Biosynthetic processing and quaternary interactions of proprotein convertase SPC4 (PACE4)

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Abstract SPC4 (PACE4), a member of the eukaryotic family of subtilisin-like proprotein convertases, is synthesized as a proenzyme (proSPC4) which undergoes proteolytic removal of N-terminal propeptide during transit through the secretory pathway. As this propeptide processing seems to be a key event in the functional expression of SPC4, we have investigated its mechanism and the intracellular site where it occurs. In transfected fibroblast cells, the 110-kDa proSPC4 undergoes slow cleavage to generate a 103-kDa mature enzyme in the endoplasmic reticulum (ER). Site-directed mutagenesis studies demonstrate that the proteolytic activation of SPC4 occurs mainly through a unimolecular autocatalytic process and propeptide cleavage is a prerequisite for its export from the ER. Sedimentation velocity and chemical cross-linking analysis demonstrate that the precursor protein in the cells exists as both a monomer and a dimer-sized complex whereas mature SPC4 exists only as a monomer. These results suggest that the cleavage of the N-terminal propeptide of SPC4 plays a regulatory role in its activation and secretion through the change in its oligomeric state.

Key words: SPC4; PACE4; Processing protease; Endoplasmic reticulum; Proenzyme activation; Oligomerization

1. Introduction

SPC4 (also called PACE4) is a member of the subtilisin-like proprotein convertase (SPC) family which is responsible for proteolytic processing of many bioactive peptide precursors at paired or multiple basic sites. To date, seven members of this family have been identified: SPC1/furin, SPC2/PC2, SPC3/PC1/PC3, SPC4/PACE4, SPC5/PC4, SPC6/PC5/PC6 and SPC7/PC7/PC8/LPC (for reviews see [1–3]). All members of the SPC family share certain structural features: an N-terminal signal peptide, followed by a propeptide region, a subtilisin-like catalytic domain, a conserved domain of unknown function named the HomoB domain or P-domain, and a divergent C-terminal tail. These convertases have overlapping but not identical substrate specificity, and they are differently distributed in tissues and show maximal activity in different cellular compartments. Identifying the physiological substrate of these convertases is vital if one is to understand the regulation of biosynthesis of bioactive peptides and proteins such as neuropeptides, peptide hormones, growth factors, receptors and plasma proteins.

SPC4 is expressed in a wide range of tissues or cells with relatively high expression levels in particular tissues such as pituitary and nervous systems. For human SPC4, eight different isoforms arising from alternative gene splicing events have so far been reported [4–7]: they are SPC4A-I, SPC4A-II, SPC4B, SPC4C, SPC4CS, SPC4D, SPC4E-I, and SPC4E-II. Previous studies demonstrated that these isoforms show distinct patterns of tissue distribution [8–10], and different properties of secretion [7]. Evidence that implicates SPC4 in development and pathogenesis, such as limb development [11], establishment of the olfactory system [10], and tumor progression [12], is accumulating. Recently, we reported the gene structure of human SPC4 [13]. The sequence of its 5′-flanking region revealed characteristic potential regulatory elements such as an E-box cluster, suggesting the dynamic regulation of SPC4 expression during development.

Studies have been performed to demonstrate the processing activity of SPC4 against several precursor proteins using heterologous expression systems in cultured cell lines. However, in most cases, SPC4 showed no or extremely low levels of activity compared to other convertases, and a promising candidate for the physiological substrate has not been identified. One reason for this weak activity of SPC4 could be the inappropriateness of the expression systems employed in the experiments. Since SPC4 is synthesized as an inactive precursor proSPC4 and then converted into the mature active protein in the cells before its secretion [7,14], the rate of the removal of the N-terminal propeptide of SPC4 could conceivably represent a mechanism by which the cell could control the efficiency of substrate processing. Previous studies did not give sufficient consideration to the maturation efficiency of the enzyme in the cells despite the potential importance of the propeptide cleavage as a possible regulatory step. In this context, here we have clarified the mechanism of intracellular maturation and secretion of human SPC4, which is critically important to the understanding of the regulation of proprotein processing by this enzyme.

