

Zymogen Activation and Subcellular Activity of Subtilisin Kexin Isozyme 1/Site 1 Protease^{*[5]}

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Background: Zymogen activation of proprotein convertases requires excision of their prodomain for activity.

Results: SKI-1/S1P matures into a plethora of forms that retain prodomain fragments and are active in different subcellular compartments.

Conclusion: SKI-1/S1P appears as a unique proprotein convertase with an unusual mechanism of zymogen activation.

Significance: Our study provides insights into how SKI-1/S1P matures and exerts its cellular activities, which is crucial for understanding its biological role.

The proprotein convertase subtilisin kexin isozyme 1 (SKI-1)/site 1 protease (S1P) plays crucial roles in cellular homeostatic functions and is hijacked by pathogenic viruses for the processing of their envelope glycoproteins. Zymogen activation of SKI-1/S1P involves sequential autocatalytic processing of its N-terminal prodomain at sites B'/B followed by the herein newly identified C'/C sites. We found that SKI-1/S1P autoprocessing results in intermediates whose catalytic domain remains associated with prodomain fragments of different lengths. In contrast to other zymogen proprotein convertases, all incompletely matured intermediates of SKI-1/S1P showed full catalytic activity toward cellular substrates, whereas optimal cleavage of viral glycoproteins depended on B'/B processing. Incompletely matured forms of SKI-1/S1P further process cellular and viral substrates in distinct subcellular compartments. Using a cell-based sensor for SKI-1/S1P activity, we found that 9 amino acid residues at the cleavage site (P1–P8) and P1' are necessary and sufficient to define the subcellular location of processing and to determine to what extent processing of a substrate depends on SKI-1/S1P maturation. In sum, our study reveals novel and unexpected features of SKI-1/S1P zymogen activation and subcellular specificity of activity toward cellular and pathogen-derived substrates.

The subtilisin/kexin proprotein convertases (PCs)³ are a family of calcium-dependent serine endoproteases with nine

identified members. The basic convertases PC1/3, PC2, furin, PC4, PACE4, PC5/6, and PC7 have similar *consensus* sequences defined by clusters of basic amino acids ((K/R)_n(K/R)↓), where *n* = 0, 2, 4, etc., whereas subtilisin kexin isozyme-1 (SKI-1)/site-1 protease (S1P) cleaves after small/hydrophobic residues in the motif (K/R)X(hydrophobic)X↓, and mature PCSK9 remains associated with its inhibitory prodomain and hence is catalytically inactive (1–3). The mechanism of activation of furin, the prototypic PC, has been described in detail and involves the removal of the N-terminal prodomain from the inactive zymogen. Cleavage occurs in a temporally and spatially orchestrated manner, first at the prodomain C terminus, keeping the enzyme in a latent form. A subsequent second processing event cleaves the prodomain into two parts, allowing substrates to access the active site (4). During the folding process, the prodomain covers the catalytic pocket, providing a chaperone-like function. Removal of the N-terminal domain results in ER retention and lack of enzymatic activity (5). As all secretory PCs, SKI-1/S1P is likewise synthesized as an inactive zymogen (proSKI-1/S1P), whose activation requires autocatalytic removal of the N-terminal prodomain that assists the correct folding of the protease. SKI-1/S1P plays a key role in the regulation of lipid metabolism through the processing of the sterol regulatory element-binding proteins (SREBP-1a, -1c, and -2) (6, 7). Interference with SREBPs activation results in significant serum cholesterol decrease. Other cellular substrates of SKI-1/S1P are members of the CREB family, including the activating transcription factor 6, involved in the unfolded protein response (8), and LUMAN/CREB3, which mediates the Golgi stress response (9). In addition to transcription factors, SKI-1/S1P cleaves *N*-acetylglucosamine-1 phosphotransferase, a Golgi-resident enzyme involved in the sorting of proteins to lysosomes (10), and the pro-brain-derived neurotrophic factor (11). SKI-1/S1P is further hijacked by highly pathogenic viruses of the Arenavi-

precursor; BTMD, before the transmembrane domain; PEI, polyethylenimine; Endo H, endoglycosidase H; PNGase F, peptide:*N*-glycanase F; RLU, relative luminescence unit(s); TCEP, Tris (2-carboxyethyl) phosphine; LASV, Lassa virus; LDLR, LDL receptor; GLuc, *Gaussia* luciferase.

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³ The abbreviations used are: PC, proprotein convertase; SKI-1, subtilisin kexin isozyme 1; S1P, site 1 protease; GP, glycoprotein; ER, endoplasmic reticulum; CREB, cAMP-response element-binding protein; GPC, glycoprotein

Partially Mature SKI-1/S1P Forms Are Enzymatically Active

TABLE 1

Primers used for the generation SKI-1/S1P mutants and sensors

Use	Primer name	Sequence (5' → 3')
Generation of soluble form of SKI-1/S1P (deletion Val ¹¹⁵⁹ –Val ¹²¹³)	BTMD_For	CTGGCCGCTACAACCAGGAGGGTAAGCCTATC
	BTMD_Rev	GAGAGGGTTAGGGATAGGCTTACCTCCTGGTTG
Generation of C site mutant (R183E,R184E)	Cmut_Fwd	GAAGGCATTCGAGCGAGGAGCTGCTGAGAGCCATC
	Cmut_Fwd	GATGGCTCTCAGCAGCTCCTCGCTCGAATGCCTTC
Generation of C' site mutant (R163E,R164E)	C'mut_For	TCATCACGTCCCCTGGAAGAAGCCAGCCTCTCCCT
	C'mut_Rev	AGGGAGAGGCTGGCTTCTTCCAGGGGACGTGATGA
Generation of C'' site mutant (R1160E)	C''mut_For	GGCAATCATCAGAGCCCTGCGAAG
	C''mut_Rev	CTTCGACGGGCTCTGATGATTGCC
SKI-1/S1P sensor (SS) cloning	XhoI_Gluc_For	CGCTCGAGATGGGAGTCAAAGTTCTGTTTG
	SacI_BspEI_Gluc_R	CGGAGCTCTCCGAGTCAACACCGCCCTTGATCT
	SacI_SpeI_Stump_F	GCGAGCTCACTAGTATTGACCTGGACAAGGTGGTGTAC
	BamHI_Stump(V5)_R	GCGGATCCTCAGTGAATCGAGACCGAGGAGAG
SS-LASV	FwD	CCGGAATTATATTAGTAGAAGATTGTTGGGCA
	REV	CTAGTGCCCAACAATCTTCTACTAATAATAAATT
SS-uLASV	FwD	CCGGAATTATATTAGTAGAAGATTGTTGGGCA
	REV	CTAGTGCCCAACAATCTTCTACTAATAATAAATT
SKI-1/S1P cloning into pcDNA3.1 and removal of V5	pIR_Fwd	TGACGCAATGGGCGGTAGG
	A102_1A_Fwd	ATGATGGCTAGGCCACCATGAAGTGTGCAACATCTGGC
	A102_1B_Rev	GAGGGCGGATCCTCACACCGAAGGGTCTTTGGCG
	A102_1B_Fwd	ATGATGGCTAGGCCACCATGGGAGTCAAAGTTCTGTTTG

rus and Bunyavirus family for the proteolytic processing of their envelope glycoprotein precursor (GPC) that is crucial for productive infection. Inhibition of GPC maturation by SKI-1/S1P blocks assembly of infectious particles and results in a potent anti-viral effect (12–16). Its proven role in human diseases and its nature as an enzyme makes SKI-1/S1P a promising target for therapeutic intervention. Here, we investigated SKI-1/S1P zymogen activation and subcellular specificity of activity toward cellular and pathogen-derived substrates revealing novel and unexpected features.

EXPERIMENTAL PROCEDURES

Antibodies—mAb 83.6 mouse anti-LCMV GP (1:1000) was produced and purified as described previously (17). Mouse anti-V5 mAb (1:5000) was purchased from Invitrogen, rabbit anti-*Gaussia* Luciferase (1:6000) was from New England Biolabs, and mouse anti- α -tubulin mAb (1:10000) was obtained from Sigma. Secondary antibodies conjugated to HRP, polyclonal rabbit anti-mouse (1:3000), and polyclonal rat anti-rabbit IgG (1:3000) were purchased from Dako, goat anti-mouse IgG rhodamine RedX (1:1000) was from Jackson ImmunoResearch Laboratories, and goat anti-mouse IgG Alexa 488 (1:1000) was from Molecular Probes.

Plasmids and Constructs—Plasmids coding for WT full-length SKI-1/S1P and a His₆-tagged soluble SKI-1/S1P variant truncated before the transmembrane domain (BTMD), the catalytically inactive mutant H249A, B'/B, B' mutants of SKI-1/S1P were previously described (18). Mutants at sites B, C, C', C'', C'/C, and C''/C were generated by site-directed mutagenesis using SKI-1/S1P WT as a template employing a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol using the specific oligonucleotides listed in Table 1. V5-tagged BTMD-SKI-1/S1P constructs were generated by deletion mutagenesis using PCR amplification with specific primers (Table 1) to delete the Val¹¹⁵⁹–Val¹²¹³ region of the corresponding SKI-1/S1P full-length constructs. The expression plasmid pC-LASV GPC has been previously described (15), as has the plasmid coding for pLDLR-Luc (19). A plasmid expressing *Renilla* luciferase (pGL4.74) was obtained from Promega.

