Competitive Inhibition of the Endoplasmic Reticulum Signal Peptidase by Non-cleavable Mutant Preprotein Cargos*

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Background: Signal peptidase (SPase) excises the signal peptide of secretory precursors. Results: A variant preproinsulin with a proline following the signal peptide cleavage site binds to and inhibits SPase.

Conclusion: Inhibition of SPase impairs, in trans, the intracellular processing, trafficking, and maturation of secretory proteins and viral polypeptides.

Significance: Our findings suggest eukaryotic SPase as a potential antiviral target.

Upon translocation across the endoplasmic reticulum (ER) membrane, secretory proteins are proteolytically processed to remove their signal peptide by signal peptidase (SPase). This process is critical for subsequent folding, intracellular trafficking, and maturation of secretory proteins. Prokaryotic SPase has been shown to be a promising antibiotic target. In contrast, to date, no eukaryotic SPase inhibitors have been reported. Here we report that introducing a proline immediately following the natural signal peptide cleavage site not only blocks preprotein cleavage but also, in trans, impairs the processing and maturation of co-expressed preproteins in the ER. Specifically, we find that a variant preproinsulin, pPI-F25P, is translocated across the ER membrane, where it binds to the catalytic SPase subunit SEC11A, inhibiting SPase activity in a dose-dependent manner. Similar findings were obtained with an analogous variant of preproparathyroid hormone, demonstrating that inhibition of the SPase does not depend strictly on the sequence or structure of the downstream mature protein. We further show that inhibiting SPase in the ER impairs intracellular processing of viral polypeptides and their subsequent maturation. These observations suggest that eukaryotic SPases (including the human ortholog) are, in principle, suitable therapeutic targets for antiviral drug design.

Most secreted proteins are synthesized with an amino-terminal signal peptide (SP)6 that directs the newly synthesized polypeptide to and across the cell membrane of prokaryotic cells or the endoplasmic reticulum (ER) membrane of eukaryotic cells (1,2). Many SPs are ~20–30 amino acids in length, with similar structural features: an amino-terminal positively charged “N region,” a central hydrophobic “H region,” and a carboxy-terminal polar “C region” (3). In prokaryotic cells, upon secretory protein translocation across the cell membrane, the SP is excised by the highly specific, membrane bound, type I signal peptidase (SPase I), which is essential for bacterial growth and viability and is a promising antibiotic target (4–7). Unlike prokaryotic SPase I, the mammalian ER SPase is a complex composed of five integral membrane protein subunits, designated signal peptidase complex subunit 1 (SPCS1), SPCS2, SPCS3, SPCS1A, and SEC11C (8). Their stoichiometry and individual contributions to SPase function have not yet been fully delineated. However, SEC11A and SEC11C are homologs of yeast Sec11p, containing catalytic activity that is absolutely required for SPase activity (9).

Impaired SP cleavage because of naturally occurring mutations at the −3 (P3) and −1 (P1) positions relative to the cleavage site has been shown to cause multiple human diseases (10–12), suggesting that efficient and proper SP cleavage is critical for the function of many protein-secreting cells. In addition, internal SPs are often found in viral polypeptides, including those encoded by hepatitis C virus (HCV), HIV, influenza virus, rubella virus, and mouse mammary tumor virus (MMTV). Mutations at the SP cleavage site of these polypeptides impair their processing and targeting, in turn decreasing virus production (13–19). These observations suggest that the ER SPase of host cells is required for viral packaging and/or assembly.

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6 The abbreviations used are: SP, signal peptide; ER, endoplasmic reticulum; SPase, signal peptidase; HCV, hepatitis C virus; MMTV, mouse mammary tumor virus; preProPTH, preproparathyroid hormone; pPI, preproinsulin; prePOMC, prepro-opiomelanocortin; IP, immunoprecipitation; PNGase F, peptide-N-glycosidase F; ISP, insulin signal peptide; ProIAPP, pro-islet amyloid polypeptide; PI, proinsulin.
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SPase is resistant to known protease inhibitors of the four standard classes of proteases (20, 21). Although certain types of β-lactam compounds have been found to inhibit bacterial SPase I (22, 23), to our knowledge, no inhibitor of eukaryotic ER SPase has been described. In this article, we report that variants of preproinsulin or preproparathyroid hormone (preProPTH), designed to contain a proline substitution at the +1 (P′) position relative to the SP cleavage site, bind to the catalytic subunits of human SPase and inhibit its activity in a dose-dependent manner. Furthermore, the variant preproinsulin inhibits intracellular processing of a viral structural protein (derived from HCV) and a protein that regulates the export and translation of mRNA (Rem, derived from MMTV). Therefore, expressing secretory proteins containing (P′) proline mutations not only provides a useful tool to study the intracellular consequences of defective SP processing in mammalian cells but also creates a rationale for targeting SPase in antiviral therapy.

