Development of Protein-based Inhibitors of the Proproteins of Convertase SKI-1/S1P

PROCESSING OF SREBP-2, ATF6, AND A VIRAL GLYCOPROTEIN*

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Processing of membrane-bound transfection data showed that out of numerous mutant candidates, the preprosegment of the basic amino acid specific convertases (e.g. furin and PC5) or α₁-PDX, a variant of α₁-antitrypsin (α₁-AT) exhibiting an RIPR^STVS sequence at the reactive site loop, were shown to potently inhibit these secretory proteinases. Accordingly, we tested the SKI-1-inhibitory potential of various point mutants of either the 198 amino acid prosegment of SKI-1 (1-198) or α₁-AT. Transient transfections data showed that, out of numerous mutants studied, the R134E prosegment mutant or the α₁-AT reactive site loop variants RVRVL^STVS, RRYL^STVS and RRLC^STVS are the best specific cellular inhibitors of SKI-1. The observed inhibition of the processing of endogenous SREBP-2, exogenous ATF6 and a PDGF-A (RRRL^STVS) variant were >55% and reach -80% in stable transfectants. We also show that SKI-1 forms SDS-stable complexes with these α₁-AT variants, but not with wild-type α₁-AT or α₁-PDX. Finally, these inhibitors were also shown to affect the processing and stability of the Crimean-Congo hemorrhagic fever virus glycoprotein.

Proteins and peptides that are biologically active are often generated by intracellular limited proteolysis of inactive precursors. The mammalian proprotein convertases (PCs)* of the secretory pathway are calcium-dependent serine proteinases related to bacterial subtilisin that cleave various precursors at the general consensus motif (K/R)(X)_n(K/R)↓, where n = 0, 2, 4, or 6 and X is any amino acid (1–3). The PC family counts seven basic amino acid-specific kexin-like convertases: furin, PC1/3, PC2, PC4, PACE4, PC5/6, and PC7/LPC (4). The eighth member is the recently discovered pyrolysin-like SKI-1/S1P that cleaves at the consensus motif (R/K)(X)Y(X)_n(Z)↓, where Z is variable (5), while the last member (6, 7) NARC-1 cleaves the sequence VFAQ^STVS↓ in its prosegment (8, 9).

More PCs contain an N-terminal signal sequence, followed by a prosegment, a catalytic domain and a P-domain. In addition, PCs possess a C-terminal segment that varies between the different members. The critical role of PCs in the proteolytic maturation of multiple proproteins, their implication in various pathologies (1, 10, 11), and their unidentified specific and/or redundant functions, make them attractive targets for the development of potent and selective inhibitors. The various successful approaches include: active site-directed chloromethyl ketone inhibitors (12, 13), reversible peptide-based inhibitors (14–17), plant derivatives (18), and several engineered variants of protein-based inhibitors that possess a furin-like motif. These include α₂-macroglobulin (α₂-MF) (19), α₁-antitrypsin (α₁-AT) Portland (α₁-PDX) (20–22), proteinase inhibitor 8 (PI8) (23), the turkey ovomucoid third domain (24), and eglin C (25, 26). However, these effective inhibitors directed against the basic amino acid-specific members lack selectivity. Furthermore, α₁-PDX was shown to inhibit all the basic amino acid-specific PCs within the constitutive secretory pathway (21, 27), whereas PI8 and α₂-MF can inhibit many other proteases in addition to the PCs.

Subtilisin-, kexin-, and furin-based studies established that the prosegment of these enzymes could act both as an intramolecular chaperone and a potentiating agent (28–31). The prodomain of PCs acts as a competitive inhibitor (31–34), whereas the prodomain of the yeast kexin behaves as a mixed inhibitor with an IC₅₀ of -160 nm (29). The wild-type prosegment of SKI-1 was also shown to inhibit this enzyme in vitro, albeit at micromolar concentrations (35). Finally, it was shown that ex vivo overexpression of the proproregers of furin (ppfurin), PC7 (ppPC7), and PC5 (ppPC5) resulted in potent but moderately selective cellular inhibition of their parent enzyme (31, 33, 36).

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‡ The abbreviations used are: PC, proprotein convertase; SKI-1, subtilisin kexin isozyme-1; ppSKI-1, prosegment of SKI-1 enzyme; S1P, site-1-protease; pro-PDGF, precursor of platelet-derived growth factor; ER, endoplasmic reticulum; CHO, Chinese hamster ovary; WT, wild type; EGFP, enhanced green fluorescent protein; SCAP, sterol cleavage-activating protein; SREBP, sterol regulatory element-binding protein; nSREBP, nuclear SREBP; ATF6, activating transcription factor 6; EGFP, platelet-derived growth factor; DMEM, Dulbecco’s modified Eagle’s medium; HMAF, hyperimmune mouse ascites fluid.

§ The abbreviations used are: PC, proprotein convertase; SKI-1, subtilisin kexin isozyme-1; ppSKI-1, prosegment of SKI-1 enzyme; S1P, site-1-protease; pro-PDGF, precursor of platelet-derived growth factor; ER, endoplasmic reticulum; CHO, Chinese hamster ovary; WT, wild type; EGFP, enhanced green fluorescent protein; SCAP, sterol cleavage-activating protein; SREBP, sterol regulatory element-binding protein; nSREBP, nuclear SREBP; ATF6, activating transcription factor 6; EGFP, platelet-derived growth factor; DMEM, Dulbecco’s modified Eagle’s medium; HMAF, hyperimmune mouse ascites fluid.

