Biosynthesis and Cellular Trafficking of the Convertase SKI-1/S1P

ECTODOMAIN SHEDDING REQUIRES SKI-1 ACTIVITY*

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Subtilisin kexin isozyme-1 (SKI-1)/site 1 protease is a mammalian subtilase composed of distinct functional domains. Among the major substrates of SKI-1 are the sterol regulatory element-binding proteins, regulating cholesterol and fatty acid homeostasis. Other substrates include the stress response factor activating transcription factor-6, the brain-derived neurotrophic factor, and the surface glycoproteins of highly infectious viruses belonging to the family of Arenaviridae. Domain deletion and/or point mutants were used to gauge the role of the various domains of SKI-1. Biosynthesis, cellular trafficking, and sterol regulatory element-binding protein-2 cleavage activity were used as diagnostic tools. Results revealed that Arg130 and Arg134 are critical for the autocatalytic primary processing of the prosegment and for the subsequent efficient exit of SKI-1 from the endoplasmic reticulum. Functional mapping of the growth factor cytokine receptor motif suggested a folding role within the endoplasmic reticulum. Microsequencing of the remaining membrane-bound stub following ectodomain shedding of SKI-1 localized the shedding site to KHQKL(K/R)(3) . Site-directed mutagenesis, in vitro cleavage of a synthetic peptide containing the shedding site, and inhibitor studies favor an autocatalytic event occurring at a non-canonical SKI-1 recognition sequence, with P2 and P1 Leu being very critical. In conclusion, multiple domains ensuring optimal functional characteristics control SKI-1 activity and cellular trafficking.

Limited proteolysis at single and pairs of basic residues has now been recognized to be a fundamental process in living organisms. The major mammalian enzymes that catalyze this reaction, known as the proprotein convertases (PCs),1 are serine proteinases related to yeast kexin and bacterial subtilisins. Mammalian PCs are implicated in the processing of numerous precursors, ranging from polypeptide hormones to growth factors, receptors, and enzymes as well as viral surface glycoproteins. Seven members of the PC family have so far been identified and shown to process these various precursors usually within the consensus sequence (R/K)X(Z)? , where Xi is the number of spacer amino acids (0, 2, 4, or 6) and X is any amino acid, but not Cys (1, 2).

Cellular processing of precursors could also occur at sites not occupied by basic residues. The search for enzymes responsible for such cleavages has recently led to the identification of a novel, widely expressed, membrane-bound, type I subtilase known either as SKI-1 (3, 4) or S1P (5, 6). We (4) and others (6) have shown that pro-SKI-1 undergoes autocatalytic activation in the endoplasmic reticulum (ER) through sequential cleavages at Arg134-Ser-Leu-Lys137-Tyr-Ala and Arg163-Arg-Leu-Leu186-Arg-Ala. In vitro analyses suggested that, for human SKI-1, an additional site may also exist at Arg130-Lys-Val-Phe133-Arg-Ser (4). Furthermore, membrane-bound SKI-1 is shed into the medium as an active soluble enzyme (sSKI-1) (3). However, neither the shedding site nor the cognate “sheddase” is yet known.

In our laboratory, SKI-1 was shown to process pro-brain-derived neurotrophic factor (Table I) (3) possibly to prepare the truncated form (28 kDa) of this neuronal precursor to enter the regulated secretory pathway (7). In addition, SKI-1/S1P activity was shown to play a key role in the regulation of lipid metabolism and cholesterol homeostasis through the processing of sterol regulatory element-binding proteins SREBP-1 and SREBP-2 (Table I) (5), which occurs in the early Golgi apparatus (8). The isolation of Chinese hamster ovary (CHO) SKI-1/S1P−/− (termed SRD-12B) cells (9) was a major determinant in that study that allowed the cloning of hamster SKI-1/S1P cDNA.

SRD-12B cells were also used in the recent demonstration that SKI-1/S1P cleaves another transcription factor, the cellular stress response factor activating transcription factor-6, which plays a major role in the unfolded protein response (Table I) (10). Finally, in a very recent work, SKI-1 was shown to process the surface glycoprotein (glycoprotein C) of Lassa virus, one of the hemorrhagic fever viruses belonging to the family of Arenaviridae (Table I) (11). This was the first case where SKI-1 was directly implicated in an infectious disease. From these results and mutagenesis studies (12, 13), it can be surmised that SKI-1 processes precursors exhibiting the consensus motif (R/K)X(hydrophobic)Z , where Z is any amino acid, preferentially Leu or Thr, but excluding Val, Pro, Gln, Asp, or Cys.

* This work was supported in part by Canadian Institutes of Health Research Grant MOP86496 and Group Grant MGC-11474, the Protein Biosynthesis and Cellular Trafficking of the Convertase SKI-1/S1P family of Arenaviridae. Domain deletion and/or point mutants were used to gauge the role of the various domains of SKI-1. Biosynthesis, cellular trafficking, and sterol regulatory element-binding protein-2 cleavage activity were used as diagnostic tools. Results revealed that Arg130 and Arg134 are critical for the autocatalytic primary processing of the prosegment and for the subsequent efficient exit of SKI-1 from the endoplasmic reticulum. Functional mapping of the growth factor cytokine receptor motif suggested a folding role within the endoplasmic reticulum. Microsequencing of the remaining membrane-bound stub following ectodomain shedding of SKI-1 localized the shedding site to KHQKL(K/R)(3) . Site-directed mutagenesis, in vitro cleavage of a synthetic peptide containing the shedding site, and inhibitor studies favor an autocatalytic event occurring at a non-canonical SKI-1 recognition sequence, with P2 and P1 Leu being very critical. In conclusion, multiple domains ensuring optimal functional characteristics control SKI-1 activity and cellular trafficking.

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1 The abbreviations used are: PCs, proprotein convertases; SKI-1, subtilisin kexin isozyme-1; sSKI-1, soluble SKI-1; hSKI-1, human SKI-1; S1P, site-1 protease; ER, endoplasmic reticulum; SREBP, sterol regulatory element-binding protein; nSREBP, nuclear SREBP; GFCR, growth factor cytokine receptor; BM-domain, before transmembrane domain; CT, cytosolic tail; Ab, anti-SKI-1 N terminus antibody; AbP, anti-SKI-1 prosegment antibody; Tricine, N[2-hydroxy-1,1-bis(hydroxymethyl)glycine; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; RP-HPLC, reverse-phase high performance chromatography; TGN, trans-Golgi network.
The emerging critical implication of SKI-1 activity in various cellular functions and in certain pathologies begs for a better understanding of its biosynthesis and activation as well as elucidation of the mechanism of its shedding and the functional contribution of specific domains within its structure. In this study, we aimed first to define the role of presegment processing in the control of the cellular trafficking and activity of SKI-1. Our data clearly demonstrate the critical importance of Arg130 and Arg134 in the autocalyveat cleavage of pro-SKI-1 (see Fig. 1). We next investigated the shedding site of SKI-1 and showed that the cognate sheddase requires SKI-1 activity. Finally, we attempted to define the possible involvement of two critical residues, viz. Asp592 and Trp591, within the growth factor cytokine receptor (GFCR)-like motif (see Fig. 1) in the control of the rate of cellular trafficking of SKI-1.