Experimental Procedures

Materials—Rabbit anti-Myc and anti-GFP were from Immunology Consultants Laboratories. Guinea pig anti-porcine insulin was from Millipore. Monoclonal anti-HCV core clone 6G7 was a gift from Dr. Harry Greenberg (Stanford University). Zysorbin was from Zymed Laboratories Inc. 35S-labeled amino acid mixture (Met + Cys) was from ICN. DTT, protein A-agarose, anti-FLAG, and all other chemical reagents were from Sigma-Aldrich. PNGase F was from New England Biolabs. 4–12% NuPage gel, Met/Cys-deficient DMEM, and all other tissue culture reagents were from Invitrogen.

Constructs and Mutagenesis—cDNAs of FLAG-tagged human SPCS1 and SEC11A were amplified using total RNA isolated HEK293T cells by real-time PCR using a forward primer (5′ ATGCTGGAGCATGAGCTGCTCTG) and a reverse primer (5′ TCACCTATCGTGCTAATCTGTGATAC- ATTATTTGAGCATGCTCTTAAT) for SPCS1 and a forward primer (5′ ATGCTGTCTCTAGACTTTTGGGAC) and a reverse primer (5′ TTACCTATCGTGCTAATCTGTGATAC- ATTTATTTGAGCATGCTCTTAAT) for SEC11A and a forward primer (5′ ATGCTGTCTCTAGACTTTTGGGAC) and a reverse primer (5′ TTACCTATCGTGCTAATCTGTGATAC- ATTTATTTGAGCATGCTCTTAAT) for SEC11A. The cDNAs were subcloned into the pTarget vector (Promega). The cDNA of Myc-tagged human preProPTH was synthesized by gene synthesis by Integrated DNA Technologies. The cDNA of Myc-tagged human preProPTH was synthesized by gene synthesis by Integrated DNA Technologies. The cDNA was subcloned into the pTarget vector. All mutations of preproteins used in this study were generated by site-directed mutagenesis (Stratagene) using corresponding plasmids encoding human WT preproinsulin (pPI) or preProPTH as described previously (24). All resulting plasmids were confirmed by direct sequencing. The plasmid encoding FLAG-tagged human WT prepro- opiomelanocortin (prePOMC) was a gift from Drs. Anna Maria Di Blasio and Monica Mencarelli (Istituto Auxologico Italiano, Italy). The plasmid encoding Myc-tagged MMTV Rem protein was a gift from Dr. Katja Kapp (Max Planck Institute of Molecular Cell Biology and Genetics, Germany). The HCV structural proteins core, E1, and E2 were subcloned from the J6 genotype 2a HCV strain (25) downstream of the CMV promoter of plasmid pMD2.G (a gift from Didier Trono, Addgene plasmid no. 12259), replacing the original vesicular stomatitis virus G protein (VSV-G).

Cell Culture and Transfection, Metabolic Labeling, Immunoprecipitation (IP), co-IP, and Image Quantification—HEK293T cells plated onto 12-well plates 1 day before transfection were transfected with 1–2 µg of plasmids encoding WT or mutant preproteins with Lipofectamine (Invitrogen). 48 h after transfection, cells were pulse-labeled with [35S]Met/Cys for 15 min, with or without chase as indicated. The cells were lysed with lysis buffer containing 100 mM NaCl, 1% Triton X-100, 0.2% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, 25 mM Tris (pH 7.4), and protease inhibitor mixture (Roche). The cell lysates and chase medium were precleared with zysorbin for 1 h, subjected to IP with the appropriate antibodies, and analyzed by NuPage or Tris-Tricine-urea-SDS-PAGE as described previously (27, 30). For co-IP, the cells were lysed with co-IP buffer containing 100 mM NaCl, 25 mM Tris (pH 7.0), 0.1% Triton X-100, 5 mM EDTA, and protease inhibitor mixture. The lysates were split in half. One half was subjected to IP with anti-insulin, and another half was subjected to co-IP with anti-FLAG followed by analysis using NuPage. The bands were detected on a PhosphorImager and quantified using National Institutes of Health Image.

