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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Herein we designed, synthesized, tested, and validated fluorogenic methylcoumarinamide (MCA) and chloromethylketone-peptides spanning the Lassa virus GPC cleavage site as substrates and inhibitors for the proprotein convertase SKI-1/S1P. The 7-mer MCA (YISRRLL-MCA) and 8-mer MCA (IYISRRLL-MCA) are very efficiently cleaved with respect to both the 6-mer MCA (ISRRLL-MCA) and point mutated fluorogenic analogues, except for the 7-mer mutant Y253F. The importance of the P7 phenyllic residue was confirmed by digestions of two 16-mer non-fluorogenic peptidyl substrates that differ by a single point mutation (Y253A). Because NMR analysis of these 16-mer peptides did not reveal significant structural differences at recognition motif RRL, 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 pro-ATF6 and pro-SREBP-2 cellular processing that decanoylated ATF6 and pro-SREBP-2 cellular processing that decanoylated ATF6 and pro-SREBP-2 cellular processing that decanoylated ATF6 and pro-SREBP-2 cellular processing that decanoylated ATF6 and pro-SREBP-2 cellular processing that decanoylated ATF6 and pro-SREBP-2 cellular processing that decanoylated

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). Whereas these proteases cleave their substrates either intra- or extracellularly, 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. Whereas seven PCs (PC1/3, PC2, furin, PC4, PC5/6, PACE4, and PC7) cleave secretory precursors within the motif R-Xn_R-Xn, where n = 0, 2, 4, or 6, and X is a variable amino acid, except Cys, the other two convertases (SKI-1/S1P and NARC-1/PCSK9) cleave within the motifs R-X-(hydrophobic)-Xn (3, 5–8) and VFAQ-Xn (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: RKVF133X and RKVFRL137X, respectively. The latter products are then transported to the cis-medial Golgi whereupon they are further autocatalytically processed into a C-form at RRL186X (Fig. 1), generating the active SKI-1 enzyme devoid of its prospecegment (5, 6, 8, 10). SKI-1 was simultaneously discovered by our group (10) and Goldstein and Brown (11) who called it site 1 protease (S1P), 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 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 SCAP-SCAP complex to the Golgi (12–14). Therein, a two-step proteolytic process (SKI-1 and site 2 protease S2P) (14) releases the wild type; SREBP, sterol regulatory element-binding protein; nSREBP, nuclear sterol regulatory element-binding protein; ATF6, activating transcription factor 6; nATF6, nuclear activating transcription factor 6; RP-HPLC, reverse phase-high performance liquid chromatography; GPC, glycoprotein C of Lassa virus; MES, 2-morpholinoethanesulfonic acid; ER, endoplasmic reticulum; SCAP, SREBP-cleavage-activating protein; CREB, cAMP-response element-binding protein; LCNV, lymphocytic choriomeningitis virus; CCHFV, Crimean Congo hemorrhagic fever virus; MCA, methylcoumarinamide; LAV, Lassa virus; Fmoc, N-fluorenylmethoxy carbonyl; PDGF, platelet-derived growth factor; DMF, dimethylformamide; ESI-MS, electrospray ionization-mass spectrometry; TFE, trifluoroethanol; NOESY, nuclear Overhauser effect spectroscopy; NOE, nuclear Overhauser effect.

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2 The abbreviations used are: PC, proprotein convertase; AMC, 7-amino-4-methylcoumarin; cmk, chloromethylketone; dec, decanoyl; 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 sterol regulatory element-binding protein; ATF6, activating transcription factor 6; nATF6, nuclear activating transcription factor 6; RP-HPLC, reverse phase-high performance liquid chromatography; GPC, glycoprotein C of Lassa virus; MES, 2-morpholinoethanesulfonic acid; ER, endoplasmic reticulum; SCAP, SREBP-cleavage-activating protein; CREB, cAMP-response element-binding protein; LCNV, lymphocytic choriomeningitis virus; CCHFV, Crimean Congo hemorrhagic fever virus; MCA, methylcoumarinamide; LAV, Lassa virus; Fmoc, N-fluorenylmethoxy carbonyl; PDGF, platelet-derived growth factor; DMF, dimethylformamide; ESI-MS, electrospray ionization-mass spectrometry; TFE, trifluoroethanol; NOESY, nuclear Overhauser effect spectroscopy; NOE, nuclear Overhauser effect.
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 (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 pro-ATF6. 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 is a soluble cytokine critically dependent on the basic leucine zipper protein Luman, the cellular counterpart of herpes simplex virus VP16 (22). 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 pro-ATF6. 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).

