Signal peptide peptidase (SPP) is an intramembrane-cleaving protease identified by its cleavage of several type II membrane signal peptides after signal peptidase cleavage. Here we describe a novel, quantitative, cell-based SPP reporter assay. This assay utilizes a substrate consisting of the NH₂ terminus of the ATF6 transcription factor fused to a transmembrane domain susceptible to SPP cleavage in vitro. In cells, cleavage of the substrate releases ATF6 from the membrane. This cleavage can be monitored by detection of an epitope that is unmasked in the cleaved substrate or by luciferase activity induced by the cleaved ATF6 substrate binding to and activating an ATF6 luciferase reporter construct. Using this assay we show that (i) SPP is the first asparpatal intramembrane-cleaving protease whose activity increases proportionally to its overexpression and (ii) selectivity of various SPP and γ-secretase inhibitors can be rapidly evaluated. Because this assay was designed based on data suggesting that SPP has an orientation distinct from presenilin and cleaves type II membrane proteins, we determined whether the segment of SPP located between the two presumptive catalytic aspartates was in the lumen or cytoplasm. Using site-directed mutagenesis to insert an N-linked glycosylation site we show that a portion of this region is present in the lumen and cytoplasm. These data provide strong evidence that although the SPP and presenilin active sites have some similarities, their presumptive catalytic domains are inverted. This assay should prove useful for additional functional studies of SPP as well as evaluation of SPP and γ-secretase inhibitors.

SPP was originally identified as a ~45-kDa N-linked glycoprotein using an inhibitor-labeling approach (1). We have found that SPP exists primarily as a stable ~95-kDa homodimer within cells and that this dimer can be labeled by an active site-directed γ-secretase inhibitor (3). Another study has shown that γ-secretase inhibitors can inhibit SPP activity (4). Collectively these data suggest the active sites of SPP and PS/γ-secretase are similar. This similarity raises concerns regarding the specificity of both SPP and γ-secretase inhibitors. Although in vitro assays for γ-secretase have been developed, cell-based assays of γ-secretase activity are most widely used to monitor its activity and the effects of inhibitors. To date, the vast majority of work characterizing SPP cleavage has utilized an in vitro assay (1, 4, 5, 8). Here we describe the development of a sensitive cell-based reporter assay for SPP cleavage. This assay enables rapid evaluation of protease inhibitors and their...
SPP contains seven presumptive transmembrane domains with the NH$_2$ terminus in the lumen (1, 3). SPP contains the naturally occurring RIR sequence immediately after the transmembrane domain. SPP is conserved aspartates present in adjacent transmembrane domains (4).

**Materials and Methods**

**DNA Constructs**—Mutant SPP constructs used in the glycosylation studies were constructed by PCR-based mutagenesis. Expression plasmids encoding SPP substrates (SPP$_{ct}$) were created in two steps. A DNA encoding the fragment of the cytomegalovirus UL40 (gpUL40) reported to be necessary for SPP cleavage (5) was cloned by annealing eight overlapping oligonucleotide probes and then amplifying the full-length product with flanking primers. The resulting DNA was then digested and cloned into the pAG3 expression vector between HindIII and XhoI (3). PS1 was detected with anti-PS1 NH$_2$ terminus (852B3) at 1:1000. Anti-V5 (Invitrogen) and anti-FLAG (Sigma) antibodies were used at 1:1000. Anti-ATF6 antibody (Imgenex) was raised to the ATP6 DNA binding region coupled to the luciferase gene promoter. b, three potential SPP substrates (SPP$_{ct}$) were designed. Each substrate contains amino acids 1–373 of ATF6 at its NH$_2$ terminus fused to a fragment of gpUL40 that includes a transmembrane signal peptide sequence and a V5 His tag. SPP$_{ct}$ differs from SPP$_{ct}$ in that it contains an additional 15 amino acids at its COOH terminus that encodes a signal peptidase cleavage site. SPP$_{ct}$ differs from SPP$_{ct}$ in that it contains the naturally occurring RIR sequence immediately after the transmembrane domain. c, Western blot analysis of SPP$_{ct}$ expression. SPP$_{ct}$, are expressed transiently as ~50-kDa proteins in HEK 293T. Bands are immunoreactive for anti-SPP (shown) and anti-V5 (not shown). WT, wild type.

