two alternate B’ and B sites: RKVF↓ and RKVFRSLK↓, respectively. The latter products are then transported to the cis/medial Golgi whereupon they are further autocatalytically processed into a C-form at RRLL↓ (Fig. 1), generating the active SKI-1 enzyme devoid of its prosegment (5,6,8,10). SKI-1 was simultaneously discovered by our group (10) and that of Goldstein and Brown’s who called it site 1 protease (S1P) (11), a key enzyme in the regulation of lipid metabolism and cholesterol homeostasis that cleaves the transcription factors sterol regulatory element binding proteins (SREBP-1 and SREBP-2) (11,12). The latter are synthesized as precursors harboring two transmembrane domains separated by a short endoplasmic reticulum (ER) luminal loop and comprising N- and C-terminal cytosolic domains. These precursors are cleaved in an SREBP-cleavage-activating protein (SCAP)- and insulin-induced gene (INSIG)-dependent fashion. When cellular cholesterol levels are high, INSIG binds and retains the SCAP-SREBP complex in the ER. When cells are deprived of sterols, INSIG separates, allowing the transport of the SREBP-SCAP complex to the Golgi (12-14). Therein, a two-step proteolytic process (SKI-1 and site 2 protease S2P) (14) releases the cytosolic N-terminal segments of SREBPs from cell membranes, allowing their translocation to the nucleus (nSREBP), where they activate transcription of more than 35 mRNAs coding for proteins/enzymes required for the biosynthesis and uptake of cholesterol and unsaturated fatty acids (15).

Similar to SREBPs, the ER-anchored type-II membrane bound transcription factor ATF6 plays a major role in the unfolded protein response (UPR) (16). Under normal conditions, it is held in the ER by the chaperone BIP, with its N-terminal DNA binding domain facing the cytosol and its COOH terminus in the ER lumen (17). Accumulation of improperly folded proteins in the ER, which can be
induced by calcium depletion (thapsigargin) or inhibition of N-glycosylation (tunicamycin), leads to an ER-stress response resulting in BIP dissociation from proATF6. The latter is then translocated in a SCAP-independent fashion to the Golgi where it is first cleaved by SKI-1 and then by S2P. This releases the cytosolic N-terminal domain, which reaches the nucleus (nATF6) to activate ER stress target genes (18,19).

Other type-II membrane-bound substrates (Fig. 1) include the basic leucine zipper transcription factor Luman, the cellular counterpart of herpes simplex virus VP16 (20), and the CREB-like proteins (21). Brain-derived neurotrophic factor (BDNF) is a soluble substrate and the study of its processing led to the initial cloning of SKI-1 (10). Recently, the soluble pro-somatostatin was also shown to be cleaved by SKI-1 to release the N-terminal peptide antrin (22). Finally, SKI-1 was shown to play a major role in cartilage development in zebrafish (23) and in the processing of surface glycoproteins of infectious viruses such as Lassa (LAV) (24,25), lymphocytic choriomeningitis (LCMV) (26,27) and Crimean Congo hemorrhagic fever (CCHFV) (28) viruses (Fig. 1). In particular, Lassa fever is endemic in West Africa and is estimated to affect some 100,000 people annually. No vaccines or antivirals are available against these deadly viruses.

PC activities are routinely assayed using two types of fluorogenic substrates, peptidyl methyl coumarinamides (MCA) (29) and intramolecularly quenched fluorogenic (IQF) peptides (5,25,30,31). Processing at the peptide-MCA bond causes a fluorescence wavelength shift allowing the detection of the released fluorescent AMC group. IQF peptides contain an N-terminal fluorescent group Abz (o-amino benzoic acid) and a C-terminal quench fluorescence moiety Y(NO2) (3-nitrotyrosine). Cleavage of IQF peptides releases the quenching effect and the released N-terminal Abz containing fragment is now free to fluoresce. Recently, we designed a 16-mer IQF substrate based on the Lassa’s GPC SKI-1 site (24), namely Abz-DIYISRRLLLGFTYY-Y(NO2)-A-amide, which turned out to be the best in vitro SKI-1 substrate known so far (25). In the present work, we designed and analyzed the kinetic properties of a number of MCA-peptides based on viral glycoprotein recognition motifs as potential in vitro substrates for SKI-1 (Fig. 1).

The critical implication of SKI-1 in various cellular functions and in certain pathologies emphasizes the importance of developing specific inhibitors that modulate its activity in disease states. While SKI-1 inhibition was recently achieved by 300 μM of the general serine protease inhibitor AEBSF (32), the latter is not a specific SKI-1 inhibitor. We recently introduced protein-based ex vivo inhibitors of SKI-1 by mutagenizing the reactive site loop of α1-antitrypsin. We also optimized the prosegment-based inhibition of SKI-1 and identified a unique R134E mutant exhibiting a potent inhibitory activity (33). In this work we developed small molecule specific inhibitors of cellular SKI-1 ex vivo activity based on the best in vitro Lassa glycoprotein GPC cleavage site, coupled to an N-terminal decanoyl membrane permeable moiety and a C-terminal chloromethylketone irreversible inhibitor functionality. These could serve as first lead compounds for further refinement.

**EXPERIMENTAL PROCEDURES**

**Synthesis of viral glycoprotein-derived MCA and cmk peptides** – All Fluorogenic peptides were synthesized in house, except for succinyl-RKLL-7-amido-4 methyl coumarinamide (MCA) (GenScript Corp, Piscataway, NJ, USA). Fluorogenic peptides derived from various viral glycoproteins including LCMV’s (Armstrong and WE) MCA-GP259-265, and CCHV’s MCA-GP18-254 were synthesized in a similar fashion to Lassa virus (LAV)’s GPCs. The latter included MCA-GP252-259 [8mer WT], MCA-GP253-259 [7mer WT], MCA-GP254-259 [6mer WT] and MCA-Y253A, Y253S, Y253I, Y253F and Y253V)-GPC253-259(7mer [Ala], 7mer [Ser], 7mer [Ile], 7mer [Phe], 7mer [Val]) (Fig. 3B). The synthesis without the C-terminal amino acid was achieved by SPPS (solid-phase methods) on a semi-automated Applied Biosystems model 431A synthesizer, using standard Nα-Fluorenylmethoxycarbonyl (Fmoc) protocols and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate /N-hydroxybenzotriazole (HBTU/HOBT) as a coupling method on Fmoc-Leu-SASRIN resin (0.77 mmol/g, 0.25 mmol, Bachem, Bubendorf, Switzerland). The introduction of a decanoyl [dec, CH3-(CH2)9-] group to the N-terminus of the inhibitors was obtained by incubating the growing chain still anchored to the solid support with 4 equivalents (eq) of decanoic acid, 4 eq HBTU/HOBT, and 0.1 equivalents DMAP in DMF. The percentage of decanoylation was evaluated to be 100% by a Kaiser test. The C-terminus of α N- and side chains protected crude peptides was coupled to H-Leu-MCA (2...
eq, Bachem, Bubendorf, Switzerland) or H-Leu-chloromethylketone (H-Leu-cmk) (2 eq, Bachem, Bubendorf, Switzerland) with 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylyuronium hexafluorophosphate/1-Hydroxy-7-azabenzotriazole (HATU/HAOt) 0.45 M (1 eq) and 4-dimethylaminopyridine (DMAP) 0.1 M (0.1 eq) in dimethylformamide (DMF). For the fluorogenic peptides, after Fmoc removal (10% diisopropylethylamine (DIEA) in DMF), succinyl group was introduced by succinic anhydride (10 eq) in DMF plus DMAP 0.1 M up to adjust the pH between 7 and 8. Final crude peptides were obtained after removal of side chain protecting groups by incubation in 10 ml mixture of 95% TFA, 2.5% H2O, and 2.5% triethylsilane (TES) for 90 min. Each reaction step was monitored by reverse phase high performance liquid chromatography (RP-HPLC). After lyophilization, the crude fluorogenic products were purified by RP-HPLC on an analytical Vydac C18 column (5 μm, 300 Å, 4.6 x 250 mm), monitoring the elution (1 ml/min) at 214 nm and using as eluents A, 0.05% TFA/H2O and B, 0.05% TFA/CH3CN. The peptides were assayed for purity (>97%) by analytical HPLC and their identity confirmed by electrospray ionization-mass spectrometry (ESI-MS) (Mariner, Perkin Elmer). Regarding the inhibitors, after removal of side chain protecting groups, final crude peptides were resuspended in diethyl ether in order to eliminate excess reagents and lyophilized. The effective concentration of dec-peptide-cmk was evaluated by mass ESI-MS.

