Bioskynthetis and Enzymatic Characterization of Human SKI-1/S1P and the Processing of Its Inhibitory Prosegment*

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Biochemical and enzymatic characterization of the novel human subtilase hSKI-1 was carried out in various cell lines. Within the endoplasmic reticulum of LoVo cells, proSKI-1 is converted to SKI-1 by processing of its prosegment into 26-, 24-, 14-, 10-, and 8-kDa products, some of which remain tightly associated with the enzyme. N-terminal sequencing and mass spectrometric analysis were used to map the cleavage sites of the most abundant fragments, which were confirmed by synthetic peptide processing. To characterize its in vitro enzymatic properties, we generated a secreted form of SKI-1. Our data demonstrate that SKI-1 is a Ca2+-dependent proteinase exhibiting optimal cleavage at pH 6.5. We present evidence that SKI-1 processes peptides mimicking the cleavage sites of the SKI-1 prosegment, pro-brain-derived neurotrophic factor, and the sterol regulatory element-binding protein SREBP. Among the candidate peptides encompassing sections of the SKI-1 prosegment, the RSLK137-and RRLL186-containing peptides were best cleaved by this enzyme. Mutagenesis of the latter peptide allowed us to develop an efficiently processed SKI-1 substrate and to assess the importance of several P- and P'-residues. Finally, we demonstrate that, in vitro, recombinate prosegments of SKI-1 inhibit its activity with apparent inhibitor constants of 100–200 nM.

Over the last 30 years (1, 2), our understanding of the complex cellular processing by limited proteolysis of inactive secretory precursors into active polypeptides and proteins has greatly expanded. It is now becoming clear that, following removal of the signal peptide, precursor cleavage can occur intracellularly, at the cell surface or within the extracellular milieu. The sites of cleavage are composed of either (i) single or pairs of basic residues (Lys or Arg) within the general motif (R/K)(X)2(−)(R/K)1, where n = 0, 2, 4, or 6 and X is any amino acid (aa) except Cys, or (ii) hydrophobic (e.g. Leu, Phe, Val, or Met) and small aa such as Ala, Thr or Ser (3). The former cleavage type occurs in many growth factors and their receptors, most polypeptide hormones and neuropeptide precursors, surface glycoproteins (including adhesion and viral envelope glycoproteins), as well as a host of other secretory proteins (1, 2). The latter type of cellular processing has been implicated in the generation of bioactive peptides (4–6), proteins (7), and transcription factors (8).

Some of the proteinases involved in intracellular endopeptidolytic events that result in cleavage at specific single or paired basic residues are members of a family of calcium-dependent serine proteinases related to the yeast subtilase kexin (1–3, 9). These dibasic- and monobasic-specific “precursor convertases” (PCs), of which seven mammalian members are presently known, comprise PC1 (PC3), PC2, furin (PACE), PC4, PC5 (PC6), PACE4, and PC7 (LPC, PC8). Each of these kexin-like subtilases contains a unique N-terminal prosegment that presumably functions both as an intramolecular chaperone and a proteinase inhibitor (1–3). Acting in concert, these enzymes determine the time and cellular location at which biologically active products are derived from inactive precursor proteins (1–3).

Efforts to identify the proteinases responsible for the intracellular processing of precursors at hydrophobic or small aa have led to the recent cloning of a new subtilase called SKI-1 (10) or S1P (11), whose aa sequence is highly conserved among human and rodent species. According to Siezen and Leunissen’s classification (9), this enzyme belongs to the prolylsin branch of subtilases (compared with PCs, which are within the kexin branch). Tissue distribution analyses by both Northern blots and in situ hybridization reveal that SKI-1 mRNA is widely expressed (10, 12). We reported previously that human SKI-1 (hSKI-1) produces a 28-kDa product from the 32-kDa brain-derived neurotrophic factor precursor (proBDNF) via selective cleavage within the sequence RGLT↓SL (10). Inde-

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The abbreviations used are: aa, amino acid(s); VV, vaccinia virus; PC, precursor convertase; ER, endoplasmic reticulum; TGN, trans-Golgi network; SREBP, sterol regulatory element-binding protein; BDNF, brain-derived neurotrophic factor; BTMD, before transmembrane domain; APMSF, (4-amidinophenyl)methylsulfonyl fluoride; PMSF, phenylmethylsulfonyl fluoride; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; SBTI, soybean trypsin inhibitor, PCR, polymerase chain reaction; FP, reverse phase; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; Abs, antibody; WT, wild type; BFA, brefeldin A; s, sense; a, antisense; Fmoc, N-(9-fluorenylethoxycarbonyl); Tricine, N-tris(hydroxymethyl)methylglycine; MALDI-TOF, matrix-assisted laser desorption/time of flight; MES, 2-(N-morpholino)ethanesulfonic acid; Abz, O-amino benzenoic acid; Y(NO3)3, 3-nitrotyrosine.

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In vitro characterization of human SKI-1

DEPENDENTLY, Sakai et al. demonstrated that hamster SKI-1/S1P is responsible for the site 1 cleavage of sterol regulatory element-binding proteins (SREBPs) (11), highlighting the critical role of SKI-1/S1P in the regulation of the synthesis and metabolism of cholesterol and fatty acids. In their model, SKI-1/S1P cleaves SREBP-2 at an RSLV I SF sequence within the lumen of the endoplasmic reticulum (ER). Mutational analyses demonstrated that the presence of Arg at the P4 substrate position is critical for cleavage, whereas the P1 Leu could be replaced by a number of other aa (8).

In this work, we first present data regarding the cellular biosynthesis of membrane-bound hSKI-1 and its zymogenic processing. Then, based on our previous discovery of a secreted (shed) form of hSKI-1 (10), we produced a vaccinia virus (VV) recombinant of a soluble form of hSKI-1 with a hexa-His sequence just before the stop product, resulting in a product called 5

with the similarly digested full-length hSKI-1 cDNA 3.5-kilobase pair plaque-forming units each of VV:SKI-1 and VV:BTMD-SKI-1 recombinants. Moreover, we examined the processing of hSKI-1 in LoVo cells infected with a VV recombinant as well as in a stable transfectant of HK293 cells (10).

