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PROCESSING AND INTRACELLULAR TARGETING OF SOMATOSTATIN

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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I dedicate this thesis to my family and to all those people in my life, past and present, for their unconditional encouragement and support throughout my life experience as a student.

Also,

In memory of my mentor, my supervisor Dr. Yogesh Chandra Patel (1942-2003).

Thank you.

ABSTRACT

Mammalian prosomatostatin (PSST) yields two bioactive peptides SST-14 and SST-28 through C-terminal cleavage at basic residues by protein convertases (PCs). Additionally, a second cleavage, mediated by an unknown enzyme at a putative Lys^{13} residue at the NH_2-termius generates $PSST_{[1-10]}$ (antrin). The function of the PSST prosegment is unknown, but its high degree of sequence homology throughout vertebrate evolution argues that it may harbor information for directing PSST towards the regulated secretory pathway (RSP). Therefore, the PSST NH_2-terminal region has been extensively analyzed with the following questions asked: **Does the NH_2-terminus play a role in targeting of PSST to the RSP? Is PSST cleaved at the Lys^{13} site resulting in antrin production, and by the action of which enzyme? What is the identity of the putative receptor in the *trans*-Golgi involved in properly targeting PSST?** To test the role of antrin in sorting function, alanine scanning and deletional mutagenesis were constructed and stably expressed in AtT-20 cells. The region Pro^5 to Gln^{12} which constitutes an α-helix was identified as being important in precursor targeting with Leu^7 and Leu^{11} being critical. Earlier morphological studies have implicated immature secretory granules as the site of prohormone processing however, blockade of PSST targeting through disruption of the helix did not hinder the ability of PSST to be processed at its C-terminus. Furthermore, I find that PSST NH_2-terminal cleavage does not take place at a basic site but, most likely at the -**R**^{6}-L^7–**R**^{8}-Q^9-**F**^{10}-**L**^{11}↓ recognition motif. Overexpression experiments in COS-1 and HEK-293 cells implicate the recently cloned subtilase-kexin-isozyme 1 (SKI-1) in antrin production. Additionally, as proteins function exclusively by means of interaction with other molecules, using the PSST NH_2-terminus as a bait in yeast two-hybrid screening assay I attempted to find specific components in the *trans*-Golgi for proper sorting to the RSP. 34 positive clones were recovered 10 of which, have exact similar sequences implying a very strong interaction. The isolated clone appears to be involved in protein folding within the endoplasmic reticulum. Interestingly, we also find that carboxypeptidase E, already implicated in sorting of a number of neuroendocrine hormones, interacts with the PSST sorting signal in a yeast two-hybrid assay.