For the generation of the SKI-1/S1P sensors (SS-LASV, SS-uLASV), we used the pCMV-GLuc plasmid coding for *Gaussia* luciferase (New England Biolabs) and the pIRES2-Stump-V5 plasmid coding for the SKI-1/S1P Stump (20). GLuc coding sequence (including the signal peptide but lacking the stop codon) and SKI-1/S1P Stump-V5 (including the stop codon but not the signal peptide) were amplified from their original cDNAs and inserted into the bicistronic pIRES2-EGFP vector, using primers containing unique restriction sites at the 3' and 5' ends (Table 1). The insertion of the cleavable (IYISRRLLG) and uncleavable (IYISELLG) motifs was achieved using specific oligonucleotides coding for the designed sequence flanked by the unique restriction sites at the 3' and 5' ends (Table 1). This cassette insertion method allows for rapid generation of constructs containing different cleavable motifs. For microscopy studies, the SS-LASV and SKI-1/S1P WT constructs were subcloned into a pcDNA3.1 vector that does not contain the EGFP reporter and V5 tag of SS-LASV was removed by PCR cloning (Table 1). Detailed cloning strategies will be provided upon request. Sequences were verified by double-strand DNA sequencing.

Cell Culture and Transfection—HEK293T cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS and 100 units/ml penicillin and 0.1 mg/ml streptomycin. CHO-K1 cells were maintained in DMEM/Ham's F12 1:1 (Biochrom AG) supplemented with 10% FBS and penicillin/streptomycin. CHO-K1-derived SRD12B cells (SKI-1/S1P deficient) (21) were grown in CHO-K1 medium supplemented with 5 μ g/ml cholesterol (Sigma), 20 μ M sodium oleate (Sigma), and 1 mM sodium mevalonate (Sigma). All cell lines were cultured at 37 °C and 5% CO₂. For all experiments, except for the sensors, transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Sensor transfections were achieved using polyethylenimine (PEI). Briefly, a PEI stock was prepared by dissolving PEI (*M_r* 25,000) obtained from Polysciences in water at 1 mg/ml, adjusted to pH 7.0, filtered, and stored at –80 °C. Each transfection needed 2 μ l of PEI stock for 1 μ g of total DNA in nonsupplemented DMEM, followed by 10 min of incubation prior to addition to cells. At 4 h

TABLE 2

Inhibition of SKI-1/S1P C' site processing by protease inhibitors

To test whether C' site processing is the result of an autoprocessing event or whether other proteases are involved, a panel of broadly active protease inhibitors was tested. The WT or C site mutant versions of SKI-1/S1P were transfected in SRD12B cells. At 4 h post-transfection, fresh medium was added containing the indicated drugs or the corresponding control vehicles. At 20 h post-treatment, cell lysates were analyzed by Western blotting for enzyme expression and processing. None of the drugs impaired C' site maturation.

Compound tested	Concentration	Source
	μM	
Phosphoramidon	10	Sigma
4-(2-Aminoethyl) benzenesulfonyl fluoride	300	
Soybean trypsin inhibitor	15	Merk
Aprotinin	10	
ZnSO ₄	50	
Dec-RVLR-CMK	10	
Chymostatin	100	
Leupeptin	100	
Pepstatin A	1	Custom synthesis
Brefeldin A	50	
PMSF	50	
EDTA	200	
PF-429242	20	
E-64	100	
NH ₄ Cl	200	

post-transfection solutions were replaced by fresh medium. Transfection efficiencies were evaluated after the indicated time points by detection of the EGFP reporter.

Drug Treatments—Induction of genes regulated by SREBP2 was triggered by treatment with Ham's F-12 DMEM 1:1, 5% delipidated FBS, 50 μM sodium mevalonate, 50 μM mevastatin (Enzo Lifescience) for 18 h, as reported previously (22). DMSO (Sigma-Aldrich) was used as a negative control. To assess SKI-1/S1P C' cleavage dependence on proteases, SRD12B cells expressing SKI-1/S1P Cmut or WT underwent drug treatment at the concentrations indicated in Table 2. Drugs were added 4 h post-transfection. The cells were lysed at 44 h post-treatment and analyzed by Western blotting. SKI-1/S1P sensor cleavage dependence on SKI-1/S1P was verified using 20 μM of PF-429242 (23) (custom synthesis; Shanghai Boyle Chemical Co.). Drug was added 4 h post-transfection. At the indicated time points, conditioned medium was collected for luciferase assays and cell lysates analyzed by Western blotting.

Confocal Microscopy—SRD12B cells were seeded on glass slides in 24-well plates and co-transfected with the indicated plasmids. At 48 h post-transfection, cells were fixed with 2% (w/v) formaldehyde/PBS for 15 min at room temperature and washed with PBS. The cells were permeabilized for 30 min at room temperature with 0.1% (w/v) saponin, 10% (v/v) FBS in PBS. Primary and secondary antibodies were diluted in 0.1% (w/v) saponin and 1% (v/v) FBS/PBS and incubated for 1 h at room temperature. Slides were mounted using VECTASHIELD® mounting medium (Vector Labs). Image acquisition was performed with a Zeiss LSM710 Quasar confocal microscope equipped with a Plan Apochromat lens (63 $\times$, 1.2 numerical aperture [NA] objective), 405-nm diode laser, argon lasers (458, 476, 488, and 514 nm), and 561-nm diode-pumped solid state laser. All images for each data set were acquired the same day with the same microscope settings. Images were analyzed using Fiji software (24), and co-localization analysis was performed with a Coloc2 plugin (Fiji).

Western Blot Analysis—At 24–48 h post-transfection, media were collected, and cells were washed twice with cold PBS and lysed with cell Lytic buffer (Sigma) supplemented with Mini Complete® protease inhibitor mixture (Roche) following the manufacturer's instructions. Cell lysates were cleared by centrifugation (15,000 rpm, 10 min), and supernatants were transferred to new tubes. Conditioned media were centrifuged (12,000 rpm, 10 min) to remove cellular debris, and supernatants were transferred to new tubes. Samples were mixed 1:1 with 2 $\times$ SDS-PAGE sample buffer containing 100 mM DTT and boiled (5 min at 95 °C). The samples were separated by SDS-PAGE and blotted on nitrocellulose membranes. Membranes were blocked in 1% (w/v) skim milk in TBS, and proteins were detected with primary antibody after overnight incubation at 4 °C using HRP-conjugated secondary antibodies for 1 h at room temperature as described (15). Membranes were developed by chemiluminescence using LiteABlot kit (EuroClone) or Amersham Biosciences ECL Prime Western blotting detection reagent (GE Healthcare). Signal acquisition was performed with ImageQuant LAS 4000Mini (GE Healthcare) or by exposure to x-ray films. Quantification of Western blot results was performed with ImageQuant TL (GE Healthcare) (15).

Endoglycosidase Assay—To examine glycosylation patterns of SKI-1/S1P variants, HEK293T cells were transfected as previously described. At 48 h post-transfection, cell lysates were split in three parts and treated with either Endo H (New England Biolabs) or PNGase F (New England Biolabs) or left untreated for 2 h according to the manufacturer's instructions. Digested and control samples were separated by SDS-PAGE followed by Western blot analysis.

Reporter Gene Assays—SRD12B cells were co-transfected with a ratio of 50:50:1 of plasmids encoding for SKI-1/S1P variants, the gene reporter plasmid (LDLR-Luc) and the normalizer reporter containing *Renilla* (PGL4.74), respectively, using Lipofectamine luciferase 2000. At 4 h post-transfection, SREBP2 activation was induced following the protocol described above. After 18 h, cells were lysed with Passive lysis buffer (Promega) and analyzed for firefly and *Renilla* luciferase activity using the Dual-Glo luciferase assay system (Promega). Luminescence was detected in a TriStar LB 941 multimode microplate spectrofluorometer (Berthold Technologies).

SKI-1/S1P Sensors Assays—To quantify secreted *Gaussia* luciferase activity in conditioned media, cells were seeded in 24-well plates and transfected with SKI-1/S1P sensors the following day. The media were collected at the indicated time points and centrifuged (12,000 rpm for 10 min) to remove cellular debris. Luciferase assays were carried out in a TriStar LB 941 Berthold microplate reader using 2.5 μl of medium and 60 μl of freshly prepared substrate (1:1000 coelenterazine stock (Molecular Probes), 160 $\mu\text{g}/\text{ml}$ in acidified methanol) diluted in PBS using white half-volume 96-well plates (Costar). Relative luminescence units (RLU) were detected after 0.5 s of shaking for 2 s. All assays were performed in biological triplicate.

