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An Internal Signal Sequence Directs Intramembrane Proteolysis of a Cellular Immunoglobulin Domain Protein*[S]

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Precursor proteolysis is a crucial mechanism for regulating protein structure and function. Signal peptidase (SP) is an enzyme with a well defined role in cleaving N-terminal signal sequences but no demonstrated function in the proteolysis of cellular precursor proteins. We provide evidence that SP mediates intraprotein cleavage of IgSF1, a large cellular Ig domain protein that is processed into two separate Ig domain proteins. In addition, our results suggest the involvement of signal peptide peptidase (SPP), an intramembrane protease, which acts on substrates that have been previously cleaved by SP. We show that IgSF1 is processed through sequential proteolysis by SP and SPP. Cleavage is directed by an internal signal sequence and generates two separate Ig domain proteins from a polytopic precursor. Our findings suggest that SP and SPP function are not restricted to N-terminal signal sequence cleavage but also contribute to the processing of cellular transmembrane proteins.

Proteolysis is one of the central mechanisms regulating tissue morphogenesis and cellular functions. Proteolytic processing underlies protein degradation and the regulated release of functionally active protein domains. Classic experiments performed in the 1970s by Blobel *et al.* (see Ref. 1 for review) showed that signal peptidase (SP),[4] an enzyme of the endoplasmic reticulum (ER), co-translationally cleaves the short leader chains that direct transmembrane and secreted proteins through the ER membrane. SP is an integral membrane protease that cleaves in the juxtamembrane region of its substrate, removing the membrane-bound signal sequence from its parent protein (2). SP recognizes small N-terminal sequences that display well characterized patterns of hydrophobicity and charge (3).

Recently, a companion enzyme to SP was identified by its ability to cleave membrane-embedded signal peptides previously processed by SP in the ER (4). This enzyme was named signal peptide peptidase (SPP) and later shown to be the founding member of a larger protease family.

Only a subset of SP substrates are also substrates for SPP; cleavage requires the presence of polar amino acids in the membrane-spanning sequence (5). The primary purpose of this intramembrane cleavage event was thought to be the clearance of signal peptides from the ER membrane, but cytoplasmic signaling functions of the released peptides have also been proposed (6, 7). Ablation of SPP in *Caenorhabditis elegans*, *Drosophila*, and zebrafish is lethal (8–10). However, the reason for the lethality is unknown, and to date no endogenous nonsignal sequence substrates for SPP cleavage have been identified.

The SPP family of enzymes represents the most recent addition to a class of structurally related intramembrane proteases, including rhomboids, the Site-2-Protease S2P, and presenilins, which mediate regulated intramembrane proteolysis, an important theme in proteolysis-dependent cell signaling (11–13). Through cleavage within transmembrane domains, these proteases trigger the release of extracellular and intracellular protein domains. Many of these fragments then exert signaling functions that can contribute to a diverse array of processes including cell differentiation, lipid metabolism, cytokine production, and the response to unfolded proteins (14–17).

SPP and the several known signal peptide peptidase-like proteins (SPPLs) localize to distinct subcellular compartments where they may target different substrates (10, 18). In contrast to presenilins and rhomboids, which cleave type I oriented transmembrane domains, SPP family proteins target type II-oriented membrane domains (15, 18, 19). For example, the transmembrane domain of the type II-oriented tumor necrosis factor α is a substrate for cleavage by SPP-like proteins SPPL2a and SPPL2b in endosomes and the plasma membrane (20, 21).

Some viruses utilize the proteolytic activity of SP and SPP in the ER for viral polypeptide biosynthesis (22–24). For example, membrane proteins of Semliki forest virus are translated as one large polyprotein precursor from which mature p62, 6k, and E1 proteins are released by SP cleavage (25). In contrast to signal peptides in cellular proteins, the SP cleavage sites are found not at the N terminus but rather internally within the polyprotein sequence. Similarly, an internal signal sequence targets the hep-

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[4] The abbreviations used are: SP, signal peptidase; SPP, signal peptide peptidase; ER, endoplasmic reticulum; SPPL, signal peptide peptidase-like protein; HA, hemagglutinin; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PNGase, peptide *N*-glycosidase; EndoH, endoglycosidase H; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TNT, transcription/translation; MOPS, 4-morpholinepropanesulfonic acid; NTD, N-terminal domain; CTD, C-terminal domain; EGFP, enhanced green fluorescent protein.

atitis C virus polyprotein to the ER (26), where the core and envelope proteins are formed by the sequential action of SP and SPP. The envelope protein E1 becomes a transmembrane protein that is targeted to the plasma membrane, whereas the core protein is released into the cytoplasmic matrix where it associates with lipid droplets (27). Despite the small size of the peptide fragment released by SPP, functional SPP cleavage is necessary for productive viral infection *in vivo* (28). Because internal cleavable signal sequences are thought to be restricted to viral polyproteins, it has been assumed that polyprotein precursors of cellular proteins cannot be similarly converted into functionally distinct proteins by SP and SPP processing.

We investigated the biosynthesis and processing of IgSF1 (p120/inhibin-binding protein), an Ig domain protein that is highly expressed in the pituitary and brain (29–32). Several previous findings highlighted potential functions of IgSF1 in cell signaling. Pituitary *Igsf1* mRNA levels are dynamically regulated during the rat estrous cycle (29), and the IgSF1 protein has been proposed to associate with transforming growth factor β receptors (33). Moreover, in recent studies we observed that IgSF1 expression was dramatically up-regulated in the developing pontocerebellar system during neuronal synapse formation (34). Sequence analysis suggested that IgSF1 is a type I transmembrane protein with an N-terminal signal sequence, followed by 11 Ig domains, a single transmembrane domain, and a short cytoplasmic tail (30–32) (see Fig. 1*A*). We show here that two additional hydrophobic domains in the center of the IgSF1 polypeptide act as stop transfer and internal signal sequences. Sequential proteolytic processing by SP and SPP generates two Ig domain proteins, each with a single transmembrane domain. Our results demonstrate that processing of internal signal sequences is not restricted to viral polyproteins and expand SP and SPP function to the generation of cellular proteins.