RÉSUMÉ

La prosomatostatin (PSST) mammifère est coupée aux résidus basiques à l'extrémité C-terminale par les prohormones convertases (PCs) pour générer deux peptides bio-actifs, le SST1-4 et le SST-28. La PSST est aussi coupée par une enzyme non-identifiée au résidu Lys^{13} à l'extrémité N-terminale pour produire $PSST_{[1-10]}$ (antrin). La fonction du prosegment de PSST est inconnue mais le haut degré d'homologie de sa sequence à travers l'évolution des vertébrés suggère que le prosegment pourrait contenir l'information lui permettant de se diriger vers la voie de sécrétion régulée (VSR). Ainsi, nous avons analysé la région à l'extrémité N-terminale de PSST avec le but de répondre aux questions suivantes : **Est-ce que l'extrémité N-terminale est impliquée dans l'adressage de PSST à la VSR? Est-ce que PSST coupée au résidu Lys^{13} mène à la production d'antrin, et par l'action de quelle enzyme? Quel est l'identité du récepteur putatif dans le *trans*-Golgi qui serait impliquée dans l'adressage de PSST?** Dans le but de tester le rôle d'antrin dans l'adressage de PSST, des mutants furent crées et exprimées de façon stable dans les cellules AtT-20. La région à l'extrémité N-terminale contenant un α-hélix (Pro^5 à Gln^{12}) fut identifiée comme étant importante pour l'adressage du précurseur peptidique et les résidus Leu^7 et Leu^{11} sont critiques pour cette fonction. Des études morphologiques antérieures avaient impliqué les granules immatures de sécrétion comme le site de maturation protéolytique de précurseurs peptidiques. Cependant, disruption de cet hélix qui bloque l'adressage de PSST n'a pas nui à sa maturation protéolytique à l'extrémité C-terminale. De plus, le site de coupage de l'extrémité N-terminale de PSST n'est pas un site basique, mais serait probablement le motif de reconnaissance **-R^6**-L^7–**R^8**-Q^9-**F^{10}-L^{11}**↓. Des études de surexpression de subtilase-kexin-isozyme 1 (SKI-1) dans les cellules COS-1 and HEK-293 impliquent cette enzyme nouvellement clonée dans la production d'antrin. Puisque les protéines fonctionnent seulement en interagissant avec d'autres molecules, nous avons essayer d'identifier les éléments spécifiques du *trans*-Golgi qui seraient responsables pour l'adressage de PSST au VSR en utilisant l'extrémité N-terminale de cette protéine pour des expériences de double-hybrid chez la levure. En utilisant cette technique, nous avons identifié 34 clones positifs, parmi lesquels, 10 possédait exactement la même séquence, suggérant une forte interaction. Les clones isolés sont probablement impliqués dans repliement de protéines à l'intérieure du reticulum endoplasmique. De façon intéressante, nous avons trouvé que le signal d'adressage de PSST interagit avec carboxypeptidase E, une protéine impliquée dans l'adressage de plusieurs hormones neuroendocrinielles.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor of five years, Dr. Y.C. Patel who unfortunately passed away January 8 'th 2003. When I started working in the lab, I barely knew how to hold a pipette. Working under your supervision and in your lab has given me the opportunity to literally try my absolute best at all fronts needed for me to become a successful scientist. Your love and dedication to science, even when you were extremely ill, are both highly inspirational.

I would like to thank Dr. Lillian Puebla, a post-doc from Spain, the first person who trained and gave me a crash course in HPLC and RIA when I initially started working in the lab in 1997. Her patience, despite her busy schedule, will always be appreciated and never forgotten.

I would like to thank Dr. R. Sasi who taught me the ABCs in molecular biology in a stepwise fashion. With the solid base acquired from him I became very confident and capable of working independently.

I would like to acknowledge the technical assistance of the best tissue culture technician Wei Wang, who helped and trained me when it came to techniques related to tissue culture work.

In particular, I would like to acknowledge the unconditional support, friendship and especially encouragement of Dr. Jose L. Ramirez who always believed in me.

I would like to acknowledge the help of Dr. H Bennett and his then research assistant James S. for making their laboratory and knowledge available any time needed especially, in my first years when I was attempting to master HPLC and gel permeation techniques. Your help will always be appreciated.

Particularly, I would like to thank Dr. Seidah NG for his support, invaluable comments, instructions, feedback and discussions throughout my work on the SKI-1 project.

I would like to thank Adallah A and, especially Bassel for helping me set-up and standardize the yeast two-hybrid technique in the lab.

Additionally, I would like to thank Dr. Turcotte B. for giving me the freedom to use the equipment in his laboratory at any time needed.

I wish to thank members of my thesis committee for their always very generous comments and feedback: Drs. Liu JL, Maysinger D, Bennett H, and Ribeiro Da-Silva A.

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I appreciate the help of Dr. Aruna Venkasteswarna and Ujendra Kumar when needed for advice related to immunocytochemistry techniques.

In the last few months leading towards the end of my degree, I am grateful to Dr. CB Srikant and Maria Correia for their support.

I wish to thank all other members, not mentioned earlier, past and present, from my lab Fraser laboratories, and my department Pharmacology and Therapeuitcs.