2. Materials and methods

2.1. Expression plasmids and site-directed mutagenesis

For transient expression of SPC4 in mammalian cells, human SPC4A-I cDNA was subcloned into mammalian cell expression vector pALTER-MAX (Promega). Site-directed mutagenesis was performed on this plasmid and the following mutants were generated using Altered sites II mammalian mutagenesis system (Promega); RQ149 ($Arg^{149} \rightarrow Gln$), KR144 ($Lys^{144} \rightarrow Arg$), DN205 ($Asp^{205} \rightarrow Asn$), SA420 ($Ser^{420} \rightarrow Ala$) and SPC4Δ680 (introduction of a stop codon

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after Thr679). The sequences of the mutagenic primers used were 5′-TTCGCACCTGTTGCTTCACCCTTC-3′ (RQ149), 5′-CACCCTTCGTCTCACTTCCTG-3′ (KR144), 5′-GCCATCATTAAGGATGG-3′ (DN205), 5′-GGGCAGAGACTGCGGTCCCAGTGTG-3′ (SA420), and 5′-GAAGATTACACATAGCAATCCACCC-3′ (SPC4Δ680), with the mutated nucleotides underlined. The expression plasmid for mouse *Ren-2* prorenin mutant, $M2R^{-6}R^{-4}$, was kindly provided by K. Nakayama and K. Murakami (University of Tsukuba, Japan) [15].

2.2. Preparation of antibodies

To generate a polyclonal antibody to SPC4, the HomoB region of SPC4 (456–688) with an amino-terminal hexa-histidine was prepared by inserting the appropriate region of human SPC4 cDNA into pQE-30 vector (Qiagen). After expression in *Escherichia coli* strain XL1-Blue cells, the protein was purified from inclusion bodies under denaturing conditions by affinity chromatography using Ni-nitrilotriacetic acid agarose (Qiagen) as described by the manufacturer. The protein was eluted in 8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris (pH 4.5), and then injected into rabbits for immunization.

For the production of monoclonal antibodies specific for SPC4A, BALB/c mice were immunized by intraperitoneal injections of peptide TGFTQLGTSCITNHTCSNAD (corresponding to amino acids 920–939 of SPC4A-I) conjugated with keyhole limpet hemocyanin. The isolated spleen cells were fused with SP2/0 myeloma cells. Colonies were selected by immunoblotting and immunoprecipitation against SPC4A-I overexpressed in COS-1 cells. The tissue culture supernatants from the clone A121G09 cell line were used for the experiments.

2.3. Cell culture and transfection

HEK293 cells and COS-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% FBS. HEK293 cells were transfected by the calcium phosphate method using the CellPhect transfection kit (Pharmacia) as described by the manufacturer and subjected to radiolabeling at 40 h post-transfection. Transfection of COS-1 cells was performed by the DEAE-dextran method as described previously [7].

2.4. Radiolabeling and immunoprecipitation

Transfected cells were washed with PBS, and then switched for 30 min to a methionine- and cysteine-free medium supplemented with 10% dialyzed FBS. Subsequently, cells were labeled for 6 h with [^{35}S]methionine/cysteine (100 μCi/ml) (>1000 Ci/mmol, Dupont-New England Nuclear). In experiments performed with brefeldin A (Sigma), the cells were preincubated for 1 h, and then labeled for 6 h and the drug was used throughout the preincubation and labeling period at a final concentration of 5 μg/ml. At the end of the incubation period, the media were removed and cells were disrupted in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin and 2 μg/ml pepstatin A) by incubating at 4°C for 30 min. The media and cell lysates were precleared in two steps using normal rabbit serum and protein A-Sepharose 4 Fast Flow (Pharmacia), and then incubated with antiserum for 1 h. Immune complexes were isolated by shaking overnight with protein A-Sepharose 4 Fast Flow and the beads were washed twice with buffer 1 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA), three times with buffer 2 (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA), three times with buffer 3 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate (SDS), 1 mM EDTA), and twice with buffer 4 (10 mM Tris-HCl, pH 7.5, 0.1% SDS). Endoglycosidase H (Boehringer Mannheim) and peptide *N*-glycosidase F (New England Biolabs) digestions were performed overnight at 37°C according to the manufacturers' instructions. The immunoprecipitation products were resolved by SDS-polyacrylamide gel electrophoresis followed by fluorography.

2.5. Sedimentation velocity analysis

Transiently transfected COS-1 cells expressing wild-type SPC4 was metabolically labeled with [^{35}S]methionine/cysteine in serum-free DMEM and then solubilized in 0.5% Triton X-100, 0.2 M KCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 μg/ml leupeptin and 10 μg/ml E-64. The culture medium was concentrated to 0.2 ml by ultrafiltration. The cell lysate and concentrated medium were applied to the top of linear 5–20% (w/v) sucrose gradients and centrifuged for 17 h at 4°C in a SW41 rotor at 35 000 rpm. Each 450 μl fraction was collected and subjected to immunoprecipitation with the polyclonal antibody to SPC4.