**EXPERIMENTAL PROCEDURES**

**Human SKI-1 and Its Mutants—** All oligonucleotides used in the various constructions are listed in Table II. Wild-type human SKI-1 cDNA (3) was subcloned into the pIRE2-EGFP expression vector (CLONTECH) following NotI-BamHI digestions. A V5 epitope (GPKPPLPLGLDST) (Invitrogen) was fused at the extreme C terminus of SKI-1 by PCR using the sense (S) and antisense (AS) oligonucleotide pair S1/A1 (Table II). Two-step PCRs were used to introduce point or deletion mutants, except when indicated. The mutated fragments were cloned using the pCR-Topo TA cloning kit (Invitrogen) and verified by sequencing. For the deletion corresponding to SKI-1 exon EII site at the 5'-end is underlined in Table II). To obtain the double mutant R130E/R134E, we used the R134E construct as a template for PCR that was mutagenized to K948A/L952A/L953A using S2/AS11 and S10/AS3. A deletion corresponding to SKI-1 exon EII site at the 5'-end is underlined in Table II). To obtain the final fragment, we mixed the two purified PCR products and when indicated. The mutated fragments were cloned using the pCRII-Topo TA cloning kit (Invitrogen) and verified by sequencing. For the deletion corresponding to SKI-1 exon EII site at the 5'-end is underlined in Table II). To obtain the double mutant R130E/R134E, we used the R134E construct as a template for PCR that was mutagenized to K948A/L952A/L953A using S2/AS11 and S10/AS3. A deletion corresponding to SKI-1 exon EII site at the 5'-end is underlined in Table II). To obtain the double mutant R130E/R134E, we used the R134E construct as a template for PCR that was mutagenized to K948A/L952A/L953A using S2/AS11 and S10/AS3. A soluble form of SKI-1 ending at amino acid 997, just before the predicted transmembrane domain (BTMD), was made using S2/AS12 (the BamHI site at the 5'-end is underlined, and the stop codon is shown in boldface in Table II). For the KDEL construct (dashed sequence in Table II), S2/AS13 and S2/AS15 (the KDEL site at the 5'-end is underlined in Table II). To obtain the double mutant R130E/R134E, we used the R134E construct as a template. PCRs were performed using S1/AS16 and S13/AS15. The oxyanion mutant N338A was made using S1/AS17 (the PstI site at the 5'-end is underlined in Table II) and S15/AS18 (the BglII site at the 5'-end is underlined). The active-site mutant H249A was constructed using S2/AS16 and S16/AS18. Deletion of the cytosolic tail (ΔCT, Δlys524–val579) was undertaken using S2/AS20. In the latter mutant, the V5 epitope was used using the antisense primer GGAGATCCCTACGTTAGATCGGACCGGAGAGGGATTGGAGATGGTTATCGTCTGAGGGCGGAG (the BamHI site at the 5'-end is underlined, the stop codon is in boldface, and the V5 nucleotide sequence is in italics). The V5 epitope-tagged versions of the above-mentioned mutants (except for ΔCT) were realized by digesting the 304-nucleotide pIRE2-WSK1I-1.5V-EGFP construct either with PvuI-BamHI and cloning it into untagged forms of mutant SKI-1 cDNAs inserted in pIRE2-EGFP or with NruI-BamHI (827 nucleotides) for the gen and exon X1 mutant.

**Transfections and Bioisotonic Analyses—** Transfections of human embryonic kidney cells (HEK293) were made as previously described (14) using 0.6 μg of human pIRE2-SKI-1-EGFP constructs. Two days post-transfection, the cells were washed and then pulse-labeled with either 200 μCi/ml [35S]Met or 400 μCi/ml Na2[35S]SO4, [3H]Leu, or [3H]Val (DuPont). The media and cell lysates (14) were immunoprecipitated using anti-SKI-1 antibodies (1:200) directed against amino acids 634–651 (AbN) or amino acids 17–188 (AbP) (see Fig. 1) (3). Anti-V5 monoclonal antibody (Invitrogen) was used at 1:500. Immunoprecipitates were resolved by SDS-PAGE on Tricine gels (8 or 14% T, 3% C) and autoradiographed as described (14).

**Calnexin/hSKI-1 Interactions—** To study the interactions of the SKI-1 with the ER chaperone calnexin, 2 days post-transfection, HEK293 cells were transfected with wild-type or mutant hSKI-1 constructs. Twenty-four hours post-transfection, cells were washed twice with phosphate-buffered saline and incubated for 7 h in Dulbecco’s modified Eagle’s medium without serum. The media were concentrated 10-fold using a Microcon-10 (Millipore). Western blot analyses of concentrated media were carried out with AbN (1:1000), followed by chemiluminescence detection (ECL, Amersham Biosciences, Inc.). To detect the SKI-1 stub after its ectodomain shedding, SRD-12B cells devoid of SKI-1/SIP activity were used as described (9). Cells were transfected (7–8 × 105 cells/P6 dish (60-mm dish)) using LipofectAMINE 2000 (Invitrogen) with 6 μg of hSKI-1 construct and DNAs. Two days post-transfection, the cells were lysed in SDS buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1% SDS) and incubated for 30 min on ice, and the cleared lysate was immunoprecipitated using anti-V5 antibody (1:500). Following SDS-PAGE on 12% glycin gel, Western blot analyses were carried out using horseradish peroxidase-coupled anti-V5 (Antigen) and anti-V5 antibody (1:5000 dilution). For the SHED-2 processing in CHO Cells—SRD-12B cells in P10 (100-mm) dishes were transfected with 3 μg of hSKI-1 constructs using Effectene (QIAGEN Inc.). Two days post-transfection, cells were washed twice with phosphate-buffered saline and incubated for 6 h in medium containing 9% (w/v) hydroxypropl-β-cyclodexrin (Sigma) (16) and 0.5% (v/v) ethanol. Cells were then washed twice, lysed in 200 μl of SDS sample buffer, electrophoresed through a 25-gauge needle, and the cleared cell lysates were analyzed for SHED-2 following SDS-PAGE on 6.5% gel using anti-IgG monoclonal antibody 7D4 (1:300) (17).

**N-terminal Sequence Analysis of the Immunoprecipitated hSKI-1 Stub—** Transfected HEK293 cells were pulse-labeled with 400 μCi/ml [3H]Leu or [3H]Val. Lysates were immunoprecipitated with anti-V5 antibody, resolved by SDS-PAGE (8% Tricine gel), and autoradiographed. The identified radioactive SKI-1 SI and SI1 stub bands (13 and 14 kDa, respectively) were excised from the gel, and the underlying proteins were microsequenced as previously described (18) on an Applied Biosystems Procise CLC Protein Sequencer.

**Effect of Selected Protease Inhibitors on hSKI-1 Shedding—** Protease inhibitors were tested on HEK293 stable clone (heparin) expressing hSKI-1 (3). Cells (6–7 × 105 cells/P6 dish) were grown overnight in Dulbecco’s modified Eagle’s medium supplemented with 7.5% heat-inactivated fetal calf serum, washed, and then incubated for 6 h in Dulbecco’s modified Eagle’s medium containing different protease inhibitors. Conditioned media were collected 10-fold on a Microcon-10. Western blot analyses were carried out following SDS–8% PAGE using AbN. Metalloprotease and matrix metalloprotease inhibitors were tested at various concentrations: phosphoramidon (10 μM), o-phenanthroline (0.4 or 0.8 mM), TIMP1 (0.4 or 0.8 mM), TIMP2 (18 mM) (Peptide International Inc.) and GM6001 (25 μM) (Chemicon International, Inc.). Pepstatin (3 μM) was used as a specific protease inhibitor. Serine/cysteine protease inhibitors tested on SKI-1 shedding were soybean trypsin inhibitor (0.65 μM), leupeptin (25 μM), and benzamidine.