EXPERIMENTAL PROCEDURES

DNA Constructs and Antibody Reagents—A construct encoding full-length human IgSF1 with a triple HA tag on the C terminus was a gift of Dr. Teresa Woodruff (Northwestern University). Mouse SHPS1 and CD47 cDNAs were isolated by reverse transcription-PCR from hippocampal total RNA.[5] An amyloid precursor protein expression construct was kindly provided by Dr. Tae-Wan Kim. All of the expression constructs contained the cytomegalovirus promoter for eukaryotic expression. See the supplemental material for details on the construction of DNA vectors.

Two anti-peptide antibodies were raised for detection of IgSF1. N-terminal domain antibodies were generated in guinea pigs using a synthetic peptide representing amino acids 21–35 (SLAVESQPELWIESN), and central domain antibodies were generated against a synthetic peptide representing amino acids 559–575 (ALTEEIEIVMPTKPEL). Each peptide was synthesized with an additional cysteine at its C terminus for coupling to keyhole limpet hemocyanin, and antibodies were affinity-purified on the antigen coupled to Sulfo-link beads (Pierce). Rat anti-HA and mouse anti-Myc antibodies were obtained from Roche Applied Science and Zymed Laboratories Inc., respectively. Mouse anti-actin antibodies were from Sigma. Secondary antibodies raised in donkey were obtained from Jackson Immunoresearch.

Cell Culture, Transfection, and Tissue Preparation—Human embryonic kidney (HEK293), Chinese hamster ovary (CHO), and COS7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin (Invitrogen). Transfections were performed using Lipofectamine Plus reagent according to the manufacturer's instructions (Invitrogen).

All animal use was approved by and according to the Institutional Animal Care and Use Committee at Columbia University. IgSF1$^{-/-}$ mutant mice were bred and genotyped as described previously (35). Adult mice were sacrificed by CO_2 inhalation. Fresh pituitaries were dissected on ice and solubilized in phosphate-buffered saline (PBS) supplemented with 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 2 mM dithiothreitol, and complete protease inhibitor mixture (Roche). Protein concentration was determined by BCA assay (Pierce) prior to SDS-PAGE analysis.

Protein Analysis Methods—For deglycosylation experiments, the protein concentration of cell lysates was determined by BCA assay (Pierce). For each sample, lysate containing 20 μg of protein was denatured in SDS-containing buffer (New England Biolabs) for 10 min. The lysates were then treated with 500 units of endoglycosidase H or PNGase F (New England Biolabs) for 1–2 h at 37 °C and analyzed by Western blotting. Because the N-terminal IgSF1 fragment tends to aggregate upon boiling, the protein samples were only heated to 37 °C before analysis by SDS-PAGE.

$(ZLL)_2$-ketone and Compound E (Calbiochem) were added to cells 10–12 h before analysis at 100 μM and 10 nM, respectively. To separate SPP-cleaved and uncleaved N-terminal fragments, the protein samples were separated on 15% Tris-Tricine gels.

For pulse-chase analysis, transfected HEK293 cells were starved for 2 h in cysteine/methionine-free Dulbecco's modified Eagle's medium (Invitrogen) and then pulsed for 10 min with 14.3 μCi of ^{35}S-radiolabeled cysteine/methionine (ProMix; Amersham Biosciences). The cells were washed once and left in media supplemented with 40 μg/ml each of unlabeled cysteine and methionine. After the indicated chase times, the cells were washed once in ice-cold PBS and collected in lysis buffer (PBS, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 2 mM dithiothreitol; Complete protease inhibitor mixture). Proteins were immunoprecipitated from cleared lysates with control or anti-HA antibodies, antibody complexes were collected with protein G beads (Amersham Pharmacia Biosciences), beads were washed five times in lysis buffer, and precipitated proteins were eluted in Laemmli buffer and separated by SDS-PAGE. The proteins were visualized by autoradiography.

For immunofluorescence analysis, the cells were fixed for 10 min with phosphate-buffered 4% paraformaldehyde. The cells were permeabilized with 0.1% Triton X-100 or left unpermeabilized, blocked for 1 h with PBS containing 10% goat serum, and incubated with primary antibodies overnight at 4 °C. For detection, cross-absorbed CY2- and CY3-conjugated secondary antibodies were used (Jackson Immunoresearch),

[5] B. Chih, unpublished observations.

and the cells were imaged on a Zeiss LSM 510meta confocal microscope.

In Vitro Translocation Assays—Wild-type and 4xY mutant IgSF1, in which four residues near the SP cleavage site have been replaced by tyrosines (see supplemental material for details) were synthesized *in vitro* using 500 ng of plasmid DNA and complete amino acid mix, following the TnT coupled reticulocyte lysate system protocol (Promega). For translocation assays, canine pancreatic microsomes (Promega) were included in the reactions (1.8 μl/25-μl reaction; increasing the amount of microsomes did not increase the efficiency of translocation; data not shown). IgSF1-containing TnT lysates were deglycosylated with PNGaseF or EndoH following the manufacturer's protocol, with minor changes. Wild-type TnT-IgSF1 (with microsomes) samples were denatured at 100 °C for 10 min in the denaturation buffer provided by the manufacturer. 4xY mutant TnT-IGSF1 (with microsomes) samples were denatured at 70 °C for 20 min to reduce aggregation of the protein.

The proteins were resolved on NuPAGE 4–12% BisTris gradient gels in MOPS buffer (Invitrogen), following the manufacturer's protocol with minor alterations. Deglycosylated samples were reheated at 37 °C prior to loading onto the gels. Nondeglycosylated samples were heated at 70 °C for 10 min. The proteins were transferred to nitrocellulose membranes (30 V, 1 h), and the membranes were blocked with 5% milk in TBST (0.05% Tween in TBS). The blots were incubated overnight at 4 °C in mouse anti-HA (1:40,000, Sigma). The following day, the membranes were washed three times for 15 min in TBST and incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature (Bio-Rad; 1:3,000 in 5% milk). The membranes were washed three times for 15 min in TBST, followed by incubation in Amersham ECL Plus reagent and exposure to x-ray film (GE Healthcare).