2.6. Chemical cross-linking

Transiently transfected COS-1 cells expressing wild-type SPC4 or SA420 mutant were solubilized in 1% Triton X-100, 150 mM NaCl and 50 mM borate (pH 8.1). The serum-free cultured medium was concentrated by ultrafiltration and conditioned to 50 mM borate (pH 8.1). The cleared cell lysates and medium were incubated at room temperature with 1.2 mM dimethyl suberimidate (DMS) for 3 h. The cross-linked samples were subjected to Western blot analysis using the monoclonal antibody to SPC4A.

3. Results

3.1. Maturation of SPC4 occurs in the endoplasmic reticulum

In this study, transient expression in fibroblast cells was used to precisely analyze the biosynthetic processing of SPC4 (PACE4). Expression plasmids for SPC4A-I were transiently transfected into HEK293 cells. Cells were radiolabeled for 6 h with [^{35}S]methionine/cysteine and then the cell extracts and conditioned medium were subjected to immunoprecipitation with the anti-SPC4 antiserum. In the cell extracts, SPC4A was detected as two bands, a major 110-kDa precursor protein and a minor 103-kDa mature protein, and the latter was secreted into the medium as previously described [7] (Fig. 1A, lanes 1 and 4). Since human SPC4A contains three potential *N*-glycosylation sites, sensitivity to endoglycosidase digestion was used to determine the intracellular site of the maturation of SPC4. Immature oligosaccharide chains were found on both the precursor and mature form of SPC4 in the cell extract: the products of peptide *N*-glycosidase F (PNGase F) and endoglycosidase H digestion exhibited similar apparent molecular masses (Fig. 1A, lanes 2 and 3). Endoglycosidase H treatment never diminished the apparent molecular mass of

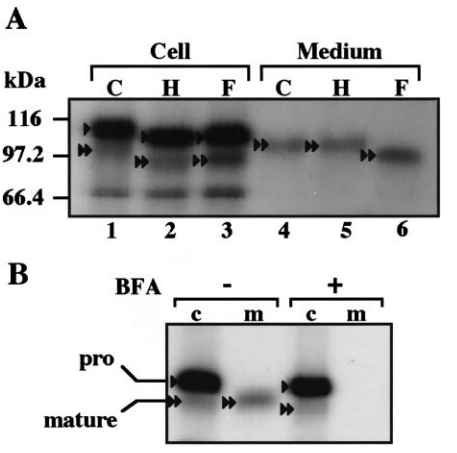

Fig. 1. Analysis of the intracellular site for the maturation of SPC4. A: *N*-Glycosylation analysis of SPC4. HEK293 cells transiently expressing SPC4A-I were biosynthetically labeled with [^{35}S]Met/Cys for 6 h. Samples were immunoprecipitated with anti-SPC4 antiserum and then treated with buffer only (lanes C), endoglycosidase H (lanes H) or protein *N*-glycosidase F (lanes F) before analysis by SDS-PAGE. B: Effects of brefeldin A on the maturation and secretion of SPC4. HEK293 cells transiently expressing SPC4A-I were biosynthetically labeled with [^{35}S]Met/Cys for 6 h in the presence or absence of brefeldin A. Cell extracts (lanes c) or media (lanes m) were immunoprecipitated with anti-SPC4 antiserum, followed by resolution by SDS-PAGE. The arrowheads indicate the proSPC4 protein and the double arrowheads indicate mature SPC4 protein.

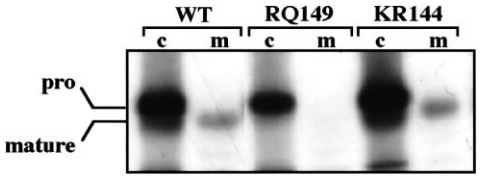

Fig. 2. Role of the propeptide cleavage of SPC4 in secretion. HEK293 cells transiently expressing SPC4A-I (WT) or its propeptide cleavage site mutants (RQ149 and KR144) were biosynthetically labeled with [^{35}S]Met/Cys for 6 h. Cell extracts (lanes c) or media (lanes m) were immunoprecipitated with anti-SPC4 antiserum, followed by resolution by SDS-PAGE.

secreted SPC4 to the same extent as did PNGase F treatment (Fig. 1A, lanes 5 and 6), indicating the protein had acquired endoglycosidase H-resistant oligosaccharide after the conversion from proSPC4. Since the processed SPC4 protein in the cell extract contained endoglycosidase H-sensitive oligosaccharide, while the secreted SPC4 protein did not, the endoproteolytic cleavage converting proSPC4 to the mature protein must have occurred at a site before the medial Golgi.

