A Conserved α-Helix at the Amino Terminus of Prosomatostatin Serves as a Sorting Signal for the Regulated Secretory Pathway

Rania Mouchantaf‡§, Ujendra Kumar‡, Traian Sulea¶, and Yogesh C. Patel‡¶

From ‡ Fraser Laboratories, Departments of Medicine and Neurology and Neurosurgery, Pharmacology, and Therapeutics, McGill University, Royal Victoria Hospital and Montreal Neurological Institute, Montreal, Quebec H3A 1A1, Canada, and the ¶ National Research Council Biotechnological Research Institute, Montreal, Quebec H4P 2R2, Canada

Mammalian prosomatostatin (PSST) contains the bioactive peptides SST-14 and SST-28 at the COOH-terminal end of the molecule and a putative sorting signal in the propeptide segment for targeting the precursor to the regulated secretory pathway. The NH₂-terminal segment of PSST consists of an amphipathic α-helix, which has been totally conserved throughout vertebrate evolution. We have analyzed the PSST-(3–15) region for sorting function by alanine scanning and deletional mutagenesis. Mutants created were stably expressed in AtT-20 cells. Regulated secretion was studied by analyzing basal and stimulated release of SST-14 LI and by immunocytochemistry for staining of SST-14 LI in punctate granules. Deletion of the PSST-(9–15) segment blocked regulated secretion and rerouted PSST for constitutive secretion as unprocessed precursor. Alanine scanning mutagenesis identified the region Pro⁵–Gln¹² as being important in precursor targeting, with Leu⁷ and Leu¹¹ being critical. Molecular modeling demonstrated that these two residues are located in close proximity on a hydrophobic surface of the α-helix. Disruption of the α-helix did not impair the ability of PSST to be processed at the COOH terminus to SST-14 and SST-28. Processing, however, was shifted to the early compartments of the secretory pathway rather than storage granules and was relatively inefficient.

Secretory cells such as neuroendocrine, exocrine, and mast cells contain two distinct pathways for protein secretion, a constitutive secretory pathway (CSP) that transports proteins to the cell surface by bulk flow and a regulated secretory pathway (RSP) that releases secretory proteins from a granular storage pool in response to specific stimuli (1, 2). Proteins destined for secretion are initially synthesized as precursors on ribosomes, translocated into the lumen of the endoplasmic reticulum, and transported through the Golgi stacks to the trans-Golgi network (TGN). Here the protein is sorted via clathrin-coated vesicles into the RSP consisting of dense core secretory granules or the CSP through small nonclathrin-coated vesicles, which exit from the TGN and rapidly migrate to the plasma membrane (2–6). A major unanswered question is the mechanism for sorting prohormone and propeptide precursors in the TGN into either the CSP or RSP (4–6). Sorting is an active process that requires some form of recognition of the secretory protein (2, 4–6). It is one step in a multistep cascade during which the prohormone is concentrated over 100-fold, packaged with other granular proteins, extruded into budding secretory vesicles, and proteolytically processed into smaller mature products. These events may be interdependent, and their temporal and spatial relationship remains poorly understood (5, 6). Three models have been proposed to explain how proteins are sorted to the RSP. The first proposes that regulated secretory proteins possess an intrinsic ability to form aggregates leading to packaging of condensed products into secretory granules, thereby sorting them away from soluble proteins that are carried off by bulk flow in small vesicles. Support for this model comes from the tendency of a number of secretory granule proteins such as prolactin, growth hormone, the chromogranins, carboxypeptidase E (CPE), and prohormone convertase 2 (PC2) to aggregate at the mildly acidic pH in the TGN (7–12). However, other proteins, such as fibronectin, that aggregate easily are not targeted into the RSP, and modifications on proteins such as chromogranin B and insulin-like growth factor-1 result in missorting without affecting aggregation (13–15). Furthermore, GH does not aggregate in the acidic environment of the TGN in COS-7 cells but does so in AtT-20 cells, and blockade of acidification with chloroquin and bafilomycin A1 is without effect on the ability of these hormones to aggregate in secretion granules in GH₃C₁ cells, suggesting that aggregation alone is not sufficient for sorting into secretory granules (7). The second model assumes that regulated secretory proteins contain sorting signals in the form of specific sequence motifs or conformational epitopes that allow them to be sorted from constitutive secretory proteins by a receptor-mediated mechanism at the level of the TGN (1, 2). The third model combines features of the first two mechanisms and assumes that there is initial interaction of the regulated secretory protein with a receptor, which then triggers the formation of an aggregate that is packaged into secretion granules. Several lines of evidence suggest that the propeptide is recognized by the sorting apparatus and that the structural domains that serve as recognition signals are dominant, since fusion of a constitutively secreted protein to a hormone (e.g. GH) targets the hybrid protein to the RSP and deletion of sorting signal domains results in mistargeting to the CSP (16–22). A sorting sequence domain has been described in the prosegment of...
Acid sequence and secondary structure of the PSST NH2-terminal region has been implicated in generating the decapeptide terminal segment with that of 14 other prohormones that have been shown experimentally to be sorted to secretory vesicles in vitro and prohormone processing may occur constitutively but is relatively inefficient in the absence of correct precursor targeting to the RSP.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic peptides were obtained as follows. SST-14 and SST-28 were from Bachem (Marina del Rey, CA); Tyrφ-SST-14 was from Peninsula Laboratories (Belmonte, CA); acetonitrile and trifluoroacetic acid were purchased from Fisher; heptadecanoic acid was obtained from Pierce; peptatin-A, 12-O-tetradecanoylphorbol-13-acetate (TPA) and phenylmethylsulfonyl fluoride were from Sigma. Forskolin (FSP) was purchased from Calbiochem; Dulbecco’s modified Eagle’s medium and fetal bovine serum were purchased from Life Technologies, Inc. Ser-X-tend was obtained from Irvine Scientific (Santa Anna, CA). All other reagents were of analytical grade and were obtained from various suppliers.