**SPP Reporter Assay**—The luciferase reporter assays were performed by transiently transfecting HEK 293T cells. HEK 293T cells were plated at 70% confluency and transiently transfected using 100 µl of serum-free Opti-MEM® (Invitrogen), 8 µl of FuGENE, 0.02 µg of pRl-SV40 Renilla expression plasmid (Promega), 0.25 µg of pGL3 5’-ATF6 reporter plasmid, 0.25 µg of pAG3 SPP$_{ct}$ plasmid, and the indicated amount of an SPP expression plasmid or control plasmid to total 2 µg of DNA in each well of a 12-well plate. Cells were incubated with the transfection reagent for 6–12 h, after which the serum-deficient medium was replaced with Dulbecco’s modified Eagle’s supplemented with 8% normal calf serum (Cambrex), 2% fetal bovine serum (Hyclone). In the experiments where inhibitors were tested, the inhibitors were added after the initial removal of the transfection reagent, and the cells were harvested 6–8 h later by lysis with passive lysis buffer (Promega). Firefly and Renilla luciferase activities were measured using the Dual-luciferase® kit (Promega) and a Veritas microplate luminometer (Turner Biosystems) with Veritas 2.0.40 software package. Transfections were performed in triplicate. Results were normalized to the Renilla luciferase activity control. In some experiments, where substrate was analyzed by Western blotting, transfections were modified such that 2 µg of SPP$_{ct}$ and 0.2 µg of SPP$_{ct}$/FLAG plasmids were used.

**Antibodies and Western Blotting**—The anti-SPP$_{ct}$ and anti-SPP$_{ct}$ antibodies have been described previously and were used at a dilution of 1:1000 (3). PS1 was detected with anti-PS1 NH$_2$ terminus (52BS3) at 1:1000. Anti-V5 (Invitrogen) and anti-FLAG (Sigma) antibodies were used at 1:1000. The anti-ATF6 antibody (Imgenex) was raised to the first 273 amino acids and recognized the NH$_2$-terminal, pre-transmembrane portion of our substrate. This antibody was used at 1:250. Anti-β-actin antibody (Sigma) was used at 1:750.

**Subcellular Fractionation**—HEK 293T cells were transiently transfected using a calcium phosphate transfection method (29). 45 µg of SPP$_{ct}$ plasmid and 5 µg of SPP$_{ct}$/FLAG plasmid were added to each 15-cm plate. Cells were lysed in 150 mM sodium carbonate, pH 11.0, by nitrogen cavitation for 1 h. The nuclei were then spun down at 1000 × g for 10 min. The supernatant was spun for 1 h at 300,000 × g, and the supernatant (S2)-containing soluble proteins and the pellet (P2)-con-
Glycosylation Experiments—HEK 293T cells were transiently transfected with either plasmids encoding SPP CTV5his or glycosylation mutant and FuGENE as described above. Cells were lysed after 48 h in 300 μl of 1% Triton X-100 in TBS and 1/100 complete protease inhibitor (Roche Applied Science). 20-μl lysate samples were treated with or without peptide N-glycosidase F (New England Biolabs) according to the manufacturer’s instructions. Samples were then analyzed by SDS-PAGE.

Data Analysis—Data were analyzed using Sigma Stat. For comparison of multiple experimental values relative to controls an analysis of variance was performed using a Dunnet’s post hoc t test. Variance is reported as the S.E.