As another approach to investigate the cellular site of SPC4 maturation, radiolabeling studies in the presence or absence of the fungal metabolite brefeldin A were performed with SPC4A-I transfected HEK293 cells (Fig. 1B). Brefeldin A causes accumulation of secretory proteins in the endoplasmic reticulum (ER) [16,17]. In the absence of brefeldin A, the precursor proSPC4 and the processed mature SPC4 were observed in the cell extract and the mature SPC4 was secreted into the medium. In the presence of brefeldin A, the secretion of the processed mature SPC4 was completely inhibited although the conversion of proSPC4 occurred in the cells, indicating that this drug effectively blocked transport of proteins in the secretory pathway of these cells but not proSPC4 maturation which takes place in the ER.

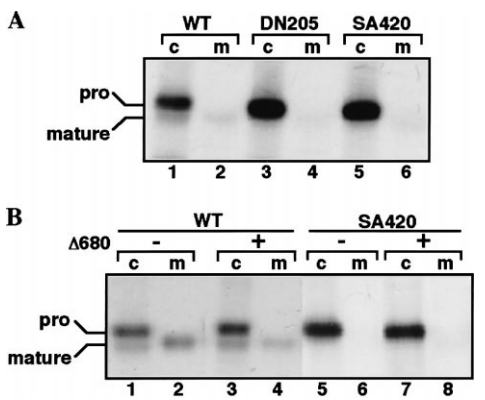

Fig. 3. Processing of active site mutant of SPC4. A: HEK293 cells transiently expressing SPC4A-I (WT) or its active site mutants (DN205 and SA420) were biosynthetically labeled with [^{35}S]Met/Cys for 6 h. Cell extracts (lanes c) or media (lanes m) were immunoprecipitated with anti-SPC4 antiserum, followed by resolution by SDS-PAGE. B: SPC4A-I (WT) or its active site mutant (SA420) was expressed in combination with C-terminally shortened SPC4 (Δ680) in HEK293 cells, and then cells were biosynthetically labeled with [^{35}S]Met/Cys for 6 h. Cell extracts (lanes c) or media (lanes m) were immunoprecipitated with anti-SPC4 antiserum, followed by resolution by SDS-PAGE.

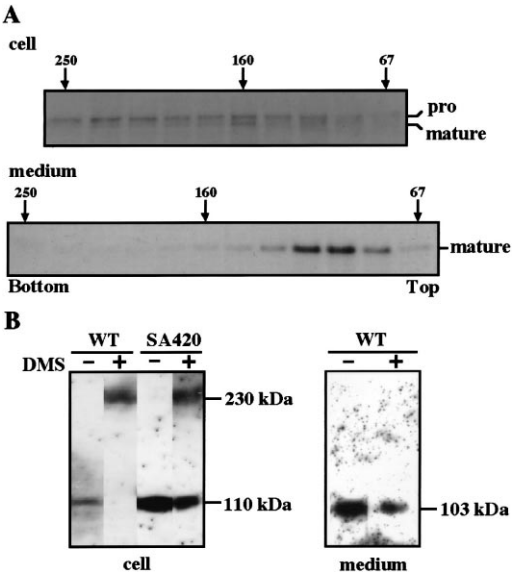

Fig. 4. Analysis of the oligomerization state of SPC4. A: COS-1 cells were transiently transfected with SPC4A-I. Conditioned medium or detergent lysate of the cells were fractionated by sedimentation on 5–20% sucrose gradients for 17 h. Individual gradient fractions were immunoprecipitated with anti-SPC4 antiserum, and then analyzed by SDS-PAGE and fluorography. Bovine serum albumin (~67 kDa), aldolase (~160 kDa) and catalase (~250 kDa) were used as internal size standards. B: COS-1 cells were transiently transfected with wild-type SPC4 or the active site mutant SA420. Conditioned media or detergent lysates of the cells were cross-linked with 1.2 mM of DMS. Samples were analyzed by Western blotting using the anti-SPC4A monoclonal antibody.