Finally, I am most grateful to the Research Institute, Department of Medicine of the Royal Victoria Hospital, FCAR-FRSQ, and the Canadian Institute of Health Research for providing me with studentship support throughout my entire PhD studies.

A few words in memory of my supervisor.....

It was the summer of 1997, the hour was 4:30 pm in the afternoon and the place was the United Arab Emirates. The phone rings and I run to pick it up. My sister calls from Montreal to inform me that someone by the name of Maria a secretory in a lab called Fraser labs would like me to come for an interview with a professor named Dr. Patel. There was a problem, I was on the other side of the Atlantic ocean. So, the next day I decide to call Maria and after explaining to her my situation we decide that the best solution is to have a phone interview with Dr. Patel. A couple of days later I pass the interview, and become a new member of Fraser labs commencing September 2nd 1997. Looking back now on that day and other wheels of days which followed, almost five years later, it seems like yesterday. Spending five years in the same place is not something to belittle at all and I thank you Dr. Patel for accepting me to be a part of your team. These past five years don't only translate me into a PhD student, but have also allowed me to acquire a lot of other useful skills for which I will use in the remainder of my career. Particularly, the one thing I will value the most is the amount of self-confidence which you have built in me.

You have accomplished a lot so far, one of which is 25 years of pioneering research on a hormone that you have naturally grown so attached to, somatostatin. The first paper, ever published, by you regarding this prohormone was in 1976 (Alpert LC, Brawe JR, Patel YC, Reichlin S. Somatostatinergic neurons in anterior hypothalamus: immunohistochemical localization. Endocrinology 1976; 98: 255-258.) Later, in 1977, you moved to Montreal and became head of the Endocrinology Division of the McGill University Hospital Center (MUHC), and director of the Royal Victoria Hospital's Fraser Laboratories. Then, in 1978, you developed a radioimmunoassay for somatostatin with a paper appearing in *Endocrinology*. From then on you published a total of 161 articles, receiving over 6000 citations. In addition, you continuously received recognition for all that you have accomplished, one of which is the Medical Research Council of Canada's distinguished Scientist Award and the Queens Jubilee Medal. So, congratulations.

In general, I suppose that we all have some of the outback in us, a deep interior where thoughts blow free, and the soul goes walking about on an endless dreamtime journey. This outback I talk about is your memories from this world which lies just on the other side of the land that is beyond Goodbye. In your trip you have probably said goodbye to a lot of students, technicians and post docs from the lab. It is really, really, unfortunate that it is now me saying Goodbye to you.

You will be really missed,
Thank you
Your student, Rania.

PREFACE

This thesis is organized in a manuscript-based structure, according to the provisions specified by the Guidelines of the Faculty of Graduate Studies and Research of McGill University, as listed in the following paragraphs:

> Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted for publication, or the clearly duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.
> If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.
> The thesis must still conform to all requirements of the "Guidelines for Thesis Preparation". The thesis must include: a table of contents, abstract in English and French, an introduction, which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary and a thorough bibliography or reference list.
> Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.
> In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral defense. Since the task examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all authors of the co-authored papers. Under no circumstances can co-author or any component of such a thesis serve as an examiner of that thesis.

This thesis is based on two manuscripts already accepted (1) or under review (2) for publication:

1. Mouchantaf R, Kumar U, Sulea T, and Patel YC. A conserved α helix at the amino terminal of prosomatostatin serves as a sorting signal for the regulated secretory pathway. *J. Biol. Chem.* 2001; **276**: 26308-26316. [CHAPTER B]

2. Mouchantaf R, Sulea T, Seidah NG, and Patel YC. Prosomatostatin is proteolytically processed at the amino terminal segment by the subtilase SKI-1. *J. Biol.Chem.* 2003. [CHAPTER C]

In addition, CHAPTER D is composed of preliminary results describing a yeast two-hybrid screening carried out in an attempt to elucidate the presence of a sorting receptor.