Protein Purification and MS Analysis—Secreted SKI-1/S1P WT and C mutant bearing a C-terminal His₆ tag were purified from culture medium by immobilized metal affinity chromatography using Co-TALON resin (GE Healthcare) and eluted with 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 300

mM imidazole. Reduction of disulfide bridges was achieved with Tris (2-carboxyethyl) phosphine (TCEP), at a molar ratio Cys:TCEP of 1:10. *S*-carboxymethylation was performed with iodoacetamide at a molar ratio Cys:iodoacetamide of 1:10. The sample was dissolved in 20 mM Tris-HCl, pH 8.5, containing 6 M guanidinium HCl and treated with TCEP for 30 min at 37 °C, followed by treatment with iodoacetamide, and the sample was kept in dark for 30 min at room temperature. The sample was then subjected to reverse phase HPLC to remove the excess of reagents. Reverse phase HPLC analysis was conducted using a C4 column (150 × 4.6 mm) with elution by a linear gradient (10–70%) of aqueous trifluoroacetic acid/acetonitrile solvent. The chromatographic fractions corresponding to the major peaks were desiccated in a Thermo Scientific SpeedVac concentrator, dissolved in 50% acetonitrile, 0.1% formic acid, and directly injected in the electrospray ionization source. Mass measurements were performed with a quadrupole TOF spectrometer (Waters, Manchester, UK) (capillary voltage, 2800–3000 V; cone voltage, 45 V; scan time, 1 s; interscan, 0.1 s). The spectra were analyzed using MASSLYNX software (Micromass, Wythenshew, UK).

Statistical Analysis—Analysis was performed using the GraphPad Prism software package. One-way analysis of variance was used for multiple comparisons, and a *p* value of 0.05 was set as threshold for significance.

RESULTS

The Prodomain Is Crucial for Proper Folding and Trafficking of SKI-1/S1P—SKI-1/S1P undergoes autocatalytic maturation by sequential cleavages of the N-terminal prodomain first at sites B'/B (RKVF ↓ RSLK¹³⁷ ↓), followed by site C (RRLL¹⁸⁶ ↓), with crucial roles for R and V/L residues at P4 and P2 (fourth and second residue upstream the scissile bond, respectively) (18). The end product form C of SKI-1/S1P represents the fully mature enzyme (Fig. 1A). Earlier studies suggested that the prodomain is essential for the exit of SKI-1/S1P from the ER (18). However, its role as chaperone to assist SKI-1/S1P folding and activity remained unclear. To address these questions, we used recombinant full-length SKI-1/S1P lacking the entire prodomain (ΔAC SKI-1/S1P FL) and a corresponding soluble version. The soluble form of SKI-1/S1P was truncated BTMD and comprised the ectodomain, followed by a C-terminal V5 tag (ΔAC SKI-1/S1P BTMD) (Fig. 1A). WT and ΔAC SKI-1/S1P FL were expressed in the SKI-1/S1P-deficient CHO cell line SRD12B, which lacks active endogenous enzyme (21), and recombinant SKI-1/S1P autoprocessing was analyzed by Western blotting. To monitor protein transport and maturation, we assessed resistance of the SKI-1/S1P variants to the *N*-glycosidase Endo H, which is acquired by most glycoproteins upon exit from the ER (25). Endo H was able to digest only the A and B forms of SKI-1/S1P, whereas the majority of mature C form co-migrated with the B form upon Endo H treatment, indicating its localization in the Golgi. In contrast, ΔAC SKI-1/S1P, which was present as a single form, was sensitive to both Endo H and PNGase F, suggesting that the protein does not traffic beyond the ER (Fig. 1B). As expected, WT Pro-SKI-1/S1P (A) and its processed forms (B and C) were sensitive to PNGase F, resulting in a shift of the apparent molecular mass upon treat-

ment, in agreement with previous reports (18, 26). We next analyzed the ability of the truncated soluble form (ΔAC SKI-1/S1P-BTMD) to be secreted. When compared with WT SKI-1/S1P-BTMD, secretion of the ΔAC mutant was not detected in the culture media (Fig. 1C), confirming that the prodomain is indeed required for trafficking, in line with an earlier report (18).

Next, we investigated whether the expression of the SKI-1/S1P pro-domain in *trans* was able to interfere with SKI-1/S1P maturation. Previous studies had demonstrated that expression of WT SKI-1/S1P prodomain in *trans* perturbed the maturation of a SKI-1/S1P cleavable variant of pro-PDGF, pro-PDGF-A*, but not SREBP-2. The prodomain mutant B (R134E) but not B' (R130E) exerted inhibitory activity against SKI-1/S1P autocleavage (22). Here, WT SKI-1/S1P prodomain, the mutant R134E, and the AB fragment of the prodomain were co-expressed with WT SKI-1/S1P or the catalytically inactive SKI-1/S1P mutant H249A. Western blot analysis of cell lysates revealed a marked overall reduction of SKI-1/S1P protein expression caused by co-expression of WT and mutant prodomain in *trans*, independent of the catalytic activity of the enzyme. Despite the overall reduction in SKI-1/S1P expression, the presence of the WT prodomain in *trans* did not affect the ratio of the incompletely matured A and B *versus* mature C forms of the enzyme, suggesting that normal maturation occurs. In contrast, the prodomain mutants R134E and AB seemed to partially interfere with SKI-1/S1P autoprocessing, specifically the generation of the B'/B form, whose band appears weaker when compared with the A form (Fig. 1D).

Previous studies revealed that the envelope GPC of the Arenavirus Lassa virus (LASV) is processed by SKI-1/S1P in early compartments (ER/*cis*-Golgi) of the secretory pathway (12). We therefore used LASV GPC as a substrate to assess a possible catalytic activity of the ΔAC SKI-1/S1P variant in the ER. Briefly, LASV GPC was co-expressed with ΔAC SKI-1/S1P in SRD12B cells using the WT enzyme and the catalytically inactive SKI-1/S1P H249A mutant as positive and negative controls, respectively. Western blot analysis of cell lysates showed no detectable processing of LASV GPC by ΔAC SKI-1/S1P (Fig. 1E), indicating a lack of catalytic activity. The data confirm that the prodomain is required for proper SKI-1/S1P folding and activity, similar to other members of the PC family (3).

SKI-1/S1P Autoprocessing Involves a Novel Site C'—Earlier work revealed that a single mutation (R134E) at the B autoprocessing site allowed maturation comparable with the WT, whereas double mutations at the B'/B site (R130E/R134E) prevented autoprocessing (18). Extended mutagenesis performed on a region proximal to the C site resulted in normal SKI-1/S1P maturation at the B'/B intermediate state (26), suggesting that processing at B'/B either precedes or occurs independently of C site cleavage. To gain further information about the specific roles of the individual SKI-1/S1P autoprocessing events and their role in maturation and activation of the enzyme, we generated a complete set of mutants, including the B' (R130E) and C (R183E/R184E) mutants (Fig. 2A). Mutant and WT SKI-1/S1P were expressed in SRD12B cells, and their autoprocessing profile was assessed. As a negative control, the catalytically inactive mutant SKI-1/S1P H249A was included. In Western