RESULTS

SPP Reporter Assay Development—Potential SPP substrates were created by fusing the NH2 terminus of human ATF6 (amino acids 1-373) with a region of cytomegalovirus gpUL40 (Fig. 1, a and b). The gpUL40 sequences include the transmembrane domain and flanking residues. A contiguous V5 and six-histidine epitope tag was inserted within the cytoplasmic region of gpUL40. Three substrates that differ at their COOH termini were generated. The first, SPPsub1, contains two point mutations in the gpUL40 (T-I-T) that were shown to be necessary for cleavage by SPP in vitro (5). The second, SPPsub2, is identical to the first except it has a 15-residue extension at its COOH terminus that includes a signal peptidase cleavage site. The third, SPPsub3, uses the wild type gpUL40 (RIR) sequence instead of the mutant T-I-T sequence. This substrate was constructed because the wild type gpUL40 was refractory to SPP cleavage in vitro (Fig. 1b) (5). After transient transfection each substrate can be detected just above the 50-kDa marker by Western blotting with an antibody to the NH2 terminus of ATF6 (Fig. 1c) or anti-V5 antibody (not shown).

SPPsub Activity Increases with Increasing SPP Concentration—To determine whether SPPsub1 could be cleaved by SPP and activate the ATF6 luciferase reporter construct (Fig. 1a), we examined whether increased expression of SPP would increase the luciferase expression. For these studies, we transiently transfected 293T cells with a constant amount of the SPPsub1, 5′ATF6 luciferase reporter, and Renilla expression constructs. Varying amounts of empty vector or a SPPNTFLAG expression vector were cotransfected such that the total amount of DNA in each transfection was constant. Luciferase activity was normalized to Renilla activity to control for transfection efficiency, and activity was expressed as the percent of
normalized luciferase activity relative to the amount of activity seen in cells expressing endogenous levels of SPP (SPP activity % endogenous). Transfection of increasing amounts of the SP-PNTFLAG expression vector resulted in a marked increase in \( \sim \)95-kDa SPP dimer up to 100-fold higher than the level of endogenous SPP (Fig. 2a). A small amount of monomer at \( \sim \)45 kDa was observed at higher levels of SPP expression. Because SPP dimer levels increased at low SPP expression levels, a corresponding linear increase in luciferase activity (expressed as percent of endogenous SPP levels) was also observed (Fig. 2b). At the highest SPP dimer expression level, there was a clear deviation from linearity. The fitted line corresponds to best fit of the data to a rectangular hyperbola, which appears linear at the points corresponding to low SPP overexpression levels, consistent with the saturation behavior of \textit{in vivo} enzyme systems. Because only the relative SPP levels and activity were plotted, the fitted values do not have physical meaning and were, therefore, not included. The effects of increasing SPP dimer expression on the SPP$_{\text{sub1}}$ were directly examined by Western blotting with anti-V5 and anti-ATF6 antibodies (Fig. 1c). The level of SPP$_{\text{sub1}}$ detected by anti-ATF6 did not change significantly with increasing amounts of SPP expression. However, as SPP levels and luciferase activity increased, the amount of SPP$_{\text{sub1}}$ detected by anti-V5 increased dramatically (Fig. 2c and d). Based on this observation we postulated that the V5 epitope was inaccessible in the intact substrate but unmasked by SPP cleavage. As SPP cleavage would release the cytoplasmic, NH$_2$-terminal domain of SPP$_{\text{sub1}}$ from the membrane, we examined whether the anti-V5 immunoreactive material was soluble or membrane-bound. Anti-V5 immunoreactive SPP$_{\text{sub1}}$ was almost exclusively present in the soluble (S2) fraction, whereas the ATF6-immunoreactive SPP$_{\text{sub1}}$ was equally distributed between the soluble (S2) fraction and the integral membrane protein (P2) fraction. PS1 and SPP, which are integral membrane proteins, were almost exclusively localized in the P2 fraction. Thus, it appears that release of SPP$_{\text{sub1}}$
from the membrane unmasks the V5 epitope, indicating that the unmasking of the V5 epitope is a good indicator for cleavage and solubilization of SPPsub1.

SPPsub1 Is Not Cleaved by PS1—To further demonstrate that the SPP-dependent increase in reporter activity and unmasking of the V5 epitope of SPPsub1 was a specific and sensitive indicator of SPP activity, we performed a number of additional studies. First, we tested for specificity by determining whether PS1, another aspartyl intramembrane-cleaving protease, increased luciferase activity in this assay. Neither wild type PS1 nor a FAD mutant, M139V, altered luciferase activity compared to endogenous SPP levels (Fig. 3a), despite increased PS1 expression levels (Fig. 3b). In addition to the SPPNTFLAG construct, we also examined two additional SPP constructs, a wild type SPP expression construct and a V5 His COOH-terminal-tagged SPP construct (SPPPVT5his). All three constructs resulted in significant increases in luciferase activity (Fig. 3a).