The following are a few additional publications published, and abstracts presented at conferences during the time of my PhD:

PUBLICATIONS

3. Mouchantaf R, Patel YC. Processing and intracellular targeting of somatostatin. Endocrine update, series-monograph on somatostatin. Kluwer Academic Publishers, New York USA. (in preparartion)

4. Ramirez JL, Mouchantaf R, Kumar U, Corchon VO, Rubinstein M, Low MJ, Patel YC. Brain somatostatin receptors are upregulated in the somatostatin deficient mouse. *Mol. Endo.* 2002; **16**:1951-63.

5. Puebla L, Mouchantaf R, Khare S, Bennett H, James S, Patel YC. Processing of rat preprocortistatin in mouse AtT-20 cells. *J. Neurochemistry* 1999; **73**: 1273-1277.

ABSTRACTS

1. Mouchantaf R, and Patel YC. A yeast two-hybrid system used to identify a potential interacting receptor for the regulated secretory pathway. 42nd Annual Meeting of the American Society for Cell Biology. (San Francisco, California, USA) December 14-18, 2002. **Poster presentation**.

2. Mouchantaf R, Sulea T, MingHong X, and Patel YC. Targeting of somatostatin at the level of the *trans*-Golgi. 9th Annual Life Sciences International Conference and Exhibition. (Ottawa, Canada) November 4-6, 2002. **Poster presentation**.

3. Mouchantaf R, Seidah NG, and Patel YC. Processing of prosomatostatin (PSST) to $PSST_{[1-10]}$ by the mammalian proteinase SKI-1/S1P. 30'th Annual Meeting Society for Neuroscience. (New Orleans, Louisiana, USA) November 4-9, 2000. **Oral presentation**.

4. Ramirez JL, Sasi R, Kumar U, Mouchantaf R, Otero V, Rubinstein M, Low MJ, and Patel YC. Brain somatostatin receptors (SSTRs) are upregulated in the somatostatin deficient mouse. 29'th Annual Meeting Society for Neuroscience (Miami Beach, Florida, USA) October 23-28, 1999. **Poster presentation**.

5. Mouchantaf R, Shida M, Sasi R, Puebla L, Bennett H, and Patel YC. Identification of a putative sorting signal motif of prosomatostatin (PSST) for precursor targeting to the regulated secretory pathway (RSP). 81'st Annual Meeting of the Endocrine Society (San Diego, USA) June 11-15, 1999. **Poster presentation**.

6. Puebla L, Khare S, Sasi R, Bennett H, Mouchantaf R, Patel YC. Processing of rat preprocortistatin. 80'th Annual Meeting of the Endocrine Society (New Orleans, USA) June 24-27, 1998. **Poster presentation**.

The contributions of the co-authors in the two papers that constitute my thesis are as follows:

Paper #1: Fig. 4: I performed the immunocytochemistry experiments however, Dr. Kumar U carried out the confocal microscopy work.

Fig. 7: Dr. Sulea T was consulted and, provided invaluable information pertaining to the secondary structure predictions of the PSST α-helix.

I performed all the other experimental work presented in the paper. The manuscript was written by both my supervisor Dr. Patel, and me.

Paper #2: Fig. 8C: Dr. Sulea T provided the molecular modeling figure for both PSST and hpro-BDNF.

Dr. Seidah provided invaluable information required for the completion of this work and was consulted in all matters related to this paper. Additionally he provided the following cells: a) transiently transfected CHO cells with rPPSST, hα$_1$-PDX with or without hSKI-1, b) vaccinia virus infections of COS-1 cells and HEK-293 cells with rPPSST, hSKI-1 and with or without hα$_1$-PDX. I carried out all the HPLC, RIA, mutagenesis constructs, stable transfections in AtT-20 cells and data analysis described in this paper. The paper was written by Dr. Seidah, Dr. Patel and me.

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CHAPTER B: A conserved α-helix at the amino terminal of prosomatostatin serves as a sorting signal for the regulated secretory pathway.

CHAPTER C: Prosomatostatin is Proteolytically Processed at the Aminoterminal Segment by the Subtilase SKI-1.