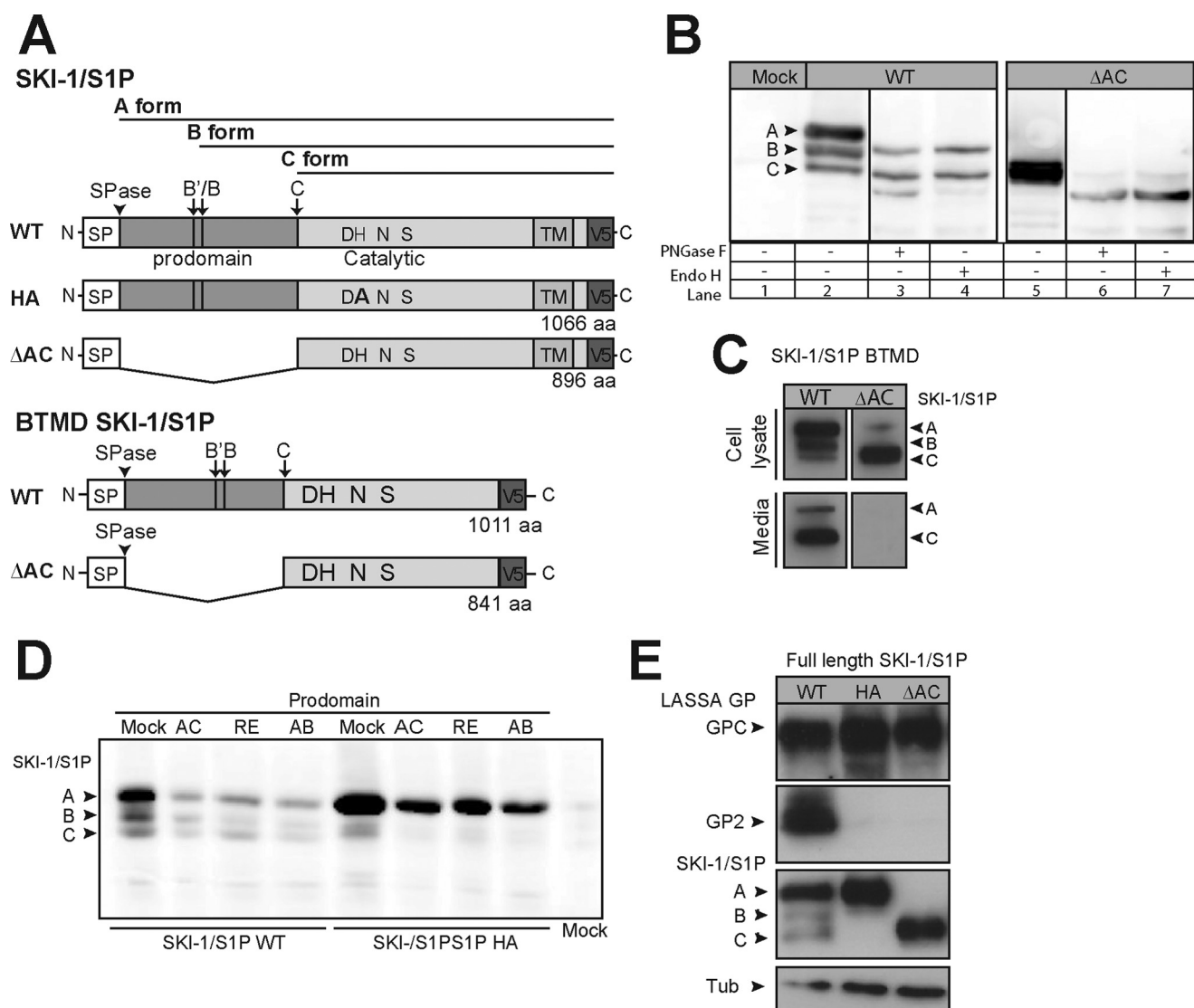


FIGURE 1. Role of SKI-1/S1P prodomain in maturation and folding of the enzyme. *A*, schematic representation of the full-length and soluble (BTMD) SKI-1/S1P WT and Δ AC constructs lacking the complete prodomain showing the signal peptide cleavage site (*SPase*) and the autocatalytic processing B'/B and C sites. The catalytic triad and oxyanion hole residues of the catalytic domain, the transmembrane domain (TM), and V5 tag are also indicated. The SKI-1/S1P HA mutant corresponds to a catalytically inactive enzyme where the residue His²⁴⁹ has been replaced by Ala. *B*, analysis of N-linked carbohydrates of SKI-1/S1P WT and Δ AC in SRD12B cells. Cells expressing either SKI-1/S1P WT or Δ AC were lysed 48 h post-transfection, and lysates were subjected to digestion with PNGase F and Endo H for 2 h prior to Western blot analysis using an anti-V5 antibody. The relative positions of the SKI-1/S1P precursor (A) and the processed forms B and C are indicated by arrows. *C*, soluble SKI-1/S1P lacking the prodomain is not secreted. SRD12B cells expressing soluble WT or Δ AC SKI-1/S1P were harvested and lysed 48 h post-transfection. Cell lysates (upper panels) and conditioned supernatants (lower panels) were analyzed by Western blot as in *B*. *D*, inhibition of SKI-1/S1P maturation by SKI-1/S1P WT and R134A prodomain. *E*, LASV GPC is not cleaved by rAC SKI-1/S1P. SRD12B cells were co-transfected with WT or Δ AC SKI-1/S1P combined with LASV GPC. Cell lysates at 48 h post-transfection were analyzed by Western blot using mAb 83.6 to LASV GP2 and anti-V5 as in *B*. The A, B, and C forms of SKI-1/S1P, as well as the LASV GPC and GP2 are indicated. Tub, tubulin.

blot analysis, the B' and B mutants showed maturation similar to the WT, whereas the B'/B double mutant remained largely unprocessed (Fig. 2B), in line with previous reports (18). Rather unexpectedly, the R183E/R184E mutations at the C site in SKI-1/S1P did not affect maturation but resulted in the appearance of a "C-like" form with an apparent molecular mass slightly higher than the mature WT C form (Fig. 2B). This suggested the presence of an additional cleavage event occurring upstream of C and that the mature SKI-1/S1P C form may have resulted from a double cleavage event (C'/C). Processing of the C site mutant was not inhibited by a panel of broadly active protease inhibitors (Table 2), suggesting autocatalytic cleavage.

Analysis of the primary structure of the prodomain upstream to the known C site RRLL₁₈₆ ↓ pinpointed two possible cleavage sites, matching the consensus motif RX(hydrophobic)X ↓ of SKI-1/S1P (27): R¹⁶⁰PLR¹⁶³ ↓ (C')R¹⁶⁴AS ↓ (C') LSLGSGF-WHATGRHSSRRLL¹⁸⁶ ↓ (C), with the latter previously suggested (26). The putative C' and C" sites were subjected to mutagenesis, resulting in the SKI-1/S1P R163E/R164E C' mutant and the R160E C" mutant. We further generated C'/C and C''/C double mutants (Fig. 2A). When expressed in SRD12B cells, the C' mutant was partially impaired in maturation, indicated by the accumulation of the incompletely matured B'/B form (Fig. 2B). The combined mutation at the C and C' sites

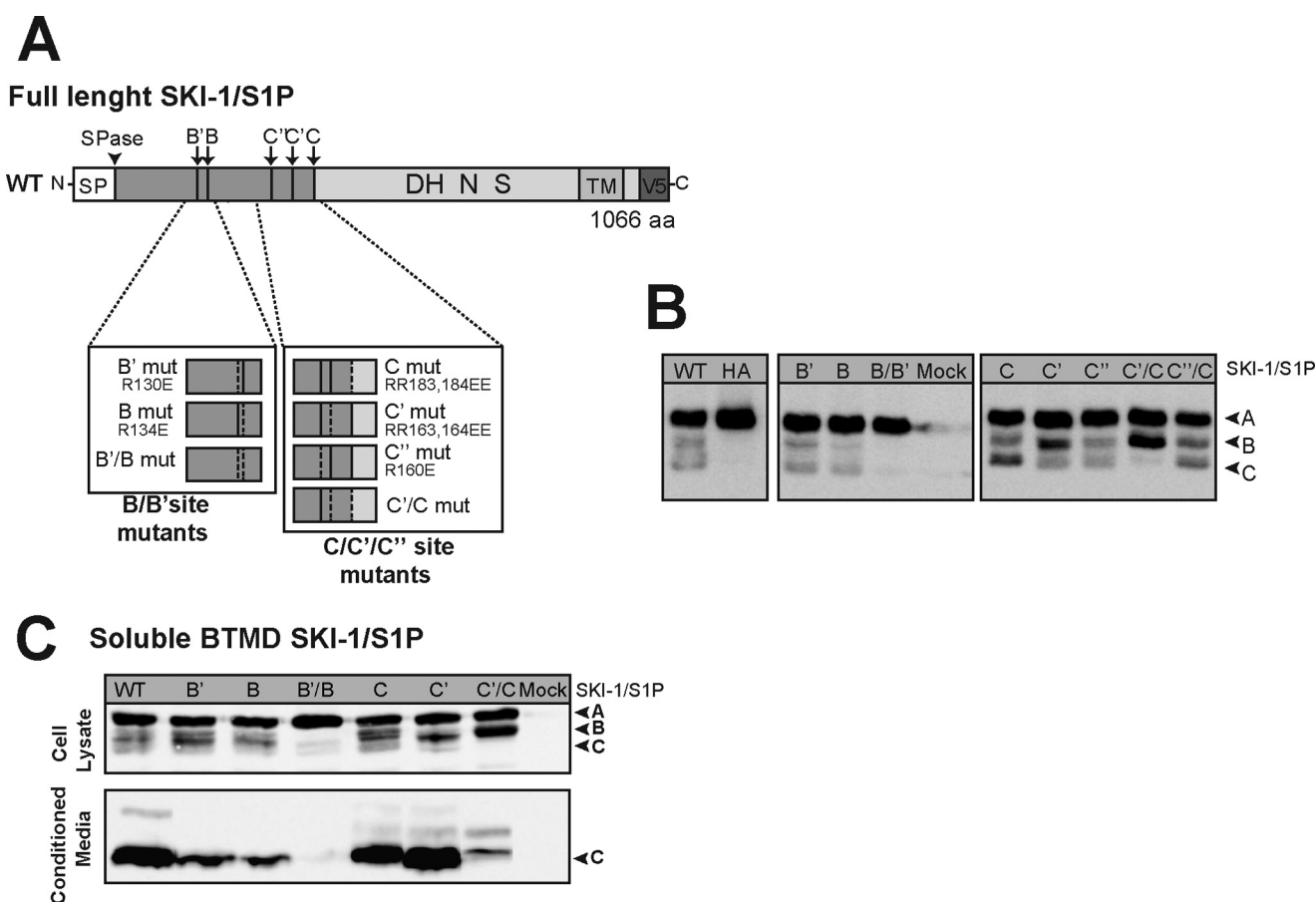


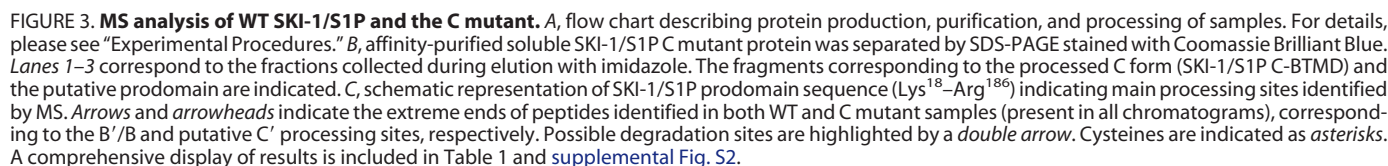
FIGURE 2. Analysis of SKI-1/S1P prodomain autoprocessing sites. *A*, schematic representation of SKI-1/S1P prodomain processing sites and corresponding mutants as in Fig. 1*A*. The cleavage sites for signal peptidase (*SPase*) as well as a cleavage site, B'/B, C, C', and C'' are indicated. *Insets* indicate the specific mutations. *B*, full-length SKI-1/S1P prodomain mutants expression and autoprocessing. SRD12B cells expressing SKI-1/S1P mutants were lysed and analyzed at 48 h post-transfection by Western blot as in Fig. 1*B*. The different maturation forms of SKI-1/S1P (A, B, and C) are indicated. *C*, expression, autoprocessing, and secretion of soluble SKI-1/S1P prodomain mutants. Mutant and WT SKI-1/S1P were transiently expressed in HEK293 cells. After 48 h, cell lysates and supernatants were analyzed by Western blot as in Fig. 1*C*.

resulted in marked reduction of the mature C form indicating that, similar to B'/B, mutation of both C and C' processing sites was required to prevent maturation (Fig. 2*B*). Mutations at site C'', either alone or in combination with the C site, did not interfere with SKI-1/S1P maturation (Fig. 2*B*). The present data revealed the presence of a yet unknown C' cleavage site that is essential for the proper maturation of the enzyme in combination with the previously identified C site.