PC activities are routinely assayed using two types of fluorogenic substrates, peptide MCA methyl coumarinomides (MCA) (29) and intramolecularly quenched fluorogenic peptides (5, 25, 30, 31). Processing at the peptide-MCA bond causes a fluorescence wavelength shift allowing the detection of the released fluorogenic AMC group. Intramolecularly quenched fluorogenic peptides contain an N-terminal fluorescent group o-aminobenzoic acid and a C-terminal quench fluorescence moiety 3-nitrotyrosine. Cleavage of intramolecularly quenched fluorogenic peptides releases the quenching effect and the released N-terminal o-aminobenzoic acid containing fragment is now free to fluoresce. Recently, we designed a 16-mer intramolecularly quenched fluorogenic substrate based on the processing site of GPC SKI-1 in Lassa (24), namely o-aminobenzoic acid-DIYISRRL ↓GTFWT-3-nitrotyrosine-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. Whereas SKI-1 inhibition was recently achieved by 300 μM of the general serine protease inhibitor 4-(2-aminoethylbenzenesulfonyl fluoride (32)), the latter is not a specific SKI-1 inhibitor. We recently introduced protein-based ex vivo inhibitors of SKI-1 by mutating the reactive site loop of α,α-antitrypsin. We also optimized the protease-domain-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-RRLL-7-amido-4-MCA (GenScript Corp., Piscataway, NJ). Fluorogenic peptides derived from various viral glycoproteins, including LCMV (Armstrong and WE), MCA-GP252–265, and CCHV MCA-GP252–265 were synthesized in a similar fashion to Lassa virus (LAV) GPCs. The latter included MCA-GPC252–259 (8-mer WT), MCA-GPC252–259 (7-mer WT), MCA-GPC254–259 (6-mer WT) and MCA-GP254A,255Y-GPC254–259 (7-mer WT), GPC252–259 (7-mer WT), MCA-GPC254–259 (6-mer WT) and MCA-GPC252–259 (7-mer WT). 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 N-fluorenylmethoxycarbonyl (Fmoc) protocols and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/N-hydroxybenzotriazole 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)₁₂) 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 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/N-hydroxybenzotriazole, and 0.1 eq of 4-dimethylaminopyridine in dimethylformamide (DMF). The percentage of decanoylation was evaluated to be 100% by a Kaiser test. The C terminus of αN- and side chain-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 0.45 M (1 eq) 2-(7-aza-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxy-7-azabenzotriazole (HATU/HAOt) and 0.1 M 4-dimethylaminopyridine (0.1 eq) in DMF. For the fluorogenic peptides, after Fmoc removal (10% diisopropylethylamine in DMF), the succinyl group was introduced by succinic anhydride (10 eq) in DMF plus 0.1 M 4-dimethylaminopyridine 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 of a mixture of 95% trifluoroacetic acid, 2.5% H₂O, and 2.5% triethylsilane for 90 min. Each reaction step was monitored by reverse phase-high performance liq-
uid 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 × 250 mm), monitoring the elution (1 ml/min) at 214 nm and using as eluents, 0.05% trifluoroacetic acid/H2O (A), and 0.05% trifluoroacetic acid/CH3CN (B). The peptides were assayed for purity (>97%) by analytical HPLC and their identity confirmed by electrospray ionization-mass spectrometry (ESI-MS) (Mariner, PerkinElmer Life Sciences). Regarding the inhibitors, after removal of side chain protecting groups, final crude peptides were resuspended in diethyl ether 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 GPC250–265mut, Fig. 3C) was performed using SPPS and N*-t-butyloxycarbonyl (Boc) amino acids on Boc-Thr(Bzl)-PAM resin (NovaBiochem, La Jolla, CA, 0.34 mmol/g). After removal of the formyl protecting group (10% piperidine in DMF, 0 °C for 2 h), peptide cleavage and side chain deprotection were achieved using 12 ml of a mixture of 82% hydrofluoric acid, 9% thioanisole, and 9% dimethyl sulfoxide (Me2SO) at −5 °C for 1 h. The crude products were purified by RP-HPLC on a semi-preparative Vydac C18 column (5 μm, 300 Å, 10 × 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 8-mer WT, dec-YISRRL-cmk, and dec-RRLL-cmk that were dissolved in Me2SO.