3.2. Proteolytic maturation is a prerequisite for SPC4 to leave the ER

To determine whether the propeptide cleavage has influence on the trafficking of SPC4 in cells, SPC4s harboring a mutation at their propeptide cleavage site were constructed. The processing site of proSPC4 contains the multiple basic amino acids Lys-Arg-Arg-Val-Lys-Arg^{-1} which is a consensus signal for cleavage by SPC family proteases including SPC4 itself. Thus, the Arg residue in the P1 position of the processing site of proSPC4A-I was replaced by Gln to generate Lys-Arg-Arg-Val-Lys-Gln^{-1} so that the consensus processing motif was disrupted (RQ149). HEK293 cells expressing the wild-type proSPC4 or the RQ149 mutant were metabolically labeled, and their maturation and secretion were compared (Fig. 2). Unlike the wild-type SPC4, the RQ149 mutant did not undergo any detectable endoproteolytic cleavage as expected, nor was secretion detectable. A Lys to Arg mutation at the P6 position to generate Arg-Arg-Arg-Val-Lys-Arg^{-1} processing site (KR144), which does not harm the consensus motif, did not affect the efficiency of either the maturation or secretion of SPC4. Taken together, these results show that the propeptide processing of SPC4 and its export from the ER are sequential events and the propeptide cleavage is prerequisite for SPC4 to exit from the ER.

3.3. Intramolecular autoactivation of SPC4

To examine whether a mutant form of proSPC4 that should not yield active enzyme could have its propeptide region cleaved, the Asp residue in the catalytic triad of SPC4 was mutated to Asn (DN205) or the Ser residue was mutated to Ala (SA420). When these active site mutants were expressed in

HEK293 cells (Fig. 3A), the SPC4 maturation was almost blocked and an accumulation of 110-kDa proSPC4 proteins was observed in the cells, although in an overexposed autoradiogram small amounts of mature proteins were also observed in the media with both mutants (data not shown). To examine whether SPC4 itself might activate the proSPC4 in an intermolecular manner, co-transfection of the processing-defective SPC4 mutant and an active SPC4 were performed. To this aim, a catalytically active mutant SPC4Δ680, lacking the entire C-terminal cysteine-rich region, was constructed, to enable two simultaneously expressed SPC4 proteins to be distinguished based on their molecular weights. The integrity of the truncated SPC4 in cleaving substrate molecules was confirmed by co-expression study with mutant prorenin $M2R^{-6}R^{-4}$ [15] (data not shown). Despite the simultaneous expression of catalytically active SPC4Δ680, the biosynthetic processing of neither the wild-type nor active site mutant SA420 was stimulated (Fig. 3B). These results indicate that SPC4 maturation involves intramolecular autocatalytic removal of the propeptide, whereas other proteases may contribute to this process to only a small extent.

3.4. Oligomeric structure of SPC4

Until now, the oligomeric state of SPC family proteases has not been thoroughly characterized. We therefore used sucrose density gradient velocity centrifugation of metabolically radiolabeled cells transiently expressing SPC4A to determine relative complex size (Fig. 4A). When a lysate of COS-1 cells expressing SPC4A-I was analyzed, the mature protein both in the cell extract and culture medium sedimented at a size compatible with it being a monomer. In contrast, the precursor form of SPC4 in the cells migrated not only as a monomeric form but also as larger complexes, including a dimer-sized complex. A more accurate estimation of the size of SPC4 was obtained by chemical cross-linking with dimethyl suberimidate (DMS) followed by immunoblotting (Fig. 4B). When lysates of transfected cells were analyzed, substantial fractions of both the wild-type and the active site mutant SA420 which accumulates in the cells as a precursor were cross-linked into a dimer-sized ~230-kDa species. On the other hand, mature SPC4 secreted into the culture medium was not cross-linked under the same conditions. The fact that the complex size observed in the cells fits a SPC4 dimer suggests the existence of such an oligomeric structure, although we can not exclude the possibility that some other cellular protein of a similar size associates with proSPC4. These findings demonstrate that the precursor of SPC4 assembles into a larger complex, probably a dimer, in the ER whereas the proteolytically processed mature SPC4 exists in a monomeric form. This suggests that the propeptide cleavage of SPC4 in the ER affects its tertiary or quaternary structure.