EXPERIMENTAL PROCEDURES

Vaccinia Virus Recombinant of BTMD-SKI—The preparation of a soluble form of hSKI-1 involved the initial amplification by polymerase chain reaction (PCR) of a 1250-base pair product encompassing nucleotides 491–1740 of the hSKI-1 cDNA (12), which includes the initiator methionine. The sense (s) and antisense (as) oligonucleotides were 5’-GGTACCACTGAGGCTGCTGACC-3’ and 5’-ACACTGGTCGCTGACCATGGGCA-3’, respectively. This completely sequenced fragment, which had been inserted into the PCR2.1 TA cloning vector (Invitrogen), was then digested with NorI and AacI. It was then ligated with the similarly digested full-length hSKI-1 cDNA 3.5-kilobase pair product, resulting in a product called 5’-hSKI-1-FL. In order to obtain a soluble form of hSKI-1 with a hexa-His sequence just before the stop codon, PCR amplification was carried out using the sense and antisense oligonucleotides: 5’-ATTGACCTGGACAAAGGCTGGT-3’ and 5’-GGAATTCCAGAGACTGGTAGCTGACCATGGGCA-3’. This resulted in a 185-base pair fragment encoding the C-terminal sequence PRQNYQEH-H+ (10). Following digestion with 5’- EcoN1 and 3’ XbaI, the product was ligated to the aforementioned and similarly digested 5’- hSKI-1-FL. This CDN, coding for BTMD-SKI-1 ending with a hexa-His sequence, was then transfected to the Bsd/Hixba site of the (VV) transfer vector PMJ60I. A recombinant was then isolated as previously reported (10). The VV recombinant of full-length hSKI-1 has been described (10).

Biochemical Analyses—Sixteen hours following infection with 2 plaque-forming units each of VV:SKI-1 and VV:BTMD-SKI-1 recombinants, human LoVo cells (3 x 10⁶) were radiolabeled with 500 µCi of [³H]Leu for 2 h or pulsed for 15 min followed by a chase of 2 h, in the presence or absence of 5 µg/ml fungal metabolite brefeldin A (BFA) as described by the manufacturer. After two washes with 5 m M imidazole, the protein was eluted with 200 m M imidazole and tested for enzymatic activity and immunoreactivity by Western blot (see below).

Purification, N-terminal Sequencing, and Mass Spectrometric Analysis of the Secreted Recombinant Prosegment(s) of hSKI-1—Concentrated media obtained from either VV:BTMD-SKI-1-infected BSC40 cells or from a stable transfectant of full-length hSKI-1 in HK293 cells (10) were loaded onto an RP-HPLC 5-µm C4 column (0.94 x 25 cm) (Vydac). Proteins were eluted at 2 ml/min using a 1%/min linear gradient (15–70%) of 0.1% aqueous trifluoroacetic acid/CH₃CN with monitoring at 210 nm. The proteins were analyzed by Western blotting, and aliquots of the immunoreactive fractions were used for a N-terminal sequence (10). The samples were used for MALDI-TOF mass spectrometry using the 3-matrix 3,5-dimethoxy-4-hydroxycinnamic acid (Aldrich). For N-terminal sequencing, fraction IV proteins (Fig. 3A) were separated by SDS-PAGE, transferred to Immobilon-P membranes, and stained with Ponceau Red. The 14- and 5-kDa bands were excised and sequenced using an Applied Biosystems model 492A protein sequencer.

Synthesis of Peptide Substrates—All Fmoc amine acid derivatives (t-form), the coupling reagents, and the solvents for peptide synthesis were purchased from PE Biosystems Inc. (Framingham, MA, USA), Calbiochem (San Diego, CA), or Richelieu Biotechnologies (Montreal, Quebec, Canada). The various linear synthetic peptides and internally quenched fluorogenic (Q)-substrates were synthesized as described by the manufacturer. The 50–60, KAGSRGLTSLIYTFE (ID hSKI-1P20–450), GGAHDSSQHPISGRSSGLVFESGGG; (III) hSKI-1-174–191, WHRTGRHSSRRLLRAIPR; (IV) hSKI-1-174+18, LEHATGRH-SRRLLRALE; (V) hSKI-1-182–188+18, SEERRLLRALE; (VI) hSKI-1-156–172, WQSSRPLLRASLGSG; (VII) hSKI-1-187–201, RAIRPQARATLAQDV; (VIII) hSKI-1-128–138, PQRVFVRSL; (IX) hSKI-1-128–142, PQRVFVRSLKAESD; (X) Q-hSKI-1-132–142, Abz-VFRSLKAESD-Y(NO2)-A; (XI) Q-hSKI-1-134–142, Abz-VFRSLKAESD-Y(NO2)-A; (XII) Q-hSKI-1-138–142, Abz-VFRSLKAESD-Y(NO2)-A. Except for the first two peptides, which were purchased from the Sheldon Biotechnology Institute (McGill University, Quebec, Canada), all other peptides were synthesized with the C terminus in the amide form. Peptides III–XI were prepared on a solid phase peptide synthesizer (Pioneer model, PE Biosystems) using either 2-(1H-benzo[α]zimidazol-1-yl)-1,1,3,3-tetramethylboronium hexafluorophosphate/N-hydroxybenzotriazole or O-[7-azabenzotriazol-1-yl]-1H,1,2,4-triazole-3-olate (HBTU) (15). Although we managed to produce limited quantities of partially purified SKI-1 using metal chelating resins, there was insufficient enzyme to carry out full kinetic analyses. However, since the medium of WT virus-expressing (or control vector-expressing) cells produced no significant peptide hydrolysis (with the exception of peptides VIII and XI), the reaction mixtures used to assess the hydrolytic activity of recombinant VV:BTMD-SKI-1 were not further fractionated with VV:BTMD-SKI-1. Thus, the metal chelation-purified enzyme served mainly to verify that the enzyme from concentrated media behaved similarly to this form. We therefore confirmed all of the peptide cleavage sites, the SREBP-2 pH optimum, and the Ca²⁺ requirement presented below.
tetramethyluronium hexafluorophosphate/disopropyl ethyl amine-mediated Fmoc chemistry with polyamide-linker-polyethylene glycol (PALPEG) unloading resin and the standard side chain protecting groups (16). For the incorporation of the two unnatural amino acids (Abz and Y(NO2)), an extended coupling cycle was used instead of either the standard or fast cycles.