All of the experiments described in this study were repeated at least three times. The repetitions yielded highly similar results, and the data included in the manuscript are representative for these independent experiments.

RESULTS

IgSF1 Is Subject to Internal Proteolytic Cleavage—We raised polyclonal antibodies to study the biosynthesis and processing of IgSF1. Specific antisera were obtained for two peptide epitopes: one located at the mature N terminus of the protein and one located in the presumptive center of the coding sequences. Surprisingly, affinity-purified antisera detected proteins of different molecular masses from mouse pituitary. The N-terminal domain (NTD) antibody detected a protein migrating at ~60–70 kDa, whereas the C-terminal domain (CTD) antibody detected a doublet migrating at ~130–150 kDa. These apparent molecular masses reflect the masses of glycosylated proteins and thus are larger than the predicted molecular masses as illustrated in the diagram (Fig. 1*A*). This fact will be further examined below (see Fig. 3).

Immune reactivity for both products was absent or strongly reduced in lysates of pituitary tissue from knock-out mice lacking the first (noncoding) exon of the *Igsf1* gene (35), demonstrating the specificity of the antibody detection (Fig. 1*B*). Multiple IgSF1 transcripts have been identified in pituitary (29, 35). To examine whether the NTD and CTD proteins are the products of separate transcripts or processing of a single polypeptide, we probed cell lysates of HEK293 cells transfected with an expression construct encoding the full-length human IgSF1 mRNA (Fig. 1*B*). As observed for the endogenous protein, two protein species were generated from this cDNA, strongly supporting the idea that proteolytic processing of an IgSF1 precursor gives rise to the two protein isoforms. We further probed the subcellular localization of the IgSF1 products by immunofluorescence analysis of COS7 cells transfected with the same full-length IgSF1 expression construct (Fig. 1*C*). Staining of permeabilized cells with the NTD and CTD antibodies revealed partially nonoverlapping localization of these proteins. Whereas both antibodies detected intracellular pools of

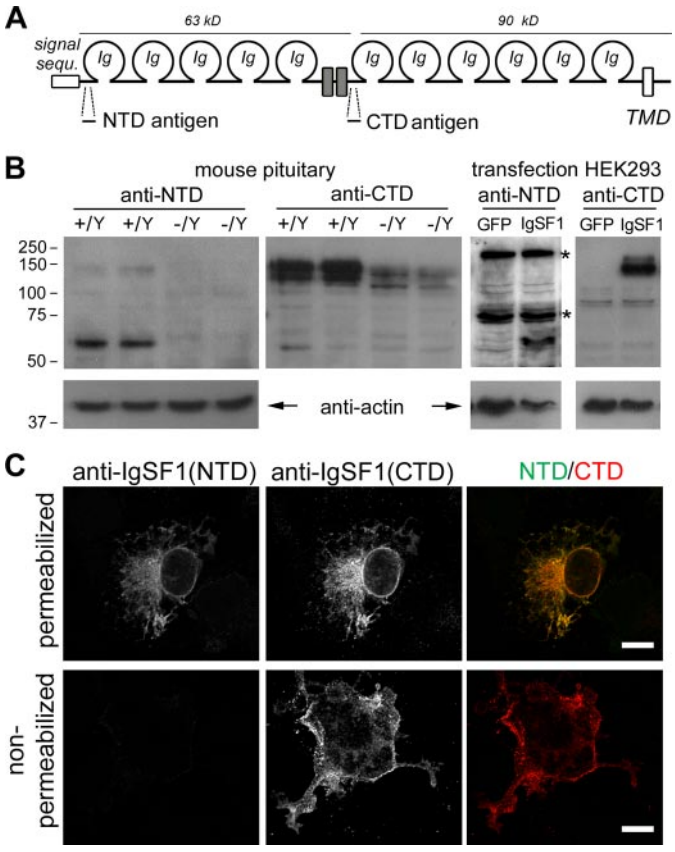

FIGURE 1. **Endogenous IgSF1 is proteolytically processed.** *A*, predicted domain organization of IgSF1. *Open circles* indicate Ig domains; *rectangles* indicate hydrophobic segments. Previous work suggested the presence of an N-terminal signal sequence and a single transmembrane domain (*TMD*). Antipeptide antibodies were generated against epitopes located at the NTD and CTD of the protein. *B*, lysates were obtained from pituitaries of adult wild-type (*+/Y*) and mutant mice (lacking exon 1 of the *Igsf1* gene located on the X chromosome (*−/Y*)). In addition, HEK293 cells transfected with EGFP (*GFP*) or with a full-length IgSF1 cDNA (IgSF1) were analyzed. Western blots were probed with affinity-purified antibodies directed against the NTD (*left*) or the CTD (*right*) of the IgSF1 protein. With the NTD antibodies, two nonspecifically cross-reacting bands are observed in the EGFP and IgSF1-transfected cells (marked with *asterisks*). Equal sample preparation and loading was confirmed by reprobing of the nitrocellulose membranes with anti-actin antibodies (*bottom*). *C*, antibodies to the NTD and CTD of IgSF1 detect protein in different cellular compartments. Permeabilized (*top*) or nonpermeabilized (*bottom*) COS7 cells transiently transfected with full-length IgSF1 expression constructs were double-labeled with guinea pig anti-NTD (*green*, *left*) and rabbit anti-CTD antibodies (*red*, *middle*). Untransfected cells showed no specific staining with either antibody. Overlays are at the *right*. *Scale bars*, 10 μm.