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 SKI-1 and 100 μl of buffer, 50 mM Succ-IYISRRLL-MCA as substrate, in a 96-well microtiter plate at 37 °C under the above conditions. IC50 values were calculated by using GraFit version 4.09 software.

In Vitro Inhibition of Pyr-RTKR-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 37 °C. After 2 h, peptide cleavage and side chain deprotection were achieved using 12 ml of a mixture of 82% hydrofluoric acid, 9% thioanisole, and 9% dimethyl sulfoxide (Me2SO) at −5 °C for 1 h. The crude products were purified by RP-HPLC on a semi-preparative Vydac C18 column (5 μm, 300 Å, 10 × 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.

Determination of Vmax and Km Kinetic Parameters—For measurement of Vmax and Km kinetic parameters, 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 37 °C under the above conditions. Vmax and Km values were estimated by GraFit 4.09. (Erithacus Software Ltd., Staines, United Kingdom). 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 rates were evaluated for each peptide at different concentrations using the 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 preincubation at 37 °C (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 6 h (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 37 °C. The solutions, containing 30 μl of the hSKI-1 preparation and 5 μl of inhibitors at different concentrations (0–1.5 × 103 μM, dec-RERRL-cmk; 0–38 μM, dec-ISRRRL-cmk; 0–36 μM, dec-YISRRLL-cmk) were incubated 20 min at 37 °C before adding 50 μM Succ-IYISRRLL-MCA as substrate. Enzymatic activity measurements were performed by measuring the liberated AMC group with a Spectra MAX GEMINI EM microplate spectrofluorometer, Molecular Devices (λex, 360 nm; λem, 460 nm).

In Vitro Enzymatic Assays—Each reaction was carried out in a 100-μl buffer (25 mM Tris-HCl, 25 mM MES, and 1 mM CaCl2 adjusted to pH 7.0 or 6.0) at 37 °C. The solutions, containing 1 μl of either concentrated human furin medium or 10 μl of mouse PCS or human PACE4 preparations (36) and 5 μl of inhibitors at different concentrations (0–15 μM, dec-RERRL-cmk; 0–15 μM, dec-YISRRLL-cmk) were incubated 20 min at 37 °C before adding 50 μM Succ-IYISRRLL-MCA as substrate. Enzymatic activity measurements were performed by measuring the liberated AMC group with a Spectra MAX GEMINI EM microplate spectrofluorometer, Molecular Devices (λex, 360 nm; λem, 460 nm).

Determination of in Vitro Inhibitor Constant IC50 Values—For measurements of IC50 values, hSKI-1 (30 μl) was incubated with increasing concentrations (0–1.5 × 103 μM, dec-RERRL-cmk; 0–38 μM, dec-ISRRRL-cmk; 0–36 μM, 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 37 °C under the above conditions. IC50 values were calculated by using GraFit version 4.09 software.