**Synthesis of Lassa GPC derived non fluorogenic peptides** - The synthesis of the non-fluorogenic peptides (GPC250-265 and GPCmut, Fig. 3C) was performed using SPPS and N-α-t-Butyloxy carbonyl (Boc) amino acids on Boc-Thr(Bzl)-PAM resin (NovaBiochem, La Jolla, USA, 0.34 mmol/g). After removal of formyl protecting group (10% piperidine in DMF, 0°C for 2h), peptide cleavage and side chain deprotection were achieved using 12 ml mixture of 82% hydrofluoric acid (HF), 9% thioanisole, and 9% dimethyl sulfoxide (DMSO) at -5°C for 1h. The crude products were purified by RP-HPLC on a semi-preparative Vydac C18 column (5 μm, 300 Å, 10 x 250 mm) in the above reported conditions. The peptides were assayed for purity (100%) by analytical RP-HPLC and their identity confirmed by ESI-MS.

**Peptide preparation** - All lyophilized peptides were dissolved in H2O except the 8mer WT, dec-YISRRLL-cmk and dec-ISRRLL-cmk that were dissolved in DMSO.

**Source of recombinant hSKI-1** - Soluble human SKI-1, lacking the transmembrane domain and cytosolic tail (BTMD-SKI-1) and containing a C-terminal Hexa-histidine tag was isolated from overnight media of HEK 293 cells stably expressing this BTMD-SKI-1, as described (6,33). The presence of SKI-1 activity was measured with the 7mer WT (optimal at pH 7.5), its ability to be inhibited with 300 μM AEBSF (32), 10 mM EDTA, or 1 mM Zn2+ (25), and the absence of basic-aa-specific furin-like cleavages tested with the pyroglutamic acid-RTKR-MCA (Pyr-RTKR-MCA, Peptide Institute Inc.) as described (34), were confirmed.

**In vitro enzymatic assays** - Each reaction was carried out in a 100 μL buffer (25 mM tris (hydroxymethyl) aminomethane (Tris-HCl), 25 mM 2-morpholinoethanesulphonic acid (MES) and 1 mM CaCl2 adjusted to pH 7.5) at 37ºC. The reactions contained 100 μM MCA-peptide and 30 μL of hSKI-1 preparation. Enzymatic activity measurements with MCA-conjugated peptidyl substrates were performed by measuring the liberated 7-amino-4-methyl-coumarin (AMC) with a Spectra MAX GEMINI EM microplate spectrofluorometer, Molecular Devices, (λex, 360 nm; λem, 460 nm). Enzymatic activity on non fluorogenic peptidyl substrates were monitored by RP-HPLC under the following conditions: Varian analytical C18 (5 μm, 100 Å, 4.5 x 250 mm) column; flow, 1 ml/min, eluent A, 0.1% TFA/H2O, eluent B 0.1% TFA/CH3CN; detector, 214 nm and 280 nm.

**Determination of kinetic parameters Vmax and Km** - For measurements of kinetic parameters Vmax and Km, hSKI-1 (30 μL) was incubated with increasing concentrations (20-200 μM) of each fluorogenic peptide in 100 μL of buffer in a 96-well microtiter plate at 37ºC under the above conditions. Vmax and Km values were estimated by Grafit 4.09 http://www.erithacus.com/grafit/. The software fits data to the Michaelis-Menten equation, where the rate is plotted as a function of the concentration of the substrate. Initial estimates are provided by use of linear fitting.
using the Scatchard rearrangement. Initial rate were evaluated for each peptide at different concentration using SOFTmaxPro 4.1 program based on the linear portion of the curve. We did not consider in the calculation the lag phase, which is probably due to the presence of inactive SKI-1 that might require time to be activated. In fact, the lag time is greatly reduced upon pre-incubation at 37ºC \((not\ shown)\). We estimate that at least 5-10% of the fluorogenic peptide was digested within the analysis period. A similar phenomenon was also observed for the convertase PC1, where the lag phase could last up to 6h (35).

**In vitro inhibition of Succ-IYIRRLL-MCA processing** - Each reaction was carried out in a 100 µL buffer (25 mM Tris-HCl, 25 mM MES and 1 mM CaCl₂ adjusted to pH 7.5) at 37ºC. The solutions, containing 30 µL of hSKI-1 preparation and 5 µL of inhibitors at different concentrations \((0-1.5 \times 10^5 \mu M, \text{dec-RRLL-cmk}; 0-38 \mu M, \text{dec-ISRRLL-cmk}; 0-36 \mu M, \text{dec-YISRRLL-cmk})\) were incubated 20 min at 37ºC before adding 50 µM Succ-1YISRRL-MCA as substrate. Enzymatic activity measurements were performed by measuring the liberated AMC group with a Spectra MAX GEMINI EM microplate spectrofluorometer, Molecular Devices, \((\lambda_{ex}, 360 \text{ nm}; \lambda_{em}, 460 \text{ nm})\).

**Determination of in vitro inhibitor constants IC\textsubscript{50s} - For measurements of IC\textsubscript{50s}, hSKI-1 (30 µL) was incubated with increasing concentrations \((0-1.5 \times 10^5 \text{ nM, dec-RRLL-cmk}; 0-38 \mu M, \text{dec-ISRRLL-cmk}; 0-36 \mu M, \text{dec-YISRRLL-cmk})\) of each inhibitor in 100 µL of buffer, 50 µM Succ-IYISRRLL-MCA as substrate, in a 96-well microtiter plate at 37ºC under the above conditions. \(IC_{50s}\) were calculated by using GraFit Version 4.09 software (Erithacus Software Ltd, Staines, UK).