4. Discussion

The biosynthetic processing and intracellular transport of SPCs are thought to provide these enzymes with opportunities for regulating the efficiency of substrate processing. For instance, SPC1 (furin), SPC6B (PC6B) and SPC7 (LPC/PC8) localize to the trans-Golgi network (TGN) through their hydrophobic transmembrane regions, and thus can process precursor proteins that pass through this compartment. On the other hand, SPC2 (PC2), SPC3 (PC3) and SPC6A (PC6A) are known to be sorted into the secretory granules of endocrine or neuroendocrine cells and process precursor proteins there [3,18]. Thus, the proprotein processing by SPCs can be controlled through the compartmentalization of both the enzymes and substrates. SPC4A (PACE4A) is expressed in both the regulated and non-regulated cells and does not have any membrane anchoring sequence, suggesting that SPC4A is secreted to the extracellular space mainly through the constitutive secretory pathway. In this case, the enzyme would only temporarily access substrates during the intracellular transport, or it would play a role in the extracellular milieu after its secretion. In either case, the availability of the active enzyme for substrate processing is limited by the propeptide cleavage in the ER. Since the maturation of SPC4 seems to be extremely slow and inefficient ([14] and our unpublished results), this step seems to play a rate-determining role for both the generation and release of the active enzyme.

Concerning SPC1, SPC3 and SPC7, the processing of the precursor proteins is known to occur via an autocatalytic intramolecular mechanism in the ER [19–24]. A recent study with SPC1 (furin) showed that the initial propeptide cleavage in the ER is not sufficient for the activation of SPC1 and a second cleavage within the propeptide occurring in the TGN is required to dissociate the propeptide from the rest of the molecule for full activation [25]. In the case of SPC3 (PC1/PC3), not only propeptide cleavage, but also C-terminal cleavage in the secretory granules is required for maximal activation [26–29]. The activation of SPC2 (PC2) is rather unique among the SPCs: it occurs slowly after the exit of SPC2 from the ER by an intermolecular autocatalytic mechanism and chaperonin-like protein 7B2 is involved in this process [23,30–34]. Thus the SPC activation does not appear to be a simple event, and besides the common mechanism, each SPC may have its own activation mechanism to achieve strict regulation of its activities. The current study demonstrates that proSPC4 processing occurs largely through an autocatalytic unimolecular reaction in the ER and is prerequisite to its export from this compartment. However, some processing still occurs even when the active site residues of SPC4 are mutated (Fig. 3A), indicating the partial involvement of another mechanism in this process. Both the autoactivation and activation by other proteases *in trans* may be needed for maximum efficiency of SPC4 maturation. Recently, Mains et al. reported accelerated maturation of rat SPC4 on truncation of its C-terminal cysteine-rich region [14]; however, this was not observed for human SPC4 (data not shown). Some differences in activation may exist between human and rat SPC4.

Many bioactive proteins are primarily synthesized as proproteins with N-terminal propeptides. Several functions of propeptides have been described, such as stabilization or secretion of active proteins, multimer assembly, sorting to subcellular organelles, inhibition of enzymatic activities, and guiding of correct protein folding. In most SPCs, the cleavage of the N-terminal propeptide occurs in the ER and is required for the export of the protein from the compartment. However, the underlying mechanism for the acquisition of the export competence remains to be established. A possible explanation is that the propeptide of the SPCs harbors a specific signal for retention in the ER. Alternatively, export of SPCs from the ER could be mediated by a change in the physical-chemical properties of the protein which is induced by the propeptide cleavage. Our observations that some proSPC4 in the cells

exists as larger complexes whereas the mature protein is only in a monomeric form suggest some relationship between the propeptide cleavage in the ER and tertiary or quaternary structure of the protein. Co-expression studies of SPC4A with mutant prorenin $M2R^{-6}R^{-4}$ in the presence of BFA indicate that SPC4 is catalytically inactive against substrate proproteins *in trans* in the ER even though its propeptide has been cleaved (data not shown). Thus, the conformational change and subsequent export from the ER induced by the propeptide cleavage may be important for the regulation of the efficiency of substrate processing by SPC4. In addition, the oligomeric complex of proSPC4 formed in the ER may have some implications for the mechanism of self-cleavage of its propeptide. Although it is not clear if the same is applicable to other members of the SPC family, the precise relationship between this oligomeric structure and the export of the protein from the ER is of considerable interest.

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