**TABLE I**

| Precursor protein | Cleavage site sequence |
|-------------------|------------------------|
| Human pro-SKI-1/SIP | P8 P6 P4 P2 P1 P1’ P2’ P4’ P6’ P8’ |
| Site A | R K V F R S L K Y A E S D P T V |
| Site B’ | V T P Q R K V F R S L K Y A E S |
| Site C | R H S S R L L R A I R P Q V A |
| Human pro-BDNF | K A G S R G L T S L K A D T F E H |
| Human SREBP-2 | S G S G R S L V S F E S G G S |
| Human SREBP-1a | H S P G R N V L G T E S R D G E |
| Human ATF6 | A N Q R H L L G F S A K E A Q |
| Lassa virus glycoprotein | I Y I S R R L L G T F T W T L S |

*Pro-BDNF, pro-brain-derived neurotrophic factor; V5, activating transcription factor-6.*
(0.16 mm) (Roche Molecular Biochemicals). The effect of phorbol 12-myristate 13-acetate (10 μM) on SKI-1 shedding via protein kinase C activation was also tested. Tumor necrosis factor-α-converting enzyme protease inhibitor was used at 10 μM; the oxidative enzyme guanidino oxidase was used at 6 units/ml; and finally, ion chelators EDTA and EGTA (Sigma) were used at 2 mM. Other protease inhibitors used for in vitro digestion analysis (see below) were the serine protease inhibitors orpan and captopril (a gift from Dr. G. Boileau, University of Montreal).

In vitro hSKI-1 Digestions—Synthetic peptides containing the shedding site of hSKI-1 (L946WKHQKLL) and the cap site (S182RRLL) (Sheldon Biotechnology Center), or the prosegment fragments of 26 (p26) and 14 (p14) kDa and hence of its shedding (Fig. 2A) revealed a partial (50%) processing inhibition at the B2 (SV2) and C sites as well as the shed form secreted (S11). In addition, we observed an association of p26/p14 with sSKI-1 is affected by this mutation. It seems that prosegment cleavage was reported to be autocatalytic, because prosegment cleavage was observed in hamster S1P (6).

To determine the importance of the B and C sites, we analyzed the processing of wild-type pro-SKI-1 and its P4 Arg mutants R134E and R130E/R134E. We selected these mutants because prosegment cleavage was reported to be autacatalytic, and Arg at P4 has been found to be critical for SKI-1-mediated cleavages (5, 3, 2, 10). When replaced by Glu, the positively charged Arg residue effectively blocked SKI-1-mediated cleavage. We also mutated either the active-site His249 or the oxyanion hole Asn338 to Ala. We expected the former mutant to be inactive, by analogy to furin (21). SKI-1 N338A could conceivably still autacatalytically cleave its prosegment in cis, but be inactive in trans on other substrates. The results from the analysis of processing of SKI-1 and its mutants in HEK293 cells are shown in Fig. 2B. The cells were pulse-labeled with [3H]Leu, and both the media and cell extracts were immunoprecipitated with either Ab:N or Ab:P (4). As previously reported (3), immunoprecipitation with Ab:N revealed the presence of four SKI-1-related forms: full-length pro-SKI-1, also recognized in cells (Fig. 2A), and three processing products, viz., SKI-1 cleaved at the B2 and C sites as well as the shed form secreted (S1). The latter formed a complex with the prosegment fragments of 26 (p26) and 14 (p14) kDa and hence co-immunoprecipitated with them when using Ab:P (Fig. 2B, Media), as previously reported (4). The B site mutant R134E revealed a partial (~50%) processing inhibition at the B2/C sites and of its shedding (Fig. 2A).


table II

| Mutant       | Sense | Antisense |
|--------------|-------|----------|
| SKI-1-V5     | ATGAGCTTGGACAGATGTTGCTG | GTGACCTTTCACTTTCTCCAG |
| S1/AS1       | 5′-EcoRI and 3′-BamHI underlined | GCCCTTACACGAGAAGGTCTTGGGCG |
| S2/AS2       | GTCCTCGGCCCGCCTTCCACCGT | CAGAAAGCCCAAGAGGCTG |
| S3/AS3       | GACTGCTTGCACATTCTCTG | GTTCCTGACTTTCGACAG |
| S4/AS4       | CGACGAGGACCAGGTTCCTCCACTCTGGAT | ATCCGACAGGGCCAAAGCTGGCCTTCG |
| S5/AS5       | GCTGAGGTCGAGGAGGACTCGTCTGGATCCTCCCAAG | GCCCTGACACTCAGACTCTGGGGC |
| S6/AS6       | GCTGAGGTCGAGGAGGACTCGTCTGGATCCTCCCAAG | GCCCTGACACTCAGACTCTGGGGC |
| S7/AS7       | CTCAGACAGGCTCCTACCTTG | CCTCTAGATGCCAACAGGG |
| S8/AS8       | ATCAGACAGGCTCCTACCTTG | CTCTCAGATGCCAACAGGG |
| S9/AS9       | ATCAGACAGGCTCCTACCTTG | CTCTCAGATGCCAACAGGG |
| S10/AS10     | AATCCCTGGGACTCAGCAAGAG | ATCAGACAGGCTCCTACCTTG |
| S11/AS11     | GGGCCGGAGTTGGAGAGGACCTG | ATCAGACAGGCTCCTACCTTG |
| S12/AS12     | GTTCTGACATCTCCTCAAAG | GTTCTGACATCTCCTCAAAG |
| S13/AS13     | GCCCAAGAAAGACTCTTGGACTCAT | GCCACAGTTCACTTTCTCCACAG |
| S14/AS14     | CAGACACTGCGGAGC | GCCACAGTTCACTTTCTCCACAG |
| S15/AS15     | GCTATGTCGCGCCAGGC | GCCACAGTTCACTTTCTCCACAG |
| S16/AS16     | TGGAAACATGCGTAACTTCTC | GTCCTGACACCAATAGG |
| S17/AS17     | TGGAAACATGCGTAACTTCTC | GTCCTGACACCAATAGG |
| S18/AS18     | AAAATCAGAGCTCCTTACCTCC | GTCACATGATGAAGG |
| S19/AS19     | CAGAAGCTATGCTTCAACAGC | GCTACATGATGAAGG |
| S20/AS20     | CAGAAGCTATGCTTCAACAGC | GCTACATGATGAAGG |
| S21/AS21     | TGGAAACATGCGTAACTTCTC | GCTACATGATGAAGG |
| AS22         | GAGGAGTACAGGGCTTCTGATTTT | GCTACATGATGAAGG |
| AS23         | GTCAATGAGACTGCTTCTG | GCTACATGATGAAGG |
| AS24         | GAGGAGTACAGGGCTTCTGATTTT | GCTACATGATGAAGG |
| S22/AS25     | CGCCATCCGCCCCAGAAAAAAGTCT | GAGAAGCTTTTGCTGGG |

Results

Importance of Arg330 and Arg324 in the Autocatalytic Processing of the Prodomain of SKI-1—We previously reported three cleavage sites within the prosegment of hSKI-1 (Fig. 1A) at sequences B2 (RKVF133), B2 (RKVF133), and C (RRLL130) (4). The last two sites were independently proposed for hamster S1P (6).