In Vitro Enzymatic Assays—Each reaction was carried out in a 100-μl buffer (25 mM Tris-HCl, 25 mM MES, and 1 mM CaCl2 adjusted to pH 7.0 or 6.0) at 37 °C. The solutions, containing 1 μl of either concentrated human furin medium or 10 μl of mouse PCS or human PACE4 preparations (36) and 5 μl of inhibitors at different concentrations (0–15 μM, dec-RERRL-cmk; 0–15 μM, dec-YISRRLL-cmk) were incubated 20 min at 37 °C before adding 50 μM Pyr-RTKR-MCA as substrate. Enzymatic activity measurements were performed by measuring the liberated AMC group with a Spectra MAX GEMINI EM microplate spectrofluorometer, Molecular Devices (λex, 360 nm; λem, 460 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 K. Samples were prepared by dissolving weighted amounts of each peptide in [D3]TFE (99% isotopic purity, Aldrich) and H2O, for final concentrations of ~1.3 mM. Chemical shifts were referenced to...
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internal sodium 3-(trimethylsilyl)[2,2,3,3-2H4] propionate (TSP). Two-dimensional experiments, such as total correlation spectroscopy, nuclear Overhauser effect spectroscopy (NOESY), rotating frame Overhauser effect spectroscopy, and double quantum-filtered correlated spectroscopy were measured with standard pulse sequences (37, 38). According to Wüthrich (39), identification of amino acid spin systems was performed by comparison of total correlation spectroscopy and DQF-COSY, although sequential assignment was obtained by the analysis of NOESY spectra. NOE analysis was achieved by means of NOESY spectra, acquired with 200- and 300-ms mixing times. NOE intensities were evaluated by integration of cross-peaks from the 200-ms NOESY using NMRVIEW (40) software and then converted into inter-proton distances 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 Pro-SREBP-2 and Pro-ATF6 —For SREBP-2 analyses, CHO K1 cells were incubated overnight with various concentrations of decanoylated chloromethylketone inhibitors, namely the 7-mer-cmk (dec-YISR-RLL-cmk, 0–110 \( \mu \)M), 6-mer-cmk (dec-ISRLL-cmk, 0–110 \( \mu \)M), and commercial 4-mer-cmk (dec-RRLL-cmk; Bachem, 0–150 \( \mu \)M). 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 Pro-PDGF-A —On day 0, a stable PDGF-A-V5 construct in HEK 293 cells was plated on 35-mm 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 1\( \times \) phosphatase-buffered saline and serum-free medium was added along with varying concentrations of dec-YISRRLL-cmk (0–110 \( \mu \)M), dec-ISRRLL-cmk (0–110 \( \mu \)M), or dec-RRLL-cmk (0–150 \( \mu \)M), and incubated overnight. On day 3, medium was analyzed by running the samples on 12% SDS-PAGE and immunoblotted using V5-horseradish peroxidase 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\( \downarrow \)6), almost all sites contain an Arg at position P4 and hydrophobic residues at P2 (Fig. 1).

![FIGURE 1](http://www.jbc.org/content/281/33/23474/F1)

**FIGURE 1.** Amino acid sequences at the SKI-1/S1P cleavage site of mammalian and viral precursors. The alignment shows the sequences between amino acid positions P4 and P6 of the 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.

**FIGURE 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), Succ-SGSRRLL-MCA (CCHFV-derived) (IV), and Succ-FLTRRLS-MCA (V). Inset, comparison of the fluorescence released from Succ-YISRRLL-MCA (I) versus that of Succ-RRLL-MCA (III). B, summary of the cleavage preferences of the above peptides by SKI-1, +++, much better cleavage than +, and − meaning no cleavage.

![FIGURE 2](http://www.jbc.org/content/281/33/23474/F2)

**FIGURE 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), Succ-SGSRRLL-MCA (CCHFV-derived) (IV), and Succ-FLTRRLS-MCA (V). Inset, comparison of the fluorescence released from Succ-YISRRLL-MCA (I) versus that of Succ-RRLL-MCA (III). B, summary of the cleavage preferences of the above peptides by SKI-1, +++, much better cleavage than +, and − meaning no cleavage.
as the wild type Succ-WKHQKLL-MCA, and its P5 mutants Succ-WKRQKLL-MCA, Succ-WKQKQLL-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 were important for SKI-1 recognition. Therefore, to test for potential in vitro SKI-1 substrates lacking P and/or GP-1 derived from the SKI-1 recognition site. 