To further characterize the SKI-1/S1P mutants, we generated the corresponding panel of soluble variants of the enzyme (Fig. 2*C*) and expressed them in HEK293T cells. Soluble WT SKI-1/S1P, as well as the C and C' mutants, was detected in similar amounts as a single band in the supernatant (Fig. 2*C*), whereas the B'/B mutant was undetectable (Fig. 2*C*, *fourth lane*) in line with previous studies (18). Notably, protein secretion was markedly impaired for the B and B' single mutants, and the double C'/C mutant was barely detectable in conditioned media (Fig. 2*C*, *second, third, and seventh lanes*), suggesting that these specific point mutations likewise affect normal trafficking.

Mature SKI-1/S1P Retains Prodomain Fragments of Different Lengths—We next sought to investigate SKI-1/S1P processing by MS analysis. For this purpose, we used a WT and C mutant

form of the soluble variant SKI-1/S1P-BTMD described above. Proteins were expressed in HEK293 cells and purified from conditioned cell supernatant by immobilized metal affinity chromatography using imidazole for elution (Fig. 3*A*). Purified protein was analyzed by SDS-PAGE, revealing a major band with an apparent molecular mass corresponding to the processed C form (Fig. 3*B*). In addition, a currently undefined low molecular mass band of ~11 kDa was detected (Fig. 3*B*). Based on its apparent molecular mass, we speculated that this band co-purifying with the mature C form of the enzyme might correspond to prodomain fragments. Purified protein was fractionated by reverse phase HPLC and analyzed by electrospray quadrupole TOF MS (Fig. 3*A*). The wild-type and C mutant forms of SKI-1/S1P-BTMD were found associated with prodomain fragments cleaved at site B' and B, as well as smaller fragments resulting from processing at downstream sequences (Fig. 3*C* and *supplemental Table S1*). In both WT and C mutant, these fragments ended within the BC region around the LSLGSG¹⁷² and GRHSSRRL¹⁸⁶ motifs. However, neither the WT nor the C mutant showed an *m/z* value compatible with fragments ending at 133 (B') or 186 (C site), suggesting nonspecific removal of the C-terminal residues. Although cleavage events were visible in at least two distinct sites of the BC region,



Incompletely Matured Forms of SKI-1/S1Ps Are Active and Differentially Process Cellular and Viral Substrates—Our studies of SKI-1/S1P maturation revealed a more complex pattern of autocatalytic activation than anticipated. Because alteration in the maturation of SKI-1/S1P affected its trafficking (Fig. 1), we next sought to investigate the activity of SKI-1/S1P prodomain mutants toward either cellular or viral substrates. The classical SKI-1/S1P cellular substrates, SREBP1 and SREBP2 are transcription factors activated by tightly regulated intramembrane proteolysis first by SKI-1/S1P, followed by site 2 protease (29). To assess the activity of SKI-1/S1P variants

A Cell-based Sensor to Monitor Endogenous SKI-1/S1P Activity—Quantitative studies of endogenous SKI-1/S1P activity require a robust and reliable cell-based assay, allowing the

Partially Mature SKI-1/S1P Forms Are Enzymatically Active

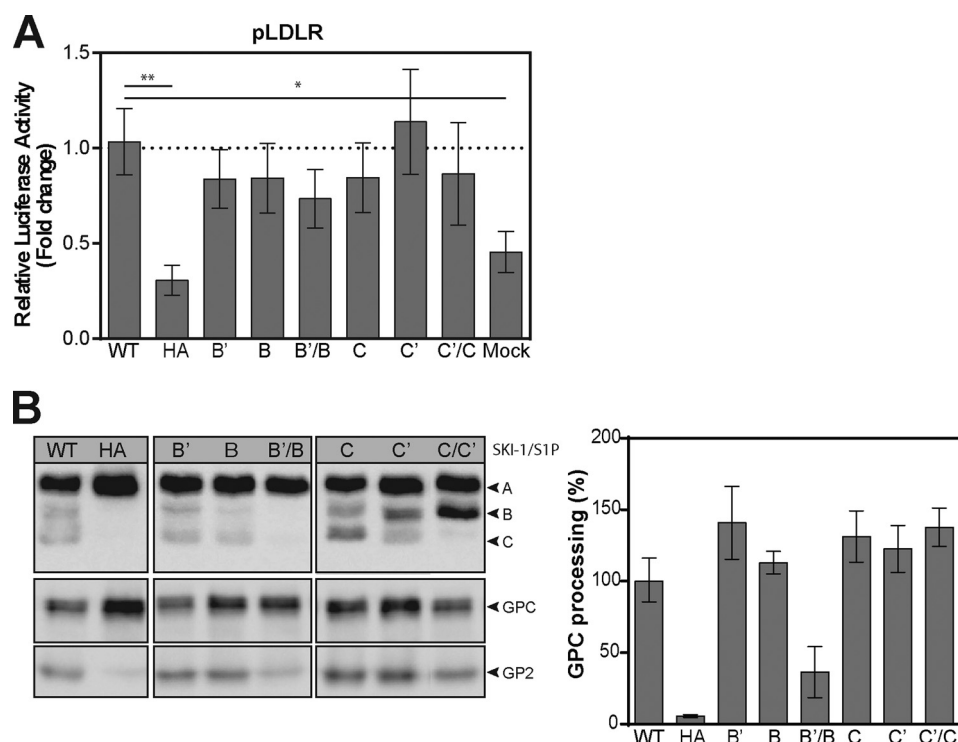


FIGURE 4. Characterization of WT SKI-1/S1P and prodomain mutants activity toward cellular and viral substrates. *A*, activity of SKI-1/S1P WT and mutants toward SREBP2. SRD12B cells were co-transfected with the indicated SKI-1/S1P variants and the pLDLR reporter construct containing a firefly luciferase reporter gene, using an expression plasmid for *Renilla* luciferase for normalization of transfection. At 48 h post-transfection, cells were lysed, and luciferase activities were analyzed. The data indicate firefly luciferase activity corrected for *Renilla* luciferase activity and are expressed as fold change, setting the SKI-1/S1P WT as 1.0. The data are means $\pm$ S.D., $n = 3$. Statistical significance was assessed by one-way analysis of variance with $p < 0.01$ (**) and $p < 0.05$ (*) indicated. *B*, activity of SKI-1/S1P WT and mutants toward LASV GPC. SRD12B cells were co-transfected with the indicated SKI-1/S1P variants and LASV GPC as in Fig. 1*D*. GPC processing and SKI-1/S1P expression were analyzed at 48 h post-transfection by Western blot as in Fig. 1*D*. SKI-1/S1P forms A, B, and C, as well as LASV GPC and GP2 are indicated. Western blot quantification of GPC processing into GP2 was assessed in three independent experiments, and the means are presented in the bar diagram in the right panel, setting GPC cleavage by SKI-1/S1P WT at 100%.

detection of SKI-1/S1P-mediated processing of cleavable peptides derived from cellular and viral substrates in the authentic subcellular context. The first cell-based molecular sensor to monitor endogenous SKI-1/S1P activity had been reported by Sakai *et al.* (31). The sensor construct was comprised of human placental alkaline phosphatase, fused to a C-terminal fragment of SREBP-2 starting at six residues upstream of the SKI-1/S1P recognition site (RSVL). Efficient and specific processing by endogenous SKI-1/S1P was observed after co-expression with SREBP cleavage-activating protein (31). We extended these previous studies aiming at the development of a sensor for endogenous SKI-1/S1P activity that allows 1) monitoring of processing of a broad range of known and putative SKI-1/S1P substrate sequences and 2) optimal interaction with the enzyme without requiring co-factors. As shown in Fig. 5*A*, our sensor construct is based on a chimeric protein composed of the reporter *Gaussia* luciferase (GLuc) anchored to the membrane-associated SKI-1/S1P stump separated by a cleavable peptide derived from known and putative SKI-1/S1P recognition sites of various substrates. SKI-1/S1P stump is the protein fragment of the enzyme remaining attached to the membrane following shedding (Fig. 5*A*).