Western Blotting, Real-time PCR, and Sodium Carbonate Extraction—For Western blotting, 10–20 µg of total protein lysates was boiled in SDS sample buffer with or without 100 mM DTT, resolved by 4–12% NuPage gel, Met/Cys-deficient DMEM, and all other tissue culture reagents were from Invitrogen.

Statistical Analyses—Paired, two-tailed Student’s t tests were used to compare two samples. Analysis of variance was performed to compare multiple samples. Results are presented as mean ± S.E. p < 0.05 was considered statistically significant.

Results

A P′ Proline Impairs Preproinsulin Processing in Mammalian Cells—From a database of 1877 eukaryotic secretory protein SPs (29), we analyzed the frequency of specific amino acid residues at the P′ position. From this analysis, we observed that alanine occurred most frequently at the P′ position, whereas proline is at least 50-fold less common at this location (Fig. 1A). To examine the efficiency of SP cleavage with different residues at P′, we exploited a dozen different human preproinsulin mutants, replacing the phenylalanine at this position (Fig. 1B). Each construct was expressed individually in 293T cells, and the SP cleavage efficiency of newly synthesized
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mutant preproinsulin was analyzed. A well known diabetes-causing mutant in the P1 rather than in the P1’ position was used as a positive control for unprocessed preproinsulin (26). Although a threonine substitution at P1’ of the M13 procoat protein has been reported to interfere with SP cleavage by bacterial SPase I (31), pPI with a Thr substitution at P1’ was processed comparably with that of other functional residues (Fig. 1C). However, a proline substitution inhibited SP processing of pPI-F25P to proinsulin (Fig. 1C). By contrast, neither pPI-V26P nor pPI-N27P inhibited SP processing of preproinsulin (Fig. 1C, right panel), indicating that the inhibitory effect of Pro was limited to the P1’ position.

A P1’ Proline Does Not Affect Preproinsulin Translocation across the ER Membrane—Impaired processing of pPI-F25P, shown in Fig. 1C, could represent either a cleavage defect or a defect of targeting/translocation across the ER membrane (31). An earlier study showed that the M13 procot protein with a proline substitution at the P1’ position failed to translocate across the cytoplasmic membrane. We therefore utilized two independent approaches to examine whether pPI-F25P was translocated into the ER lumen. First, we analyzed preproinsulin disulfide bond formation, which occurs in the oxidizing environment of the ER lumen, using Tris-Tricine-urea-SDS-PAGE under nonreducing and reducing conditions (27, 33). Preproinsulin WT and pPI-A24D, both of which are translocated into the ER lumen (26), were used as positive controls of oxidative folding. As shown in Fig. 2A, during a 15-min metabolic labeling, pPI-F25P underwent rapid oxidative folding, similar to pPI-A24D. Second, we introduced a predicted N-linked glycosylation site at the 74th position of pPI-F25P, generating the double mutant pPI-F25P/A74N. Indeed, both newly synthesized pPI-A24D/A74N and pPI-F25P/A74N were glycosylated, as confirmed by mobility shift after deglycosylation with peptide-N-glycosidase F (PNGase F) (Fig. 2B). These data indicate that at least 76 residues of newly synthesized pPI-F25P were accessible on the luminal side of the ER membrane. Together, the results shown in Fig. 2 indicate that a P1’ Pro substitution does not interfere with the translocation of nascent preproinsulin across the ER membrane.

pPI-F25P Blocks SP Cleavage of Co-expressed Secretory Proteins in a Dose-dependent Manner—To examine the fate of uncleaved pPI-F25P, we performed pulse-chase experiments and found that 2Myc-tagged pPI-WT folded and trafficked normally in cells (data not shown). However, both 2Myc-tagged pPI-A24D and pPI-F25P mutants failed to be secreted from cells after a 2-h chase (Fig. 2C). Remarkably, pPI-F25P, but not pPI-A24D, could also impair the SP cleavage of co-expressed preproinsulin-WT (Fig. 2C, lane 14). The unprocessed preproinsulin-WT was partially degraded during a subsequent 2-h chase but without evidence of further processing to proinsulin (Fig. 2C, lanes 15 and 16). To examine whether pPI-F25P affects the translocation of preproinsulin-WT, we co-expressed pPI-F25P with pPI-A74N and found that almost all of the pPI-A74N became glycosylated, although the SP remained unprocessed, as confirmed by deglycosylation with PNGase F (Fig. 3A, solid and open arrows indicate glyco-pPI and preproinsulin, respectively). Therefore, pPI-F25P inhibits SP cleavage of co-expressed preproinsulin-WT but does not affect its translocation.