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SKI-1/S1P Inhibition

Subtilisin kexin isozyme-1 (SKI-1) (5, 7) also known as Site 1 protease (6) represents the first mammalian member of secretory subtilisin-like processing enzymes that cleaves after hydrophobic residues. It is synthesized as an inactive precursor (1,052 amino acids) that undergoes three sequential autocatalytic processing events of its prosegment (amino acids 17–186). The signal peptidase cleavage generates a A form (amino acids 17–1052) that is subsequently autocatalytically cleaved in the endoplasmic reticulum (ER) at two alternate B’ and B sites: RKVF[134] (SKI-1-134–1052) and RKVRFSKL[137] (SKI-1-138–1052), respectively. The latter products are then transported to the cis/medial Golgi whereupon they are further autocatalytically processed into a C-form at RRLL[186], generating SKI-1-187–1052 (5, 7, 35, 37).

SKI-1 plays a crucial role in the regulation of lipid metabolism and cholesterol homeostasis through the processing of the sterol regulatory element-binding proteins, SREBP-1 and SREBP-2, which occurs in the cis/medial Golgi (38, 39). Other type-II membrane-bound substrates include ATF6 that plays a major role in the unfolded protein response (UPR) to enhance the protein folding or refolding capacity of the secretory pathway (40, 41), and the basic leucine zipper transcription factor Luman, the cellular counterpart of herpes simplex virus VP16 (42). Brain-derived neurotrophic factor (BDNF) is a soluble substrate and the study of its processing led to the initial cloning of SKI-1 (5). Mutation of proproletat-derived growth factor A (pro-PDGF-A) at its furin-cleavage site (RRKR[1166] to RRRL[1176]) (pro-PDGF-A*) resulted in a SKI-1 artificial substrate (43). Finally, SKI-1 was shown to play a major role in the processing of surface glycoproteins of infectious viruses such as Lassa (44, 45), lymphocytic choriomeningitis (LCMV) (46, 47) and Crimean Congo hemorrhagic fever (CCHF) (48) viruses. Whereas SKI-1 inhibition was recently achieved with 300 μM of the general serine protease inhibitor AEBSF (55), it was not a specific SKI-1 inhibitor. In the present study, we introduced SKI-1 recognition motifs into the reactive site loop (RSL) of α-AT (P1-P4 positions) as one approach to the development of protein-based inhibitors. We also optimized the prosegment-based inhibition of SKI-1 and identified a unique R134E mutant exhibiting a potent inhibitory activity. These inhibitors represent protein-based inhibitors designed to specifically block intracellular SKI-1 activity.

EXPERIMENTAL PROCEDURES

Construction of Human α-AT Variants by Site-directed Mutagenesis—The pRES2-EGFP vector (Clontech) with the human α-AT cDNA containing the wild-type sequence (AIPM[506]) in the reactive site loop (RSL) was used as template to introduce mutations. The various P1-P4 reactive site loop variants were generated by a two-step PCR using Elongase (Invitrogen, Life Technologies) using sense (S) and antisense (AS) oligonucleotides. The oligonucleotides used for introducing mutants were S1/AS1, S2/AS2, S3/AS3, S4/AS4, S5/AS5, and S6/AS6. S7/AS7, S8/AS8, S9/AS9, S10/AS10, S11/AS11, and S12/AS12, respectively (Table I). The various α-AT variants were subsequently amplified using S22/AS22, and the PCR products were cloned into the pCRII-TOPO TA-cloning vector (Invitrogen) and sequenced. The PstI/SacII cDNA fragments replaced that of the wild-type α-AT sequence in the pRES2-EGFP, resulting in the mutant α-AT recombinants RRLL, RKVL, RRLL, RKVF, RRLE, RRIL, RRVL, RRFL, RRRL, and KLLL.

Human Prepro-SKI-1 and Its Mutants—The N-terminal fragment of hSKI-1 (wild type) (1–198 amino acids) was amplified by PCR using S13/AS13 and cloned into pCRII-TOPO TA-cloning vector. Subsequently, the amplified cDNA was digested by Xhol/BamHII and sub-cloned into pRES2-EGFP. Site-directed mutagenesis was carried out using the wild-type construct as the template using the pairs of oligonucleotides (Table I): S13/AS14, S15/AS15, S16/AS16, S17/AS17, S18/AS18, S19/AS19, S20/AS20, S21/AS21. This generated the preproSKI-1 (pS13/AS13) cDNA variant expressing either pSKI-1 cleavage B or B’ site mutants RRKVFSKL[137]–stop, R134E, R134K, K137V, K191A, K130A (amino acids 1–198)–KDEL, and the double mutant K130A/R131A. All mutant cDNAs were sequenced and sub-cloned into pRES2-EGFP.