**Construction of Wild Type and Mutant PSST cDNAs**—cDNA for wild type rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8. Using rat prepro-SST as template, a series of mutants were created by the PCR overlap extension technique (35) (Fig. 2): (i) alanine scanning of rat prepro-SST was constructed in the expression vector pTEJ8![](https://www.bjoc.org/).
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were verified by sequencing of double-stranded DNA (University Core DNA Service, University of Calgary, Alberta, Canada), and at least two independent clones of each mutant were independently transfectected.

Cell Culture and Transfection—AtT-20 mouse anterior pituitary cells were cultured in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum supplemented with 10% HGF, 20% CO2, and 95% air in a humidified incubator at 37 °C. Cells were plated in 100 × 20-mm Petri dishes and transfected at 50% confluence with 3–5 μg of the appropriate plasmid construct by LipofectAMINE (Life Technologies) and stable G418 (0.861 mg/ml)-resistant nonclonally selected cells were propagated for study.

Secretion Studies—Stably transfected AtT-20 cells were cultured in 35-mm diameter six-well plastic Petri dishes and grown to 80–90% confluence, after which they were prepared for studies of basal and stimulated secretion of immunoreactive SST-14 (SST-14 LI). Following removal of the feeding medium, groups of five wells were incubated with Dulbecco’s modified Eagle’s medium plus 1% bovine serum albumin containing phenylmethylsulfonyl fluoride and peptidase-A (20 μg/ml each). To study regulated secretion, cells were incubated with 20 μM FSK or standards, and at 1 h, Medium serum albumin-coated charcoal separation method was further extracted by sonication followed by centrifugation at 10,000 × g for 5 min. The supernatant was stored at −20 °C for RIA and HPLC analysis.

HPLC—Poole acidified secretion media and cell extracts were diluted 1:7 with 0.1% trifluoroacetic acid and concentrated using Waters Sep-Pak C18 cartridges. The adsorbed peptides were analyzed by HPLC on a C18 μBondapak reverse phase column using a Waters HPLC system as previously described (30, 31). The column was eluted at room temperature (21 °C) at 1 ml/min with 12–55% acetonitrile and a 0.2% heptafluorobutyric acid gradient over 150 min. The column effluent was monitored for UV absorbance at 214 and 280 nm. Fractions were spiked with 10 μl of 10% bovine serum albumin, and at stored at −20 °C until further use. 30–100-μl aliquots from each fraction were rotary-evaporated with a Speedvac and assayed for SST-14 LI by RIA.