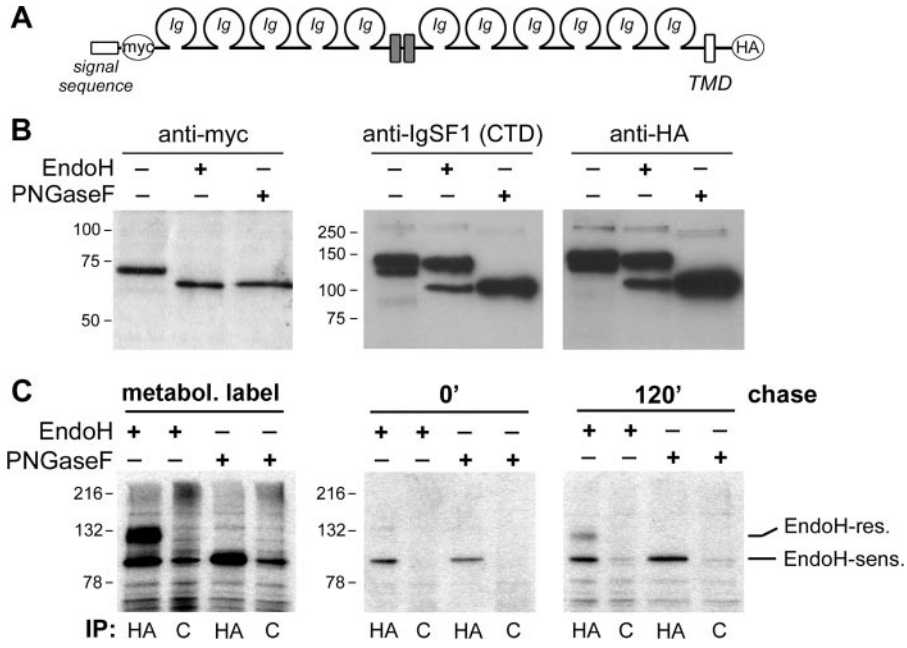

FIGURE 2. **Cleavage of IgSF1 occurs in the endoplasmic reticulum.** *A*, diagram of dually epitope-tagged IgSF1 containing an N-terminal Myc tag and a C-terminal HA tag (Myc-IgSF1-HA). *B*, lysates from HEK293 cells transiently expressing Myc-IgSF1-HA were treated with either vehicle (−), EndoH, or PNGase F. The proteins were analyzed by Western blotting with anti-IgSF1 (CTD) (*left*), anti-HA (*middle*), or anti-Myc (*right*) antibodies. Note the different molecular mass markers in the *left panel* and the *two right panels*. *C*, HEK293 cells expressing Myc-IgSF1-HA were radiolabeled overnight (*metabol. label*, *left*) or were pulse-labeled for 5 min with [^{35}S]cysteine and methionine and chased in presence of excess unlabeled cysteine and methionine for 0 or 120 min (*right*). IgSF1 protein was immunoprecipitated with anti-HA (*HA*) or control antibodies (*C*). Immunoprecipitates were treated with EndoH or PNGaseF as indicated and analyzed by autoradiography.

the protein in the ER, only the CTD antibody detected protein in nonpermeabilized cells. This suggests that the N-terminal protein product might be retained intracellularly, whereas the CTD protein is targeted to the plasma membrane.

The apparent processing of IgSF1 was further examined using a dually tagged IgSF1 cDNA (Fig. 2*A*). A Myc tag was introduced after the N-terminal signal sequence, and a triple HA epitope was inserted at the C terminus. Immunoblotting with anti-Myc and anti-HA antibodies confirmed that IgSF1 underwent proteolytic cleavage (Fig. 2*B*). The HA-tagged (C-terminal) fragment had the same molecular mass as the protein detected with the CTD antibody, whereas the Myc-tagged fragment corresponded in size to the protein detected by the NTD antibody. Taken together with the findings on the endogenous protein, this indicates that IgSF1 is cleaved into at least two fragments, one containing the C terminus and the other containing the N terminus of the original polyprotein. Interestingly, the protein fragments also differed in their carbohydrate modifications (Fig. 2*B*). A substantial portion of the HA-tagged (C-terminal) protein acquired EndoH-resistant *N*-glycans, whereas the glycans on the Myc-tagged (N-terminal) protein remained fully EndoH-sensitive consistent with its predicted retention in the ER.

The retention of the NTD in the ER (Fig. 1*C*) and the lack of EndoH-resistant carbohydrates suggested that cleavage of the IgSF1 precursor protein might occur early in the secretory pathway. To explore the kinetics of IgSF1 precursor cleavage, transfected HEK293 cells were pulsed with radiolabeled methionine and cysteine for 5 min, and IgSF1 was immunoprecipitated either with antibodies against the C-terminal HA tag or with a control antibody (Fig. 2*C*). Precipitates were treated with either EndoH or PNGase F to discriminate size variations caused by changes in the protein backbone and *N*-glycosylation. Even when proteins were precipitated immediately after the pulse (0 min), no IgSF1 precursor was recovered. After the 120-min chase, 30% of IgSF1 had acquired complex carbohydrates, indicating transport to the Golgi complex, and ~75% had acquired EndoH resistance in samples metabolically labeled overnight (Fig. 2*C*). These findings strongly suggest that proteolytic cleavage of IgSF1 occurs co-translationally or very soon after translation of the full-length protein.

IgSF1 Contains an Internal Signal Sequence—Based on the apparent molecular masses of the N- and C-terminal fragments, as well as the location of the epitope localized by the CTD antibody, we predicted that the cleavage site should be located within amino acids 400–560 of full-length IgSF1. This region contains two hydrophobic domains, the second of which bears a strong similarity to that of an N-terminal signal peptide. It contains a positively charged n-region, a central hydrophobic h-region, and a polar C-terminal c-region (predictions with Signal P 3.0; Fig. 3*A*) (3, 36, 37). To test whether this hydrophobic region was indeed capable of functioning as a signal peptide, we constructed a truncated IgSF1 expression construct (IgSF1-ΔN) where the N-terminal signal sequence, the first five Ig domains, and the first hydrophobic domain were deleted (Fig. 3*B*). When expressed in COS7 cells, IgSF1-ΔN acquired complex EndoH–resistant carbohydrate modifications and was efficiently transported to the plasma membrane (Fig. 3, *B* and *C*). This demonstrates that the second internal hydrophobic domain in IgSF1 represents a functional signal sequence when placed at the N terminus of a protein and is sufficient to direct translocation and subsequent cleavage by SP. We speculated that this sequence also might direct cleavage of the full-length protein.