In vitro hSKI-1 Digestions—Synthetic peptides containing the shedding site of hSKI-1 (L946WKHQKLL) and the P1 Val mutant (L946WKHQKL) (Sheldon Biotechnology Center), or the prosegment fragments of 26 (p26) and 14 (p14) kDa and hence the serine protease inhibitors orpan and captopril (a gift from Dr. G. Boileau, University of Montreal).
cells were transiently transfected with wild-type SKI-1 (WT) or the R130E, R134E, H249A, and N338A cDNAs. Transfectants were labeled for 2 h with 400 μCi/ml Na235SO4 and cell lysates (A) and conditioned media (B) were immunoprecipitated with Ab:N and then resolved by SDS-PAGE on 8% Tricine gels. Arrow/arrowheads indicate different processed forms of SKI-1, and molecular masses are given in kilodaltons. p26 and p14 represent the proteolytic fragments of pro-SKI-1 encompassing amino acids 17–137 and 17–186, respectively (4). pSKI, pro-SKI-1.

FIG. 1. Schematic representation of human SKI-1/S1P. A, the legend to protein domains is depicted at the bottom. B/B’ and C cleavage sites in the N-terminal proregion are shown with arrows. Conserved and mutagenized residues (from Cys849 to Gln868) within the GPCR-like motif are underlined. B, amino acid length and molecular masses are given for each processed form of SKI-1 and also for the BTMD mutant form. CRM, cytokine receptor-like motif.

FIG. 2. Autocatalytic zymogen processing of SKI-1. HEK293 cells were transiently transfected with wild-type SKI-1 (WT), R134E, R130E/R134E (R130,134E), H249A, and N338A cDNAs. Two days post-transfection, the cells were labelled for 3 h with 400 μCi/ml [3H]Leu. Cell lysates and conditioned media were immunoprecipitated with either Ab:N (A) or Ab:P (B) and analyzed by SDS-PAGE on 8% Tricine gels. Arrow/arrowheads indicate different cleaved forms of SKI-1, and molecular masses are given in kilodaltons. p26 and p14 represent the proteolytic fragments of SKI-1 encompassing amino acids 17–137 and 17–186, respectively (4). pSKI, pro-SKI-1.

FIG. 3. The Arg130/Arg134, His249, and Asn338 mutants are mostly localized to the ER. Sulfate labeling was carried out with HEK293 cells transfected with wild-type SKI-1 (WT), R134E, R130E/R134E (R130,134E), H249A, and N338A cDNAs. Transfectants were labeled for 2 h with 400 μCi/ml Na235SO4, and cell lysates (A) and conditioned media (B) were immunoprecipitated with Ab:N and then resolved by SDS-PAGE on 8% Tricine gels and autoradiographed. In C, CHO SKI-1/S1P cells were transfected with the V5 epitope-tagged forms of the above-mentioned cDNAs in the absence (−) or presence (+) of wild-type SKI-1 cDNA. Immunoprecipitated materials (Ab:V5) were resolved by SDS-PAGE on 6% glycine gel, and horseradish peroxidase-coupled anti-V monoclonal antibody was used for Western blot analysis. In D, immunocytochemical analysis was carried out with CHO SKI-1/S1P cells transfected with the V5 epitope-tagged versions of wild-type, H249A (H/A), and R130E/R134E (RR/E) cDNAs. Co-localization of calnexin (Cx; red upper row) with either SKI-1 or its mutants (SKI; white middle row) is shown in the lower yellow/white row. The level of V5 background is presented in the control lane (CTL), where cells were transfected with the empty vector pIRE2-EGFP. Enhanced green fluorescent protein labeling is from the bicistronic vector pIRE2-EGFP. Bar = 5 μm. pSKI, pro-SKI-1.

product was still seen (albeit to a lesser extent) in the B site mutant R134E (estimated cellular processing of 20% compared with 25% for wild-type SKI-1) (Fig. 2A) supports the notion that an alternative cleavage may occur at the B’ site (4). Indeed, the R130E/R134E double mutant almost totally abolished the B/B’ cleavage product and caused the cellular accumulation of pro-SKI-1. In addition, no sSKI-1 was detected in the medium. These data suggest that shedding of cellular SKI-1 may require prior removal of its prosegment. Autocatalytic intramolecular zymogen processing is supported by the fact that the active-site His249A mutant remained as pro-SKI-1 that was barely processed, likely in trans by endogenous SKI-1 in HEK293 cells (Fig. 2). Interestingly, a similar conclusion was reached with the oxyanion hole N338A mutant, thus differentiating SKI-1 prosegment processing from that of furin (21).

To define the cellular trafficking of SKI-1 and its prosegment mutants, we first analyzed the sensitivity of the forms presented in Fig. 2 to digestion by endoglycosidases H and F. The results showed that all cellular and secreted forms were sensitive to both enzymes (data not shown), indicating the persistence of high-mannose glycosylation sites as with other convergent forms. SKI-1 is sulfated on one or more of its sugar moieties (3). As sulfotransferases are located in the trans-Golgi network (TGN) (22, 23), the degree of sulfation was used to estimate the ability of the various SKI-1-related products to reach the TGN. HEK293 cells transiently transfected with SKI-1 and its mutants were pulse-labeled for 2 h with Na235SO4, and the media and cell extracts were immunoprecipitated with Ab:N (Fig. 3, A and B). Although the wild-type sulfated pro-SKI-1, C, and sSKI-1 forms could be readily detected in cells and only the latter in the media, sulfated B/B’ cellular forms were barely detected. This suggests that the majority of the processing of the intermediate B/B’ form into the C form occurs between the ER and the TGN, as proposed (3, 4, 6). Furthermore, blocking the B site (R134E mutant) still did not result in the accumulation of the sulfated B’ form. Only a relatively small portion of the R130E/R134E double mutant reached the TGN and became sulfated, but was mostly unprocessed (Figs. 2A and 3A, Cells). This is consistent with Western blot analyses showing only pro-SKI-1 in cells (Fig. 3C) and no secretion (data not shown). In contrast, both H249A and N338A mutants could exit the ER and reach the TGN, and some did get processed into a C form, possibly in trans by endogenous wild-type SKI-1. Western blot analyses of SKI-1 and its mutants in CHO SKI-1/S1P cells (9) revealed that, upon coexpression of these mutants with wild-type SKI-1 (Fig. 3C, +), only the C forms appeared, albeit...
FIG. 4. B' processing is not a prerequisite for B site cleavage. HEK293 cells were transfected with the empty control vector (CTL) or the V5 epitope-tagged versions of wild-type SKI-1 (WT) and mutants R130E, R134E, and R130E/R134E (R130,134E). A, 1 days post-transfection, cell lysates were immunoprecipitated with anti-V5 monoclonal antibody. B and C, conditioned media were immunoprecipitated with either Ab:N or Ab:P, respectively, and analyzed by SDS-PAGE on 6, 8, or 12% glycine gel. Arrows indicate different processed forms of SKI-1, and molecular masses are given in kilodaltons. pSKI, pro-SKI-1.

Confocal microscopy co-localization of SKI-1 and its mutants was used to further support the biochemical conclusions regarding their cellular trafficking (Fig. 3D). The data show that, although the SKI-1 H249A and R130E/R134E mutants co-localized with the ER marker calnexin, wild-type SKI-1 exhibited a more diffuse pattern of expression, with only a fraction of the total perinuclearly co-localizing with calnexin. These morphological data confirm that the majority of the H249A and R130E/R134E mutants and the N338A mutant (data not shown) remain in the ER.