Next we co-expressed a fixed amount of pPI-WT with increasing amounts of pPI-F25P in HEK293T cells. We found that the SP cleavage defect of preproinsulin-WT became increasingly severe as the amount of pPI-F25P was increased (Fig. 3B, quantified in C), indicating dose-dependent inhibition of preproinsulin-WT SP cleavage in trans by pPI-F25P. To establish competitive inhibition of pPI-F25P on SPase, we co-expressed a fixed amount of pPI-F25P with an increasing amount of preproinsulin-WT and found that the ratio of proinsulin/preproinsulin increased gradually (Fig. 3D), suggesting that the inhibitory effect of pPI-F25P can be overcome by an increased concentration of preproinsulin-WT substrate. These data indicate that pPI-F25P acts as a competitive inhibitor of SPase.

Inhibition of SP Cleavage in Trans by Mutants with P1’ Proline Does Not Depend on a Specific Signal Sequence or a Specific Secretory Polypeptide—First, we asked whether pPI-F25P could inhibit SP processing of other secretory preproteins that they share the same preproinsulin signal sequence. We constructed a fusion protein in which the insulin SP (ISP) was fused with a Myc-tagged pro-islet amyloid polypeptide (Pro1APP) (named ISP-Pro1APP-Myc; Fig. 4A, top panel).

When this fusion protein was co-expressed with pPI-F25P, excision of its SP was inhibited (Fig. 4, A and B). Next, we examined the effect of pPI-F25P on SP cleavage of preproteins that do not share the same signal sequence. Indeed, pPI-F25P inhibited SP cleavage of all tested secretory proteins, including the...
small unglycosylated secretory proteins preProAPP (Fig. 4, C and D) and preProPTH (Fig. 4, E and F) as well as the relatively large secretory glycoprotein prePOMC (Fig. 4G), and GFP fused with insulin SP in a dose-dependent manner (data not shown).

Because proline is the least favored residue at the P1' position (Fig. 1A), we asked whether other secretory proteins bearing Pro at the P1' position also inhibit Spase. Indeed, preProPTH-K26P was also uncleaved in 293T cells (Fig. 5A; the preProPTH-C18R hypoparathyroidism-causing mutant (34) served as a positive control for uncleaved preProPTH). In addition, preProPTH-K26P in trans also impaired SP cleavage of co-expressed proinsulin-WT (Fig. 5B). Together, these results suggest that preproteins with a Pro at the P1' position (e.g. pPI-F25P and preProPTH-K26P) may be used as general SPase inhibitors, impairing SP processing and intracellular trafficking of secretory proteins.

Uncleaved pPI-F25P Is Integrated into the ER Membrane, and Its Inhibition of Spase Depends on Its Membrane Topology—Upon translocation across the ER membrane, pPI-F25P can either remain associated with the ER membrane or be released freely into the ER lumen. To examine this, we transfected Myc-tagged pPI-F25P into HEK293T cells and performed a sodium carbonate wash to separate integral membrane proteins from lumenal and peripheral membrane proteins (35). At steady state, ~30% of pPI-F25P molecules were processed to proinsulin that was fully extracted (in parallel with binding immunoglobulin protein, an ER-lumenal protein control). In addition, a small fraction of uncleaved pPI-F25P that might be more weakly associated with the ER membrane or released gradually to the ER lumen was also extracted by carbonate. However, the majority of uncleaved pPI-F25P molecules was not extracted and was recovered from the membrane fraction in parallel with calnexin, an ER-integral membrane protein control (Fig. 6A).
All five subunits of SPase are integral membrane proteins with different membrane topologies (36, 37). Because the majority of uncleaved pPI-F25P is anchored in the ER membrane, we asked whether the inhibitory effect of pPI-F25P depended on its topology. We have reported recently that a positive charge in the N region and charge gradient flanking the H region of SP plays a critical role in orienting the SP of preproinsulin during translocation. Indeed, two SP mutations, pPI-R6C and pPI-R6C/D20R, cause ~50% and ~90%, respectively, of preproinsulin to become misoriented in the ER membrane during translocation (28). We therefore introduced F25P into pPI-R6C or pPI-R6C/D20R, generating double and triple mutants, respectively (Fig. 6B). When pPI-R6C/F25P was co-expressed with preproinsulin-WT, its inhibitory effect on the
SP cleavage of preproinsulin-WT was decreased significantly (Fig. 6C, quantified in E) compared with that of pPI-F25P, and the inhibitory effect was decreased further in the triple mutant pPI-R6C/D20R/F25P (Fig. 6D, quantified in E). These results suggest that pPI-F25P exerts its inhibitory effect on the luminal side of the ER membrane and that the inhibition requires a specific orientation of its SP.