**Fig. 3. Assay validation.** a, three different constructs of SPP increase SPP reporter assay activity to 5–10-fold above endogenous SPP activity. Neither PS1 nor PS1 FAD mutant, M139V, show any increase in SPP reporter assay activity. *, p < 0.05 analysis of variance. b, SPP overexpression was confirmed by SDS-PAGE analyses, which were probed by both anti-SPPNT and anti-SPPCT antibodies, and in the case of the tagged SPP constructs anti-FLAG and anti-V5, antibodies were used as indicated. wt, wild type. PS1 Western blot analysis was performed using anti-PS1 NTF (852B3). c, SPP inhibitor (Z-LL)2 ketone and two other γ-secretase inhibitors (III-31-C and LY411,575) inhibit SPP reporter assay activity when SPPNTFLAG is transiently overexpressed. As seen in the in vitro assay system (1), compound E and DAPT do not inhibit SPP reporter activity. d and e, the amount of SPPsub1 detectable with anti-V5 decreases with increasing SPP inhibitors (Z-LL)2, ketone and LY411,575. However, a relatively constant amount of anti-ATF6 detectable SPPsub1 is detected in each experiment. Quantification of the amount of anti-V5 detectable SPPsub1 shows the decrease in the anti-V5 signal as a result of inhibition by (Z-LL)2 ketone or LY411,575. f, each of the SPPsub1 is active in the presence of endogenous SPP (1), and a significant increase in reporter activity is observed with each when SPPNTFLAG is overexpressed (2). Additionally, the activity of each substrate is inhibitable by LY411,575 and (Z-LL)2 ketone (3). SPP activity for each sample is plotted as the % greater than the background activity or “% control.”
Effects of SPP and γ-Secretase Inhibitors—Having demonstrated that the SPP$_{\text{sub}1}$ reporter luciferase activity is proportional to SPP expression but not altered by two other family members, we evaluated several protease inhibitors for their effect on reporter activity. Inhibition of activity was assessed in cells expressing endogenous SPP and cells transiently overexpressing SPP$_{\text{NTFLAG}}$ (Fig. 3, c and d). Similar results were seen for both conditions. An SPP selective inhibitor (Z-LL)$_2$ ketone and a γ-secretase inhibitor, LY411,575, previously shown to inhibit SPP (4) inhibited reporter activity with $IC_{50}$ values of $\sim 0.14 \text{ M}$ for (Z-LL)$_2$ ketone and $\sim 0.73 \text{ M}$ for LY411,575 (Fig. 3c). III-31-C, a transition state γ-secretase inhibitor (30), inhibited reporter activity with an $IC_{50}$ of $\sim 3 \text{ M}$. This compound is the parent compound to III-63, which we have shown binds the SPP homodimer (3). Consistent with studies using in vitro SPP cleavage assays (4), two other γ-secretase inhibitors, DAPT and Compound E, did not inhibit reporter activity (Fig. 3c). The effects of (Z-LL)$_2$ ketone and LY411,575 on SPP$_{\text{sub}1}$ were also assessed by Western blotting (Fig. 3d). Both compounds significantly inhibited the unmasking of the V5 epitope, providing further evidence that the V5 epitope is unmasked by SPP cleavage. In numerous experiments, cells either expressing endogenous SPP or transiently overexpressing SPP$_{\text{NTFLAG}}$, the maximal inhibition observed of luciferase activity, was $\sim 80\%$ of the uninhibited activity.