LIST OF ABBREVIATIONS

α	alpha
AC	adenylate cyclase
ACTH	adenocorticotropin
AD	activation domain
AF	anglerfish
AP	adaptor or assembly protein
ARF	adenosine diphosphate-ribosylation factor
AtT-20 cells	mouse pituitary tumour cells
β	beta
BD	binding domain
BDNF	brain-derived neurotrophic factor
BiP	binding protein
CCVs	clathrin-coated vesicles
CF	catfish
CGA	chromogranin A
CGB	chromogranin B
CNS	central nervous system
CPE	carboxypeptidase E
CRE	cAMP responsive element
CREBP	cAMP responsive element binding protein
CSF	cerebrospinal fluid
CSP	constitutive secretory pathway
CST	cortistatin
DAG	diacylglycerol
DAMP	3-(2,4-Dinitroanilino)-3' amino-N-methyldipropyl-amine
DTT	dithiothreitol
DPD	dipyridyl
DR	dopamine receptor
ER	endoplasmic reticulum
EST	Expressed Sequence Tag
ERD	ER retention defective
ERGIC	ER-Golgi intermediate compartment
FRET	fluorescence energy transfer
Fsk	forskolin
GABA	γ-aminobutyric acid
GAPS	GTPase activating proteins
GEFS	guanine nucleotide exchange factors
GGA	Golgi-localized γ-ear-containing ADP-ribosylation factor-binding protein
GH	growth hormone
GH4 cells	rat pituitary tumour cells
GFP	green fluorescent protein

G protein	guanine-nucleotide-binding protein
gpαF	glycosylated pro α factor
GPCR	G-protein coupled receptor
grp	glucose regulated protein
GST	glutathione-S-transferase
HEK-293 cells	human embryonic kidney cells
HFBA	heptafluoro-butyric acid
Hsp	heat shock protein
htgs	High Throuput Genome Sequence
IP3	inositol triphosphate
IP3R	inositol-1,4,5-triphosphate receptor
KDEL	Lys-Asp-Glu-Leu
Kir	inward rectifying potassium
KO	knock-out
MAPK	mitogen activated protein kinase
MCF7	mammalian breast cancer cells
M6PR	mannose-6-phosphate receptor
NGF	nerve growth factor
nr	non redundant
PACS	phosphofurin acidic cluster sorting protein
PC	protein convertase
Pc	procollagen
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulphonyl fluoride
POMC	pro-opiomelanocortin
PPCST	preprocortostatin
PPSST	preprosomatostatin
$PSST_{[1-0]}$	antrin
PTP	phosphotyrosine phosphatase
PTX	pertussis toxin
RIA	radioimmunoassay
RIN T3 cells	rat islet tumour cells
SREBP	serum responsive element binding protein
RSP	regulated secretory pathway
RSV(s)	regulated secretory vesicle(s)
S1P	subtilase-1 P
SCAP	SREBP cleavage-activating protein
SEAP-His	His-tagged secreted alkaline phosphatase
SKI-1	subtilisn-kexin-isozyme I
SRIF	somatotropin release inhibiting factor
SRP	signal recognition particle
SST	somatostatin
SST-14	somatostatin 14

SST-28	somatostatin 28
SSTR	somatostatin receptor
tER	transitional ER
TFA	trifluoroacetic acid
TGN	*trans* Golgi
TPA	tetradecanoylphorbolacetate
TRAM	translocating chain associated membrane
UPR	unfolded protein response
vs	versus
VSV-G	vesicular stomatitis virus glycoprotein
VTCs	vesicular tubular clusters
wt/WT	wildtype

LIST OF AMINO ACIDS

NAME	SYMBOL
Alanine	Ala, A
Arginine	Arg, R
Asparagine	Asn, N
Aspartic acid	Asp, D
Cysteine	Cys, C
Glutamic acid	Glu, E
Glutamine	Gln, Q
Glycine	Gly, G
Histidine	His, H
Isoleucine	Ile, I
Leucine	Leu, L
Lysine	Lys, K
Methionine	Met, M
Phenyalanine	Phe, F
Proline	Pro, P
Serine	Ser, S
Threonine	Thr, T
Tryptophan	Trp, W
Tyrosine	Tyr, Y
Valine	Val, V