**In vitro inhibition of Pyr-RTKR-MCA processing** - Each reaction was carried out in a 100 µL buffer (25 mM Tris-HCl and 1 mM CaCl₂, adjusted to pH 7.0 or 6.0) at 37ºC. The solutions, containing 1 µL of either concentrated human furin medium or 10 µL of mouse PC5 or human PACE4 preparations (36) and 5 µL of inhibitors at different concentrations \((0-15 \mu M, \text{dec-RRLL-cmk}; 0-15 \mu M, \text{dec-YISRRLL-cmk})\) were incubated 20 min at 37ºC before adding 50 µM Pyr-RTKR-MCA as substrate. Enzymatic activity measurements were performed by measuring the liberated AMC with a Spectra MAX GEMINI EM microplate spectrofluorometer, Molecular Devices, \((\lambda_{ex}, 360 \text{ nm}; \lambda_{em}, 460 \text{ nm})\).

**Nuclear magnetic resonance (NMR) analysis -** NMR experiments were carried out on a Varian Inova 500 MHz. NMR characterization was performed in trifluoroethanol (TFE)/H₂O 90:10 (v/v) and 70:30 (v/v) at 298 degrees Kelvin. Samples were prepared by dissolving weighted amounts of each peptide in \([D_3]\text{TFE} (99\%\ isotopic purity, Aldrich) and H₂O, for final concentrations of \(~1.3\ \text{mM}.\) Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)[2,2',3,3'-d₄] propionate (TSP). Two dimensional (2D) experiments, such as total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), rotating frame Overhauser effect spectroscopy (ROESY), double quantum-filtered correlated spectroscopy (DQ-FCOSY) were measured with standard pulse sequences (37,38). According to Wüthrich (39) identification of amino acid spin systems was performed by comparison of TOCSY and DQF-COSY, while sequential assignment was obtained by the analysis of NOESY spectra. NOE analysis was achieved by means of NOESY spectra, acquired with a 200, and 300 msec mixing times. NOE intensities were evaluated by integration of cross-peaks from the NOESY 200 ms using the program CALIBA of the DYANA (41) package. Geminal protons, not stereospecifically assigned, were substituted by pseudo atoms (42).

**Ex vivo inhibition of the SKI-1/S1P processing of proSREBP-2 and proATF6 -** For SREBP-2 analyses, CHOK1 cells were incubated overnight with various concentrations of decanoylated chloromethylketone inhibitors, namely the 7mer-cmk (dec-YISRRLL-cmk, 0-110 µM), 6mer-cmk (dec-ISRRLL-cmk, 0-110 µM), and commercial 4mer-cmk (dec-RRLL-cmk; Bachem, 0-150 µM). The cell lysates were then analyzed for endogenous SREBP-2 or exogenously expressed ATF6 immunoreactivity by Western blots, as described (33). For ATF6 analyses the cells were first transiently
transfected with ATF6 cDNA and then incubated the next day with the inhibitors.

**Ex vivo inhibition of the furin-like processing of proPDGF-A**  – On day 0, stable PDGF-A-V5 construct in HEK293 cells, were plated on 35mm plates in (Dulbecco's modified Eagle's medium containing 100 units/ml gentamycin) supplemented with 5% heat inactivated fetal calf serum. On day 1, cells were washed twice with phosphate buffered saline (PBS) and serum free medium was added along with varying concentrations of dec-YISRRLL-cmk (0-110 µM) or dec-ISRRLL-cmk (0-110 µM) or dec-RRL-cmk (0-150 µM), incubated overnight. Day 3, medium was analyzed by running the samples on 12% SDS-PAGE and immunoblotted using V5-HRP antibody 1:5000.

**RESULTS**

**Design and evaluation of SKI-1 fluorogenic peptide substrates**  – One of the major aims of the present study was to develop small MCA-containing fluorogenic substrates of SKI-1. Alignment of the recognition motifs of all known SKI-1 substrates revealed that except for the autocatalytic shedding sequence (KHQKLL↓) (6), almost all sites contain an Arg at position P4 and hydrophobic residues at P2 with Leu and Val being most prevalent (exceptions include Ile for CREB4 and Phe for prosomatostatin) (Fig. 1). *In vitro* analysis showed that SKI-1 cleaves a 12mer synthetic peptide (LWKHQKLL↓SIDL) derived from its shed site (6). However, when we analyzed at various pHs the processing of MCA-containing peptides mimicking this site, such as the wild type Succ-WKHQKLL-MCA, and its P5 mutants Succ-WKRQKLL-MCA, Succ-WKKQKLL-MCA, or Succ-WKAQKLL-MCA, none were significantly processed (*not shown*). This suggested that for this substrate particularly, either extended P and/or P' residues are important for SKI-1 recognition. Therefore, in order to test potential *in vitro* SKI-1 substrates lacking P' sites, we decided to synthesize a series of MCA-containing substrates with an Arg at P4 and a Leu at P2, based on the processing site of viral glycoproteins (GP) exhibiting potential SKI-1 recognition motifs, such as those found in Lassa virus (LAV) (24), Crimean Congo hemorrhagic fever virus (CCHFV) (28,33), and Lymphocytic Choriomeningitis virus (LCMV) (26) (Fig. 2B). The glycoprotein GPC of LAV was shown to be very well cleaved *in vitro* using a 14mer quenched fluorogenic substrate (25). In the present study, the analysis of the processing of these viral derived peptides revealed that the order of SKI-1 cleavage preference is: 7mer LAV GPC >> 6mer LAV GPC ≈ 4mer LAV GPC (inset Fig. 2A) ≈ 7mer CCHV GP (Fig. 2A, B), whereas the chosen LCMV-derived substrates were not cleaved (Fig. 2A,B). Since SKI-1 processing usually occurs in the medial Golgi where the pH is close to 6.4, we tested the cleavage of the peptides IV, V and VI (see Fig. 2B) at pHs 7.5, 6.7, 6.3 and 6.0. Results showed that these peptides are not cleaved *in vitro* at acidic pHs (*not shown*), even though their parent glycoprotein precursors are likely to be cleaved *in vivo* in the Golgi. It is not excluded that *in vitro* cleavage of the latter peptides may require extended P or the presence of P' residues, as it is the case of the LCMV *in vivo* processing (26).