**Purification, Analysis, and Digestion of Peptide Substrates**—The crude peptides were purified by RP-HPLC using a semi-preparative Chromatographic Sciences Co. Inc. (CSC)-Exsil C18 column (0.94 × 25 cm). Monitoring at 210 nm, the peptides were eluted with a 1%/min linear gradient of aqueous 0.1% trifluoroacetic acid/CH3CN at 2 ml/min. The peptide purity and concentration were determined by quantitative amino acid analysis (16). The identity of each purified peptide was confirmed by MALDI-TOF spectrometry using the matrix α-cyano-4-hydroxycinnamic acid (Aldrich).

For digestions, each peptide was typically reacted at 37 °C with 10 µl of the concentrated enzyme preparation in a buffer consisting of 50 mM HEPES (ICN Biomedicals Inc), 50 mM MES (Sigma), and 3 mM Ca2+-acetate (pH 6.5). The digestion products were separated by RP-HPLC on a Beckman 5-µm Ultrasphere C18 column (0.46 × 25 cm) and eluted with a 1%/min linear gradient of aqueous 0.1% trifluoroacetic acid/CH3CN (5–45%) at a flow rate of 1 ml/min. The collected peptides were characterized by mass spectrometry and amino acid composition, which was also used to quantitate the amount of various substrates and products. The digestions of the quenched fluorogenic peptides were analyzed by RP-HPLC using a dual UV (210 nm) and fluorescence (excitation and emission wavelengths of 320 and 420 nm, respectively) detector (Rainin).

**pH Optimum, Calcium Dependence, and Inhibitor Profile**—The protocols used were essentially the same as reported previously (13). Stocks of the buffer described above were adjusted to pH 5.0–8.5 at 0.5-unit increments by addition of either acetic acid or sodium hydroxide. In order to investigate the calcium requirement of SKI-1, increasing concentrations of Ca2+-acetate were used ranging from 0 to 10 mM. For inhibition studies, the enzyme in the reaction buffer was preincubated with the desired agents for 30 min prior to addition of peptide II. Determinations—Following digestion reactions with increasing substrate concentrations, the products were separated by RP-HPLC. The rate of substrate hydrolysis was obtained from the integrated peak areas of the chromatograms. Values were estimated using nonlinear regression analysis (Enzfitter software; Elsevier Biosoft, Cambridge, United Kingdom) of plots of the hydrolysis rate versus the substrate concentration. For apparent inhibitor constant determinations, variable inhibitor concentrations within the range of 15–70% inhibition were used at three concentrations of peptide IV ranging from 0.6 to 3.5 times the value. For the two quenched peptides, kinetic parameters were determined as described (17).

**RESULTS**

**SKI-1 Overexpression, Purification, Biosynthesis, and Prosegment Processing**—We have previously shown that overexpression of full-length SKI-1 (FL-SKI-1) in HK293 cells results in shedding of a 98-kDa form (sSKI-1) of this enzyme into the medium (10). Based on this finding, we engineered a soluble form of SKI-1 (BTMD-SKI-1), ending at residue 997, to which we added a hexa-His sequence at the C terminus (Fig. 1A). In a comparative biosynthetic analysis, shown in Fig. 1B, LoVo cells were infected with the SKI-1 virus constructs VV:FL-SKI-1, VV:BTMD-SKI-1, and wild type virus (VV:WT). After labeling the cells for 3 h with [35S]Cys, proteins in the media were immunoprecipitated with an antisera directed against either the prosegment of SKI-1 (Ab:P) or an internal SKI-1 sequence (Ab:S). In both cases, a protein of ~14 kDa co-immunoprecipitated with the 98-kDa sSKI-1 or the 96-kDa BTMD-SKI-1 (bSKI-1, Fig. 1B) that was not seen with VV:WT infections. Since the 14-kDa standard of the SKI-1 prosegment peptide and has been shown previously to detect the SKI-1 zymogen (10), we concluded that the ~14-kDa peptide is most likely derived from the cleaved prosegment (the full-length prosegment is ~24 kDa; see below). The fact that it co-immunoprecipitated with the enzyme under denaturing conditions suggests a strong interaction between SKI-1 and this enzyme.