RIA of SST-14 LI—RIA for SST-14 LI was performed using a rabbit anti-SST antibody (R149), [125I]Tyro-35 SST-14 radioligand, synthetic SST-14 standards, and 10% bovine serum albumin-coated charcoal separation method (30, 31). Antibody R149 is directed against the central segment of SST-14 and detects SST-14 as well as the molecular forms extended at the amino terminus of the peptide such as SST-28 and PSST.

Immunofluorescence Microscopy—The cellular localization of SST-14 LI in AtT-20 cells expressing wild type and mutant PSST forwarded was determined by immunofluorescence microscopy (32). Stably transfected AtT-20 cells were plated at 1.25 × 10^5 cells/well in 24-well plates coated with 50 mg/ml polyornithine. On day 3 at ~60–70% confluence, cells were washed twice in PBS and fixed in 2% paraformaldehyde (0.1% PBS) for 20 min on ice. Cells were then permeabilized with 0.2% Triton X-100 in 0.1% PBS for 5 min at room temperature, washed three times in PBS and incubated with R149 anti-SST-14 antibody (diluted in PBS) for 1 h at 4 °C. The cells were washed with PBS and incubated for 90 min at 20 °C with Cy3-conjugated goat anti-rabbit secondary antibody (1:200). For staining the Golgi apparatus, cells were washed twice in PBS and incubated for 5 h with wheat germ agglutinin conjugated to fluorescein (1:1000). Finally, cells were washed twice with PBS, mounted with immunofluor and viewed under a Zeiss LSM 410 confocal microscope. Images were obtained as single optical sections taken through the middle of cells and averaged over 32 scans/frame.

Secondary Structure Prediction and Model Building—The secondary structure of rPSST (residues Ala1–Cys25) was predicted with the NPS@ consensus secondary structure prediction algorithm (37) using 11 secondary structure prediction methods: SOPM, SOPMA, HNN, DPM, DSC, GOR-I, GOR-III, GOR-IV, PHD, PREDATOR, and SIMPA96. A structural model of the predicted a-helical region Pro1–Thr3 was constructed using the structures of the BIC protein (2F5V) and SYBYL 6.6 molecular modeling software (Tripos Inc., St. Louis, MO). NH2 and COOH termini were blocked with acetyl and methylamino groups, respectively. Structural refinement was carried out by energy minimization using an AMBER 4.1 all-atom force field (38) and a distance-dependent (4R) dielectric constant.

Statistical Analysis—Results are expressed as mean ± S.E. Statistical analysis was carried out by one-way analysis of variance followed by the post hoc Bonferroni’s test for multiple comparisons.
RESULTS

Basal and Stimulated Release of WT PPSST—AtT-20 cells expressing WT PPSST released total SST-14 LI at a low basal rate of 0.54 ± 0.08 ng/ml/4 h representing 8.3% of total cell content (Fig. 3A). FSK stimulated SST-14 LI secretion 1.9-fold, whereas TPA demonstrated a 3.3-fold stimulation of SST-14 LI release. By immunocytochemistry, SST-14 LI displayed punctate localization in vesicular structures in both the main cell body throughout the cytoplasm as well as in cell processes (Fig. 4, A–C). These results provide both morphological and functional evidence that PPSST is properly sorted to the RSP in AtT-20 cells displaying low basal secretion and positive response to secretagogue stimulation, thereby making these cells an appropriate model for studying PPSST sorting to the RSP.