The Internal Signal Sequence in IgSF1 Is Sufficient to Direct Site-specific Cleavage of Membrane Proteins—To test directly whether the internal signal sequence in IgSF1 is sufficient to confer proteolytic cleavage, we generated a series of chimeric molecules using sequences from IgSF1 and SHPS-1 (SHP substrate 1), a type I transmembrane protein with an N-terminal signal sequence, three extracellular Ig domains, and a single transmembrane domain. In the chimera SHPS1-IgSF1, the two hydrophobic segments and the adjacent c-region of IgSF1 were fused into SHPS-1 (Fig. 4*A*). As a negative control, we trans-

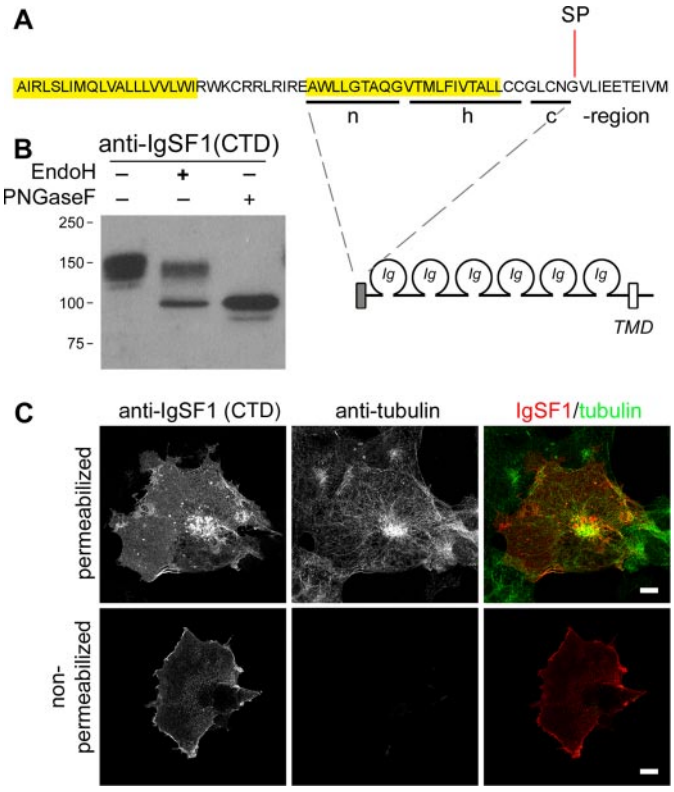

FIGURE 3. **An internal peptide sequence in IgSF1 represents a functional signal peptide.** *A*, analysis of internal IgSF1 sequences with SignalP reveals a candidate internal signal peptide. The amino acid sequences of the two internal hydrophobic domains are marked in *yellow*, predicted n-, h-, and c-regions are *underlined*, and the predicted SP cleavage site is marked. *B*, diagram of a truncated IgSF1 construct where the first five Ig domains and first hydrophobic domain were deleted, placing the predicted internal signal sequence at the N terminus. The *gray box* represents the n-, h-, and c-regions shown in *A*. HEK293 cells transiently transfected with this construct were lysed, treated with vehicle, EndoH, or PNGase F, and analyzed by Western blotting with anti-IgSF1 (CTD) antibodies. *C*, permeabilized or nonpermeabilized COS7 cells transiently transfected with the truncated IgSF1 expression construct were double-labeled with rabbit anti-IgSF1CTD (*red*) and mouse anti-tubulin antibodies (*green*). *Scale bar*, 10 μm.

planted two hydrophobic segments (a stop transfer and a start transfer sequence) of the polytopic plasma membrane protein CD47 in the same position (SHPS1-CD47). The hydrophobic domains of IgSF1 were further dissected in three additional chimeras. In CD47/IgSF1, the second hydrophobic domain of IgSF1 and the adjacent c-region were combined with the stop transfer sequence of CD47. In IgSF1Δc the c-region of IgSF1 was deleted, and in IgSF1 4xY four amino acids near the predicted SP cleavage site were mutated to tyrosines.

HEK293 cells were transfected with these constructs, and processing of their protein products was monitored using a C-terminal HA tag for detection. All of the samples were deglycosylated with PNGaseF to eliminate any size differences caused by alterations in carbohydrate modifications. Using this detection system, noncleaved proteins have an apparent molecular mass of 70–80 kDa, whereas proteolytic cleavage produces C-terminal fragments of about 55 kDa (Fig. 4*B*).

Analysis of the chimeric molecules strongly supported the idea that the internal signal sequence in IgSF1 directs site-specific protein processing. Transfer of the two hydrophobic domains and the c-region of IgSF1 into SHPS1 was sufficient to convert SHPS1 into a cleavage substrate. Whereas the first hydrophobic domain of IgSF1 could be replaced by the stop transfer sequence of CD47 without affecting processing (CD47/IgSF1), cleavage was abolished in chimeras that contained only CD47 transmembrane domains (CD47) or that lacked the c-region of the internal IgSF1 signal peptide (IgSF1Δc). Mutation of amino acids in the c-region of signal sequences has been demonstrated to block processing by SP in several proteins (3, 25). Importantly, similar mutations in the internal IgSF1 c-region strongly reduced internal cleavage (IgSF1 4xY), supporting the idea that this site is processed by SP or by a closely related enzyme.