![In Vitro Characterization of Human SKI-1](image)

**Fig. 1.** A, schematic representation of the structure of FL-SKI-1 and its truncation mutant BTMD-SKI-1. The various SKI-1 domains depicted are, respectively, the signal peptide, pro-segment, catalytic domain, and the C-terminal region comprising a cytokine receptor/growth factor motif, a transmembrane domain, and a cytosolic tail. The positions of polypeptides used to produce SKI-1-specific antisera (Ab: P, N, and S) are also displayed. B, biosynthetic analysis of SKI-1. WT, VV:FL-SKI-1, BTMD-SKI-1 (bSKI-1) or control VV:WT-infected LoVo cells were pulse-labeled with [35S]Cys for 3 h. Media were immunoprecipitated with either Ab:S or Ab:P and then resolved by SDS-PAGE on an 8% gel, followed by autoradiography. Arrows point to the migration positions of the 100-kDa BTMD-SKI-1 (bSKI-1), the 96-kDa shed form (sSKI-1), as well as the 14-kDa presegment product. C, Western blot analysis of the overexpressed BTMD-SKI-1. Samples from VV:WT- or BTMD-SKI-1-infected BSC40 cells (left and middle panels) were processed as described under “Experimental Procedures” and run on an 8% SDS-PAGE reducing gel. Following electrophoresis to polyvinylidene difluoride membranes, protein bands were visualized via ECL detection using primary rabbit antisera Ab:S or Ab:N. Purified BTMD-SKI-1 (right panel, *) was obtained from a Ni2+ affinity resin as described under “Experimental Procedures,” then processed as described above. A mixture of Ab:S and Ab:P were used as primary antisera. Elution buffer was used as a control (CTL).
region of its prosegment. The actual stoichiometry of enzyme-to-prosegment is not clear from this experiment, since it was carried out using two different antisera and denaturing conditions. We also observed that some of the 100-kDa BTMD-SKI-1 is cleaved into a 98-kDa form similar to that found with FL-SKI-1 (Fig. 1B). This conversion is presumably carried out by endogenous “shedding enzymes” (10, 18) that can act on both SKI-1 (Fig. 1) and endogenous “shedding enzymes” (10, 18) that can act on both prosegments could still be detected. Since the fungal metabolite BFA is believed to disassemble the Golgi complex and cause the ER to fuse with the cis, medial, and trans-Golgi (but not the trans-Golgi network, TGN) (19), this result strongly implies that the initial zymogen processing of proSKI-1 occurs early along the secretory pathway. Possible locations include the ER or cis-Golgi, as previously reported (10). Moreover, further processing of the prosegment into yet smaller fragments also occurs in these organelles.

To further characterize the prosegment of SKI-1, we took advantage of a stable transfectant of FL-SKI-1 in human HK293 cells that we had made previously (10). This system has the added advantage that the possibility of VV overexpression artifacts influencing the processing of the prosegment is eliminated. Concentrated culture medium from these cells (serum-free) was purified via RP-HPLC using first a semi-preparative C4 column (data not shown) followed by an analytical C4 column (Fig. 3A). The eluted fractions were analyzed by Western blot using Ab:P (Fig. 3B). Immunoreactive peptides ranging from ~4.5 to 24 kDa were apparent. N-terminal sequencing of the very abundant ~14-kDa protein in fraction IV (Fig. 3C) revealed a major sequence starting at Gly140 of pre-proSKI-1 (10, 12). This clearly defines the signal peptidase cleavage site as LVVLLC174/GKKHLG, which is 1 aa before that predicted by signal peptidase cleavage site algorithms (10, 11). The N-terminal sequence of the ~4.5-kDa peptide (Fig. 3D) revealed that it starts at Pro145, indicating a cleavage at the sequence KYAESD142/PTRVCNPRWSQK. This fragment is most likely the product of cleavage between Asp and Pro that may be caused by the acidic conditions encountered in either RP-HPLC, Edman sequencing (20), or sample preparation for SDS-PAGE analysis (21). An unexpected benefit of this cleavage was our finding that phenylthiohydantoin (PTH)-Asn148, which occurs in the putative N-glycosylation site Asn-Glu-Thr in FL-SKI-1 was readily detected in this sequence. Thus, the predicted N-glycosylation site Asn148 within the prosegment of SKI-1 is not employed, at least in this expression system. This conclusion was also supported by the prosegment’s resistance to endo H and endo F digestion (data not shown). Of the two eukaryotic subtilases known to contain a potential N-glycosylation Asn-Glu-Thr site, i.e. kexin (22) and SKI-1 (10), it appears that at least the latter’s prosegment is not N-glycosylated. Finally, the separation of the above prosegment fragments from mature SKI-1 using RP-HPLC (Fig. 3, A and B) and non-reducing SDS-PAGE (data not shown), suggests that none of the Cys residues in the prosegment (10) are linked by disulfide bridges to the rest of the enzyme.

As a preliminary means of characterizing the SKI-1 prosegment fragments, MALDI-TOF analysis (Fig. 3E) of fraction IV from Fig. 3B was carried out. Three major molecular ions of masses 13,351, 13,518, and 13,685 Da were detected, with an expected error of ±25 Da for this mass range. Combined with the previous N-terminal sequencing results of the ~14-kDa peptide (Fig. 3C), these mass values indicate that this peptide
has heterogeneous C termini that are derived from cleavages near the sequence RKVFRSLK\textsuperscript{137}, as indicated in Fig. 3E. In fact this region contains three potential SKI-1 cleavage sites (8) with an Arg or Lys at the P4 position and an Phe, Arg, or Lys at the P1 position. Although the calculated molecular masses of 13,339, 13,496, and 13,696 for the polypeptides G\textsuperscript{17}KK—RKVF\textsuperscript{133}, G\textsuperscript{17}KK—RKVF\textsuperscript{134} and G\textsuperscript{17}KK—RKVFRSLK\textsuperscript{136}, respectively, match within experimental error (∓22 Da) the observed masses in Fig. 3E, these assignments should only be taken as a first indication (see below). Moreover, the predicted G\textsuperscript{17}KK—RKVF\textsuperscript{136} fragment does not correspond to the expected SKI-1 cleavage motif of a basic residue at the P4 position. Hence, this secreted peptide could result either from cleavage at G\textsuperscript{17}KK—RKVF\textsuperscript{136} or, more likely, at G\textsuperscript{13}KK—RKVF, followed by basic carboxypeptidase cleavage of the C-terminal Lys (23). Since we were unable to obtain consistent mass spectra of the ~4.5-kDa polypeptide that was sequenced in Fig. 3D, we could not use this technique to approximate its C terminus, which presumably corresponds to the C terminus of the processed SKI-1 pro-segment. We therefore resorted to synthetic peptide cleavage as a tool to accurately define potential prosegment cleavage sites.