ΔPPSST, KR, and RTKR Substitution Mutants—To assess the sorting function of the NH₂-terminal domain of PPSST, we created a deletion mutant in which the Ser 3 to Leu 15 residues were removed. In addition, two other mutants were created, replacing the putative monobasic Lys 13 processing site with RTKR (a classic furin motif) or the dibasic motif KR to enhance NH₂-terminal PPSST cleavage endogenously by the prohormone convertase furin or PC1/PC2, respectively. AtT-20 cells stably expressing the ΔPPSST mutant released SST-14 LI at a high basal rate (7.1 ± 0.33 ng/ml/4 h) representing ~50% of total cell content (Fig. 3B). Release was unresponsive to FSK or TPA stimulation during a 4-h incubation (7.12 ± 0.38 and 7.83 ± 0.35 ng/ml SST-14 LI, respectively) (Fig. 3B). Similar results were obtained with the RTKR and KR substitution mutants, which showed even higher basal release of SST-14 LI of 72 and 81% of cell content, respectively, with no response to FSK and TPA stimulation (Fig. 3, C and D). These results were correlated with immunocytochemistry. Contrary to WT PPSST expression in AtT-20 cells, SST-14 LI in cells expressing the KR substitution mutant was localized to a perinuclear area that was immunopositive for wheat germ agglutinin (WGA) and corresponded to the TGN (Fig. 4, D–F). Similar results were obtained with ΔPPSST and RTKR mutants. Constitutive secretion, absence of secretagogue responsiveness, and lack of SST-14 LI staining in punctate granules suggest that the PPSST-(1–15) domain harbors important information that is essential for sorting PPSST correctly to the RSP.

Alanine Substitution Mutants—Having found that the amino-terminal 3–15 domain of PPSST contains a potent sorting signal, we proceeded to map specific amino acid residues involved by alanine scanning mutagenesis. Mutants were stably expressed in AtT-20 cells and characterized for basal and regulated secretion, and the results were correlated with immunocytochemistry. Basal release of SST-14 LI from the Ser 3, Asp 4, Lys 13, Ser 14, and Leu 15 mutants was ~10% of total cellular content, comparable with that of WT PPSST (Fig. 5). Pro 5, Arg 6, Arg 8, Gln 9, Phe 10, and Gln 12 mutants, however, exhibited somewhat higher levels of basal SST-14 LI release compared with wild type (~15% of cell content/4 h). Substitution of the Leu 7 and Leu 11 residues with Ala resulted in a dramatic increase in basal secretion to 72 and 70% of total cell content, respectively, comparable with the amounts found with the
AtT-20 cells transfected with Ala substitutions displayed various effects on SST-14 LI release and immunofluorescent staining patterns:

- **WT**: Full-length PSST accounted for 68% of total SST-14 LI, with a 25–31% peak corresponding to full-length PSST, accounting for 10% of the total intracellular immunoreactivity.

- **Ser3** and **Asp4**: These mutants showed a 2-fold increase in secretion in response to FSK and TPA. Like WT PSST, the Lys13, Ser14, and Leu15 mutants displayed a punctate pattern of staining throughout the cytoplasm similar to WT PSST, implying proper PSST targeting to secretory granules.

- **Gln9**: SST-14 LI released basally consisted of SST-14, SST-28 (retention time 73 min), and PSST (retention time 111 min), representing 65, 28, and 7% of total immunoreactivity, respectively (Fig. 6B). SST-14 LI released basally consisted entirely of two peaks corresponding to SST-14 (70%) and SST-28 (30%) (Fig. 6B). A similar ratio of SST-14 to SST-28 was obtained in FSK and TPA stimulated release medium (data not shown). The Leu15 to Ala mutant displayed comparable HPLC profiles to WT PSST in both cell extracts and media. Thus, this mutant was efficiently processed intracellularly to SST-14 and SST-28 (67 and 24% of SST-14 LI, respectively). SST-14 and SST-28 were also the principal immunoreactive species released into the medium; the peak corresponding to PSST released from these cells comprised 9% of the total released immunoreactivity. In contrast, mutants characterized by diversions of PSST from the RSP to the CSP (ΔNPSST, Lys13 to KR, Lys13 to RTKR, Leu6 to Ala, Leu11 to Ala) displayed a different HPLC profile of SST-14 LI, SST-28, and unprocessed PSST (Fig. 6, C–F, Table II). In the case of the Leu6 to Ala mutant, despite the missorting, PSST to the RSP, the precursor was efficiently cleaved intracellularly to SST-14 and SST-28 (59 and 31%, respectively); a third peak corresponding to full-length PSST accounted for 10% of the total intracellular immunoreactivity (Fig. 6E, left panel). In contrast to cell extracts, however, the HPLC profile of SST-14 LI released basally in the medium was very different, with only small amounts of processed SST-14 and SST-28 (14 and 18%, respectively); the major product released into the medium of these cells was full-length PSST, accounting for 68% of total SST-14 LI (Fig. 6E, right panel). As expected, the pattern of release after FSK or TPA stimulation was identical to that of basal release, since neither secretagogue provoked regulated release from these cells (data not shown). Similar results were observed in the case of the Leu11 to Ala mutant and the ΔNPSST, Lys13 to KR, and Lys13 to RTKR mutants, all of which displayed efficient intracellular PSST processing to SST-14 and SST-28 (62 and 25–31%, respectively), with a small 7–13% peak corresponding to unprocessed PSST. However, the major form released into the medium both basally and in response to secretagogue stimulation was unprocessed PSST, accounting for 51–73% of total released immunoreactivity. These results indicate that PSST that fails to be targeted to the RSP can still be processed to SST-14 and SST-28 in TGN compartments. However, PSST targeting is critical for efficient processing of the releasable pool of SST-14 and SST-28.