First, we generated a SKI-1/S1P sensor containing the LASV GPC cleavage site IYISRRLL $\downarrow$ G (SS-LASV). To test the specificity of the sensor, it was co-expressed in SRD12B cells with WT SKI-1/S1P or the catalytically dead SKI-1/S1P H249A, as

well as empty vector as negative controls. Efficiency of processing by the endogenous enzyme was evaluated in CHO-K1 cells. At 48 h post-transfection, media were collected, and cells were lysed to verify expression of the sensor and the extent of cleavage. Western blot analysis of SS-LASV showed robust expression and the cleavage product (cGLuc) appearing only in the presence of either heterologous (SRD12B) or endogenous (CHO-K1) SKI-1/S1P, but not the H249A mutant. Luminescence measurements of GLuc secreted in the supernatants matched the Western blot analysis (Fig. 5*B*). In addition, an uncleavable variant of the LASV sensor (SS-uLASV) was generated by insertion of the mutant IYISEELL $\downarrow$ G sequence. Its cleavability was assessed in SRD12B, CHO-K1, and HEK293 cells, and compared with that of SS-LASV. Upon expression in CHO-K1 and HEK293 cells, the uncleavable sensor SS-uLASV showed markedly reduced levels of GLuc protein and luminescence activity in the supernatant, similar to the background levels observed in SKI-1/S1P-deficient SRD12B cells, suggesting negligible specific processing of SS-uLASV by SKI-1/S1P (Fig. 5*C*). Accordingly, in supernatants of CHO cells transfected with SS-uLASV and SRD12B cells expressing SS-LASV, the expected GLuc fragment resulting from specific processing by SKI-1/S1P was undetectable. However, a smaller fainter band was detected whose appearance correlated with significant background luminescence (Fig. 5*C*). The significant shift in apparent molecular mass compared with the specific GLuc

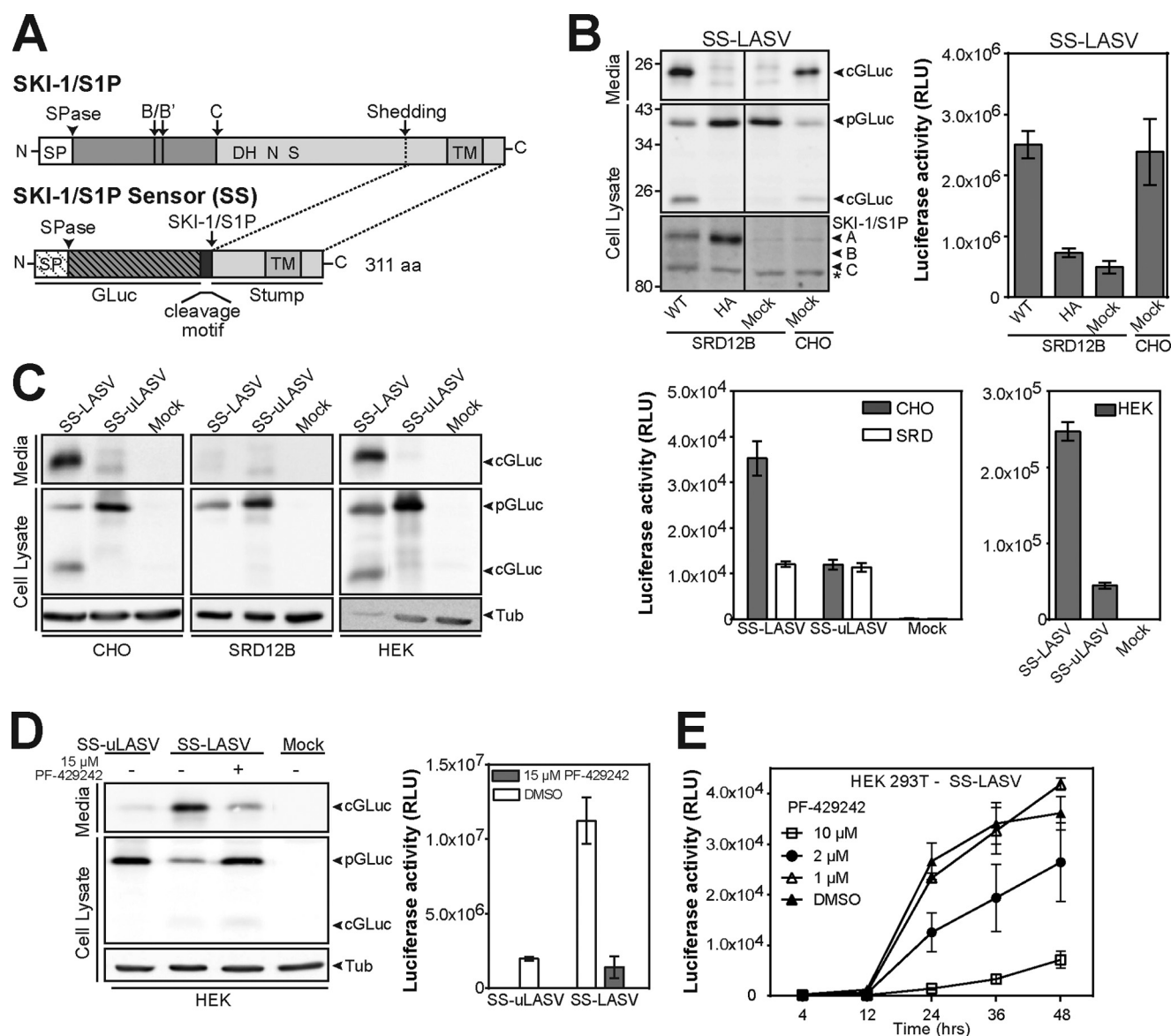


FIGURE 5. Characterization of the cell-based SKI-1/S1P sensor. A, schematic representation of the SKI-1/S1P sensor composed of signal peptide (SP), the GLuc reporter, a variable SKI-1/S1P cleavable motif and the ectodomain of SKI-1/S1P (stump) that arises from processing of full-length SKI-1/S1P at a shedding site. B, characterization of SKI-1/S1P sensor containing the LASV GPC-derived cleavage motif IYISRRLL ↓ (SS-LASV) in SRD12B and CHO-K1 cells. Cells were co-transfected with SS-LASV and either SKI-1/S1P WT, the inactive HA mutant, or empty vector (Mock). At 48 h post-transfection, cell lysates and supernatants were collected and analyzed by Western blot using an anti-GLuc antibody (left). The uncleaved sensor precursor (pGLuc) and cleaved sensor (cGLuc) and SKI-1/S1P are indicated. Expression of SKI-1/S1P variants was confirmed by Western blot using an antibody to V5 (bottom panel). The asterisk indicates a nonspecific band. Conditioned media were analyzed for *Gaussia* luciferase activity by addition coelenterazine substrate (right). The data are shown as RLU (means + S.D.; n = 3). C, characterization of SS-LASV and the uncleavable form of SS-LASV (SS-uLASV) containing the mutated cleavage site IYISEELL ↓. Sensors and empty vector (Mock) were transfected into SRD12B, CHO-K1, and HEK293T cells. At 24 h post-transfection, media were collected and analyzed by Western blot or luciferase assay as in B. D, inhibition of processing of SS-LASV and SS-uLASV with the SKI-1/S1P inhibitor PF-429242. HEK293T cells were transfected with the indicated SKI-1/S1P sensors. After 4 h, PF-429242 or vehicle (DMSO) was added. At 44 h post-treatment, media and cell lysates were collected and analyzed as in B. E, characterization of SS-LASV sensitivity to PF-429242 inhibitor. HEK293T cells were transfected with SS-LASV and drug or vehicle (DMSO) added 4 h post-transfection. At the indicated time points, media samples were collected and analyzed as in Fig. 5B. The data shown are RLU (means + S.D., n = 3).

product suggests ectopic cleavage of the sensor by as yet unknown other protease(s) present in the secretory pathway, different from SKI-1/S1P. This nonspecific background varied among different cell lines; for example, please compare CHO cells with HEK293 cells (Fig. 5C). Treatment with the SKI-1/S1P inhibitor PF-429242 reduced processing of SS-LASV to background levels observed with SS-uLASV (Fig. 5D), and inhibition was dose-dependent (Fig. 5E), confirming SKI-1/S1P-dependent processing of the sensor.

In our design, the SKI-1/S1P sensors contain the transmembrane domain and cytosolic tail of SKI-1/S1P to allow targeting of the chimera to the same subcellular compartment as the WT enzyme. We next sought to verify the subcellular distribution of our sensors by confocal microscopy. To this end, SS-LASV was transiently co-expressed with V5-tagged SKI-1/S1P in CHO-K1 cells. At 48 h post-transfection, the cells were fixed and stained with anti-GLuc antibody (sensor) and anti-V5 (SKI-1/S1P). In permeabilized cells, we observed a