Analysis of Synthetic Prosegment-derived Peptide Cleavages—Based on our detection of ~26- and 24-kDa SKI-1 prosegment products (Fig. 2), as well as on a mutagenesis study of SREBP-2 cleavage sites (8), we synthesized three SKI-1 prosegment peptides encompassing potential, C-terminal, autocatalytic cleavage sites (10, 11). All contain Arg at P4 and either Leu or Ala at P1 (peptides III, VI, and VII shown in Table I). Of these peptides containing only native sequences, the only one with detectable cleavage by SKI-1-containing concentrated medium (from either VV:BTMD-SKI-1-infected BSC40 cells or SKI-1-transfected HK293 cells) was peptide III (WHATGRHSSRLK\textsuperscript{130} RAIPR) (see Table I). No cleavages were observed when VV:WT-infected or empty vector-transfected media were used (data not shown). Metal chelation chromatography-purified enzyme further supported that this cleavage is effected by SKI-1 (Fig. 4A; peptide IV), and the products were positively identified via mass spectrometry.

Similarly, based on the mass spectrometry data in Fig. 3E, we synthesized two peptides (VIII and IX) encompassing the putative internal processing site(s) of the SKI-1 prosegment. Both were cleaved at multiple locations by SKI-1-containing concentrated medium from HK293 transfectants (data not shown). Further analysis revealed that one of these cleavages, corresponding to PQ\textsuperscript{R}KVF\textsuperscript{133} RSL, was as prevalent in empty vector-transfected HK293 medium as in SKI-1-containing medium (see Table I, peptide VIII). In contrast, the PQ\textsuperscript{R}KVF\textsuperscript{137} YAESD cleavage was only seen in SKI-1-containing medium. This cleavage was also confirmed using metal chelation chromatography-purified enzyme (Fig. 4B;
Peptides were first reacted with approximately equal quantities of BTMD-SKI-1 medium for 2–18 h as described under “Experimental Procedures.” When cleavage was not detected, a 10-fold concentrated enzyme preparation was tested. Arrow thickness is a qualitative estimate of the cleavage efficacy.

### Table I: Synthetic peptide substrates

| Peptide   | P16 | P12 | P8 | P4 | P1 | P4’ | P8’ |
|-----------|-----|-----|----|----|----|-----|-----|
| I         | KAGSRGLT | SLADTF |     |    |    |     |     |
| II        | GGAHDSQHPHS | SGRSRLS |     |    |    |     |     |
| III       | WHATGRHSSRL | RAIPR  |    |    |    |     |     |
| IV        | WHATGRHSSRL | RALE   |    |    |    |     |     |
| V         | SRRRL | RALE   |    |    |    |     |     |
| VI        | WQSSRPLRASLLSGLSG |    |    |    |    |     |
| VIIb      | RAIPIRQVA | QTLQA |    |    |    |     |
| VIIIb     | PKRFV | RSL |    |    |    |     |
| IXb,c     | PKRFVF | RSLK | YAESD |    |    |     |
| X         | Abz-VF | RSLK | YAESD |    |    |     |
| XI        | Abz-R | SLK | YAESD |    |    |     |

* No cleavage detected even with a 10-fold excess of enzyme.
* Cleavage detected but not attributable to SKI-1.
* Kinetic determinations of this peptide were not attempted due to the presence of multiple cleavages.
* Y(NO2).

### Figure 4: Processing of proSKI-1 autocatalytic prosegment candidate sequences by purified, shed SKI-1.

The proposed prosegment C-terminal mutant 17-aa peptide IV (A) and 15-aa peptide IX (B) were digested for 18 h with metal chelation chromatography-purified BTMD-SKI-1. The cleavage products were separated by RP-HPLC using a 5-μm analytical Ultrasphere C18 column (Beckman) as described under “Experimental Procedures.” The peptides contained in all but two peaks were identified by mass spectrometry. The unidentified peaks are attributable to contaminating activities seen in WT/empty vector controls.

Residual contaminating proteases in our purified SKI-1 preparations (minor bands were visible on colloidal gold-stained membranes of SKI-1 preparations). Thus, while we are confident that SKI-1 cleaves its prosegment at the C-terminal WHATGRHSSRL | RAIPR site and at the internal PQVKFVRSLK | YAESD site, our data do not allow us to rule out SKI-1-mediated cleavage at the PQVKF133 | - RSLKYAESD site.

Comparing the simple cleavage rates of the SKI-1 prosegment internal and C-terminal sites, we observed that the former was vastly superior to the latter (data not shown). We also noticed that the peptides best processed by SKI-1 contain an acidic residue at the P3’ or P4’ substrate site, whereas those that did not, appeared to be cleaved poorly or not at all (Table I). Moreover, we had previously established that SKI-1 does not cleave the fluorogenic peptides RGLT-MCA, RGLTTT-MCA, and RSVL-MCA (10), which lack P’ residues. Based on these observations, we asked if replacing the Ile and Pro residues at P3’ and P4’ of the C-terminal prosegment processing site would significantly improve the SKI-1-mediated cleavage of peptide III. Thus, we synthesized two mutants of this peptide (peptides IV and V, the latter truncated by 8 aa at the N terminus) in which the Ile and Pro residues at P3’ and P4’ were replaced by Leu and Glu, respectively. As shown in Table II, this change significantly improved the processing of these peptides, such that we were able to determine V_{max}(app)/K_{m}(app) values. The approximately 2-fold difference in these values for peptides IV and V further suggests that determinants N-terminal to the P4 position may also play a role in substrate specificity. The SKI-1 specificity of these peptide cleavages was also verified using metal chelation chromatography-purified enzyme (when VV:WT-infected or empty vector-transfected media were used, no peptide processing was observed).