Inhibition of Pre-PDGF-A and Pre-PDGF-A* Processing ex Vivo—Chinese hamster ovary CHO-K1 (4 x 10^6) cells (in a 60-mm plate) were transfected using LipofectAMINE 2000 (Invitrogen). A total of 6 μg DNA was used for each transfection in a ratio of 1:4 (substrate:inhibitor) expressing either pRES2-EGFP-V5 alone, pRES2-EGFP-pro-PDGFA-A5 (WT), or pRES2-EGFP-pro-PDGFA-A5 (mutant). Co-transfection with various mutants constructs such as α-PDX (RIPR[285]) WT, various α-AT, and ppSKI-1 variants were used to inhibit the processing of wild-type pre-PDGF-A or its RSLR[285] mutant (pre-PDGFA-A5). After 24 h of incubation at 37 °C in DMEM/10% fetal calf serum (Invitrogen, Life Technologies), the cells were rinsed with PBS and incubated in serum-free DMEM for another 24 h. 48-h post-transfection, the medium was resolved on a 12% SDSPAGE gel. Detection by Western blotting was done with monoclonal

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antibody directed against the V5 epitope fused to the C-terminal end of pro-PDG-F-A (1:5000 dilution) (Invitrogen).

**Transient Transfection and Immunoblot Analysis of Endogenous Hamster SREBP-2**—Chinese hamster ovary cells (CHO-K1) were set up at a density of 4 × 10^5 cells per 60-mm plate for transfection. On day 1, cells were transiently transfected with 6 μg of cDNA using LipofectAMINE 2000, and cultured overnight in medium B (1:1 mixture of Ham’s F-12 medium and Dulbecco’s modified Eagle’s medium containing 100 μg/ml streptomycin sulfate) supplemented with 5% fetal calf serum. On day 2, cells were washed with PBS and then switched to medium with 5% lipoprotein deficient serum (LPDS) with 50 μg complexes and 50 μm sodium mevalonate in the absence of the presence of sterols for 18 h. Thereafter, the cells received N-acetyl-leucinyl-leucinal-norleucinal (ALLN, Sigma) at a final concentration of 25 μg/ml, a calpain and proteasome inhibitor that blocks the degradation of the mature form of SREBP-2, and the cells harvested 1-h later. Cells were then washed twice with PBS, lysed in 200 μl of SDS buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1% SDS, and 25 mM δ mercaptopropionic acid) and centrifuged. The lysates were analyzed by immunoblotting using a 1:5000 dilution of anti-FLAG M2 monoclonal antibody (Prizm) using ImageQuant program. In experiments involving infection with CCHF virus, CHO-K1 cells stably expressing α1-AT, RRVL358, RRYL358, and R134E were maintained in 1:1 proportion of DMEM and Ham’s F-12 medium supplemented with 5% fetal calf serum. Heat-inactivated mouse ascites fluid (HMAF) for CCHF virus proteins were kindly provided by T. Ksiazek, Centers for Disease Control and Prevention (Atlanta, GA). The antibody used in this study was raised in rabbits against KLH-conjugated peptide sequences present in the mature Gn (amino acids 540–551 of CCHF virus 1bAr 10200 strain: EHBGDNYYGPPOG) and was detected using goat anti-rabbit IgG (1:1000 dilution) (Invitrogen).

**CHF Virus Pre-Gn to Gn Processing and Inhibition**—SW13 cells were maintained in DMEM supplemented with 10% fetal calf serum. SW13 cells stably expressing α1-PDX and RRVL256 were developed and maintained in the presence of G418 (300 μg/ml). CHO-K1 derived cells expressing α1-AT, RRVL256, RRVL256, and R134E were maintained in 1:1 proportion of DMEM and Ham’s F-12 medium supplemented with 5% fetal calf serum. Hyperimmune mouse ascites fluid (HMAF) for CCHF virus proteins were kindly provided by T. Ksiazek, Centers for Disease Control and Prevention (Atlanta, GA). The antiprotein antibody used in this study was raised in rabbits against KLH-conjugated peptide sequences present in the mature Gn (amino acids 540–551 of CCHF virus 1bAr 10200 strain: EHBGDNYYGPPOG) and was competed under contract with Research Genetics Inc, Huntsville, AL. NuPAGE ready-made gels (7% or 3–8%) and recommended buffers were purchased from Invitrogen.

Because the expression of CCHF virus glycoprotein required T7 RNA polymerase to drive the T7 promoter, wt-7 vaccinia virus expression system was used as performed before (48). Briefly, 5 × 10^6 cells were seeded onto 6-well plates a day before transfection and were infected for 1 h with vaccinia virus expressing T7 RNA polymerase. Upon removal of the virus, cells were transfected with plasmid DNA encoding the wild type CCHF virus glycoprotein or in combination with other inhibitor expressing plasmids at 1:1 or 1:4 ratio. After 12 h, cells were labeled for 30 min with [35S]cysteine and chased for 3 h. While using cells stably expressing α1-AT variants using 1-AT, RRVL358, RRYL358, and R134E were infected with them with vaccinia virus and transfected with 5 μg of plasmid DNA expressing CCHF virus glycoprotein. Immunoprecipitated proteins were resolved in NuPAGE gels and proteins were visualized by autoradiography. The protein levels were quantified using a PhosphorImager (ABI Prizm) using ImageQuant program. In experiments involving infection with CCHF virus, SW13 cells stably expressing α1-AT, RRVL256, RRVL256, or R134E were infected with CCHF virus and labeled with [35S] in the Biosafety Level 4 laboratory at the CDC, Atlanta. Proteins were immunoprecipitated using HMAF, resolved in 7% NuPAGE, and bands visualized by autoradiography.