The above results revealed that the cleavage rate of the 6mer Lassa’s GPC (Succ-ISRRLL-MCA) is comparable to that of a 7mer peptide (Succ-SGSRRLL-MCA) mimicking the CCHFV glycoprotein processing site, but that the 7mer Lassa’s GPC (Succ-YISRRLL-MCA) was a more than 14-fold better substrate than either of the above, even though all contain the common P5-P1 SRRLL sequence (Fig. 2). Thus, we hypothesized that the Tyr at the P7 position within the LAV’s GPC sequence plays a critical role. Accordingly, we synthesized MCA-substrates with mutated P7 Tyr into either Phe, Ile, Val, Ser, or Ala, as well as an 8mer wild type sequence Succ-IYISRRLL-MCA (Fig. 3). Based on the optimal pH for the 7mer peptide processing (Fig. 4, inset) and the absence of a [Ca^{2+}] effect on the SKI-1 activity from 0.5-20 mM (*not shown*), we chose to compare the SKI-1 cleavage of these peptides at pH 7.5 and 1 mM Ca^{2+}. The data revealed that the 8mer is the best substrate for SKI-1, exhibiting a ~2.4-fold better V_{max}/K_{m} versus the 7mer, whereas only the Phe mutant 7mer is relatively well cleaved (Fig. 4, Table 1). Thus, within the context of the LAV GPC sequence only the aromatic amino acid Phe (also found at P7 in LCMV; Fig. 1) can somewhat replace Tyr, but not the other tested hydrophobic amino acids such as Val or Ile or even Ala or the hydroxyl-containing Ser. Thus, while Val occupies the P7 position in the cleavage sites of CREB-like and Luman, Ala in
proBDNF and Ser in CCHFV and SREBP-1 (Fig. 1), these amino acids are not acceptable within the LAV GPC sequence. We conclude that the 8mer and 7mer wild type LAV GPC sequences represent the best MCA-containing SKI-1 substrates.

**Enzymatic assays of non-fluorogenic SKI-1 peptide substrates derived from the LAV GPC sequence** – MCA-peptides provide a highly sensitive approach for monitoring enzymatic cleavage C-terminal to an amino acid attached to the MCA moiety, as fluorescence is detected only when the AMC group is released. On the other hand, the analysis of the possible contribution of P' residues to catalysis requires the use of a different approach, including quenched fluorogenic substrates (25) or non-derivatized peptides (6). Consequently, we synthesized two 16mer non-fluorogenic substrates comprising six P' positions mimicking the LAV glycoprotein processing site, namely GPC250-265 and its Y253A P7 mutant (GPCmut; Fig. 3). As shown by RP-HPLC analysis, the wild type GPC250-265 peptide is much better processed over 16h by SKI-1 as compared to the GPCmut (Fig. 5). We estimated that 26% processing of GPC250-265 occurred within 5h, whereas a similar extent of cleavage is not achieved for GPCmut even after 16h incubation. These data independently confirm that the P7 position in the LAV GPC sequence plays a critical role in substrate recognition.

**NMR analysis of the GPC250-265 and GPCmut** – In an attempt to rationalize the importance of the P7 residue in the LAV GPC processing site, we determined the NMR structure of the wild type and mutant 16mer peptides. Such an analysis was performed in 70/30 TFE/H2O and 90/30 TFE/H2O. TFE is well known to favor solvent-shielded amide conformations, thus promoting ordered structures. It has been reported that the local conformation of native proteins is usually better reproduced for structure calculations of the peptide GPC250-265 and 108 experimental constraints from NOE data (69 intra-residual, 34 sequential, 5 medium-range) for GPCmut. The best 10 structures for GPC250-265 with residual restraint energy lower than -112 kcal/mol and for GPCmut lower than -182 kcal/mol were selected to represent the solution structure of these peptides (Fig. 6A). For both peptides, a 3_10 helix turn is found in the segment Ile^254-Arg^257 (root mean square deviations, RMSDs ~ 0.50 ± 0.20 Å). A superimposition of the average solution conformations of GPC250-265 (black) and GPCmut (grey) is shown in Fig. 6B. These data suggest that the cleavage site follows a helical segment and that the presence of Ala instead of Tyr at P7 does not seem to perturb this secondary structure. However, even though the N-terminal segment, which includes the Y253A mutation, is quite flexible (Fig. 6A), the data suggest that the wild type and mutant conformations are not identical around Tyr^253 versus Ala^253 (Y253/A253 in Fig. 6B). It is thus likely that the observed preference of SKI-1 for phenylic structures at the P7 position of LAV GPC substrates may be better understood within the context of the enzyme-substrate complex than within that of the substrate alone.

**Development of potent cell-permeable peptidyl chloromethylketone SKI-1 inhibitors** – The aim here was to develop potent cell-permeable, N-terminal decanoylated, (dec) irreversible peptide inhibitors of SKI-1 activity containing a C-terminal Leu-chloromethylketone (cmk). On the basis of the above results, we compared the ex vivo inhibitory potential of an untested, yet
commercially available, dec-RRLL-cmk to two other synthetic peptides made by us, namely the 6mer dec-ISRLL-cmk and 7mer dec-YISRRL-cmk (Figs. 7, 8). The selected ex vivo SKI-1 precursor substrates were the membrane-bound transcription factors proSREBP-2 (46) and proATF6 (17,18), whose Golgi-associated cellular processing into their nuclear form nSREBP-2 and nATF6 can be inhibited by protein-based SKI-1 inhibitors (33). We first compared the inhibition of the processing of endogenous proSREBP-2 by the 6mer versus the 7mer cmk-peptides (Fig. 7). The data showed that both peptides are potent ex vivo inhibitors of this SKI-1-generated cleavage with an estimated 50% inhibition at ~7 µM and ~20 µM for the 7mer-cmk and 6mer-cmk, respectively (Fig. 7). In another experiment we found that both 4mer-cmk and 7mer-cmk peptides are almost equipotent in inhibiting the processing of the overexpressed proATF6 into nATF6 following ER-stress induced by overnight tunicamycin treatment of CHO-cells (17,18,33). We estimate that 50% inhibition occurs at ≤ 1 µM of either cmk-peptide (Fig. 8). Accordingly, we can conclude that the dec-4mer-cmk, dec-6mer-cmk and dec-7mer-cmk cell permeable SKI-1 inhibitors are almost equipotent ex vivo.

We next defined the selectivity of the above cmk-peptides for inhibition of SKI-1 versus other convertase-generated processing reactions. Accordingly, we opted to test the ability of the 7mer cmk-peptide to inhibit the processing of the precursor of the platelet derived growth factor proPDGF-A into PDGF-A by furin-like basic-aa-specific convertases (47), and to compare its potential inhibitory effect to that of the frequently used commercially available furin-like convertase inhibitor dec-RVKR-cmk (48). The data showed that in HEK293 cells stably expressing proPDGF-A the processing of this precursor by endogenous furin-like convertases is completely inhibited by ~3 µM dec-RVKR-cmk (Fig. 9A), whereas it would take >100 µM to inhibit less than 2% of this reaction by the 7mer dec-YISRRL-cmk (Fig. 9B), or the 4mer dec-RRLL-cmk (not shown).