In Vitro Kinetic Properties of SKI-1: Comparative Analysis of Synthetic Peptide Cleavages—In a previous report (10), sSKI-1 was shown to cleave the 32-kDa proBDNF into a 28-kDa form at the RGLT | SL sequence in vitro with a pH optimum close to neutrality. Similar to PCs (1–3), we suggested that SKI-1 might be a Ca^{2+}-dependent enzyme since the calcium ionophore A23187 inhibited the ex vivo cleavage of proBDNF (10). In order to obtain kinetic analyses of defined SKI-1 substrates, we examined a 14-aa peptide spanning the hproBDNF processing site (10), K^{50}AGSRGLT | SLADTF^{65} (peptide I) and a 27 aa hSREBP-2-related peptide (8), G^{50}GAHSDQHPHSGGS-RSVL | SFEESGSGG^{530} (peptide II). Concentrated SKI-1-containing medium (from either VV:BTMD-SKI-1-infected BSC40
cells or SKI-1-transfected HK293 cells) was reacted with these peptides at pH 6.5, followed by MALDI-TOF mass spectrometric analysis of the RP-HPLC-purified products. The expected cleavages were confirmed and did not occur using WT/empty vector-derived media (Fig. 5). Again, the metal chelation chromatography-purified enzyme generated the same products as the concentrated media (data not shown). We then demonstrated that the optimal pH and calcium concentrations for efficient cleavage of the hSREBP-2 peptide (II) are pH 6.5 and 2 mM Ca\(^{2+}\), respectively (Fig. 6). Interestingly, the pH optimum observed with the proBDNF peptide (I) is sharper than that obtained with peptide II. In the former case, the enzyme still retains about 30% of its activity at pH 5.0 and 55% of its activity at pH 8.5 (Fig. 6A). Similar results for the pH optimum of peptide II cleavage were obtained with metal chelation-purified BTMD-SKI-1 (data not shown). In contrast, however, the pH optimum of peptide IX with the purified enzyme was 8.0, with no activity detectable below pH 5.5.

A summary of the kinetic analyses of the synthetic proBDNF (peptide I) and SREBP-2 (peptide II) cleavages by SKI-1 is shown in Table II. Both peptides are cleaved at comparable kinetic efficiencies with \( V_{\text{max(app)}}/K_m(\text{app}) \) values of 0.002 and 0.004 h\(^{-1}\), respectively. In comparison, the \( V_{\text{max(app)}}/K_m(\text{app}) \) value estimated with peptide IV is 5–10-fold higher than those obtained with peptides I and II (Table II). The N-terminal truncation of peptide IV from 17 to 9 aa (peptide V, Table I) caused a 2-fold reduction in catalytic efficiency (Table II).

Table III shows the inhibitor profile of SKI-1, in which it is clear that this enzyme is quite sensitive to metal chelators such as EDTA and to the calcium chelator EGTA. In addition, the transition metals Cu\(^{2+}\) and Zn\(^{2+}\), but not Ni\(^{2+}\) or Co\(^{2+}\), inhibit the enzyme at mM concentrations. As reported using the 32-kDa proBDNF (10), assays with the synthetic SREBP-2 peptide demonstrated that the metal chelator o-phenanthroline becomes inhibitory at concentrations above 1 mM. The other non-chelator inhibitors tested had minimal or no effects on SKI-1 activity.

In order to develop a convenient \textit{in vitro} assay for SKI-1, we designed a number of internally quenched fluorogenic substrates and tested their cleavage efficacy by SKI-1. The two best peptides encompassed the processing site RSLK \( \downarrow \) within the hSKI-1 prosegment (peptides X and XI, Table I). Mass spectrometric analysis confirmed that both peptides were cleaved at the RSLK \( \downarrow \) site by shed SKI-1 derived from HK293 cell transfectants, but not by medium obtained from HK293 empty vector transfectants. This processing generated the fluorescent N-terminal peptides Ahz-VFRSLK or Ahz-RSLK, and a non-fluorescent C-terminal peptide YAEDY(NO\(_2\))-A (data not shown). Measurements of kinetic parameters demonstrated that peptides X and XI are about 20- and 200-fold better substrates than the C-terminal prosegment peptide IV (Tables II and IV), suggesting that the shorter peptide XI may be the best SKI-1 substrate tested to date. This cleavage was completely abolished in the presence of 10 mM EDTA, in agreement with the Ca\(^{2+}\) dependence of SKI-1 activity (Fig. 6B).

**SKI-1 Inhibition by Its Prosegment**—One important question remaining is whether the SKI-1 prosegment functions as an inhibitor of its enzymatic activity, analogous to the prosegments of other subtilases (3). We thus prepared prosegment constructs, designated ending near the proposed C-terminal processing site RRLIL (Fig. 4A): PS1, extending to Leu\(^{189}\); PS2, extending to Ala\(^{188}\); and PS3, extending to Leu\(^{197}\) (Fig. 7). To each C terminus we coupled a hexa-His tag. These prosegment constructs were expressed in bacteria and purified by Ni\(^{2+}\)-chelation chromatography followed by RP-HPLC (see “Experimental Procedures”). The purity of these prosegments was confirmed by SDS-PAGE/Coomassie staining (Fig. 7B) and aa analysis (data not shown). A summary of the inhibitory potency of each prosegment using peptide IV as a substrate is shown in Table V. Kinetic analysis using Dixon plots (15) indicated a competitive inhibition mechanism (data not shown). Although PS2 exhibits the best apparent inhibitory constant (\( K_{i(\text{app})} = 97 \) nM), PS3 (\( K_{i(\text{app})} = 127 \) nM) and PS1 (\( K_{i(\text{app})} = 182 \) nM) are similarly potent SKI-1 inhibitors. When PS2 was digested with carboxypeptidase B to eliminate the His tag, its inhibitory potency was not affected (data not shown), confirming that this tag is not responsible for the observed inhibition. We also tested the inhibitory activity of the RP-
HPLC-fractionated native prosegment (see Fig. 3). Only the material from fraction IV, which included the full-length 24-kDa prosegment, was inhibitory, whereas that of the others, including the 14-kDa peptide alone or in combination with smaller fragments, were not inhibitory (data not shown).