**Molecular Modeling of rPSST**—We constructed a structural model of the Pro5 to Thr19 sequence of rPSST based on the
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Secondary structure prediction data (Fig. 7A). This model reveals an amphipathic α-helix with a hydrophobic face formed by the side chains of Leu^7, Phe^10, Leu^11, and Leu^15 residues and a polar face comprising the side chains of Arg^6, Arg^8, Gln^9, Gln^12, and Lys^13 residues (Fig. 7B). The side chains of Leu^7 and Leu^11 residues that are essential for high activity are located in close proximity to each other on the hydrophobic surface of the α-helical structure. It is noteworthy that for all of the point mutants created for this study that contain a single amino acid residue mutated to alanine, the α-helical structure is highly probable due to the strong propensity of alanine to adopt the α-helical conformation (39). The inactivity of the Leu^7 to Ala and Leu^11 to Ala mutants can be attributed to the removal of critical side chains from the hydrophobic surface, which probably forms a binding interface for the propeptide, rather than to global conformational changes introduced by the mutations.

DISCUSSION

In this study, we have shown that the PSST-(3–15) segment, which comprises an amphipathic α-helix, acts as a sorting signal for directing PSST to the RSP and that residues Leu^7 and Leu^11 separated by one turn on the α-helix are critical determinants of precursor sorting. Disruption of the NH2-terminal α-helix does not impair the ability of PSST to be processed at the COOH terminus to SST-14 and SST-28. Processing, however, is shifted to early compartments of the secretory pathway instead of storage granules and is relatively inefficient.

TABLE II

Comparison of the percentage of SST-14, SST-28 and unprocessed PSST derived from HPLC chromatograms of cell extracts and media from AtT-20 cells expressing WT or mutant PSST

|                  | Cell extract | Medium          | Unprocessed PSST |
|------------------|--------------|-----------------|------------------|
|                  | SST-14       | SST-28          |                  |
| WT               | 65 ± 5       | 28 ± 7          | 7 ± 2            |
| Δ NPSSST         | 61 ± 2       | 26 ± 3          | 13 ± 3           |
| Lys^13 → KR      | 62 ± 5       | 26 ± 6          | 13 ± 6           |
| Lys^15 → RKR     | 62 ± 11      | 31 ± 5          | 7 ± 1            |
| Ser^3 → Ala      | 70 ± 8       | 23 ± 4          | 7 ± 1            |
| Leu^7 → Ala      | 58.6 ± 6     | 30.8 ± 3        | 10.6 ± 5         |
| Leu^11 → Ala     | 62.2 ± 7     | 28 ± 8          | 9 ± 2.2          |
| Lys^13 → Ala     | 68.3 ± 10    | 22 ± 5          | 9.7 ± 4          |
| Leu^15 → Ala     | 67 ± 8       | 24 ± 3          | 9 ± 2            |