Finally, the data in Fig. 3 show that inhibiting cleavage at Lys137 (B processing site), although reducing somewhat the overall processing of pro-SKI-1, still produced products migrating at the B'/B (likely at the B' processing site) and C sites. Fig. 4A shows that the R130E and R134E mutants behaved similarly. Here, HEK293 cells were transfected with the V5 epitope-tagged forms of wild-type SKI-1 and its R130E, R134E, and R130E/R134E mutants. Cell lysates were immunoprecipitated using anti-V5 monoclonal antibody and analyzed by Western blotting using the same antibody (Fig. 4A). Western blots of the media using Ab:N (Fig. 4B) or Ab:P (Fig. 4C) are also depicted. These data suggest that blocking either the B or B' site does not abrogate processing. However, as in Fig. 3, the R130E/R134E mutant was barely processed to the C form and was not significantly secreted.

Shedding of SKI-1 Requires SKI-1 Activity—SKI-1 is a transmembrane type 1 protein (Fig. 1) with a highly basic cytosolic tail (amino acids 1023–1052) (3) containing two putative phosphorylation sites at Ser1026 and Thr1049. To assess the role(s) of the cytosolic tail and transmembrane domain in cellular trafficking and secretion, we generated C-terminal SKI-1 with deletions of (i) the cytosolic tail (ΔCT); (ii) both the cytoplasmic tail and transmembrane domain (BTMD); (iii) and (iiii) a BTMD form ending with KDEL, an ER retrieval signal (24).

HEK293 cells expressing these mutants were pulse-labeled with [35S]Leu for 3 h (Fig. 5A) or with Na2[35S]SO4 for 2 h (Fig. 5B). Media and cell extracts were immunoprecipitated with Ab:N and resolved by SDS-PAGE. Deletion of either the cytoplasmic tail (ΔCT) or both the cytoplasmic tail and transmembrane domain (BTMD) did not block the processing of pro-SKI-1 into the B'/B or C form. The ΔCT form was shed into the medium and migrated with a similar molecular mass as the wild-type enzyme. The secreted BTMD form migrated with an ~5-kDa higher molecular mass compared with sSKI-1, with a minority having a mass similar to that of sSKI-1 (Fig. 5, Media). This suggests that the cytoplasmic tail does not affect shedding and that the shedding site is N-terminal to Glu997, the C-terminal amino acid of BTMD.

Although the effective retrieval of SKI-1 in the ER/cis-Golgi compartments by the KDEL sequence allowed the accumulation of the B'/B and C forms (Fig. 5A, Cells), only minute amounts of sSKI-1 could be detected (Fig. 5, A and B, Media). The latter observation suggests that a small amount of the overexpressed SKI-1 KDEL isoform does leak out of the ER and reach the TGN, where it is sulfated. We surmise that the majority of shedding occurs following exit from the ER/cis-Golgi compartments and that the transmembrane domain is needed for effective shedding.

We next determined the shedding site using a C-terminal V5 epitope-tagged form of SKI-1. Fig. 6A represents an SDS-PAGE analysis of HEK293 cell lysates immunoprecipitated with anti-V5 monoclonal antibody following pulse labeling for 4 h with [3H]Leu or [3H]Val (data not shown). The specific appearance of V5 epitope-positive polypeptides migrating at 14 (SI) and 13 (SII) kDa, not seen in control cells expressing untagged wild-type SKI-1 (data not shown), suggested that these may represent membrane-bound stubs formed following SKI-1 shedding. Microsequence analysis of both forms revealed an identical N-terminal Leu1′Leu9′ and Val′Val18′ sequence (Fig. 6B). These data clearly localize the shedding site to the HKQKLL[35S]p sequence. The difference between the SI and SII forms likely results from a post-translational modification such as phosphorylation at Ser1026 and/or Thr1049.

To delineate the class of enzyme to which the SKI-1 sheddase belongs, we measured the level of sSKI-1 by Western blotting following incubation of HEK293 cells stably expressing SKI-1 (3) with various inhibitors (Fig. 6C). The data show that, of all the inhibitors tested, only o-phenanthroline inhibited shedding. Dose dependence analysis revealed that 0.4 mM o-phenanthroline inhibited ~90% of the shedding (Fig. 6C, o-Phe*), with 100% reached at 0.8 mM (o-Phe**). This metalloproteinase
Because SKI-1 prefers to cleave following the (L/T) motif (2), the absence of Arg at P4 of the shedding site using P6 (K948A), P4 (Q950A and Q950R), P3 (K951A), P2 (L952V, L952I, and L952A), and P1 (L953V and L953E) mutants and the triple variant K948A/L952A/L953A (Fig. 7). HEK293 cell-surface biotinylation (Fig. 7A), biosynthetic (Fig. 7B), and/or Western blot (Fig. 7D) analyses demonstrated that P6 and P4 were not critical because mutations K948A, Q950A, and Q950R did not significantly affect shedding (Fig. 7D). Lys at P3 seems to play some role because shedding of mutant K951A was ~30% that of the wild-type enzyme. The L952I, L952V, and L952A mutants showed that, although Ile was well tolerated at P2, Val and Ala were barely so (Fig. 7D), suggesting that Leu and Ile are preferred at P2. The P1 L953V and L953E mutants were barely cleaved (<5%). This agrees with the specificity of SKI-1/S1P for SREBP-2. P1 Leu is much more favored compared with Val and Glu (12). The K948A/L952A/L953A triple mutant also resulted in >95% inhibition of shedding (Fig. 7B, Media; and D), with a concomitant large increase in the level of the C form of SKI-1 at the cell surface (Fig. 7A, Cells). This increase was not evident under the conditions used for fixing the cells for confocal microscopy (see "Experimental Procedures") (Fig. 7E). The primary shed form of the K948A/L952A/L953A mutant migrated with a slightly higher molecular mass (Fig. 7B, Media), suggesting the presence of minor alternative site(s) C-terminal to QKLL$. This suggests that the P2 preference was similar to that observed with SKI-1 ex vivo with pro-brain-derived neurotrophic factor,$^2$ and inhibitor has also been reported to inhibit sSKI-1 or BTMD in vitro, albeit at an ~5-fold higher concentration (3, 4, 25).

Because SKI-1 prefers to cleave following the RX/hydrophobic(L/T) motif (2), the absence of Arg at P4 of the shedding site (KHQKL$^{295}$) cells) into question whether SKI-1 could shed itself. We further defined critical residues within the shedding site using P6 (K948A), P4 (Q950A and Q950R), P3 (K951A), P2 (L952V, L952I, and L952A), and P1 (L953V and L953E) mutants and the triple variant K948A/L952A/L953A (Fig. 7). HEK293 cell-surface biotinylation (Fig. 7A), biosynthetic (Fig. 7B), and/or Western blot (Fig. 7D) analyses demonstrated that P6 and P4 were not critical because mutations K948A, Q950A, and Q950R did not significantly affect shedding (Fig. 7D). Lys at P3 seems to play some role because shedding of mutant K951A was ~30% that of the wild-type enzyme. The L952I, L952V, and L952A mutants showed that, although Ile was well tolerated at P2, Val and Ala were barely so (Fig. 7D), suggesting that Leu and Ile are preferred at P2. The P1 L953V and L953E mutants were barely cleaved (<5%). This agrees with the specificity of SKI-1/S1P for SREBP-2. P1 Leu is much more favored compared with Val and Glu (12). The K948A/L952A/L953A triple mutant also resulted in >95% inhibition of shedding (Fig. 7B, Media; and D), with a concomitant large increase in the level of the C form of SKI-1 at the cell surface (Fig. 7A, Cells). This increase was not evident under the conditions used for fixing the cells for confocal microscopy (see "Experimental Procedures") (Fig. 7E). The primary shed form of the K948A/L952A/L953A mutant migrated with a slightly higher molecular mass (Fig. 7B, Media), suggesting the presence of minor alternative site(s) C-terminal to QKLL$. This suggests that the P2 preference was similar to that observed with SKI-1 ex vivo with pro-brain-derived neurotrophic factor,$^2$ and