Even though the above 4, 6 and 7mer cmk-peptides were designed to be cell-permeable through the attachment of an N-terminal decanoylated functionality, we were still interested to define their in vitro inhibitory potency on the processing of the 8mer succ-IYISRRLL-MCA. Unexpectedly, the data show that the 4mer-cmk (IC₅₀ ~9 nM) is a ~250-fold better inhibitor than either of the 6 or 7mer-cmk peptides (IC₅₀ ~2,300 nM) (Fig. 10). This in vitro result could possibly be due to the presence of the highly hydrophobic decanoylated N-terminal moiety in these cmk-peptides. Accordingly, for the 6mer-cmk and 7mer-cmk it may be too hydrophobic and likely kinetically unfavorable, while for the 4mer-cmk it may mimic the hydrophobic patch Ile-Tyr-Ile found at the P8-P6 positions of the GPC LAV processing site. We can speculate that intracellularly, the decanoylated moiety is membrane-bound and hence may not exert such a negative effect (as compared to in vitro) on the inhibitory potency of the 7mer-cmk and 6mer-cmk peptides.

In a similar fashion we also compared the ability of the 4mer-cmk and 7mer-cmk peptides to inhibit the in vitro furin processing of the pentapeptide pyr-RTKR-MCA. Interestingly, while these cmk-peptides do not inhibit either PC5 or PACE4, they are potent in vitro inhibitors of furin only at pH 7 (and pH 7.5, not shown), but much less at pH 6 (Table 2). Therefore, we conclude that while the designed cmk-inhibitors are potent and relatively selective ex vivo inhibitors of SKI-1, they should be used with caution for in vitro reactions.

**DISCUSSION**

The main objectives of this study were centered around (i) the development of a simple fluorogenic substrate of SKI-1 that could be used in high throughput in vitro analysis of its activity, and (ii) use this information to design and synthesize a potent specific and cell-permeable SKI-1 peptidyl inhibitor.

For the first aim, we decided to test the ability of SKI-1 to cleave 7mer MCA-containing peptides mimicking the glycoprotein recognition sequence of various hemorrhagic fever viruses. Unexpectedly, while intracellularly SKI-1 cleaves reasonably well the full length glycoprotein of LCMV-WE strain (26), it could not cleave in vitro the 7mer-MCA peptides derived from either the WE or Armstrong strains (Fig. 2), suggesting that an extended sequence may be necessary for SKI-1 recognition. In contrast, the LAV GPC 7mer sequence is well processed in vitro, much better than the 7mer CCHFV mimic (Fig. 2), emphasizing the critical importance of the primary structure of the 7mer substrate. The MCA-peptides derived from the GPCs of LAV and CCHFV only differ in their
P7 and P6 positions, exhibiting Tyr-Ile and Gly-Ser residues, respectively (Fig. 2). This led us to investigate the critical residues in the LAV GPC sequence that makes it such a great substrate for SKI-1. While the wild type 8mer LAV GPC MCA-peptide is a 2-fold better substrate than the 7mer, the latter is 14-fold better than the 6mer (Fig. 4, Table 1). Interestingly, the 6mer-MCA peptide derived from LAV GPC is processed by SKI-1 to the same extent as the 7mer CCHFV peptide (Figs. 2A,B). These data suggested that the P7 Tyr is much more critical than the P8 Ile for SKI-1 cleavage. Accordingly, in order to keep the substrate as short as possible while still retaining good cleavability and in vitro solubility, we tested whether other residues could replace the P7 Tyr in the 7mer peptide by its mutation into various amino acids (Fig. 3B). The results showed that only Phe can partially replace the P7 Tyr, albeit with a loss of ~40% cleavability. All other mutants resulted in >70% loss of SKI-1 cleavability (Fig. 4, Table 1), emphasizing the importance of the P7 phenyllic group for optimal SKI-1 recognition. Independently, we confirmed the critical importance of the P7 Tyr within the context of the LAV GPC sequence by analyzing the in vitro processing of a 16mer peptide mimicking the wild type sequence from P10-P6’ (Figs. 3C and 5A) and that of a Y253A mutant (Figs. 3C and 5B). In agreement with the above data, a similar observation of the critical presence of a phenyllic group at P7 was also observed ex vivo for the cleavage of the glycoprotein of LCMV, since the replacement of the P7 Phe (Fig. 1) by Ala resulted in almost absence of SKI-1 processing (26).

While the importance of the P7 Tyr residue was deduced from MCA-substrates, the absence of cleavage by SKI-1 of the 16mer Y253A mutant may conceivably be due to additional structural features of the peptide. Preliminary Circular Dichroism analysis in aqueous solution of the non-fluorogenic 16mer wild type and its Y253A mutant did not reveal significant conformational differences between them (not shown). We next opted for the comparison of the NMR structure of these 16mer peptides. The data revealed that both peptides exhibit a helical structure from Ile$^{254}$-Arg$^{257}$, and do not significantly differ from each other at the SKI-1 cleavage site RRLL$^{259}$. However, the Y253A mutation may partially affect the solution structure of the peptide at the N-terminal segment preceding Ile$^{254}$ (Fig. 6). We conclude that the rationale behind the observed large difference in SKI-1 cleavability of these 16mer peptides (Fig. 5) does not reside in the secondary structure of the free substrate, but that likely the P7 Tyr plays a critical role in the binding of the substrate to the catalytic pocket of SKI-1.

Since we do not have an active site titrant of SKI-1, it is not possible presently to convert our Vmax data into kcat with confidence. The same applied to our previously published quenched fluorogenic substrates (QFS) (25). Since the kinetics are really different between the QFS and the MCA-containing ones proposed in this work (not shown), it is presently difficult to directly compare the two types of substrates. Nevertheless, we tested both types of substrates and observed that after two hours incubation both assays are similarly sensitive. Finally, the choice of the MCA-containing substrates was to narrow down the selectivity of the substrate to SKI-1, whereas the longer QFS ones could be cleaved by other enzymes.

Our next aim was to design irreversible peptide inhibitors of SKI-1 based on the results obtained from MCA-peptide substrate studies and incorporating a C-terminal chloromethylketone reactive moiety. Furthermore, since we aimed to obtain cell-permeable inhibitors for ex vivo studies we introduced a hydrophobic decanoyl-group at the N-terminus of the designed peptides. Indeed, results from other studies on furin inhibitors revealed the critical importance of the addition of an N-terminal decanoyl-group for cellular permeability of cmk-containing peptide inhibitors (49). On the basis of the above results we designed a decanoylated 7mer-cmk peptide mimicking the LAV GPC sequence. In order to test the importance of the peptide length for cellular permeability and inhibition of SKI-1, we also compared the ex vivo (Figs. 7,8) and in vitro (Fig. 10) inhibitory properties of the dec-7mer-cmk to that of either a 1-aa shorter (dec-6mer-cmk) or 3-aa shorter, commercially available, dec-4mer-cmk. Unexpectedly, and in contrast to the LAV-GPC-derived MCA-substrate results (Table 1), we observed that the dec-6mer-cmk peptide was almost equipotent to the dec-7mer-cmk in inhibiting the cellular processing of proSREBP-2 (Fig. 7). Furthermore, the dec-4mer-cmk peptide was equipotent to the dec-7mer-cmk in inhibiting the cellular proATF6 processing (Fig. 8). However, it must be mentioned that while the LAV GPC sequence is so far the best substrate of SKI-1, peptides
derived from either proATF6 or proSREBP-2 turned out to be much poorer SKI-1 substrates (25). In addition, different from the succinyl-MCA-substrates, the inhibitors used contain an N-terminal decanoyl group, which may influence their conformation upon membrane attachment and/or binding to the catalytic pocket of SKI-1. This is especially relevant in view of the observed poor cleavability of the 4mer succ-RRLL-MCA (Fig. 2A, inset) and the importance of the hydrophobic residues Ile-Tyr-Ile at the P8-P6 positions in the LAV GPC sequence (Figs. 3B,4). We can conclude that the dec-7mer-cmk, dec-6mer-cmk and 4mer-cmk peptides are potent SKI-1 ex vivo inhibitors.