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**Table 1**

| Primers | Sense (S) | Antisense (AS) |
|---------|-----------|----------------|
| S1/AS1  | CGCAGCTCTCTCCGATCCCGCCGAGGTCAG | GAGGAGTTCGCTGCTAAAACATGGCGCCCTGCG |
| S2/AS2  | CGCAGCTTCAATCCGATCCCGCCGAGGTCAG | TTGGAAGTTCGCTGCTAAAACATGGCGCCCTGCG |
| S3/AS3  | CGCAGCTCATCTCCGATCCCGCCGAGGTCAG | GTATGAGTTCGCTGCTAAAACATGGCGCCCTGCG |
| S4/AS4  | CGCAGCTCTCTCCGATCCCGCCGAGGTCAG | GTATGAGTTCGCTGCTAAAACATGGCGCCCTGCG |
| S5/AS5  | CGCAGCTCTCTCCGATCCCGCCGAGGTCAG | GTATGAGTTCGCTGCTAAAACATGGCGCCCTGCG |
| S6/AS6  | CGCAGCTCTCTCCGATCCCGCCGAGGTCAG | GTATGAGTTCGCTGCTAAAACATGGCGCCCTGCG |
| S7/AS7  | CGCAGCTCTCTCCGATCCCGCCGAGGTCAG | GTATGAGTTCGCTGCTAAAACATGGCGCCCTGCG |
| S8/AS8  | CGCAGCTCTCTCCGATCCCGCCGAGGTCAG | GTATGAGTTCGCTGCTAAAACATGGCGCCCTGCG |
| S9/AS9  | CGCAGCTCTCTCCGATCCCGCCGAGGTCAG | GTATGAGTTCGCTGCTAAAACATGGCGCCCTGCG |
| S10/AS10| CGCAGCTCTCTCCGATCCCGCCGAGGTCAG | GTATGAGTTCGCTGCTAAAACATGGCGCCCTGCG |
| S11/AS11| GTTTTGGAGCAGAGACCTTCGCTCG | CTTGGAGGATCAAAGGCTTTC |
| S12/AS12| GTTTTGGAGCAGAGACCTTCGCTCG | CTTGGAGGATCAAAGGCTTTC |
| S13/AS13| GTTTTGGAGCAGAGACCTTCGCTCG | CTTGGAGGATCAAAGGCTTTC |
| S14/AS14| GTTTTGGAGCAGAGACCTTCGCTCG | CTTGGAGGATCAAAGGCTTTC |
| S15/AS15| GTTTTGGAGCAGAGACCTTCGCTCG | CTTGGAGGATCAAAGGCTTTC |
| S16/AS16| GTTTTGGAGCAGAGACCTTCGCTCG | CTTGGAGGATCAAAGGCTTTC |
| S17/AS17| GTTTTGGAGCAGAGACCTTCGCTCG | CTTGGAGGATCAAAGGCTTTC |
| S18/AS18| GTTTTGGAGCAGAGACCTTCGCTCG | CTTGGAGGATCAAAGGCTTTC |
| S19/AS19| GTTTTGGAGCAGAGACCTTCGCTCG | CTTGGAGGATCAAAGGCTTTC |
| S20/AS20| GTTTTGGAGCAGAGACCTTCGCTCG | CTTGGAGGATCAAAGGCTTTC |
| S21/AS21| GTTTTGGAGCAGAGACCTTCGCTCG | CTTGGAGGATCAAAGGCTTTC |
| S22/AS22| GTTTTGGAGCAGAGACCTTCGCTCG | CTTGGAGGATCAAAGGCTTTC |
RESULTS

Inhibition of Pro-PDGF-A* (RRLL358) Processing by SKI-1 in CHO-K1 Cells—Previoulsy (43), we had shown that SKI-1 can process intracellularly an RRLL358 mutant of pro-PDGF-A (pro-PDGF-A*) into PDGF-A (Fig. 1A), paving the way for a convenient ex vivo assay of SKI-1 inhibition using an anti-V5 Western blot analysis of secreted PDGF-A. The choice of CHO-K1 cells was dictated by the availability of CHO cells lacking SKI-1 (SRD-12B, called here SKI(-) cells) (56). In CHO-K1 cells, endogenous SKI-1 processes pro-PDGF-A, whereas in SKI(-) cells such processing does not occur except in the presence of overexpressed SKI-1 (SKI(+) cells) (Fig. 1B). We used this assay to test the inhibition of intracellular SKI-1 activity in CHO-K1 cells by a series of mutants of either α1-AT (Fig. 1C) or the preprosegment of human SKI-1 (ppSKI-1; Fig. 1D).