**DISCUSSION**

Limited proteolysis of inactive precursor proteins at sites marked by paired or multiple basic residues is a widespread process (1, 2). Less common is the recent finding that bioactive peptides or proteins can also be generated by limited proteolysis after either hydrophobic or small residues (3). SKI-1 represents the first mammalian member of subtilisin-like processing enzymes with such substrate specificity (10, 11). It is a widely expressed enzyme (10) that may play a crucial role in cholesterol and fatty acid metabolism (11). Due to its very recent discovery, information regarding its enzymatic properties, substrate specificity, and the function of its proregion have only begun to be addressed.

Many peptidyl hydrolases, including subtilases, possess a prodomain that acts both as an intramolecular chaperone and a highly potent inhibitor of its associated protease (24, 25). Activation of the enzyme typically requires release of the prosegment in an organelle-specific manner. For furin (26) the release occurs in the TGN, whereas for PC1 and PC2 (27) it occurs in immature secretory granules. The data presented in this report demonstrate that SKI-1 is unique among the mammalian subtilases, since both the C-terminal and internal cleavages of its prosegment occur in the ER. Hence, this enzyme does not appear to require an acidic environment for activation, assuming, by analogy with other subtilases (3), that prosegment release is the crucial step leading tozymogen activation. We propose the following sequence of events presumably leading to SKI-1 activation. 1) The signal peptide is removed in the ER by a signal peptidase cleavage at LVVLLC17,4.3 4.3 0.4 X 1.0 1.9 4.3 XI 1.0 4.3 4.3

| Peptide | \( K_m \) (µM) | \( V_{max(app)} \) (nmol/h) | \( V_{max(app)}/K_m(app) \) (h⁻¹ liter⁻¹) |
|---------|----------------|-----------------------------|----------------------------------|
| X       | 4.3            | 1.9                         | 0.4                              |
| XI      | 1.0            | 4.3                         | 4.3                              |

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

**TABLE IV**

Kinetic constants for the hydrolysis of quenched fluorogenic substrates by shed-hSKI-1

**Effect of selected protease inhibitors on BTMD-hSKI-1 activity**

Digestion reactions using BTMD-SKI-1 medium plus peptide II were carried out as described under "Experimental Procedures." The agents were preincubated with the enzyme for 30 min.

| Inhibitor | Concentration (µM) | Hydrolysis of SREBP-2 peptide% of control |
|-----------|---------------------|-----------------------------------------|
| Control   |                     | 100                                     |
| APMSF     | 1.0                 | 95                                       |
| PMSF      | 1.0                 | 85                                       |
| TPCK      | 1.0                 | 71                                       |
| TLEC      | 1.0                 | 100                                      |
| SBTI      | 0.5*                | 100                                      |
| Cystatin  | 0.01                | 100                                      |
| Antipain  | 1.0                 | 100                                      |
| Chymostatin | 1.0               | 100                                      |
| Leupeptin | 1.0                 | 100                                      |
| Pepstatin | 0.1                 | 97                                       |
| E-64      | 0.01                | 100                                      |
| O-Phenanthline | 0.05  | 135                                      |
|           | 1.0                 | 90                                       |
|           | 5.0                 | 0                                        |
| EDTA      | 10.0                | 0                                        |
| EGTA      | 10.0                | 15                                       |
| Dithiodiethiol | 10.0  | 92                                       |
| CuSO4     | 1.0                 | 0                                        |
| ZnSO4     | 1.0                 | 0                                        |
| NISO4     | 1.0                 | 93                                       |
| MgCl2     | 1.0                 | 100                                      |
| CaCl2     | 1.0                 | 100                                      |

*Concentration in mg/ml.
rupt the complex. It is only during RP-HPLC purification (Fig. 3A), in the presence of strong acids and organic solvents, that the prosegment peptides dissociate from SKI-1. These data suggest that hydrophobic interactions may be critical, as is the case for subtilisin (24, 25).

To distinguish the SKI-1 prosegment autoprocessing sites (C-terminal and internal) from several closely situated candidate sites, we employed a combination of mass spectrometry and synthetic peptide digestion. In the case of the C-terminal site, only one of three candidate peptides (III) was processed by SKI-1 (Table I), indicating that RRLRV186↓RAIP is the most likely autoprocessing site. For the internal site, preliminary mass spectrometric data suggested three distinct cleavages occurring within the sequence PQKVFRLSLEYAESD (142) (Fig. 3B). Two of the three possible sites (PQKVFR131↓RSLKYEASD and PQKVFRL131↓SLKYEASD) appeared to satisfy the proposed SKI-1 recognition motif requiring a P4 basic residue (8). The third possibility (PQKVFRL131↓KYAESD) could be considered by assuming the cleavage actually occurred at PQKVFRL137↓YAESD, followed by endogenous, basic carboxypeptidase removal of the C-terminal Lys residue (23). Assays carried out in vitro with synthetic peptides corresponding to this region of the prosegment (peptides VIII and IX) produced the same cleavage products (data not shown), but only the PQKVFRL137↓YAESD cleavage was unique to SKI-1. Thus, we propose that the aforementioned site is the most likely internal autoprocessing site, with the qualification that PQKVFRL133↓RSLKYEASD may occur to a lesser extent (see "Results" and Fig. 4B).