Based on the processing site of viral glycoproteins exhibiting potential SKI-1 recognition motifs, such as those found in LAV (24), CCHFV (28, 33), and LCMV (26) (Fig. 2B). The glycoprotein GPC of LAV was shown to be cleaved very well in vitro using a 14-mer quenched fluorogenic substrate (25). In the present study, analysis of the processing of these viral-derived peptides revealed that the order of SKI-1 cleavage preference is: 7-mer LAV GPC ⩾ 6-mer LAV GPC ⩾ 4-mer LAV GPC (Fig. 2A, inset) = 7-mer CCHFV glycoprotein (Fig. 2, A and B), whereas the chosen LCMV-derived substrates were not cleaved (Fig. 2, A and B). Because SKI-1 processing usually occurs in the medial Golgi where the pH is close to 6.4, we tested the cleavage of peptides IV, V, and VI (see Fig. 2B) at pH values of 7.5, 6.7, 6.3, and 6.0. Results showed that these peptides are not cleaved in vitro at acidic pH values (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 Lassa 6-mer GPC (Succ-ISRRLL-MCA) is comparable with that of a 7-mer peptide (Succ-SGSRRLL-MCA) mimicking the CCHFV glycoprotein processing site, but that the 7-mer GPC Lassa (Succ-YISRRLL-MCA) was a more than 14-fold better substrate than either of the above, even though all contain the common P5-P1 SRRL sequence (Fig. 2). Thus, we hypothesized that Tyr at the P7 position within the GPC sequence of LAV plays a critical role. Accordingly, we synthesized MCA substrates with mutated P7 Tyr into Phe, Ile, Val, Ser, or Ala, as well as an 8-mer wild type sequence Succ-IYISRRLL-MCA (Fig. 3). Based on the optimal pH for the 7-mer peptide processing (Fig. 4, inset) and the absence of a [Ca2+] effect on the SKI-1 activity from 0.5 to 20 mM (not shown), we chose to compare the SKI-1 cleavage of these peptides at pH 7.5 and 1 mM Ca2+. The data revealed that the 8-mer is the best substrate for SKI-1, exhibiting a ~2.4-fold better Vmax/Km versus the 7-mer, whereas only the Phe mutant 7-mer is relatively well cleaved (Fig. 4, Table 1). Therefore, 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, whereas Val occupies the P7 position in the cleavage sites of CREB-like and Luman, Ala in pro-brain-derived neurotropic factor and Ser in CCHFV and SREBP-1 (Fig. 1), these amino acids are not acceptable within the LAV GPC sequence. We conclude that the 8-mer and 7-mer wild type LAV GPC sequences represent the best MCA-containing SKI-1 substrates.
SKI-1/S1P Fluorogenic Substrates and Inhibitors

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 P7 residues to catalysis requires the use of a different approach, including quenched fluorogenic substrates comprising six peptides (6). Consequently, we synthesized two 16-mer non-derivatized fluorogenic substrates (25) or non-derivatized peptides to catalysis requires the use of a different approach, including quenched fluorogenic substrates (25) or non-derivatized peptides (6).

TABLE 1

Kinetic parameters of MCA-peptidyl SKI-1 substrates

<table>
<thead>
<tr>
<th>Name</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-mer WT</td>
<td>14.8</td>
<td>0.155</td>
<td>10.5</td>
</tr>
<tr>
<td>7-mer WT</td>
<td>34.2</td>
<td>0.137</td>
<td>2.5</td>
</tr>
<tr>
<td>7-mer Phe</td>
<td>38.1</td>
<td>0.094</td>
<td>1.3</td>
</tr>
<tr>
<td>7-mer Ile</td>
<td>36.7</td>
<td>0.046</td>
<td>0.8</td>
</tr>
<tr>
<td>7-mer Ser</td>
<td>59.4</td>
<td>0.049</td>
<td>0.8</td>
</tr>
<tr>
<td>7-mer Ala</td>
<td>73.35</td>
<td>0.050</td>
<td>0.7</td>
</tr>
<tr>
<td>7-mer Val</td>
<td>137.8</td>
<td>0.050</td>
<td>0.4</td>
</tr>
<tr>
<td>6-mer WT</td>
<td>266.9</td>
<td>0.090</td>
<td>0.3</td>
</tr>
</tbody>
</table>

For the DG/Amber calculations a set of 116 experimental constraints from NOE data (81 intra-residual, 29 sequential, 6 medium range) was used 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 (not shown). The nuclear Overhauser effect (NOE) patterns confirm the possible occurrence of the helical structure in this region, as evidenced by the presence of diagnostic helical medium range effects of the type $\alpha N(i,i+3)$ and $\alpha \beta(i,i+3)$ in this area (39).