pPI-F25P Binds to the Catalytic Subunits of SPase—Among the five subunits of the SPases, SEC11A and SEC11C are thought to be catalytic subunits of the enzyme (9). To examine...
whether pPI-F25P inhibits SPase by binding to the catalytic subunits, we co-expressed Myc-tagged pPI-F25P with the FLAG-tagged subunits SEC11A or SPCS1. The interactions of Myc-tagged pPI-F25P with SPase subunits were analyzed using co-immunoprecipitation with anti-FLAG antibody. Neither preproinsulin-WT nor pPI-F25P was pulled down by SPCS1 (Fig. 7). However, pPI-F25P was specifically co-immunoprecipitated with SEC11A (Fig. 7), and similar results were obtained with SEC11C (data not shown), suggesting that pPI-F25P inhibits SPase activity by binding to catalytic subunits of the enzyme.

**FIGURE 5.** preProPTH-K26P not only fails to be processed by SPase but also impairs SP cleavage of co-expressed pPI-WT. A, 293T cells transfected with Myc-tagged preProPTH-WT, preProPTH-K26P, or preProPTH-C18R were labeled with [35S]Met/Cys for 15 min. The processing of newly synthesized preProPTH-WT and mutants was analyzed after anti-Myc immunoprecipitation followed by NuPage. B and C, 293T cells were co-transfected with Myc-tagged preProPTH-WT or preProPTH-K26P with or without Myc-tagged pPI-WT at a ratio of 1:1. The cells were labeled with [35S]Met/Cys for 15 min. Impaired SP cleavage of pPI-WT in the presence of preProPTH-K26P (first lane) was analyzed in B and is quantified in C. pPI-A24D was used as a positive control for uncleaved PPI.
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FIGURE 7. Unblocked pPI-F25P binds to the SPase catalytic subunit SEC11A. 293T cells were co-transfected with Myc-tagged pPI-WT or -F25P plus plasmids encoding FLAG-tagged SPCS1 or Sec11A. 48 h post-transfection, the cells were labeled with [35S]Met/Cys for 2 h and lysed with co-IP buffer as described under "Experimental Procedures." The lysates were then split into halves. One half was immunoprecipitated with anti-insulin to estimate the total amount of pPI-WT and F25P, and the other half was co-immunoprecipitated with anti-FLAG. The total amounts of pPI-WT and F25P, and the amounts co-immunoprecipitated with anti-FLAG (top panel) and the amounts co-immunoprecipitated with anti-FLAG (bottom panel) were analyzed using NuPage.

fickling, and maturation of some viral polypeptides. We therefore examined the effect of pPI-F25P on the processing of viral proteins bearing an N-terminal SP. We co-expressed pPI-F25P with Myc-tagged MMTV Rem protein, which has a signal sequence at its N terminus (19). Using metabolic labeling to follow intracellular processing and trafficking of newly synthesized proteins (Fig. 8A) and Western blotting to follow unprocessed/processed Rem protein at steady state (Fig. 8B), we found that the majority of Rem protein remained uncleaved in cells co-expressing pPI-F25P (Fig. 8, A and B), suggesting that pPI-F25P impaired intracellular processing and maturation of Rem protein. Next, to examine whether pPI-F25P affects the maturation of viral polypeptides bearing an internal SP, we co-expressed pPI-F25P with the HCV structural protein, which is composed of an N-terminal core protein, two internal SPs, and two heavily glycosylated transmembrane envelope proteins, E1 and E2 (Fig. 8C, top panel). Although the mRNA encoding HCV structural protein was expressed at a similar level in cells co-expressing preproinsulin-WT and pPI-F25P (Fig. 8D), the processed core protein detected by anti-core antibody was decreased significantly in cells expressing pPI-F25P (Fig. 8C). We were not able to detect significantly increased levels of uncleaved structural protein, either because these uncleaved proteins were unstable and degraded or because the anti-core monoclonal antibody could not efficiently recognize the uncleaved form. Interestingly, on longer exposures, we did observe high molecular weight proteins that may represent uncleaved structural protein accumulating in cells expressing pPI-F25P (data not shown).