We next addressed the issue of the selectivity of these ex vivo SKI-1 inhibitors and whether they could inhibit other convertases such as the basic-aa specific furin-like proteinases (2). This was especially relevant, since we recently observed that the RXXR motif is not always indicative of a furin-like recognition sequence, as the motif RXLR was also recognized by SKI-1 in both Luman (20) and a modified PDGF-A sequence (47). For this purpose we used a cell-based assay monitoring the processing of proPDGF-A into PDGF-A through cleavage by furin-like enzymes at the RRKR\(^{86}\) sequence (47), a process inhibitable by dec-RVKR-cmk (Fig. 9A). The data revealed that at concentrations \(\leq 100 \mu M\) of dec-7mer-cmk, which effectively inhibit SKI-1 activity ex vivo (Figs 7,8), no significant inhibition of proPDGF-A cleavage was observed (Fig. 9B). Note that at 110 \(\mu M\) of dec-YISRRL-cmk \(\sim 2\%\) inhibition of proPDGF-A ex vivo processing was observed (Fig. 9B). The fact that furin prefers basic-aa containing substrates containing a Leu at P2’ and that the presence of a Leu at P1’ prevents cleavage by this enzyme (2,50), suggests that a peptide that contains all these characteristics, e.g., RRLL found in all our dec-peptide-cmks, could conceivably inhibit furin. Thus, we can conclude that when used at concentrations \(\leq 100 \mu M\), the dec-7mer-cmk is relatively specific for SKI-1 inhibition ex vivo.

Since we did not observe toxicity of the synthesized dec-RRL-cmk on HEK293 and CHO-K1 cells, we collaborated with a group in the Center of Disease Control (Atlanta, GA) to test this inhibitor on the live Lassa virus. Unfortunately, this compound was found to be toxic to Vero cells and cannot be used on this cell line, which is the model cell line used in Lassa virus infections.

Even though the cmk-peptides were designed to work in cells, we also tested their in vitro inhibitory potency and selectivity (Figs. 10, Table 2). The data revealed that all of them are effective inhibitors of the in vitro SKI-1 cleavage of a succinyl-8mer-MCA peptide derived from LAV-GPC, especially the dec-RRL-cmk (IC\(_{50}\) \(\sim 9\) nM). The high in vitro potency of the latter as compared to the 7mer-cmk and 6mer-cmk (IC\(_{50}\) \(\sim 2,300\) nM) may in part be due to the presence of a common N-terminal decanoyl group added to an already very hydrophobic sequence within the latter two peptides, hence decreasing their solubility and/or increasing their tendency to form micelles in aqueous buffers. For comparison, the estimated in vitro IC\(_{50}\) of dec-RVKR-cmk for furin is \(\sim 2\) nM (51). We therefore tested the in vitro inhibitory selectivity of the dec-4mer-cmk and dec-7mer-cmk on the constitutively secreted convertases (2) furin at pHs 7 and 6 (since processing of both proSREBP-2 and proATF6 occurs in the medial Golgi with an intraluminal acidic pH), and PACE4 / PC5A at pH 7. The data revealed that furin is not inhibited at pH 6 (Table 2), in agreement with the ex vivo data that gauged the activity of furin on proPDGF-A in the trans Golgi network (Fig. 9B) (47,52). On the other hand, only furin is inhibited by both peptides at pH 7, while under the same conditions the activities of PC5A and PACE4 are not affected (Table 2). As mentioned above, an RRL sequence could potentially inhibit furin, but since both PC5A and PACE4 can process peptides with a P1’ Leu (53,54) the latter convertases are not expected to be inhibited by this sequence. We can discard the possibility that the observed different inhibitory potential of the cmk-peptides on furin at pH 6 and 7 is due to the degradation of the cmk-peptides, since their levels did not significantly change upon incubations at either pHs, as verified by mass spectrometry (not shown). The sizes of the inhibitor dec-RRL-cmk and the substrate pyr-RTKR-MCA are too small to assume a rigid conformation in solution. However, since the reported crystal structure of furin was obtained at pH 6.0 (55), it is a matter of speculation whether the inhibition of furin at pH 7 by the cmk-peptides may be due to a different conformation of the enzyme at acidic versus neutral pHs. Along the same reasoning, it was recently demonstrated that the serpin \(\alpha\)-PDX
and some of its mutants inhibit furin better at pH 7 than at pH 6 (56). If so, this may be an interesting avenue to pursue, as a number of proteins of mammalian, bacterial and viral origin are processed by furin at the cell surface (52).

In conclusion, the data presented here provide a new framework for the development of potent cell-permeable SKI-1 inhibitors containing the central RRLL minimal core. Modifications of this structure as well as the addition of other inhibitory functionalities (57) may lead to specific and pharmacologically useful compounds to control SKI-1 activity in vivo. The availability of the proposed small MCA-substrates should also help in following the in vitro activity of this enzyme under various physiological conditions.

FOOTNOTES

The authors are indebted to Josée Hamelin, Mikhail Ponamarev and Annik Prat for their constant and precious advice and help. We gratefully acknowledge the precious help of Martin Vincent and Eric Bergeron on experiments using Lassa virus in Biosafety lab 4 (CDC, Atlanta). The secretarial assistance of Brigitte Mary is greatly appreciated.

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The abbreviations used are: aa, amino acid; AMC, 7-amino-4-methyl-coumarin; cmk, chloromethylketone; dec, decanoyl; PC, proprotein convertase; SKI-1, subtilisin kexin isozyme-1; S1P, site-1-protease; CHO, Chinese hamster ovary; WT, wild type; SREBP, sterol regulatory element-binding protein; nSREBP, nuclear SREBP; ATF6, activating transcription factor 6; nATF6, nuclear ATF6; RP-HPLC, Reverse-Phase High Performance Liquid Chromatography; GPC, Glycoprotein C of Lassa virus; NMR, Nuclear Magnetic Resonance.

FIGURE LEGENDS

Fig. 1. Amino acid sequences at the SKI-1/S1P cleavage site of mammalian and viral precursors. The alignment shows the sequences between amino acid position P8 and P4' of natural mammalian and viral substrates of SKI-1/S1P. The arrows indicate the cleavage site. The critical recognition amino acids Arg at P4 and hydrophobic amino acid at P2 are emphasized in bold.