Other information regarding the substrate preferences of SKI-1 was obtained by replacing the P3' and P4' Ile and Pro residues of the C-terminal cleavage site peptide (III) by Leu and Glu (peptides IV and V) to create a very well processed SKI-1 substrate. While it would appear that the presence of an acidic residue at P4' significantly enhances the rate of substrate hydrolysis, it is also possible that the presence of Pro at P4' hinders efficient substrate processing. The presence of similar acidic residues at the P3' or P4' position of the two confirmed substrates of SKI-1 (peptides I and II) as well as in the prosegment internal cleavage site RRLRV186↓YAES (Table I) lends support to the first argument. In addition to these residues, others also appear to play a role in SKI-1 substrate cleavage catalysis. The peptide pairs IV/V and X/XI both point to influences of positions N-terminal to the P4 residue. Interestingly, the efficiency of the truncated C-terminal peptide V is lower than that of peptide IV, whereas that of the truncated internal (quenched) peptide XI is higher. Taken together, these data indicate the importance of aa at both the P and P' positions in SKI-1-mediated substrate hydrolysis.

The data presented in Fig. 6 indicate that SKI-1 functions most efficiently near neutral pH and at 2–3 mM Ca2+. This is in general agreement with the conditions that reportedly prevail in the ER (28, 29). However, closer examination of the data reveal that the pH optimum of SREBP-2 cleavage (peptide II, Fig. 6A) is actually 6.5, an observation that we confirmed using our purified SKI-1 preparation (data not shown). This suggests that the processing of SREBP might occur outside of the ER, perhaps in the Golgi where pH values are ~6.5 have recently been reported (30, 31). Indeed, there is now cellular evidence suggesting that SREBP cleavage may occur in the Golgi rather than in the ER (32, 33). The pH optimum of SKI-1 appears to be dependent on the substrate employed; proBDNF (10) and its related peptide (I), appear to be well cleaved even at pH 5.5, suggesting that it could cleave this (and possibly other substrates) in acidic endosome-like compartments where it was previously localized (10). On the other hand, cleavage of the internal, autocatalytic, prosegment processing site PQKVFRL137↓YAESD (Fig. 4B) is optimal at pH 8 (data not shown), implying that this event, as we concluded from our biosynthesis assays, takes place most effectively in the ER. Overall, the pH and Ca2+ profiles of SKI-1 resemble those of the constitutively secreted PCs (1, 13). The inhibitor profile of SKI-1 (Ref. 10, Table III), showing that enzymatic activity is significantly inhibited by EDTA, EGTA, and only high concentrations of o-phenanthroline, tend to discount the likelihood that SKI-1 is a transition metal-dependent protease. In fact, SKI-1 activity is inhibited by low concentrations of certain transition metals, such as Cu2+ and Zn2+.

Directed by the observation that peptides containing the primary processing site of the prosegment of PC7 are potent inhibitors of its activity, and that the C-terminal basic residues of furin and PC7 are essential for enzyme inhibition (34, 35), we assessed the inhibitory potency of three SKI-1 recombinant propeptides (Fig. 7A). All of these end at sequences near the RRLRV186↓RAIP cleavage site. Interestingly, the three
prosegments displayed comparable inhibitory potencies (Table V). Compared with proPC1 (34), pro-furin and proPC7 (35), the $K_{i\text{app}}$ values (Table V) are up to 250-fold higher. This suggests that the prosegment of SKI-1, although potentially inhibitory in vivo, may function more as a chaperone, catalyzing the productive folding of SKI-1. Indeed, since SKI-1 may be active in the ER (10, 11), whereas the PCs are not (13, 26), the lower inhibitory potency of the prosegment of SKI-1 may be adapted to the conditions prevailing in this cellular compartment. In the case of PCs, highly effective inhibition by the prosegment may be needed in order to ensure that these enzymes are activated only when they reach the TGN or secretory granules (1–3). The 14-kDa fragment, which represents the major secreted form of the prosegment, is tightly associated with SKI-1 (Fig. 1C), yet it is not inhibitory (data not shown). Accordingly, this segment may serve a chaperone-like function similar to that reported for the N-terminal 150 aa of 7B2 toward proPC2 (36, 37).

In conclusion, the present work firmly establishes that SKI-1 is a Ca$^{2+}$-dependent subtilase with a reasonably neutral pH optimum, depending on the substrate employed. We also demonstrate that SKI-1 can cleave substrates C-terminal to Thr, Leu, and Lys residues, thus providing direct, in vitro evidence that it is a candidate converting enzyme responsible for the generation of 28-kDa proBDNF (10) and SREBP-2 processing at site 1 (11). For efficient cleavage, it appears that substrates should contain a basic residue at P4 and an aliphatic one at P2 (Table I). Furthermore, aa at the P3' and P4' positions seem to exert an important discriminatory effect. The best substrate tested so far is the quenched fluorogenic substrate Abz-RSLK$^2$-DYYG$^2$-N$_2$$\text{O}_2$-Ala, thereby providing a convenient and sensitive assay for SKI-1 activity. The present data demonstrate that only the full-length SKI-1 prosegment is inhibitory. Thus, overexpression of this prosegment in cell lines may provide a novel method for inhibiting the cellular activity of this enzyme in a fashion similar to that of over-expressed profurin and proPC7 (35). Finally, it is anticipated that precursor substrates other than the sterol-regulating SREBPs (8) and the neurotrophin proBDNF (10) will be identified, thereby extending the spectrum of activity of this unique and versatile neurotrophin proBDNF (10) will be identified, thereby extending the spectrum of activity of this unique and versatile neurotrophin proBDNF (10) will be identified, thereby extending the spectrum of activity of this unique and versatile neurotrophin proBDNF (10) will be identified, thereby extending the spectrum of activity of this unique and versatile neurotrophin proBDNF (10) will be identified, thereby extending the spectrum of activity of this unique and versatile neurotrophin proBDNF (10) will be identified, thereby extending the spectrum of activity of this unique and versatile neurotrophin proBDNF (10) will be identified, thereby extending the spectrum of activity of this unique and versatile neurotrophin proBDNF (10) will be identified, thereby extending the 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