In the α1-AT variants, we mutated the reactive site loop sequence AIPM358 (P1-P4 positions) to mimic the reported SKI-1 specificity for the motif (R/K)X(hydrophobic)Z (5–7), with a preference for the RRLL cleavage site reported for the SKI-1 prosegment site C (5), and the glycoproteins of Lassa virus (44, 45) and CCHV virus (48). Co-expression of pro-PDGF-A or pro-PDGF-A* with either α1-AT, α1-PDX, or various serpin mutants are shown in Fig. 2. Western blots using an α1-AT antibody revealed that α1-AT is very efficiently processed in a 16-kDa PDGF-A product. This process is completely inhibited by α1-PDX, and most of the mutants analyzed did not have any significant inhibitory effect. However, a series of mutants RRLL358, RRYL358, RRVL358, and RRLL358, showed <30% inhibitory effect (Fig. 2A, bottom panels). The small amount of inhibition observed might be due to the presence of a dibasic motif in the reactive site loop that may inhibit the basic convertases. In contrast, the processing of pro-PDGF-A* is inhibited by the mutants RRLL358, RRVL358, RRYL358, RRIL358, and RRLE358, whereas α1-AT, α1-PDX and the other mutants tested were not inhibitory (Fig. 2A, upper panels). These results emphasize that serpins with a P2 hydrophobic and P4 Arg are very efficient in blocking SKI-1 activity, consistent with the SKI-1 recognition motif (5–7).

The mutations of the prosegment of SKI-1 (ppSKI-1) were selected to evaluate the importance of B/B’ primary autacalytic zymogen processing sites (amino acids 130–137), and the segment separating the B/B’ and the C-sites. As previously reported with in vitro data (35), wild-type ppSKI-1 effectively inhibits the intracellular processing of pro-PDGF-A* (Fig. 2B, top panels). Retention of the prosegment in the ER through a C-terminal KDEL sequence abolished its inhibitory effect (data not shown), revealing that pro-PDGF-A* is processed at a post-cis-Golgi compartment (57). Conversely, shortening of the prosegment to end at the C terminus of the B-site, i.e. at RSLK357 (STOP), resulted in an inactive protein. Of the B/B’ mutants tested, only R130A and R134E were found to be inhibitory. Following quadruplicate independent experiments with similar results, we can state that the order of inhibitory potency of the prosegments of pro-PDGF-A* processing by SKI-1 was found to be: WT-ppSKI-1 > R134E > R130A (Fig. 2B).
Inhibition of Endogenous SREBP-2 Processing by SKI-1 in CHO-K1 Cells—We next investigated the intracellular inhibitory properties of the same series of α1-AT and ppSKI-1 mutants (Fig. 1, C and D) on the processing of endogenous proSREBP-2 (SREBP-2), a SKI-1/S1P substrate that is reported to be cleaved in the cis/medial Golgi (37). As shown in Fig. 3A, the membrane-bound SREBP-2 is not significantly processed into nSREBP-2 in cells incubated in medium containing cholesterol (+), whereas its characteristic doublet nuclear N-terminal fragment (nSREBP-2) is observed in the absence of exogenous sterols (−), as previously reported (56). The latter is generated by the successive cleavage of SREBP-2 by SKI-1/S1P and S2P, and requires the removal of cholesterol (49). Thus, all inhibitors were tested in the absence of exogenous sterols. Under these conditions, and in transient transfections, the α1-AT variants that were found to be significant intracellular inhibitors (56–61% inhibition) were the RRYL 358, RRVL358, and RRIL358 mutants (Fig. 3A). In the same vein, only the R134E mutant of ppSKI-1 exhibited ~77% inhibition (Fig. 3B). In all cases equal protein levels were loaded onto the gels, as evidenced by the similar amounts of immunoreactive α1-AT (Fig. 3A) or protein loaded based on β-actin immunoreactivity (B). The estimated % inhibition are shown at the bottom of each panel.