Discussion

In eukaryotic cells, SPase catalyzes the first processing event to excise the SP of secretory proteins upon their translocation across the ER membrane. Impaired SP cleavage because of naturally occurring SP mutations has been linked to multiple human diseases, including early-onset diabetes mellitus associated with mutant preproinsulin (10, 26), central diabetes insipidus associated with mutant preprovasopressin (12), and hemophilia associated with mutant precoagulation factor X (11). These observations suggest that efficient and proper SP excision is a critical event for the intracellular processing, folding, trafficking, and maturation of secretory proteins. Despite the importance of SPase, the studies of mammalian SPase and related cellular events are rather limited, at least partially because of a lack of specific inhibitors. In this study, we report that preproinsulin bearing proline at the P1’ position (pPI-F25P) specifically binds to SPase catalytic subunits and acts as a competitive inhibitor of SPase activity.

Unlike the mammalian SPase complex, prokaryotic SPase I has been studied extensively (23, 38, 39) and shares a preference for small nonpolar residues at the P3 and P1 positions, despite some other differences (9, 40). The catalytic site of bacterial SPase I is exposed to the surface of the cell membrane, and its activity functions by a Ser/Lys catalytic dyad mechanism (unlike the mammalian ER SPase complex, which catalyzes cleavage on the luminal side of the ER membrane and employs an SHD triad for catalysis (41)). Furthermore, the bacterial SPase I has been an attractive target to develop new antibiotics (6, 7). In addition to the P3 and P1 positions of preproteins, extended subsite interactions also play a role in substrate recognition and cleavage by prokaryotic SPase I (38, 42, 43). Indeed, it has been shown that a proline or threonine substitution at the P1’ position of M13 procoat protein or maltose-binding protein impairs cleavage by bacterial SPase I (44, 45), although the proline substitution may also impair transmembrane translocation (31). We analyzed a database of eukaryotic secretory proteins and found that proline was indeed the least favored residue at the P1’ position (Fig. 1A). Moreover, although a P1’ threonine substitution was largely without effect, a P1’ proline substitution clearly impairs processing of preproinsulin to proinsulin (Fig. 1C). However, using two independent approaches, we found that pPI-F25P was translocated successfully across the ER membrane (Fig. 2), indicating that the processing defect of pPI-F25P to proinsulin was the direct consequence of a defect of SP cleavage. Importantly, uncleaved pPI-F25P also inhibited, in trans, SP cleavage of other secretory proteins (Figs. 3–5). Because another uncleaved preproinsulin, pPI-A24D, did not exert a similar inhibitory effect on co-expressed preproinsulin (Fig. 2C), this inhibition in trans is a specific effect of the proline located at the P1’ position.

All five subunits of the mammalian ER SPase complex are integral membrane proteins (9). SPCS1 and SPCS2 span the ER membrane twice, exposing the bulk of each protein to the cytosolic site of the ER membrane, and are not required for SPase activity (37, 46). In contrast, SPCS3, SEC11A, and SEC11C are single-spanning membrane proteins that are required for SPase activity (9, 47). The latter subunits anchor their amino-terminal transmembrane domains in the ER membrane, exposing much larger domains to the ER lumen (36). In this report, we found that the majority of uncleaved pPI-F25P was anchored in the ER membrane with the same topology as that of SEC11A and SEC11C (Figs. 2 and 6), and its inhibitory potency was topology-dependent (Fig. 6E). Moreover, pPI-F25P specifically co-immunoprecipitated with the catalytic subunit SEC11A (Fig. 7) but not with SPCS1. Importantly, the inhibition of pPI-F25P on SPase is dose-dependent (Fig. 3, B and C) and could be overcome by increasing the concentration of preproinsulin-WT substrate (Fig. 3D). Taken together, these data suggest that pPI-
F25P specifically binds to the SPase catalytic subunits and acts as a competitive inhibitor of SPase, impairing the cleavage of other secretory proteins entering the ER.

Signal sequences are also present in many viral polypeptides, such as HCV (14, 48), HIV (26, 32), and MMTV Rem protein (18, 19). Although host cell SPase may be necessary for the intracellular processing of these proteins, leading to virus production, no study examining the effects of inhibiting SPase on this process has yet been reported. Here we examined the effects of pPI-F25P on the processing of two viral proteins, Rem (with an N-terminal signal sequence) and HCV structural protein (with two internal signal sequences). We found that inhibiting SPase by pPI-F25P impaired the maturation of both protein precursors (Fig. 8), suggesting that SPase may be a potential therapeutic target for developing antiviral treatment. On the basis of these results, we believe that further studies are warranted for the development and targeted delivery of eukaryotic SPase inhibitors.

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