Fig. 2. SKI-1 activity on MCA-conjugated viral glycoprotein peptide substrates. Fluorescence versus hydrolysis time of: (A) Succ-YISRRLL-MCA (I) and Succ-ISRRLL-MCA (LAV-derived) (II), and Succ-SGSRRLL-MCA (CCHFV-derived) (IV), and Succ-FLTRRLS-MCA (V) and Succ-FFTRRLA-MCA (LCMV Virus derived) (VI). Inset: comparison of the fluorescence released of Succ-YISRRLL-MCA (I) versus that of Succ-RRLL-MCA (III). (B) Summary of the cleavage preferences of the above peptides by SKI-1, with the +++ meaning much better cleavage than +, and the – meaning no cleavage.

Fig. 3. Schematic representation of LAV GPC and synthetic peptides derived from the SKI-1 recognition site. (A) SP represents the signal peptide. GP-1 and GP-2 are the maturation products resulting from GPC cleavage (arrow) that occurs after the recognition motif 256RRLL259 by SKI-1. Y-shaped projections represent predicted N-linked glycosylation sites. Names and sequences of the wild type and point mutated (B) fluorogenic and (C) non-fluorogenic substrates derived from the GPC sequence.

Fig. 4. SKI-1 activity on wild type and point mutated LAV GPC derived MCA-substrates. Comparison of the AMC released fluorescence versus time for the wild type and the P7 point mutated peptides (I-VIII) derived from the glycoprotein processing site of LAV. In the inset monitoring of the SKI-1 activity on 7mer WT peptide at different pHs is reported. The higher initial rate was reached at pH 7.5.
Fig. 5. **GPC-250-265 is efficiently processed by SKI-1, whereas GPC-mut is not.** The SKI-1 enzymatic cleavage of GPC-250-265 (A) and GPC-mut (B) was monitored by RP-HPLC at both 214 and 280 nm. Note that the cleavage product GFTFTW is identified as the peptide under the major peak at 280 nm. The % processing was estimated from the equation: (100 - % remaining precursor), and the precursor levels were evaluated by integrating the area of the corresponding peak.

Fig. 6. **NMR analyses of GPC-250-265 and GPC-mut.** (A) Superposition of the backbone from residue Ile-254 to Leu-259 of the best ten structures after minimization with Amber for GPC-250-265 and GPC-mut, in TFE/H2O 70/30. (B) Backbone superposition of the molecular models (in TFE/H2O 70/30) for GPC-250-265 (black ribbon) and GPC-mut (grey ribbon) in the segment Ile-254 to Leu-259. Each structure represents the mean value over ten representative conformations after minimization with Amber.

Fig. 7. **Dec-YISRRLL-cmk and dec-ISRRLL-cmk are effective inhibitors of endogenous proSREBP-2 ex vivo processing.** CHOK1 cells were treated with medium containing delipidated serum (LPDS), 50 µM compactin and 50 µM sodium mevalonate in the absence or presence of different concentrations of (A) dec-YISRRLL-cmk or (B) dec-ISRRLL-cmk for 18 h. Western blot analyses of the cell lysates were performed using a mouse monoclonal antibody directed against the NH2-terminal domain of hamster SREBP-2. The arrows point to the migration position of the precursor proSREBP-2 and its mature nuclear form nSREBP-2. Molecular masses are given in kDa.

Fig. 8. **Dec-YISRRLL-cmk and dec-RRLL-cmk are potent inhibitors of endogenous proATF6 ex vivo processing.** Following transient transfection of CHOK1 cells with a cDNA coding for ATF6-Flag, the cells were treated with varying concentrations of (A) dec-RRLL-cmk or (B) dec-YISRRLL-cmk in the presence of 2 µg/ml tunicamycin for 12 h. Western blot analyses of the cell lysates were performed using an anti-FLAG M2 monoclonal antibody. The arrows point to the migration position of the precursor proATF6 and its mature nuclear form nATF6. Molecular masses are given in kDa.

Fig. 9. **Dec-YISRRLL-cmk is not an effective inhibitor of the ex vivo processing of proPDGF-A.** On day 1, HEK293 cells, stably expressing PDGF-A-V5 construct, were incubated overnight in serum free medium with varying concentrations of (A) dec-YISRRLL-cmk or (B) dec-RVKR-cmk. On day 3, the media were fractionated on 12% SDS-PAGE and then analyzed by Western blot using a V5-HRP antibody. The arrows point to the migration position of the precursor proPDGF-A and its mature form PDGF-A. Molecular masses are given in kDa.

Fig. 10. **Dec-RRLL-cmk, dec-ISRRLL-cmk and dec-YISRRLL-cmk are effective inhibitors of Succ-IYISRRLL-MCA in vitro processing by SKI-1.** Concentration dependent inhibition of Succ-IYISRRLL-MCA in vitro SKI-1 mediated processing by (A) dec-RRLL-cmk, (B) dec-ISRRLL-cmk and (C) dec-YISRRLL-cmk. All inhibitory constants (IC50) values were evaluated with 50 µM of substrate using GraFit version 4.09 as described in the Materials and Methods section.

**Table 1. Kinetic parameters of MCA-peptidyl SKI-1 substrates.**

**Table 2. In vitro inhibition of the processing of Pyr-RTKR-MCA by PC-like enzymes.** Each reaction was carried out in a 100 µL buffer (pH 7.0 or 6.0) at 37°C in presence of different concentrations of (A) dec-YISRRLL-cmk, or (B) dec-RRLL-cmk. The % inhibition was evaluated from initial rate values.
References

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<th>P4</th>
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<th>P2'</th>
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**Fig. 2**

Fluorescence \[\text{RFU}\times10^3\] vs. Time \[\text{Sec} \times 10^3\]

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### A

![Diagram of protein structure](https://via.placeholder.com/150)

- **PreGPC**
- **SP**
- **GPC**
- **SKI-1/S1P**
- **H<sub>N</sub>**
- **259**

### Name

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<td><strong>Succ−I−ISRRLL −MCA</strong></td>
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</tbody>
</table>

### C

- **GPC<sup>250-265</sup>**
  - **250RDIYISRRLL<sup>259</sup>−GTFTWT<sup>265</sup>**
- **GPC<sup>mut</sup>**
  - **250RDIAISRRLL<sup>259</sup>−GTFTWT<sup>265</sup>**

---

> Downloaded from [link](https://via.placeholder.com/150) by guest on September 1, 2017
Fig. 4

pH optimum 7mer WT

<table>
<thead>
<tr>
<th>Fluorescence [RFU x 10^3]</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>8mer WT</td>
<td>5</td>
</tr>
<tr>
<td>7mer WT</td>
<td>6</td>
</tr>
<tr>
<td>7mer [Phe]</td>
<td>6.5</td>
</tr>
<tr>
<td>[Ala]</td>
<td>7</td>
</tr>
<tr>
<td>[Ser]</td>
<td>7.5</td>
</tr>
<tr>
<td>[Val]</td>
<td>8</td>
</tr>
<tr>
<td>[Ile]</td>
<td></td>
</tr>
<tr>
<td>6mer WT</td>
<td></td>
</tr>
</tbody>
</table>