Fig. 3. Inhibition of the processing of endogenous SREBP-2. A and B, CHO-K1 cells were transiently transfected with the empty vector (pRES2-EGFP), α1-AT, α1-AT, or ppSKI-1 mutant cDNAs. 24-h post-transfection the cells were washed with PBS and incubated in (-sterol) medium for 18 h at 37 °C as described under “Experimental Procedures.” Cells were lysed, and total cell extracts were resolved on 6% SDS-PAGE and endogenous hamster SREBP-2 was detected using a mouse mAb IgG-7D4 (1:200). The precursor of SREBP-2 (SREBP-2) and the nuclear processed form (nSREBP-2) migrated as doublet (56) with apparent molecular masses of ~120 kDa and ~64 kDa, respectively. Stars indicate significant reduction in nSREBP-2 detection, emphasizing inhibition of SKI-1 activity. Lower panels show immunoblot analysis of the same membrane subsequently rehybridized with the α1-AT antibody to confirm the level of expression (A) and of protein loaded based on β-actin immunoreactivity (B). The estimated % inhibition are shown at the bottom of each panel.
does not require the participation of SCAP, but rather the incubation of cells under stressful conditions resulting in the activation of the UPR, such as the treatment with the Ca\(^{2+}\) ionophore thapsigargin or by the generation of a major fraction of ATF6 remains in the ER as an uncleaved \(\sim 90\) kDa precursor (40). Under normal conditions (Fig. 4A, upper panels), in the absence or presence of the chosen inhibitors the majority of ATF6 remains in the ER as an uncleaved \(\sim 90\) kDa precursor (40). However, incubation of cells with either thapsigargin or tunicamycin (Fig. 4A, lower panels) results in the generation of a \(\sim 50\) kDa nATF6 (40). From the co-transfection of ATF6 and inhibitor cDNAs, it is clear that, whereas \(\alpha_1\)-AT and \(\alpha_1\)-PDX had no effect, the \(\alpha_1\)-AT variants RRVL, RRYL, and RYL form SDS-stable complexes with \(\alpha_1\)-AT migrating at an apparent molecular mass of \(\sim 230\) kDa (Fig. 5A). A minor form migrating at \(\sim 180\) kDa was also observed. In contrast, neither WT-\(\alpha_1\)-AT nor \(\alpha_1\)-PDX form such complexes with \(\alpha_1\)-AT (Fig. 5A), and none of the serpins tested can form an SDS-stable complex with inactive \(\alpha_1\)-AT variants. Western blot analysis of cell lysates following SDS-PAGE using a monoclonal antibody that recognizes the V5-epitope at the C terminus of active \(\alpha_1\)-AT revealed that the \(\alpha_1\)-AT mutants RRVL, RRYL, and RYL form SDS- and heat-stable complexes with \(\alpha_1\)-AT migrating at an apparent molecular mass of \(\sim 230\) kDa (Fig. 5A). A minor form migrating at \(\sim 180\) kDa was also observed. In contrast, neither WT-\(\alpha_1\)-AT nor \(\alpha_1\)-PDX form such complexes with \(\alpha_1\)-AT (Fig. 5A), and none of the serpins tested can form an SDS-stable complex with inactive \(\alpha_1\)-AT (H249A) (Fig. 5B). We also noted that the propeptide mutant R134E and that the RSL mutants RRVL and RYL inhibited the formation of both the B/B and B/C forms. Western blot analysis of cell lysates following SDS-PAGE using a monoclonal antibody that recognizes the V5-epitope at the C terminus of active \(\alpha_1\)-AT revealed that the \(\alpha_1\)-AT mutants RRVL, RRYL, and RYL form SDS- and heat-stable complexes with \(\alpha_1\)-AT migrating at an apparent molecular mass of \(\sim 230\) kDa (Fig. 5A). A minor form migrating at \(\sim 180\) kDa was also observed. In contrast, neither WT-\(\alpha_1\)-AT nor \(\alpha_1\)-PDX form such complexes with \(\alpha_1\)-AT (Fig. 5A), and none of the serpins tested can form an SDS-stable complex with inactive \(\alpha_1\)-AT (H249A) (Fig. 5B). We also noted that the propeptide mutant R134E and that the RSL mutants RRVL and RYL inhibited the formation of both the B/B and the active \(\alpha_1\)-AT C-forms, suggesting that these are the best \(\alpha_1\)-AT inhibitors (Fig. 5, A and C). It should be noted that \(\alpha_1\)-PDX was reported to form similar complexes with furin (22, 61), PC5, and PACE4 (27). Similar analyses were performed in HK293 cells transiently overexpressing both \(\alpha_1\)-AT and the serpins (Fig. 6). Again, we could detect the \(\sim 230\) kDa intracellular complex between \(\alpha_1\)-AT and either \(\alpha_1\)-AT (Fig. 6A) and either \(\alpha_1\)-AT (RRVL, RRYL, and RYL) but not with \(\alpha_1\)-PDX or WT-\(\alpha_1\)-AT (Fig. 6A). Because membrane-bound \(\alpha_1\)-AT autocatalytically sheds itself releasing a soluble secreted form (5, 7), we also tested the media using an \(\alpha_1\)-AT antibody and detected similar complexes (Fig. 6B). As a further control, we also tested whether these inhibitors can form complexes with the soluble basic-amino acid-specific PC5A (62). Although not shown, only \(\alpha_1\)-PDX was shown to form a \(\sim 230\) kDa com-
Inhibition of the SKI-1-mediated Processing of the CCHF Virus Glycoprotein—We have previously shown that the mature Gn (37 kDa) of CCHF virus is processed from Pre-Gn (140 kDa) by SKI-1 following the RRL \( \downarrow \) tetrapeptide sequence (amino acids 516–519). Furthermore, a CHO-K1-derived cell line expressing a mutant form of SKI-1 (7) was defective in Pre-Gn processing (48). After screening many SKI-1 inhibitors and identifying a few potent ones effective for cellular proteins (Figs. 1–6), we wanted to analyze if those inhibitors were able to reduce or block the processing of a virus glycoprotein processed by SKI-1. For that purpose, CHO-K1 cells stably expressing \( \alpha_1\)-AT, RRVL358, RRYL358, or R134E were infected with vaccinia virus expressing T7 RNA polymerase and transfected with a T7 driven plasmid expressing CCHF virus glycoprotein and analyzed for the inhibition in Pre-Gn processing. In cells expressing the CCHF virus glycoprotein alone or in combination with inhibitors, Pre-Gn was synthesized as a ~140 kDa protein, which was processed to Gn during a 3-h chase period (Fig. 7). Comparison of the levels of CCHF virus Pre-Gn at the 0 and 3 h chase time points shows that ~35% of Pre-Gn is proteolytically cleaved during this time period in the control CHO-\( \alpha_1\)-AT cells (labeled as WT). As expected, mature Gn is undetectable at the 0 time point, but accumulated in significant amounts following 3-h chase. Comparison of the levels of mature Gn accumulating 3-h postchase in the RRVL358, RRYL358, and R134E cells relative to the control cells, indicated levels of inhibition of ~55%, ~38 and ~70%, respectively (Fig. 7). Interestingly, these SKI-1 protease inhibitors also appeared to affect the stability of the CCHF virus Pre-Gn precursor protein. Although similar amounts of Pre-Gn are detectable in the various cells during the pulse label period, less of the protein remained detectable after 3-h chase in the RRVL358, RRYL358, and R134E cells relative to the control WT cells. This instability appears specific to Pre-Gn, as a panel of nonspecific proteins (indicated by asterisk) and cellular calreticulin remained unaffected (Fig. 7).