Substrates with a Signal Peptidase Cleavage Site or Positively Charged Residues Flanking the Hydrophobic Domain Are Efficiently Cleaved by SPP in Cells—We examined cleavage of two variant substrates in the reporter assays (Fig. 3f). SPP$_{\text{sub}2}$, which includes a signal peptidase cleavage site, was an efficiently cleaved substrate. SPP$_{\text{sub}3}$ reporter activity increased upon transient overexpression of SPP, and this activity was inhibited to a similar extent as SPP$_{\text{sub}1}$ by the SPP inhibitors (Z-LL)$_2$ ketone and LY411,575. Somewhat unexpectedly, the SPP$_{\text{sub}3}$, which contains the naturally occurring RIR amino acid sequence in the COOH terminus that prevented cleavage by SPP in an in vitro assay (5), also appears to be efficiently cleaved in vivo. SPP$_{\text{sub}3}$ reporter activity is increased by SPP overexpression and is inhibitable by SPP inhibitors.

An N-linked Glycosylation Site SPP Mutant Is Active and Reveals That SPP Has a Topology Consistent with Cleavage of Type II Membrane Proteins—It has been postulated that the orientation of the opposing transmembrane domains containing the two presumptive catalytic aspartates is inverted in SPP relative to PS. The evidence for this is indirect and comes from two observations. First, the identified PS substrates are type I membrane proteins, whereas identified SPP substrates including the ATF6 fusion protein substrates used in this study are type II membrane proteins. Second, studies of N-linked glycosylation sites place the NH$_2$ terminus of SPP in the lumen (1). This finding coupled with a seven transmembrane domain
model suggested that the transmembrane domains containing the critical aspartates were oriented such that the intervening sequence would be present in the lumen (Fig. 4a) and not in the cytoplasm. To experimentally determine whether the loop between the catalytic aspartates was luminal or cytoplasmic, we generated a number of SPP mutants designed to delete endogenous or add exogenous N-linked glycosylation sites. The mutants used are shown in Fig. 4a and are numbered 1–5. Mutant 1 (N10S, N20S) is not glycosylated (Fig. 4b and c) and confirms the luminal location of the NH₂ terminus of SPP (1). The lack of glycosylation of this mutant also demonstrates that two additional endogenous N-linked glycosylation sites, one in the NH₂ terminus and one in the loop region immediately preceding the first aspartate (TMD4), are not utilized (Fig. 4a).

Four additional mutants (2–5) were then generated on the unglycosylated mutant 1 background (Fig. 4a). Mutants 2 and 5 migrated identically to mutant 1, whereas mutants 3 and 4 migrated at a higher molecular weight (Fig. 4b), suggesting that they were glycosylated. There were consistent differences in the extent of glycosylation of mutants 3 and 4. The majority of SPP mutant 3 appeared to be glycosylated, whereas only a fraction of mutant 4 was glycosylated. To confirm that the shift in M₀ was due to glycosylation, cell lysates were treated with peptide N-glycosidase F before electrophoresis and Western blotting. Both SPP CT5his and mutant 3 migrate at an identical M₀ to mutant 1 after peptide N-glycosidase F treatment (Fig. 4c). Because the SPP dimer migrates over a broader range, we focused these studies on the monomeric forms. However, all mutants did form dimers and showed changes in migration consistent with the alterations in glycosylation inferred from the study of monomer (Fig. 4b). The mutants were then evaluated in our reporter assay to ensure that they were active. As shown in Fig. 4d, all of these mutants are active, significantly increasing luciferase activity relative to cells expressing endogenous SPP.

**DISCUSSION**

We have developed a cell-based reporter assay for SPP utilizing the NH₂-terminal 373 amino acids of ATF6 fused to the SPP-cleavable transmembrane domain of gpUL40. The SPP reporter activity assay provides an excellent read-out of SPP activity. Luciferase activity increases proportional to SPP dimer overexpression and is inhibited by protease inhibitors previously shown to inhibit SPP activity. Two of the inhibitors
that decrease reporter activity, III-31-C and (Z-LL)_2 ketone, have been previously shown to effectively displace the binding of III-63, a photoaffinity probe that binds the SPP dimer, indicating that these inhibitors directly target the SPP dimer (3). Thus, these data provide additional evidence that the SPP dimer is the active form of SPP in cells. Overexpression of PS1, another member of the aspartyl intramembrane-cleaving protease family, did not increase reporter activity. Thus, under conditions where SPP is overexpressed, we can conclude that the majority of reporter activity is attributable to SPP cleavage of the substrate. However, it is possible that other uncharacterized members of the PS/SPP family could exhibit activity in this assay.