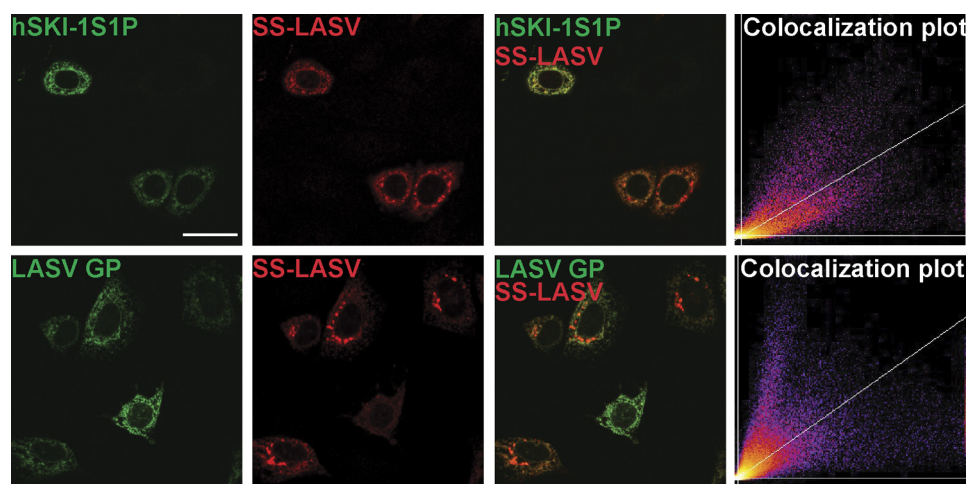


FIGURE 6. Co-localization of SKI-1/S1P sensors with SKI-1/S1P WT and LASV GPC. CHO-K1 cells were co-transfected with SS-LASV and with either the V5-tagged full-length form of SKI-1/S1P WT or with LASV GPC. At 48 h post-transfection, cells were fixed, permeabilized, and stained for *Gaussia* luciferase (red), V5 tag (green, top panels), or LASV GP2 (green, bottom panels). Specimens were examined by confocal laser scanning microscopy. Images on the left show individual stainings for SKI-1/S1P (green), LASVGP (green), and SS-LASV (red). The right image represents a merge of the signals for SKI-1/S1P (top panels) or LASV GP (bottom panels) with SS-LASV. In the scatter plots, co-localization appears in the diagonal of the plot. Bar, 20 μ m.

strong co-localization of SKI-1/S1P with SS-LASV in organelles within the perinuclear region, compatible with the presence of sensor/enzyme in compartments of the early secretory pathway (Fig. 6).

The SS-LASV sensor is a chimeric protein designed to mimic the natural SKI-1/S1P LASV GPC substrate. To investigate the cellular distribution of the sensor in comparison to LASV GP, the two proteins were co-expressed in CHO-K1 cells. At 48 h post-transfection, the cells were fixed and stained with anti-GLuc antibody (sensor) and anti-GP2 (GPC + GP2). Significant co-localization of LASV GP and SS-LASV was observed, suggesting that the sensor traffics through the same organelles as the viral GP (Fig. 6). In conclusion, our sensor appears as a novel and potent tool to quantitatively study SKI-1/S1P-mediated processing of known and suspected substrate sequences of cellular and viral origin in the authentic cellular context.

The Substrate Recognition Sequence Defines the Subcellular Location and Maturation Dependence of SKI-1/S1P Processing— Perturbation of SKI-1/S1P autoprocessing at the B'/B sites differentially affects cleavage of viral GPCs, but not cellular substrates (30). Using our novel sensor platform, we investigated the specific role of the SKI-1/S1P recognition sequences derived from LASV GPC in this specificity. Briefly, SRD12B cells were co-transfected with the SS-LASV and the different SKI-1/S1P mutants. At 48 h post-transfection, media were collected, and the cells were lysed to verify comparable expression levels of sensor and proteases. The relative activity of the mutants was compared with that of SKI-1/S1P WT and the inactive SKI-1/S1P H249A served as negative control. Across the board, processing of the LASV sensor by the SKI-1/S1P variants resembled processing of the authentic GPC with the specific impairment of the B'/B mutant of SKI-1/S1P (compare Fig. 4B, right panel, with Fig. 7A). Because of the nature of our sensors, the data imply that the eight P residues and one P1' Gly IYISRRLL ↓ G of LASV GPC are necessary and sufficient for the differential recognition by the B'/B mutant and WT SKI-1/S1P.

Unlike the known cellular substrates, LASV GPC undergoes SKI-1/S1P processing already in the ER/*cis*-Golgi (12), and there is evidence that the RRL recognition motif can influence the subcellular location of viral GPC processing (30). To test this hypothesis we co-expressed SS-LASV in SRD12B cells with either WT SKI-1/S1P or a soluble SKI-1/S1P form that is retained in the ER by a KDEL motif at its C terminus (SKI-1/S1P-KDEL). As a negative control, we included the noncleavable SS-uLASV sensor. As shown in Fig. 7B, the SS-LASV sensor was processed more efficiently by soluble SKI-1/S1P-KDEL when compared with the WT. These data suggest that the nine residues are also necessary and sufficient to define the location of LASV GPC processing in the early secretory pathway such as the ER/*cis*-Golgi.

DISCUSSION

Within the PC family, the role of prodomains as chaperones is well established (3, 5). Specifically, prodomains are thought to support enzyme protein folding by docking into the catalytic pocket (5, 32, 33). In the context of SKI-1/S1P, deletion of the entire prodomain confirmed an important function in proper folding and transport of this enzyme, similar to other members of the PC family. However, the present study revealed novel and rather unique features of the mechanisms underlying zymogen activation of SKI-1/S1P. We report for the first time the existence of a C' site of autoprocessing, indicating that both steps in prodomain processing, B'/B and C'/C, involve double cleavage events. These post-translational modifications are crucial for the subcellular localization of autoprocessing intermediates of the enzyme. The biological relevance of such double cleavages around the same region is still a matter of debate. Although our assays were limited to *in vitro* investigations, we cannot exclude the possibility that SKI-1/S1P mutants give specific *in vivo* phenotypes. Of note, the BC region that contains these C' cleavages resides in a domain highly conserved among different species (supplemental Fig. S1). However, the presence of a double pro-

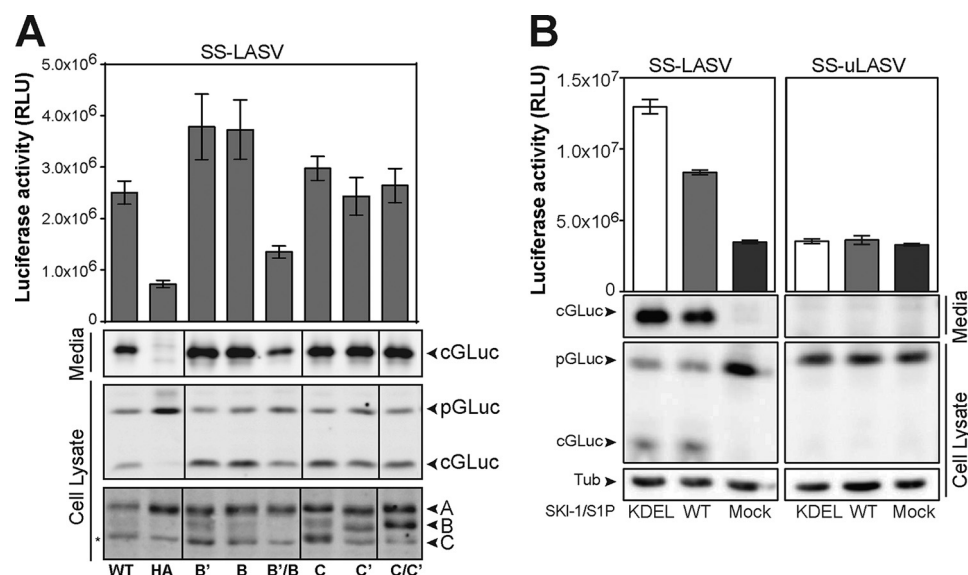


FIGURE 7. Characterization of SKI-1/S1P WT and prodomain mutants activity toward SS-LASV. A, SRD12B cells were co-transfected with the indicated SKI-1/S1P variants and the SS-LASV sensor. At 48 h post-transfection, supernatants and cell lysates were collected and analyzed as in Fig. 5B. Lanes WT and HA of the Western blot panel in A are from the same original blot than in Fig. 5B. The data shown are RLU (means $\pm$ S.D., $n = 3$). B, efficiency of processing of SS-LASV by SKI-1/S1P-KDEL. SRD12B cells were co-transfected with SKI-1/S1P WT, the inactive HA mutant, or the ER-retained form SKI-1/S1P-KDEL in combination with either SS-LASV or the uncleavable SS-uLASV. At 48 h post-transfection, conditioned media and cell lysates were collected and analyzed as in Fig. 5B. The data shown are RLU (means $\pm$ S.D., $n = 3$). The asterisk indicates a nonspecific band in the Western blot.

cessing event may simply reflect redundancy to assure the detachment of the prodomain from the catalytic groove.

Mass spectrometry analysis of a soluble form of the mature enzyme revealed its association with a complex set of prodomain fragments of different length. From our data it seems that mature SKI-1/S1P represents in fact a heterogeneous collection of prodomain enzyme (Pro-SKI-1/S1P) heterodimers rather than a monomer of defined length and composition. Apart from Pro-SKI-1/S1P complexes containing prodomain fragments processed at B'/B site, we identified Pro-SKI-1/S1P species including larger fragments not cleaved at B'/B (supplemental Table S1 and Fig. S2). This is in line with a mechanism of SKI-1/S1P activation that includes cleavage in *cis* (B'/B) followed by independent processing in *trans* (C'/C). It is conceivable that the early generated B'/B forms of the protease use either fully immature or B'/B truncated proteins as substrate to process the C site in *trans*. Goldstein and Brown and co-workers (26) first suggested that inactive SKI-1/S1P H249A could be processed to the C-form by endogenous SKI-1/S1P. Here, we demonstrate that this indeed occurs under normal conditions and that the mature enzyme includes all possible variants.