IgSF1 Is Cleaved Internally by an ER-localized Protein—To test whether the SP cleavage site in the internal signal sequence was required for processing of full-length IgSF1, we introduced the 4xY mutation into the human Myc-IgSF1-HA expression vector. The wild-type and mutant constructs were then transfected into CHO cells, and products were analyzed by Western blotting with antibodies to the C-terminal HA epitope tag. The wild-type construct resulted in a product of ~130 kDa (Fig. 4*C*), a size similar to the endogenous protein detected with a C-terminal antibody (Fig. 2*B*). In contrast, the 4xY mutant yielded a protein of ~180–200 kDa, suggesting that the mutation of four amino acids in the internal signal sequence indeed resulted in an inhibition of precursor cleavage. Importantly, the differences in electrophoretic mobility were not due to differences in carbohydrate modifications. Deglycosylated forms of the wild-type protein had an apparent molecular mass of ~90 kDa, whereas the 4xY mutant migrated at a size of ~160kDa (Fig. 4*C*), sizes that are in good agreement with the molecular masses predicted from the amino acid sequence of the protein (Fig. 1*A*).

To further examine whether processing of IgSF1 occurs during or soon after ER translocation, we employed *in vitro* TNT assays in the presence and absence of microsomes. The products were run on SDS-PAGE and subjected to Western blotting with an anti-HA antibody. In the absence of microsomes, wild-type IgSF1 generated a protein precursor of >150 kDa (Fig. 4*D*, *lane 2*). When microsomes were included in the TNT reaction, we observed a novel band at ~130 kDa (Fig. 4*D*, *lane 3*), with equivalent mobility to the form of the protein observed in CHO cells transfected with the wild-type construct (Fig. 4*C*, *lane 2*). Importantly, treatment of the proteins from the TNT/microsome assays with EndoH or PNGaseF (Fig. 4*D*, *lanes 4* and *5*) caused the disappearance of the ~130-kDa band and the emergence of the ~90-kDa band that was observed with deglycosylated IgSF1 from transfected cells (Fig. 4*C*, *lanes 3* and *4*). These data confirm that in the presence of microsomes (and resident SP), the IgSF1 precursor (*lane 2*, ~160 kDa) can be cleaved and glycosylated, yielding a ~130-kDa protein (~90 kDa after deglycosylation).

Expression of the IgSF1 4xY mutant protein in the absence of microsomes yielded a precursor protein of similar mobility as the wild-type protein. However, inclusion of microsomes caused a significant size shift to ~180–200 kDa (Fig. 4*D*, compare *lanes 7* and *8*), consistent with the carbohydrate modification of the protein in microsomes. This protein co-migrated with IgSF1 4xY protein detected in transfected CHO cells (Fig.