The studies herein further reinforce the differences between PSs and SPP. PSs are the presumptive catalytic component of a multiprotein complex that requires at least three additional factors, Aph-1, Pen-2, and Nicastrin (26). Reconstitution of γ-secretase activity in yeast requires all four components (24, 27). Although small increases in γ-secretase activity can sometimes be observed upon overexpression of individual components of the γ-secretase complex, large increases in activity in mammalian cells are not observed unless all four components are overexpressed (22–27, 31, 32). In contrast, no co-factors are necessary to reconstitute SPP activity in yeast, and (1) as shown herein, SPP activity increases proportionally over a wide range of SPP dimer levels in cells.

It has been proposed that topological differences between PS and SPP account for their respective preference for cleaving type I membrane proteins and type II membrane proteins (1, 13, 19). Although the precise topology of PS is still debated, there is a general consensus that the loop between the critical aspartate residues has a cytoplasmic localization. Here we provide evidence based on glycosylation site mutants of SPP that the corresponding region of SPP is in fact luminal. Moreover, we show that these SPP mutants are functionally active. Thus, we provide strong evidence that, as hypothesized, the active sites of SPP and PS are inverted within the membrane. Clearly, additional structural studies will be required to more precisely characterize the topology of SPP.

Although a number of results obtained with our assay correlate well with previous observations using in vitro assays to characterize SPP cleavage, we find that the substrate, SPPsub3, which contains a sequence resistant to SPP cleavage in vitro (5), is cleaved in cells by SPP. Such data suggest that the requirements for in vivo and in vitro cleavage may be distinct. Additional studies cross-comparing cleavage of various substrates will be required to resolve such discrepancies.

Although the precise SPP cleavage site or sites have not been determined for any substrate, it is clear that SPP cleavage releases the cytoplasmic domain of the target protein from the membrane. In this reporter assay, cleavage of the various SPP substrates releases the ATF6 transcription factor so that it can translocate to the nucleus and activate the luciferase reporter construct. We find that an internal V5 epitope tag appears to differ by 11 amino acids, masked in the intact substrate, but once cleaved and solubilized, the V5 tag becomes accessible. The cleaved and uncleaved SPPsub1 are predicted to differ by 11 amino acids, making it difficult to differentiate them after electrophoresis. Thus, the unmasking of the V5 epitope associated with increased cleavage and solubilization of substrate appears to be an excellent surrogate indicator for substrate cleavage. Because this assay utilizes a reporter construct that could be activated by induction of the unfolded protein response with cleavage of endogenous ATF6 by site-one and site-two proteases, both methods for assessing SPPsub cleavage should be utilized to ensure that substrate proteolysis is being monitored.

Both SPP and PS are potential therapeutic targets for human diseases. Preclinical studies in mouse models suggest that if toxicity can be minimized, inhibition of Aβ production by γ-secretase inhibitors may be an effective therapy for Alzheimer’s disease (33–36). To date at least two γ-secretase inhibitors have entered initial phase I trials for Alzheimer’s disease. Because of their role in altering cell-signaling cascades, γ-secretase inhibitors are also being explored as immune modulatory agents and as novel anti-cancer agents. In contrast to γ-secretase, SPP is a theoretical therapeutic target. Based on its known substrates, major histocompatibility complex class I, and hepatitis C virus polyproteins, SPP inhibitors could have therapeutic utility as antiviral agents or immune modulatory agents. It is clear from this and other published studies that some γ-secretase inhibitors inhibit SPP (4). Clear separation of inhibitory activities would be desirable for both SPP and γ-secretase inhibitors that are intended for clinical use. The cell-based reporter assay we have developed should prove useful in the evaluation and development of both γ-secretase and SPP selective inhibitors.

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SPP Reporter Assay