As CHO-K1 cells are refractory to CCHF virus infection, the experiments using the CHO derived cell lines could only be performed using transfected plasmids encoding the CCHF virus glycoproteins, which does not allow analysis of the effects of these inhibitors on virus glycoprotein processing in the context of actual virus infection. To address this issue, we attempted to develop similar stably expressing cell lines but derived from SW13 cells that are the common cell line used for CCHF virus growth and experimentation. SW13 cell lines stably expressing \( \alpha_1\)-PDX and \( \alpha_1\)-AT-RRVL358 were successfully obtained. As \( \alpha_1\)-PDX (furan inhibitor) does not affect CCHF virus Pre-Gn processing to mature Gn (48), the \( \alpha_1\)-PDX stably expressing cells may serve as a control for assessment of processing inhibition in \( \alpha_1\)-AT-RRVL358-expressing cells. As expected, transient expression of CCHF virus glycoprotein expressed from transfected plasmids in the \( \alpha_1\)-PDX-expressing SW13 cells resulted in the virus Pre-Gn being processed to mature Gn (Fig. 8A). Comparison of the level of accumulation of mature Gn in the \( \alpha_1\)-PDX versus \( \alpha_1\)-AT-RRVL358 expressing cells in two independent experiments demonstrated ~65–70% inhibition of Pre-Gn processing (Fig. 8A). This level of inhibition is somewhat higher than that observed in the corresponding CHO-derived cells (Fig. 7).

To determine the level of inhibition of virus Pre-Gn processing seen in the context of a CCHF virus infection, the \( \alpha_1\)-PDX and RRVL358 stably expressing SW13 cells were infected and the level of processing assessed (Fig. 8B). In CCHF virus-infected SW13-\( \alpha_1\)-PDX cells, the virus’ Pre-Gn, Pre-Gc, Gc, and nucleocapsid proteins were visualized during the 30-min pulse and Gn appeared during the 5-h chase. Similar amounts of the virus proteins were observed in the \( \alpha_1\)-AT-RRVL358 expressing virus-infected cells with the exception of mature Gn, which appeared to be reduced (relative to the \( \alpha_1\)-PDX expressing cells) by ~50–55%, when normalized to nucleoprotein content. Taken together, these data and those from the CHO-derived stably expressing cell lines strongly suggest that SKI-1 protease inhibitors may represent promising compounds for further study in the development of effective antiviral strategies for control of CCHF virus infections.

**DISCUSSION**

Cellular localization experiments revealed that SKI-1 is sorted to the cis/medial Golgi (5, 37, 63), suggesting that it is poised to process its cognate precursors therein, SREBPs (39), ATF6 (41), Luman (42), and proBDNF (5). Whereas the modified serpin \( \alpha_1\)-PDX (20, 21) and the PC-prosegments (31) inhibit the basic amino acid-specific PCs within the constitutive secretory pathway, no specific-inhibitor of SKI-1 is yet known. In *vitro*, the prosegment of SKI-1 is only a modest \( \mu \)s inhibitor of SKI-1 activity (35). Recently, Okada et al. (55) reported that the general serine protease inhibitor AEBSF inhibits the SKI-1-mediated Golgi processing of both SREBP-2 and ATF6\( \alpha \) and ATF6\( \beta \).

In this work we demonstrated by four different *ex vivo* assays using pro-PDGF-A\( ^{\alpha} \), SREBP-2, ATF6, and Pre-Gn that the best SKI-1 inhibitors are consistently ppSKI-1 R134E and the \( \alpha_1\)-AT variants RRVL358, RRYL358, and R134E. Notably, ppSKI-1 R134A was not inhibitory, whereas the R134E mutant was the most potent SKI-1 inhibitor identified in this study. Because the B’/B sequence cleaved is RKVF \( ^{134} \text{RSLK} \) (7), the R134E mutation places a Glu at the P1’ position of the B’ site (RKVF \( ^{134} \text{ESLK} \)) and at the P4 position of the B-site (134ESLK) (Fig. 1). The absence of a P4 Arg should block the B-site (7, 37) but could still allow the B’ to interact with the SKI-1 catalytic pocket. It should be noted that the R134E mutant of the full-length SKI-1 is ~50% less processed into the B’/B and C forms than the wild-type sequence (7). The importance of a P1’ acidic residue for SKI-1 activity was not studied before. Interestingly, the recently described crystal structure of furin clearly suggested that a P1’ acidic residue would greatly
favor the insertion of a substrate within the catalytic pocket (64). Indeed, a number of good furin/PC5 substrates such as their own prosegments, /H9251-integrins, and the Ebola virus glycoprotein /H11032-exhibit an acidic P1/H11032 residue (1). Although the structure of SKI-1 is yet to be unraveled, it is tempting to speculate that similar structural constraints to those reported for furin may be applicable for SKI-1.