$^2$ S. J. Mowla and N. G. Seidah, manuscript in preparation.
In vitro processing of shed and prosegment site peptides by SKI-1

Synthetic peptides containing the shedding site or the proregion of hSKI-1 were incubated in vitro with affinity-purified BTMD for 5 h and then analyzed as described under “Experimental Procedures.” AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; TAPI, tumor necrosis factor α-converting enzyme protease inhibitor.

| Inhibitor      | Inhibition of hydrolysis (%) |
|---------------|------------------------------|
|               | LWKHQKLL | SIDL | SRRLL | RALE |
| Zn²⁺          | 0.05 mM  | 14   | 54    |      |
|               | 0.1 mM   | 20   | 56    |      |
|               | 0.25 mM  | 53   | 80    |      |
|               | 0.5 mM   | 67   | 93    |      |
|               | 1.0 mM   | 85   | 100   |      |
| Ni²⁺/EDTA     | 1.0 mM   | 25   | 39    |      |
|               | 3.0 mM   | 16   | 32    |      |
|               | 30 mM    | 77   | 66    |      |
| TLCK (1.0 mM) | 3        | 0    |       |      |
| Phosphoramidon (1.0 mM) | 6 | 12 |      |      |
| Captopril (0.1 mM) | 5 | 35 |      |      |
| GM6001 (0.1 mM) | 23 | 30 |      |      |
| Thiorphan (0.1 mM) | 5 | 6  |      |      |
| TAPI (0.1 mM)  | 5        | 0    |       |      |

* Values represent averages of duplicate assays (variation is ±5%).

site mutants (see Fig. 3D) as well as to the GFCR (see below) motif mutant D858A/W861A (Fig. 7E, DW/A). These latter mutants are mainly retained in the ER, as evidenced by their major co-localization with calnexin.

To further test the possible autocatalytic nature of SKI-1 shedding, we first affinity-purified hexahistidine-tagged BTMD on a Talon-Ni²⁺ column, resulting in a homogenous preparation as evidenced by Western blotting and Coomassie Blue staining (data not shown). This preparation was then incubated in vitro with a synthetic 12-mer peptide (LWKHQKLLSIDL) encompassing the shedding site, its P1 Val mutant (LWKHQKLVSIDL), or the previously reported 9-mer prosegment substrate (SRLRLRALE) (4) in the absence or presence of agents known to inhibit SKI-1 (4, 25). The products were quantitatively analyzed by RP-HPLC and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Table III). The data show that BTMD cleaved both the 12-mer (1493 Da) and 9-mer (1113 Da) peptides exactly at the expected LWKHQKLL | SIDL and SRRLL | RALE sites, yielding peptides LWKHQKLL (1065 Da) and SIDL (446 Da) and peptides SRRLL (644 Da) and RALE (487 Da), respectively. Interestingly, SKI-1 could not cleave the 12-mer P1 Val mutant LWKHQKLY-SIDL (data not shown). The affinity-purified in vitro activity was similar to ex vivo expressed SKI-1 (Fig. 1D) because P1 L953V was not cleaved in both cases. As shown in Table III, the shedding site and prosegment peptide substrate cleavages were largely inhibited by 1 mM Zn²⁺ (85 and 100%, respectively) and less efficiently by EDTA even at 30 mM (77 and 66%, respectively), as previously described for SKI-1 (4, 25). However, much less significant inhibitions were observed with 1 mM Ni²⁺ (25 and 39%, respectively), 1 mM leupeptin, 4-(2-aminoethyl)benzenesulfonyl fluoride, TLCK, and various general or specific metalloprotease inhibitors even at elevated (0.1 mM) concentrations. We next wished to compare the processing efficiency of the shedding site peptide with that of the prosegment. Identical amounts of the partially purified SKI-1 enzyme were incubated for various periods in the presence of a 140 μM concentration of each peptide. The times required to reach 50% cleavage were found to be 160 min for the shedding site and 220 min for the prosegment peptide (Fig. 8). Finally, although we could not titrate the SKI-1 activity with ω-phenanthroline (as it eluted at a similar position on the RP-HPLC column as the products), we did so with Zn²⁺. These titration experiments demonstrated that cleavage of the better shed substrate was more difficult to inhibit than that of the prosegment (Table III).

**GFCR Motif of SKI-1—**The sequence of SKI-1 contains a partial GFCR motif (C^AAAA^LXXX^XXX^D^XXX^CXW^XXX^) split between exons XX (amino acids 812–858) and XXI (amino acids 859–901). Exon XXI is also alternatively spliced in some rat SKI-1 mRNA transcripts (3, 26). To investigate the importance of this GFCR motif, we either deleted part of exon XX (Δ849–861) or the whole of exon XXI (Δ862–901) or generated point mutants within the conserved residues (D858A and W861A). The biosynthetic consequences of such mutations were analyzed by SDS-PAGE of extracts of HEK293 cells pulse-labeled either for 3 h with [3H]Leu (Fig. 9A) or for 2 h with Na₂[35S]SO₄ (Fig. 9B). Pro-SKI-1 accumulated intracellularly for mutants Δ862–901, Δ849–861, and D858A/W861A, and their B/B, C, and sSKI-1 forms were either absent or barely detectable, with minor amounts reaching the TGN. In contrast, processing of the W861A mutant was similar to that of wild-type SKI-1 (Fig. 9, A and B).

It is well known that calnexin binds to unfolded glycosylated proteins until fully folded, whereupon it dissociates (27). The extent of folding of SKI-1 and its mutants was probed using calnexin co-immunoprecipitations in HEK293 cells. Following a short 1-h pulse with [35S]Met, immunoprecipitation of cell lysates with anti-calnexin antibody was followed by a second one of the solubilized calnexin precipitate using anti-V5 antibody (Fig. 9C, Ab:Cx/V5). Much more pro-SKI-1 (arrow) co-immunoprecipitated with calnexin for the D858A/W861A and Δ849–861 mutants compared with the wild-type and W861A forms, in agreement with the longer retention of the former mutants in the ER. Because mostly pro-SKI-1 interacted with calnexin, it could be deduced that the processed forms are folded. This point was further investigated by Western blotting with calnexin immunoprecipitates from HEK293 cell lysates transfected with the V5 epitope-tagged forms of wild-type SKI-1 or the above-mentioned mutants. Two days post-transfection, cell lysates were immunoprecipitated with anti-calnexin antibody, and the supernatants were re-immunoprecipi-
and with calnexin, the C form did not seem to, in agreement with its mostly pro-SKI-1 and some B form and the W861A and D858A/W861A mutants, although even small amounts of zymogen-activated enzyme (C form in cis–Golgi at the motif (R/K) and in some cases, this results in a partially cleaved form that is transported in early compartments of the secretory pathway such as the ER or early Golgi (3, 6, 8).