|                  | SST-14       | SST-28          |                  |
|                  | %            | %               |                  |
|                  | %            | %               |                  |
| WT               | 70 ± 9       | 30 ± 3          | 9                |
| Δ NPSSST         | 22 ± 3       | 27 ± 2          | 51 ± 4           |
| Lys^13 → KR      | 20 ± 3       | 22 ± 5          | 58 ± 9           |
| Lys^15 → RKR     | 16 ± 1       | 20 ± 4          | 64 ± 5           |
| Ser^3 → Ala      | 68 ± 5       | 25 ± 3          | 7 ± 3            |
| Leu^7 → Ala      | 14 ± 3       | 18 ± 5          | 68 ± 7           |
| Leu^11 → Ala     | 10 ± 2       | 17 ± 5          | 73 ± 11          |
| Lys^13 → Ala     | 68 ± 5       | 28 ± 6          | 4 ± 1            |
| Leu^15 → Ala     | 65 ± 6       | 26 ± 6          | 9 ± 5            |
Several previous studies have shown that the prosegment of PSST harbors a sorting signal (18, 19, 28). For instance, an SST fusion protein consisting of the signal peptide and proregion of anglerfish PSSTI fused to α-globin is sorted to the RSP in transfected GH3 cells, whereas the α-globin gene joined to the β-lactamase signal peptide is degraded in the secretory compartment (18). Anglerfish PSSTI transfected in Rin5F cells is directed to the RSP, whereas anglerfish PSSTII is mainly targeted to the CST (19). A fusion protein comprising the first 54 residues of rPSST and the last 48 amino acids of anglerfish PSSTII is correctly targeted to the RSP (19). Deletion of the PSST-(1–10) segment results in selective blockade of the mutant precursor from sorting into a TPA-responsive (but not cAMP-responsive) secretory compartment (28). These findings suggest that NH2-terminal sequences of rPSST (and probably anglerfish PSSTI but not anglerfish PSSTII) contain intracellular targeting information (19). Molecular modeling of rPSST reveals an α-helix at residues 5–19 with the side chains of residues Leu7, Phe10, Leu11, and Leu15 forming a contiguous hydrophobic patch on the helix surface (Fig. 7). This domain is highly conserved in all known vertebrate PSST molecules as well as in the SST-related precursor proctistatin (PCST) (where it is located not at the NH2 terminus but further downstream at residues 19–35) but is not present in anglerfish PSSTII consistent with the targeting data obtained for this precursor experimentally (Fig. 1). The secondary structure predictions of a dozen other prohormones that are known to be precursor experimentally (Fig. 1). The secondary structure predictions of several other prohormones that are known to be precursor experimentally (Fig. 1). The secondary structure predictions of several other prohormones that are known to be precursor experimentally.
These results provide direct evidence that an α-helix in PSST and PC1 mediates the targeting of the two proproteins to the RSP and suggest that an α-helical structure common to a number of prohormones may serve as a general sorting signal. The α-helix sorting signal differs from the disulfide bond constrained amphipathic hairpin loop structure shown to be a sorting signal for POMC (21, 24, 26). The critical elements of this motif comprise residues DLEL at the apex of the loop and Cys8/Cys20 residues that form a disulfide bridge (21). Molecular modeling has revealed a similar putative disulfide bond constrained sorting motif in proenkephalin and proinsulin (26).

If there is a sorting signal, does it bind to a specific sorting receptor? Thus far, two proteins have been proposed to function as sorting signal receptors. One is the inositol 1,4,5-triphosphate receptor, which binds chromogranin A (43). This receptor, however, is only weakly expressed in secretory granules of neuroendocrine cells and therefore is unlikely to function as a general sorting receptor. The second is membrane-associated CPE expressed in high concentrations in TGN and secretory granule membranes of neuroendocrine cells (24–27). CPE binds to the POMC sorting signal motif (24). CPE interacts at Arg-Lys basic residues with the acidic residues in the POMC sorting signal (25). Mutation of the binding site on CPE, in vitro antisense depletion of CPE, or genetic ablation of CPE in the CPE fat mouse leads to an unprocessed PSST through the CSP. The remainder of the precursor, however, was retained in the TGN, where it underwent relatively efficient processing to both SST-14 and SST-28, presumably through the action of PC1 (for SST-14) and furin/PCACE4 (for SST-28). These results are consistent with previous studies that have shown significant processing of PSST in the absence of secretory granules in TGN compartments (48, 50). Overall, this means that the NH2-terminal PSST conformation does not influence enzyme recognition and PSST cleavage at the COOH terminus. The main consequence of the blockade of PSST entry into secretory granules is incomplete precursor processing and retention of the cleaved mature products in Golgi vesicles. Targeting of PSST to secretory granules, therefore, subserves two purposes: to optimize processing and to package and store the mature products for regulated release.

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