The association of the active, mature SKI-1/S1P enzyme with a plethora of prodomain fragments is consistent with the full catalytic activity of incompletely matured forms of the protease toward cellular substrates. This is also in line with the observed lack of inhibition of SKI-1/S1P maturation by the WT prodomain provided in *trans*. In this aspect, SKI-1/S1P appears different from subtilisins, where prodomain fragments act as potent inhibitors of the catalytic activity, locking immature forms of the proteases in an inactive state (34). Indeed, we found that all incompletely matured intermediates containing partially processed prodomains show sufficient catalytic activity to process their cellular and pathogen-derived substrates, with the exception of the B'/B SKI-1/S1P mutant. The enzymatic analysis of each mutant was quantified using a novel sensitive cell-based sensor for SKI-1/S1P that allowed the reliable detection of incremental differences in activity. Our SKI-1/S1P sensor represents a further development of a prototype pioneered by Sakai *et al.* (31). In our design, the membrane anchor of the sensor mimics that of SKI-1/S1P, allowing optimal substrate enzyme recognition within the authentic subcellular compartment. The SKI-1/S1P cleavage site to be analyzed replaces the natural shedding site of the protease and is therefore located in a naturally exposed and accessible loop to favor processing. Upon processing, this sensor releases the GLuc reporter to the medium, where it can be easily detected using sensitive luciferase assays. Specific features of the sensor allow for optimal sensitivity and specificity. GLuc is a naturally secreted luciferase characterized by 1000-fold brighter bioluminescence than *Renilla* and firefly luciferase (35). Using this cell-based sensor for SKI-1/S1P, we were able to show that the nine residues P1–P8 $\downarrow$ P1' at the processing site of substrates are necessary and sufficient to define the subcellular location of processing and dependence on maturation.

Based on the data at hand, we performed molecular modeling of the catalytic domain in association with the prodomain (36). We already described a model for the catalytic domain, spanning residues from the C site to Pro⁴⁸⁰ (36) assessed by standard evaluations such as QMEAN and other software (37). Our homology modeling studies were supported by the correct positioning of the catalytic triad (Asp²¹⁸, His²⁴⁹, and Ser⁴¹⁴) if compared with other members of the subtilisin-like PCs. Based on the data at hand, we further developed a model of the pro-region and its interaction with the catalytic domain. Although the SKI-1/S1P prodomain shows only weak homology with other propeptides of the subtilisin-like protease family, a model with an acceptable level of confidence could be developed for the AB segment, either alone or in complex with the catalytic

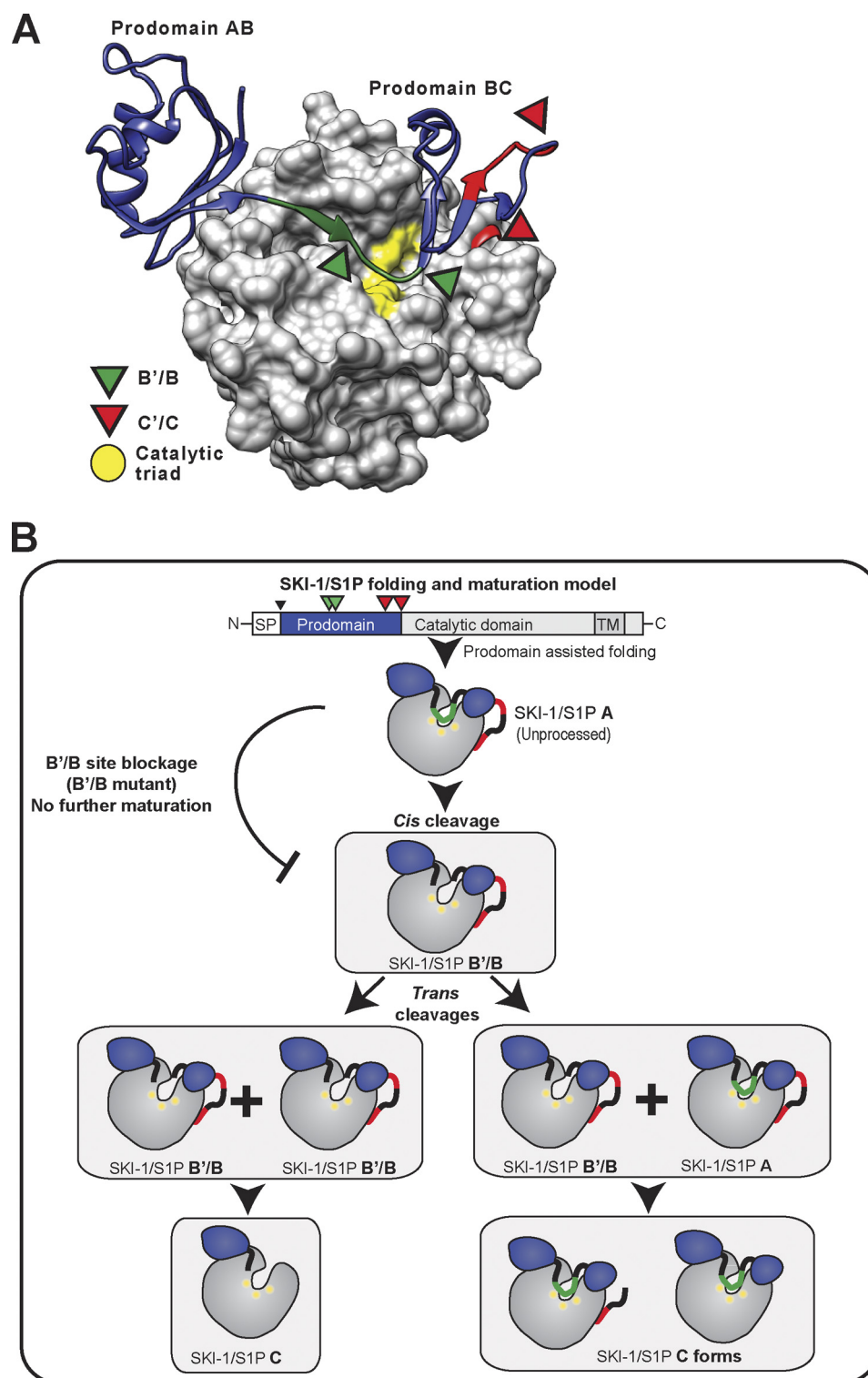


FIGURE 8. Working models for the SKI-1/S1P prodomain and zymogen activation. *A*, molecular modeling of SKI-1/S1P and its prodomain. A solid surface representation of the SKI-1/S1P model is shown with the positions of the catalytic residues Asp²¹², His²⁴⁹, and Ser⁴¹⁴ highlighted in *yellow*. The prodomain extending into the SKI-1/S1P catalytic groove is shown in *blue* with the B'/B and C'/C sites colored in *green* and *red*, respectively. The AB region of the prodomain corresponding to amino acids between sites A and the B'/B cleavage site, as well as BC (spanning residues between B'/B and C sites), are also indicated. *B*, proposed working model for SKI-1/S1P folding and maturation. Schematic representation of SKI-1/S1P forms with prodomain B'/B and C'/C site cleavages indicated in *green* and *red*, respectively. Upon translation of the SKI-1/S1P zymogen in the ER, the prodomain assists in the folding of the immature precursor, acting as a chaperone. The prodomain then follows a multistep maturation process characterized by several autoprocessing events. Autoprocessing at the B'/B sites, which, according to our model, is located above the catalytic groove, is the result of in *cis* cleavage events and is thus one of the first events to take place, generating the SKI-1/S1P B forms. The B forms can then process other SKI-1/S1P forms at the C'/C site in *trans*, generating processed and partially unprocessed SKI-1/S1P forms that might coexist both temporally and spatially. For details, please see text.

domain. In contrast, the BC region seemed to include large portions that apparently lack defined structure, which rendered modeling difficult. It is, however, conceivable that a more flexible conformation within the cleavable B'/B and C'/C sequences is required to enter the catalytic pocket and to allow the enzyme autoprocessing. In our model displayed in Fig. 8A, the prodomain of SKI-1/S1P is located above the catalytic pocket of the enzyme, with the AB region adopting an anti-parallel β -sheet structure anchored to the surface of the molecule. This arrangement is similar to what is found in the prodomains of other serine proteases. In subtilisin E, a serine protease from *Bacillus subtilis*, the anti-parallel β -sheet in the propeptide is responsible for the stable interaction with two parallel helices in the subtilisin domain (38). In the complex, the prodomain is anchored to the surface of the molecule above the catalytic pocket of the enzyme and interacts mainly via the β -sheet surface (32), whereas the residues surrounding the B'/B site are extended to span the catalytic pocket. Based on our molecular modeling and the experimental data, we propose a working model for the autocatalytic activation of SKI-1/S1P (Fig. 8B). Upon synthesis of the polypeptide chain at the rough ER and initial folding events, the immature precursor is first subject to autocatalytic processing at B'/B in *cis*. This form can process in *trans* either the A (fully immature) or B'/B form (itself) generating C'/C forms. In the resulting mature C forms, the active catalytic domain stays associated with prodomain fragments of different length and such Pro/(SKI-1/S1P) complexes may represent the mature enzyme. We propose that interference with the correct autocatalytic maturation may alter the specific composition of the Pro/(SKI-1/S1P) population, shifting the balance toward a particular form whose prodomain fragment renders the catalytic pocket more/less suitable to accommodate specific sequences, explaining impaired processing, for example of some viral substrates by incompletely matured forms of the enzyme.

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