Two days post-transfection, the cells were labeled for 3 h with 400 μCi/ml [3H]Leu (A) or for 2 h with 400 μCi/ml Na235SO4 (B). Cell lysates and conditioned media were immunoprecipitated with AbN and analyzed by SDS-PAGE on 5% Tricine gels. C, HEK293 cells were transiently transfected with the empty control vector pRES2-EGFP (CTL) or the V5 epitope-tagged versions of wild-type SKI-1, W861A, D858A/W861A, and Δ849–861. Transfectants were pulse-labeled for 1 h with 200 μCi/ml [35S]Met; cell lysates were immunoprecipitated (IP) with anti-calnexin (Cx) antibody; and the solubilized calnexin precipitate was then re-immunoprecipitated with anti-V5 antibody (Ab: Cx/V5). D, HEK293 cells were transiently transfected with the V5 epitope-tagged forms of wild-type SKI-1, W861A, D858A/W861A, and Δ849–861. Two days post-transfection, cell lysates were immunoprecipitated with anti-calnexin antibody, and the supernatant was re-precipitated with anti-V5 antibody. Western blot results with anti-V5 antibody of the immunoprecipitate (Fig. 9) revealed that, for the wild-type and the supernatant (middle panel) indicated that, for the wild-type form and the W861A and D858A/W861A mutants, although mostly pro-SKI-1 and some B/B processed form interacted with calnexin, the C form did not seem to, in agreement with its primary generation in the Golgi (6). In mutants D858A/W861A and Δ849–861, the processing of the B/B form to the C form was either highly impaired or non-detectable, respectively (Fig. 9, A and D, middle panel).

Processing of SREBP-2 by SKI-1 and Its Mutants—Processing of SREBP-2 by SKI-1 depends on the level of sterols; at low levels, the sterol sensor protein SREBP cleavage-activating protein, which binds SREBP within the ER, escorts it to the Golgi (Fig. 10A), whereby the complex meets SKI-1/S1P, and the first cleavage at RSVL, rapidly occurs, followed by a second cleavage by the metallopeptinase S2P at DRSLILL. This generates a soluble cytosolic basic helix-loop-helix transcription factor, which enters the nucleus, binds nuclear sterol regulatory elements, and up-regulates the transcription of the various mRNAs needed for cholesterol and lipid synthesis and metabolism (8, 16).

To assess the activity of SKI-1 and its multiple mutants generated in this study, we analyzed the ability of these enzymes to process the precursor of SREBP-2 into its nuclear product (nSREBP-2) in SRD-12B cells in the absence of sterols. Western blot analysis of the SREBP-2 products using the hamster-specific monoclonal antibody 7D4 (Fig. 10B) revealed that only the active-site H249A, oxianion hole N338A, and Δ849–861 (and Δ862–901; data not shown) mutants could not generate the ~64-kDa nSREBP-2 protein, similar to control cells transfected with an empty vector. This data point out that even small amounts of zymogen-activated enzyme (C form in R130E/R134E and D858A/W861A; see Figs. 3C and 9B, respectively) are enough to generate SREBP-2 cleavage activity and that only completely inactive SKI-1 will not do so. Finally, the ability of the KDEL mutant to generate nSREBP-2 is in accord with the similar profile obtained in the presence of brefeldin A for all active mutants and wild-type SKI-1 (data not shown). This confirms that at least some activity of SKI-1 can be detected in early compartments of the secretory pathway such as the ER or early Golgi (3, 8).

**DISCUSSION**

SKI-1/S1P is a novel mammalian subtilase that cleaves precursors within the cis-medial-Golgi at the motif (R/K)X(hydrophobic)Z [4], where Z is not necessarily a basic residue. These characteristics clearly differentiate this enzyme from the seven other dibasic/monobasic specific subtilases known as the proprotease convertases, which normally process precursors in the TGN, within immature secretory granules, or at the cell surface (2). Known SKI-1 substrates (Table I) are cleaved within earlier compartments of the secretory pathway than those of the PCs; and in some cases, this results in a partially cleaved form that is subsequently further processed by a FC, as is the case for pro-brain-derived neurotrophic factor (3, 7). So far, very little is known about the functional importance of the various domains of SKI-1 (Fig. 1A) and their implication in the folding, activity, or cellular trafficking of this critical convertase. In this report, we analyzed the structure-function of three segments of the molecule, viz. its prodomain, the GFCR motif, and the transmembrane domain/ cytoplasmic tail, and examined the importance of their integrity in the process of ectodomain shedding of SKI-1.

By analogy to bacterial and other mammalian subtilases, the
prosegment of SKI-1 was predicted to play an important role as an intramolecular chaperone and/or as a specific inhibitor of SKI-1. In vitro data demonstrated that the full-length wild-type prosegment is a moderate (IC\textsubscript{50} ~ 1 µM) inhibitor of the enzyme (4), in contrast to those of furin, PC7 (28), and PC1 (29), but similar to that of PC2 (30). A construct lacking the prodomain (SKI-1pro) did not exit the ER and was not shed into the medium (data not shown), indicating that the prosegment is critical for trafficking. This suggests that specific residues within this domain could affect the folding and/or cellular sorting of SKI-1. In agreement with previous studies on furin and other PCs (2), but different from yeast kexin (31), coexpression of the prosegment in trans with SKI-1pro did not restore its cellular trafficking characteristics (data not shown).

Based on our previous study suggesting the existence of two primary processing sites (B' and B) within the prosegment sequence R\textsuperscript{130}KVF R SRLK R (4), we mutated the two critical P4 Arg\textsubscript{130} and Arg\textsubscript{134} residues to Glu. Biosynthetic, Western blot, and immunological analyses (Figs. 2–4) demonstrated that, although the R130E and R134E mutants were processed similarly to the wild-type enzyme, the R130E/R134E double mutant accumulated mostly as a zymogen (pro-SKI-1) in the ER. These data confirm the importance of Arg\textsubscript{130} and Arg\textsubscript{134} in the B' and B processing sites (3). P4 Arg in substrates was predicted to interact with negatively charged residues within the S4 pocket of furin (21). Should a similar situation occur in SKI-1, we speculate that the above Arg-to-Glu mutation(s) may cause a repulsion of the prosegment away from the catalytic pocket and possibly prevent its chaperone-like function. Previous reports (3, 6), and our present sulfation experiments (Figs. 3, A and B) suggest that processing to the B and B' forms occurs in the ER and that the majority of the C form accumulates in the Golgi. This does not exclude the possibility that, given the right circumstances, such C processing can occur within the ER or early Golgi. Indeed, brefeldin A treatment (3) and the SKI-1 D858A/W861A mutants were detected using sensitive antibodies and shown to have different substrate profiles (35). Despite the biological importance of the membrane-anchored and membrane-soluble forms of these proteins is thought to be necessary for their physiological roles. A perturbation in this balance may lead to pathology such as Alzheimer's disease (34). Even though functionally (Fig. 3) and by Northern blotting (3), we know that SKI-1 is present in HEK293 cells, we have not been able so far to detect by Western blotting therein endogenous membrane-bound or membrane-soluble SKI-1. Future improvement in antibodies may resolve this problem. However, very recently, in epidermis, both shed and transmembrane forms of endogenous furin were detected using sensitive antibodies and shown to have different substrate profiles (35). Of the various metalloproteases, their mode of activation, and the structural determinants of the ectoproteins involved in this process. Shedding of SKI-1 occurs following exit from the ER (because incubation with brefeldin A blocks shedding), and sSKI-1 accumulates in the media (3). Moreover, shedding does not seem to require an acidic environment because treatment of HEK293 cells expressing SKI-1 with 50 mM NH\textsubscript{4}Cl for 3 h did not have any effect (data not shown). The data presented in this report also show that the cytosolic tail of SKI-1 (SKI-ACT) (Fig. 5) is not required for prodomain processing, cellular trafficking, or shedding. The latter observation was also reported for angiotensin-converting enzyme (36) and \(\beta\)-amyloid precursor protein (37). Compared with the wild-type enzyme, the soluble BTMD variant of SKI-1 was not effectively shed (Fig. 5), suggesting that the transmembrane domain is needed for effective shedding. Shedding of the active-site H249A and N338A mutants and the R130E/R134E double mutant was highly impaired (Figs. 2A and 3B). Because the former mutants were inactive and the R130E/R134E mutant could still process SREBP-2 (Fig. 10B), but was mostly localized to the ER (Fig. 3D), shedding is thought to require an active enzyme and not to occur in the ER. Requirement of SKI-1 activity in shedding is also demonstrated by the ability of wild-type SKI-1 to induce in trans shedding of the H249A mutant in CHO SKI-1/S1P\textsuperscript{–/–} cells (Fig. 7C). Microsequencing experiments identified the shedding site of SKI-1 as KHQKL\textsubscript{1104} D SIDL (Fig. 6, A and B). The general metalloprotease inhibitor \(\alpha\)-phenanthroline, an inhibitor of SKI-1 (3, 4, 25), effectively inhibited shedding (by ~90% at 0.4 mM) (Fig. 6C). Shedding was not affected when cells were treated with the phospholipase A2 \(\alpha\)-myristate 13-acetate, an activator of protein kinase C. This inhibition profile revealed that SKI-1