Our studies with the RSL mutants of /H9251-PDX also unraveled the selectivity of the type of P2 hydrophobic residues acceptable by SKI-1, when P1 is Leu. Thus, whereas a P2 Val, Ile, and Tyr are conducive to an inhibitory RSL, P2 Pro, Leu, Phe are not (Figs. 2–4). It may well be that the RRLL sequence that is best recognized by SKI-1 in a number of substrates (7, 44, 48) may actually be cleaved when present in the RSL of /H9251-PDX. Thus, whereas only an RSL with a P1 Leu in combination with either P2 Val, Ile, and Tyr resulted in an inhibitory serpin, we cannot eliminate the possibility that a P1 Val or Ile with a P2 Val, Ile, or Tyr may not also be inhibitory. This is especially true as site-directed mutagenesis studies of SREBP-2 revealed that whereas SKI-1 can cleave the WT-sequence RSVL it does not process an RSVV mutant (65). However, the latter sequence may well be inhibitory.

The ability of /H9251-PDX to form SDS-stable complexes with furin, PC5/6B (22, 66) and PACE4 (27), but not with PC2 (22), led to test the possibility that variants of /H9251-PDX that better fit the SKI-1 recognition motif may also form stable complexes with this enzyme. Indeed, our data revealed that the best inhibitors of SKI-1 activity, namely /H9251-PDX RRVL, RRYL, and RRIL do form such complexes with SKI-1 seen both in cells and media, whereas the non-inhibitory /H9251-PDX or /H9251-PDX do not (Figs. 5 and 6). This observation is similar to what has already been published for /H9251-PDX and furin in vitro (22) and in HK293 cells (27), and in all cases only a small portion of the total enzyme can be seen as a complex with the serpin on SDS-PAGE gels, suggesting that only a small amount of enzyme-serpin complex is truly resistant to SDS and heat. Thus, it is conceivable that the reactive site loop of the inhibitory /H9251-PDX variants exhibit typical serpin-like properties with respect to their inhibition of SKI-1.

Because SKI-1 silencing is lethal, conditional knockout experiments in liver revealed that silencing of its mRNA by 80% results in 50% decrease in circulating cholesterol and fatty acids (53). Therefore, the proposed R134E prosegment could conceivably represent a potentially useful inhibitor to reduce the activity of SKI-1 in vivo. Because the latter enzyme is also critical for the activation of some hemorrhagic fever viral surface glycoproteins (44–46, 48), we also tested our best inhibitors on the processing of Pre-Gn into Gn of CCHF virus. Unlike the other cellular proteins analyzed in this report, expression of
the glycoprotein required the need for vaccinia virus to drive the T7 promoter. Our attempts to express glycoprotein using the CMV promoter or by providing T7 RNA polymerase from a plasmid were not successful. When using vaccinia virus, we do see inhibition of Pre-Gn processing in cells expressing α1-AT-RRVL358 and ppSKI-1 R134E. The fact that the Pre-Gn becomes unstable during the chase phases makes it harder to estimate the exact level of inhibition using the vaccinia system. Most of the experiments described for the inhibition of cellular proteins were performed with 1:4 substrate and inhibitor molar ratio. When we used that ratio in CCHF virus glycoprotein experiments, we observed a complete blockade of virus synthesis and glycoprotein stability even during the 30-min pulse period. We suggest that α1-AT-RRVL358 and ppSKI-1 R134E might be good inhibitors if we could manage to get the ideal expression with improved protein stability. On the other hand, SW13-derived α1-AT-RRVL358 cells showed promising inhibition (~65–70% in transfactions and ~50–55% in CCHF virus infection assays) compared with α1-PDX cells. In CCHF virus infected cells, there were no significant differences in the levels of expression of Pre-Gn, Pre-Gc, Gc, or nucleocapsid. We are in the process of developing cells stably expressing other promising inhibitors in the context of SW13 cells. Upon successful expression of all the inhibitors and necessary control, we intend to investigate the role of processing inhibition of Pre-Gn on virus assembly, release and infectivity with two target viruses (CCHF and arena viruses) which are causative agents of severe hemorrhagic manifestations in humans and whose glycoproteins are known to be activated by SKI-1. In this context, it was recently shown that lack of SKI-1-mediated processing of the glycoprotein of lymphocytic choriomeningitis virus (LCMV) leads to non-infectious virus (47). If the lack of Pre-Gn processing negatively affects the above-mentioned parameters, then the inhibitors responsible for reducing or abrogating the processing would offer a good therapeutic potential.

In conclusion, the proposed inhibitors should be very useful in defining novel functions and/or substrates of SKI-1 in cell lines of choice as well as in vivo in various species. They could be used as prototypes for the development highly potent small molecule SKI-1 inhibitors that could find important clinical and pharmaceutical applications.

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