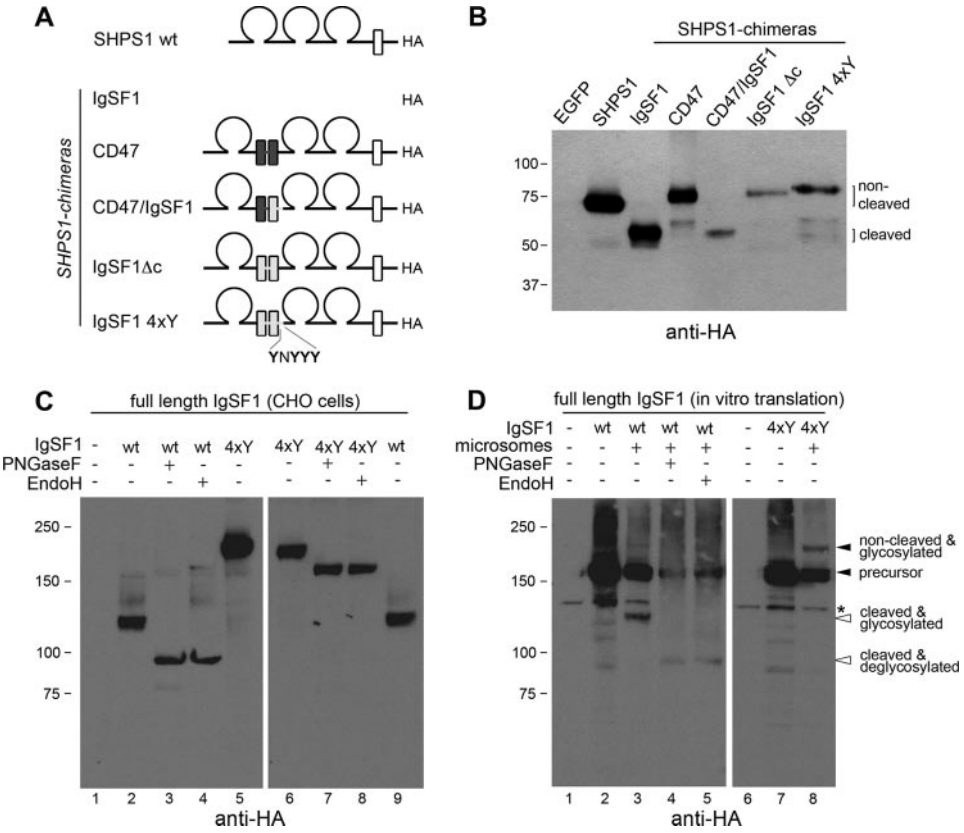

FIGURE 4. **The internal signal sequence in IgSF1 directs protein processing by a signal peptidase-like factor.** *A*, diagram of chimeric proteins. Internal hydrophobic sequences of IgSF1 (*light gray*) or CD47 (*dark gray*) were introduced into SHPS1 (*black*), a type I transmembrane protein with three extracellular Ig domains. In IgSF1Δc the two hydrophobic domains were included, but the c-region of the predicted internal signal peptidase cleavage site of IgSF1 was removed. In IgSF1 4xY four residues within the putative SP cleavage site were mutated to tyrosines. All of the constructs contained a C-terminal HA tag. *B*, HEK293 cells were transfected with cytomegalovirus-EGFP or the constructs diagrammed in *A*. All of the lysates were treated with PNGaseF and analyzed by Western blotting with anti-HA antibodies. The positions of cleaved and noncleaved products are indicated. *C*, proteins derived from transfected CHO cells were run on 4–12% gels and probed with anti-HA antibodies in Western blot analyses. Wild-type (*wt*) or 4xY mutant IgSF1 (4xY) expression vectors and deglycosylating enzymes (EndoH or PNGaseF) were included as indicated. We consistently noted lower expression from the 4xY construct in transfected cells. Therefore, 6 μg of whole cell extract from control transfected cells were run in *lane 1*, 3 μg of wild type in *lanes 2–4* and *lane 9*, and 15 μg of 4xY in *lanes 5–8*. Molecular mass markers (in kDa) are at the *left*. *D*, proteins derived from TNT assays were run on 4–12% gels and probed with anti-HA antibodies in Western blot analyses. Wild-type and 4xY mutant expression vectors, microsomes, and deglycosylating enzymes were included as indicated. *Lane 1* contains TNT lysate without DNA template. 10% (2.5 μl) of each TNT lysate was loaded in the indicated wells. The *asterisk* denotes a nonspecific band detected in TNT lysates. Molecular mass markers (in kDa) are at the *left*. The different forms of IgSF1 are marked by *arrowheads* on the *right*. Note that the protein sizes observed in the TNT assay correspond in molecular mass to the equivalent forms observed in *C* for transfected CHO cells.

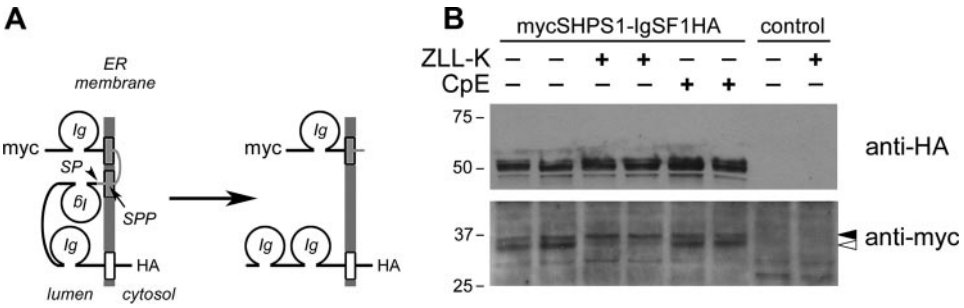

FIGURE 5. **The IgSF1 N-terminal domain is processed by SPP-like activity.** *A*, hypothetical model of sequential processing of the SHPS1-IgSF1 chimeric protein by SP and SPP. The positions of the N-terminal Myc and C-terminal HA epitopes are marked. *B*, HEK293 cells transfected with a construct encoding the Myc-SHPS1-IgSF1-HA or EGFP (control) were treated overnight with 100 μM $(ZLL)_2$-ketone, 10 nM compound E (*CpE*), or vehicle. The cells were lysed and treated with PNGaseF, and duplicate samples were separated on 15% Tris-Tricine gels and immunoblotted with anti-HA and anti-Myc antibodies to detect C-terminal and N-terminal fragments, respectively. The $(ZLL)_2$-ketone-sensitive Myc-tagged product is marked with a *white arrowhead*, and the $(ZLL)_2$-ketone-resistant form is marked with a *black arrowhead*.

4*C*, *lane 6*). These data show that the mutation (4xY) in the putative SP cleavage site inhibits proteolytic processing of full-length IgSF1. Collectively, these data support the hypothesis that IgSF1 is cleaved at an internal site by SP or an SP-like protein within the ER.

IgSF1 Is a Substrate for Intramembrane Proteolysis by SPP—Cleavage of the internal signal sequence in IgSF1 is predicted to leave the NTD of the protein attached to two transmembrane domains connected through a short cytoplasmic loop (Fig. 5*A*). Recent work on SP processing of N-terminal signal sequences and viral polypeptide precursors revealed a second intramembrane cleavage event mediated by SPP (4, 5). Interestingly, the second hydrophobic domain of IgSF1 contains several polar amino acids, similar to N-terminal signal sequences that are substrates of SPP (Fig. 3*A*). To test whether IgSF1 sequences would undergo sequential processing by SP followed by SPP, we incubated SHPS1-IgSF1 expressing cells with $(ZLL)_2$-ketone, a specific inhibitor of SPP (38, 39). As a negative control, the cells were treated with Compound E, an inhibitor of intramembrane proteolysis by the presenilin-γ-secretase complex.

Analysis of the N-terminal fragments generated by processing of SHPS1-IgSF1 revealed two specific bands that differed in apparent molecular mass by ~1 kDa (Fig. 5*B*). $(ZLL)_2$-ketone, but not Compound E, resulted in a significant loss of the smaller product, strongly supporting the idea that generation of this smaller protein product requires an SPP-like activity. However, blocking SPP activity had no visible effect on migration of the C-terminal (HA-tagged) fragment of the SHPS1-IgSF1 chimera. Similarly, we found that cleavage of full-length IgSF1 into N- and C-terminal fragments was not significantly altered by $(ZLL)_2$-ketone (supplemental Fig. S1). Interestingly, on overexposed Western blots, we could detect a small

amount of apparent full-length IgSF1 (less than 1% of the total protein), which might accumulate because of a back-up of protein in the ER membrane. This indicates that the initial cleavage step does not require SPP activity but that an SPP-like activity is required for a subsequent cleavage event in the NTD. In summary, this work reveals sequential processing of a cellular transmembrane protein by SP and SPP activities, which generates two independent Ig domain proteins from one polytopic transmembrane precursor.