\[3\] Available at www.expasy.ch/tools/\#secondary.
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sheddin not regulated by phorbol 12-myristate 13-acetate and is possibly constitutive (38). Mutagenesis of the shedding site (Fig. 7, A–D) demonstrated that, although P6, P4, and P3 residues are not critical for SKI-1 shedding, aliphatic residues at P1 (Leu/Ile) and P2 (Leu) are favored. These P1 and P2 preferences (but not the P4) resemble those preferred by SKI-1/S1P itself (Table I). The restricted SKI-1 shedding site Leu-Val (Fig. 7D), with a minor alternative one (Fig. 7B), contrasts with the presence of a number of sites in angiotensin-converting enzyme (36), ß-amyloid precursor protein (39), tumor necrosis factor-á (40) and L-selection (41). Because P4 Gln950 is not critical for SKI-1 shedding (Fig. 7D), whereas it is for other known SKI-1 substrates (Table I), it was not evident whether SKI-1 could catalyze its own shedding or whether it activates the cognate sheddase. However, because the H249A mutant was not effectively shed in either CHO SKI-1/S1P+/− or HEK293 cells (Figs. 3B and 7C) and was much more so when wild-type SKI-1 was coexpressed (Fig. 7C), the latter model would presume that, in these cells, most of the sheddase is latent, requiring SKI-1 activation. Incubation of a synthetic peptide containing the shedding site as well as a non-cleavable mutant with affinity-purified SKI-1 (Table III) revealed a single cleavage at the same site deduced from ex vivo studies. Furthermore, the profile of inhibition by metal ions and serine and metallopeptase inhibitors and the non-cleavability of the Val952 mutant (Table III) favor a model whereby SKI-1 shedding is autocatalytic. The mode of activation could be intra- and/or intermolecular. Evidence from Fig. 7C demonstrates that the intermolecular mechanism is possible because the V5 epitope-tagged H249A mutant is shed upon overexpression of wild-type SKI-1. In addition, the transmembrane domain (but not the cytosolic tail) seems to be an important determinant for shedding, as the ER-retrieved BTMD-KDEL form of SKI-1, although active (Fig. 10), was still not shed (Fig. 5). However, at this point, we cannot exclude an indirect effect of SKI-1 activity resulting in its cell-surface shedding. If SKI-1 shedding, which occurs at a site 44 amino acids N-terminal to the transmembrane domain, is autocatalytic, then why is P4 Gln950 not critical (Fig. 7D), as it is for other SKI-1 substrates (Table I)? One possibility is that SKI-1 shedding takes place either in the TGN or close to the cell surface and that the intracellular milieu at these sites is different from that of the ER or cis/medial-Golgi, where the other known substrates are processed. Alternatively, other factors may regulate shedding, including phospholipid rafts and cholesterol, the levels of which may be regulated by SKI-1/S1P substrates. There is increasing evidence that sphingolipid- and cholesterol-rich microdomains (rafts) exist in the plasma membrane (42). Specific proteins assemble in these membrane domains and play a role in signal transduction and many other cellular events. Cholesterol plays an important role in maintaining membrane rafts in a separate phase from the rest of the bilayer. Cholesterol depletion causes disassembly of the raft-associated proteins, suggesting an essential role of cholesterol in the structural maintenance and function of rafts. Although not shown, upon 7 h of incubation of HEK293 cells with 1% cyclohextrin, an agent known to remove sterols from intact cells (43), we observed an ~2-fold increase in the level of shed SKI-1. This effect was reversed by the addition of an extracellular cholesterol/25-hydroxycholesterol mixture (see “Experimental Procedures”). Future studies should investigate how SKI-1 shedding is modulated by sterols. The triple mutant of the shedding site (K948A/L952A/L953A) led to an active SKI-1 enzyme (Fig. 10B), which accumulated at the cell surface to a larger extent than the wild-type enzyme (Fig. 7A). It would also be interesting in the future to define the changes in cellular homeostasis accompanied by overexpression of this mutant in various cells, including those infectable by hemorrhagic fever viruses belonging the Arenaviridae family, the glycoprotein of which could be a substrate for SKI-1 (Table I) (11). In summary, this work shows that SKI-1 contains various critical domains that control its zymogen processing, cellular sorting, activity, and ectodomain shedding. Our data demonstrate that a drastic reduction of the active SKI-1 C form is required, e.g. for the active-site His and Asn mutants and the exon XX and XXI deletants, before significantly affecting the activity of this enzyme for SREBP-2 (Fig. 10). These data agree with a recent report demonstrating that nearly complete silencing of SKI-1/S1P is required for abrogation of its SREBP-cleaving activity (44). Thus, in mouse liver conditional knockout cells, the 5–10% of SKI-1/S1P activity still remaining is enough to process SREBPs, albeit to a lesser extent; and consequently, these mice exhibit only a 75% decline in cholesterol and fatty acid biosynthesis (44). Finally, SKI-1 ectodomain shedding requires SKI-1 activity and may be autocatalytic. Shedding may regulate the level of intracellular or cell-surface SKI-1 and/or accessibility to different substrates, as shown for furin (34), membrane-type matrix metalloproteinase-5 shed by a PC-like enzyme (45), and heparan sulfate proteoglycans (46). If SKI-1 ectodomain shedding is autocatalytic, this would be rather unusual, as most other cases involve processing by surface metallopeptinas. If so, the unique Leu-Val j P1 and P2 cleavage specificity at the shedding site suggests that, depending on its substrate and/or cellular localization, SKI-1 may exhibit a different P4 sensitivity, possibly leading to a wider spectrum of unsuspected substrates.

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