Time [sec x 10^3]

0 10 20 30 40 50

I. 8mer WT
II. 7mer WT
III. 7mer [Phe]
IV. [Ala]
V. [Ser]
VI. [Val]
VII. [Ile]
VIII. 6mer WT

Fig. 4
<table>
<thead>
<tr>
<th>Name</th>
<th>$K_m$ [μM]</th>
<th>$V_{max}$ [units $\times$ sec$^{-1}$]</th>
<th>$V_{max}/K_m$ [units $\times 10^{-3} \times$ sec$^{-1} \times$ μM$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>8mer WT</td>
<td>14.8</td>
<td>0.155</td>
<td>10.5</td>
</tr>
<tr>
<td>7mer WT</td>
<td>34.2</td>
<td>0.137</td>
<td>4.3</td>
</tr>
<tr>
<td>7mer [Phe]</td>
<td>38.1</td>
<td>0.094</td>
<td>2.5</td>
</tr>
<tr>
<td>7mer [Ile]</td>
<td>36.7</td>
<td>0.046</td>
<td>1.3</td>
</tr>
<tr>
<td>7mer [Ser]</td>
<td>59.4</td>
<td>0.049</td>
<td>0.8</td>
</tr>
<tr>
<td>7mer [Ala]</td>
<td>73.35</td>
<td>0.050</td>
<td>0.7</td>
</tr>
<tr>
<td>7mer [Val]</td>
<td>137.8</td>
<td>0.050</td>
<td>0.4</td>
</tr>
<tr>
<td>6mer WT</td>
<td>266.9</td>
<td>0.090</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Table 1
Fig. 5

A

B

Absorbance at 214 nm [mAu]

Digestion time [h] | % processing
--- | ---
0 | 0
3 | 16
5 | 26
7 | 31
16 | 77

GPC^{250-265}

250RDIAISRRLGTFTWT^{265}

GPCmut

250RDIAISRRLGTFTWT^{265}
A

GPC$^{250-265}$

$^{250}$RDI\text{Y}ISRRLL$\downarrow$GTFTWT$^{265}$

GPC$^{\text{mut}}$

$^{250}$RDIA\text{ISRRLL}$\downarrow$GTFTWT$^{265}$

B

GPC$^{250-265}$

Y253 /A253

R256

L259

cleavage site

GPC$^{\text{mut}}$
**Fig. 7**

**A**

<table>
<thead>
<tr>
<th>dec-YISRRLL-cmk [μM]</th>
<th>0</th>
<th>75</th>
<th>50</th>
<th>10</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>proSREBP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nSREBP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nSREBP-2/proSREBP-2</td>
<td>1.36</td>
<td>0.02</td>
<td>0.01</td>
<td>1.15</td>
<td>1.32</td>
</tr>
<tr>
<td>% inhibition</td>
<td>0</td>
<td>99</td>
<td>99</td>
<td>89</td>
<td>3</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>dec-ISRRLL-cmk [μM]</th>
<th>0</th>
<th>120</th>
<th>40</th>
<th>8</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>proSREBP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nSREBP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nSREBP-2/proSREBP-2</td>
<td>1.17</td>
<td>0.04</td>
<td>0.04</td>
<td>1.14</td>
<td>1.17</td>
</tr>
<tr>
<td>% inhibition</td>
<td>0</td>
<td>97</td>
<td>97</td>
<td>26</td>
<td>0</td>
</tr>
</tbody>
</table>
Fig. 8

A

<table>
<thead>
<tr>
<th>dec-RRLL-cmk [µM]</th>
<th>0</th>
<th>150</th>
<th>15</th>
<th>1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>proATF6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nATF6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nATF6/ proATF6</td>
<td>0.57</td>
<td>0.09</td>
<td>0.14</td>
<td>0.18</td>
</tr>
<tr>
<td>% inhibition</td>
<td>0</td>
<td>84</td>
<td>75</td>
<td>62</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>dec-YISRRLL-cmk [µM]</th>
<th>0</th>
<th>110</th>
<th>10</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>proATF6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nATF6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nATF6/ proATF6</td>
<td>0.59</td>
<td>0.17</td>
<td>0.20</td>
<td>0.29</td>
</tr>
<tr>
<td>% inhibition</td>
<td>0</td>
<td>71</td>
<td>66</td>
<td>51</td>
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</tbody>
</table>
Fig. 9

A

<table>
<thead>
<tr>
<th>dec-RVKR-cmk [µM]</th>
<th>0</th>
<th>110</th>
<th>10</th>
<th>1</th>
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<tbody>
<tr>
<td>proPDGF-A</td>
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<tr>
<td>PDGF-A</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kDa</td>
<td></td>
<td>30</td>
<td>20</td>
<td>14</td>
</tr>
</tbody>
</table>

% inhibition

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>99</th>
<th>99</th>
<th>95</th>
</tr>
</thead>
<tbody>
<tr>
<td>proPDGF-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGF-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>dec-YISRRLL-cmk [µM]</th>
<th>0</th>
<th>110</th>
<th>10</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>proPDGF-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGF-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kDa</td>
<td></td>
<td>30</td>
<td>20</td>
<td>14</td>
</tr>
</tbody>
</table>

% inhibition

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>2</th>
<th>0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>proPDGF-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGF-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 9
**dec-RRLL-cmk [nM]**

$IC_{50} = (9 \pm 0.2) \text{ nM}$

**dec-ISRRLL-cmk [\mu M]**

$IC_{50} = (2,300 \pm 20) \text{ nM}$

**dec-YISRRLL-cmk [\mu M]**

$IC_{50} = (2,270 \pm 10) \text{ nM}$

*Fig. 10*
### Table 2

<table>
<thead>
<tr>
<th>A</th>
<th>[dec-YISRRLL-cmk] M</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Furin (pH 6)</td>
<td>Furin (pH 7)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>0</td>
<td>47</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>0</td>
<td>62</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>0</td>
<td>51</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>0</td>
<td>42</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>67</td>
<td>75</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>[dec-RRLL-cmk] M</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Furin (pH 6)</td>
<td>Furin (pH 7)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$1.5 \times 10^{-9}$</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td>$1.5 \times 10^{-8}$</td>
<td>0</td>
<td>54</td>
</tr>
<tr>
<td>$1.5 \times 10^{-7}$</td>
<td>0</td>
<td>49</td>
</tr>
<tr>
<td>$1.5 \times 10^{-6}$</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>$1.5 \times 10^{-5}$</td>
<td>0</td>
<td>63</td>
</tr>
</tbody>
</table>
The proprotein convertase SKI-1/S1P: In vitro analysis of lassa virus glycoprotein-derived substrates and ex vivo validation of irreversible peptide inhibitors
Antonella Pasquato, Philomena Pullikotil, Marie-Claude Asselin, Manuela Vacatello, Livio Paolillo, Francesca Ghezzo, Federica Basso, Carlo Di Bello, Monica Dettin and Nabil G. Seidah

J. Biol. Chem. published online June 21, 2006

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