DISCUSSION

This study provides evidence for a novel role for SP activity in the biosynthesis of a cellular Ig domain protein. The IgSF1 full-length transcript encodes an 11 Ig domain precursor protein that is targeted to the ER by an N-terminal signal sequence. We demonstrate that a stop transfer sequence and internal signal sequence located after the first five Ig domains then direct proteolytic cleavage of the IgSF1 polypeptide, separating the first five from the remaining six extracellular Ig domains. The internal signal sequence subsequently undergoes intramembrane proteolysis mediated by SPP or an SPP-like protein in the ER. This second cleavage event presumably releases the cleaved signal peptide into the cytoplasm, leaving the stop transfer sequence as a single transmembrane domain of the N-terminal IgSF1 isoform. This sequential enzymatic processing event ultimately converts one large polytopic membrane protein into two type I proteins that are further transported independently of each other.

A role for SP and SPP in this sequential proteolytic processing is supported by the following observations. First, immunohistochemical and carbohydrate analyses demonstrate that cleavage occurs in the ER. Second, pulse-chase analysis and *in vitro* translocation assays indicate that cleavage occurs rapidly after synthesis or co-translationally. Third, when placed at the N terminus of the central domain, the internal cleavage site of the protein acts like a functional signal sequence. Fourth, mutations that abolish SP cleavage in other proteins also abrogate internal processing of the IgSF1 precursor protein. Interestingly, the sequence of the predicted internal SP cleavage site (Fig. 3*A*) matches exactly the sequence of an IgSF1 N-terminal peptide isolated from bovine pituitary membranes (30). Chong *et al.* (30) obtained the sequence NH_2-VLIEETEIVMPTPTK-PEL-COOH by Edman degradation and assumed that this internal sequence was recovered because of nonphysiological fragmentation of the protein. Our results strongly suggest that this sequence is in fact the N terminus of the C-terminal IgSF1 product that results from internal SP cleavage.

The hypothesis that a second cleavage event is mediated by SPP is based on the fact that it occurs after the SP cleavage event and is inhibited by $(ZLL)_2$-ketone. SPPL3 is another ER SPP-like protein that is inhibited by $(ZLL)_2$-ketone (10, 40). Therefore, it is possible that SPPL3 and not SPP is involved in processing of IgSF1 or that both enzymes participate.

Although the C-terminal IgSF1 cleavage product was transported to the cell surface, the N-terminal fragment was primarily detected in the ER. ER retention might be due to slower processing of the N-terminal fragment. Alternatively, this fragment might require association with additional membrane proteins for surface transport. In this case, ER retention would reflect an inability of the protein to pass ER quality control in transfected cells. Recent studies revealed that SPP can associate with misassembled transmembrane domain sequences (41) and that SPP is involved in the dislocation of cellular proteins from the ER (42). Our analysis of IgSF1 expression in pituitary tissue demonstrates that proteolytic processing of IgSF1 is a normal step in its biogenesis *in vivo* rather than a response to misfolding, although the limited quality of our antibody reagents has so far precluded histological studies to determine the localization of the N- and C-terminal domains in the pituitary.

Our data on internal signal sequence processing of IgSF1 reveal clear parallels with intramembrane cleavage events in other cellular compartments (11). Intramembrane cleavage of type I oriented cell surface proteins such as Notch and amyloid precursor protein by presenilin/γ-secretase, as well as type II oriented sterol regulatory element-binding protein by S2P and tumor necrosis factor α by SPPL2a and SPPL2b, requires an initial cleavage by a shedding protease that removes the extracellular domain. Intramembrane proteolysis follows release of the extracellular domains and is dependent on it (5, 11, 20, 21). Similarly, full-length IgSF1 only becomes a substrate for SPP after SP cleavage of the internal signal peptide in the luminal face of the ER membrane. Consequently, the addition of the SPP inhibitor $(ZLL)_2$-ketone was not sufficient to prevent separation of the N- and C-terminal domains of IgSF1 but only inhibited the second processing step of the N-terminal fragment (Fig. 5 and supplemental Fig. S1).

The purpose of the novel cleavage event identified here remains speculative, because the biological function of IgSF-1 is poorly defined at present. The presence of the C-terminal domain at the cell surface suggests a possible function in cell-cell adhesion or signaling. The confinement of the N-terminal domain to the ER membrane in our experiments could suggest any of several possibilities. First, the N-terminal domain could, as a resident transmembrane ER protein, have ER functions. Second, it could be that, under physiological conditions, the N terminus is transported out of the ER to the cell surface or to another membrane-bound organelle. Signals that would normally mediate this process *in vivo* might be lacking in the cultured cell lines used for our studies. Both of these cases postulate an independent function of the isolated N terminus, a possibility we consider likely in view of its size, its status as a substrate for SPP family cleavage, and its stability sufficient for detection in our experiments. Either of these possibilities would reflect a novel activity of SP in processing a cellular polyprotein. It should be noted that the lack of an obvious phenotype in mice lacking upstream DNA sequences of *Igsf1* (35) implies that the function of the N-terminal protein is not required for grossly normal development and reproduction. Investigations directed toward uncovering more subtle phenotypes in these mice are ongoing.

An interesting question emerging from this work is whether internal signal sequence cleavage might be regulated in response to physiological signals. Whereas regulated cleavage of signal sequence substrates has not been described, it has been proposed that subcompartmentalization of the ER as well as differential use of signal peptides for translocation could yield functionally distinct protein pools (43, 44). In our experiments,

both for the endogenous protein *in vivo* and the protein expressed from cDNAs *in vitro*, we always observed processing of the majority of IgSF1 and did not recover a significant non-cleaved pool of full-length IgSF1. This indicates that under our experimental conditions, processing is constitutive. However, further work will be required to clarify whether regulation might occur under different conditions.

In summary, our work highlights an unexpected proteolytic processing of a cellular transmembrane protein dependent on an internal signal sequence. It is plausible that other proteins might be modified by a similar mechanism. Considering the extensive knowledge and powerful computational prediction methods for N-terminal signal sequences (36), new candidate substrates for internal signal sequence cleavage could be identified using bioinformatic approaches.

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