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On the basis of the above results, we compared the ex vivo inhibitory potential of an untested, yet commercially available, dec-RVKR-cmk to two other synthetic peptides made by us, namely the 6-mer dec-ISRRLL-cmk and 7-mer dec-YISRRLL-cmk (Figs. 7 and 8). The selected ex vivo SKI-1 precursor substrates were the membrane-bound transcription factors pro-SREBP-2 (46) and pro-ATF6 (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 pro-SREBP-2 by the 6-mer versus 7-mer 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 and ~20 μM for the 7-mer-cmk and 6-mer-cmk, respectively (Fig. 7). In another experiment we found that both 4-mer-cmk and 7-mer-cmk peptides are almost equipotent in inhibiting the processing of the overexpressed pro-ATF6 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 7-mer cmk-peptide to inhibit processing of the precursor of the platelet-derived growth factor pro-PDGF-A into PDGF-A by furin-like basic amino acid-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 HEK 293 cells stably expressing pro-PDGF-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 7-mer dec-YISRRLL-cmk (Fig. 9B) or 4-mer dec-RLL-cmk (not shown).

Even though the above 4-, 6-, and 7-mer cmk-peptides were designed to be cell permeable through the attachment of an N-terminal decanoylated moiety, we were still interested in defining their in vitro inhibitory potency on processing of the 8-mer succ-YISRRLL-MCA. Unexpectedly, the data shows 4-mer-cmk (IC$_{50}$ ~ 9 nm) as a ~250-fold better inhibitor than either the 6- or 7-mer-cmk peptides (IC$_{50}$ ~ 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 6-mer-cmk and 7-mer-cmk it may be too hydrophobic and likely kinetically unfavorable, whereas for the 4-mer-cmk it may mimic the hydrophobic patch Ile-Tyr-Ile found at the P6–P8 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 with in vitro) on the inhibitory potency of the 7-mer-cmk and 6-mer-cmk peptides.

In a similar fashion we also compared the ability of the 4-mer-cmk and 7-mer-cmk peptides to inhibit in vitro furin processing of the pentapeptide pyr-RTKR-MCA. Interestingly, whereas 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 whereas 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 7-mer MCA-containing peptides mimicking the glycoprotein recognition sequence of various hemorrhagic fever viruses. Unexpectedly, whereas intracellularly SKI-1 cleaves reasonably well the full-length glycoprotein of the LCMV-WE strain (26), it could not cleave in vitro the 7-mer-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 7-mer sequence is well processed in vitro, much better than the 7-mer CCHFV mimic (Fig. 2), emphasizing the critical importance of the primary structure of the 7-mer 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. Whereas the wild type 8-mer LAV GPC MCA-peptide is a 2-fold better substrate than the 7-mer, the latter is 14-fold better than the 6-mer (Fig. 4, Table 1). Interestingly, the 6-mer-MCA peptide derived from LAV GPC is processed by SKI-1 to the same extent as the 7-mer CCHFV peptide (Fig. 2, A and B). These data suggested that the P7 Tyr is much more critical than the P8 Ile for SKI-1 cleavage. Accordingly, 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 7-mer 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 phenylic 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 16-mer peptide mimicking the wild type sequence from P6’ to P10 (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 phenylic group at P7 was also observed ex vivo for the cleavage of the glycoprotein of LCMV, because replacement of the P7 Phe (Fig. 1) by Ala resulted in an almost absence of SKI-1 processing (26).

Whereas the importance of the P7 Tyr residue was deduced from MCA substrates, the absence of cleavage by SKI-1 of the 16-mer Y253A mutant may conceivably be due to additional structural features of the peptide. Preliminary circular dichroism analysis in aqueous solution of the non-fluorogenic 16-mer 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 16-mer peptides...
peptides. The data revealed that both peptides exhibit a helical structure from Ile254 to Arg257, and do not significantly differ from each other at the N-terminal segment preceding Ile254 (Fig. 5). We can conclude that the rationale behind the observed large difference in SKI-1 cleavability of these 16-mer 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 binding the substrate to the catalytic pocket of SKI-1.

Because we do not have an active site titrant of SKI-1, it is not possible presently to convert our $V_{\text{max}}$ data into $k_{\text{cat}}$ with confidence. The same applied to our previously published quenched fluorogenic substrates (25). Because the kinetics are really different between the quenched fluorogenic substrates 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 a 2-h 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 quenched fluorogenic substrates 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, because 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 7-mer-cmk peptide mimicking the LAV GPC sequence. To test the importance of the peptide length for cellular permeability and inhibition of SKI-1, we also compared ex vivo (Figs. 7 and 8) and in vitro (Fig. 10) inhibitory properties of dec-7mer-cmk to that of either a 1-amino acid shorter (dec-6mer-cmk) or 3-amino acid 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 cellular processing of pro-SREBP-2 (Fig. 7). Furthermore, the dec-4mer-cmk peptide was equipotent to dec-7mer-cmk in inhibiting the cellular pro-ATF6 processing (Fig. 8). However, it must be mentioned that, whereas the LAV GPC sequence is so far the best substrate of SKI-1, peptides derived from either pro-ATF6 or pro-SREBP-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 4-mer succ-RRLL-MCA (Fig. 2A, inset) and the importance of the hydrophobic residues Ile-Tyr-Ile at the P6 to P8 positions in the LAV GPC sequence (Figs. 3B and 4). We can conclude that dec-7mer-cmk, dec-6mer-cmk, and 4mer-cmk peptides are potent SKI-1 ex vivo inhibitors.

We next addressed the issue of selectivity of these ex vivo SKI-1 inhibitors and whether they could inhibit other convertases such as the basic amino acid-specific furin-like proteinases (2). This was especially relevant, because 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 pro-PDGF-A into PDGF-A through cleavage by furin-like enzymes at the RRKR67 motif (Fig. 9A). The data revealed that at concentrations ≤100 μM dec-7mer-cmk, which effectively inhibit SKI-1 activity ex vivo (Figs. 7 and 8), no sig-
significant inhibition of pro-PDGF-A cleavage was observed (Fig. 9B). Note that at 110 μM dec-YISRRRL-cmk ~2% inhibition of pro-PDGF-A ex vivo processing was observed (Fig. 9B). The fact that furin prefers basic amino acid containing substrates with a Leu at P2’ and 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. RRL found in all our dec-peptide-cmks, could conceivably inhibit furin. Thus, we can conclude that when used at concentrations ≤100 μM, the dec-7mer-cmk is relatively specific for SKI-1 inhibition ex vivo.

Because we did not observe toxicity of the synthesized dec-RRL-cmk on HEK 293 and CHO K1 cells, we collaborated with a group in the Center of Disease Control (Atlanta, GA) to test this inhibitor on 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 (IC50 ~ 9 nM). The high in vitro potency of the latter as compared with the 7-mer-cmk and 6-mer-cmk (IC50 ~ 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 IC50 of dec-RVKR-cmk for furin is ~2 nM (51). We therefore tested the in vitro inhibitory selectivity of dec-4mer-cmk and dec-7mer-cmk on the constitutively secreted convertases (2) furin at pH 7 and 6 (because processing of both pro-SREBP-2 and pro-ATF6 occurs in the medial Golgi with an intraluminal acidic pH), and PACE4/PSC5A 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 pro-PDGF-A in the trans Golgi network (Fig. 9B) (47, 52). On the other hand, only furin is inhibited by both peptides at pH 7, whereas under the same conditions the activities of PCSA and PACE4 are not affected (Table 2). As mentioned above, an RRL sequence could potentially inhibit furin, but because both PCSA and PACE4 can process peptides with 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, because their levels did not significantly change upon incubation at either pH, as verified by mass spectrometry (not shown). The sizes of the inhibitor dec-RRL-cmk and the substrate pyr-RKTRK-MCA are too small to assume a rigid conformation in solution. However, because 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 pH values. Along the same reasoning, it was recently demonstrated that the serpin α1-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 RRL 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.

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**SKI-1/S1P Fluorogenic Substrates and Inhibitors**

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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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