CHAPTER A

INTRODUCTION

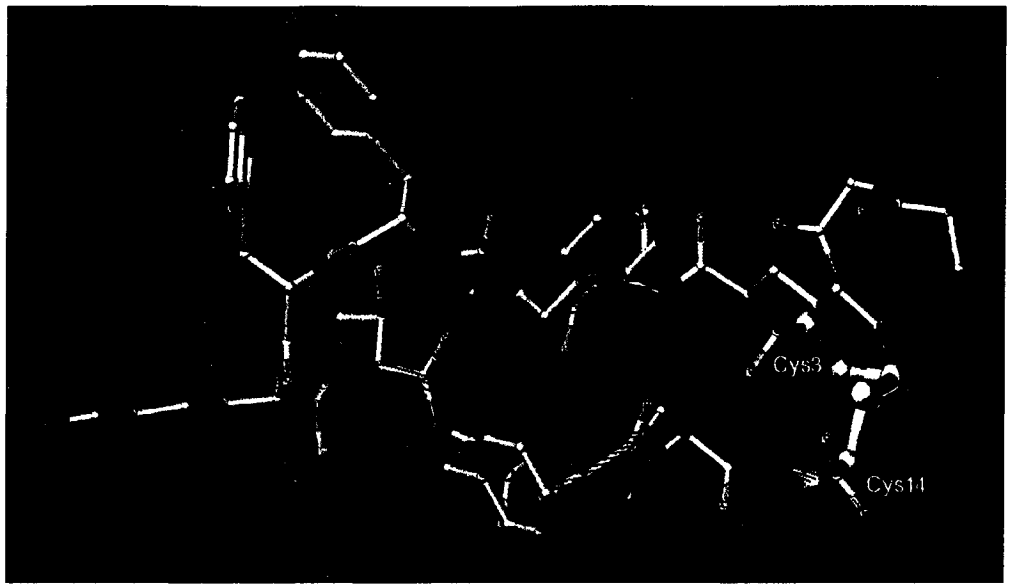
1. General background

1.1 Journey of Somatostatin

By the early 1960s, few studies had been performed considering the possibility that a component of the nervous system particularly in the hypothalamus contains a growth hormone (GH) release factor regulating GH synthesis and/or release from the pituitary (1,2). A few years later, in 1968, Krulich et al. provided the first evidence that an inhibitor of GH release is present in the hypothalamus (3). Through gel filtration experiments of either sheep or rat ovine hypothalamic extracts two zones eluted from a Sephadex G-25 column that influenced GH release *in vitro* upon its addition to incubated pituitaries. The nature of the postulated hypothalamic factor remained a mystery until 5 years later (1973), when Brazeau et al., in Roger Guillemin's laboratory, determined the amino acid (a.a) sequence of the GH release factor isolated from ovine hypothalamic tissue and called it somatostatin (SST) or, somatotropin release inhibiting factor (SRIF) (4,5).

At the time, SST was the third hypothalamic hormone to be discovered after thyrotrophin (6) and leuteinizing releasing hormone (7). Structurally, it was found to be a cyclic tetradecapeptide containing a disulfide bond and was designated SST-14 (Fig.1A). Synthetic analogs of SST-14 which could be iodinated were soon made available in which the synthetic molecule had similar characteristics to the natural form (8,9). This made possible the production of antibodies to SST, and in turn permitted the development of radioimmunoassays (RIAs) (10,11,12) as well as the application of immunofluorescent and immunocytochemical techniques for studies related to its cellular localization (13,14,15).

A. Ala_1-Gly-**Cys**-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-thr-Ser-**Cys_{14}**



B. Ser_1-Ala-Asn-Ser- Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-**Cys**-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-ThrSer-**Cys_{28}**

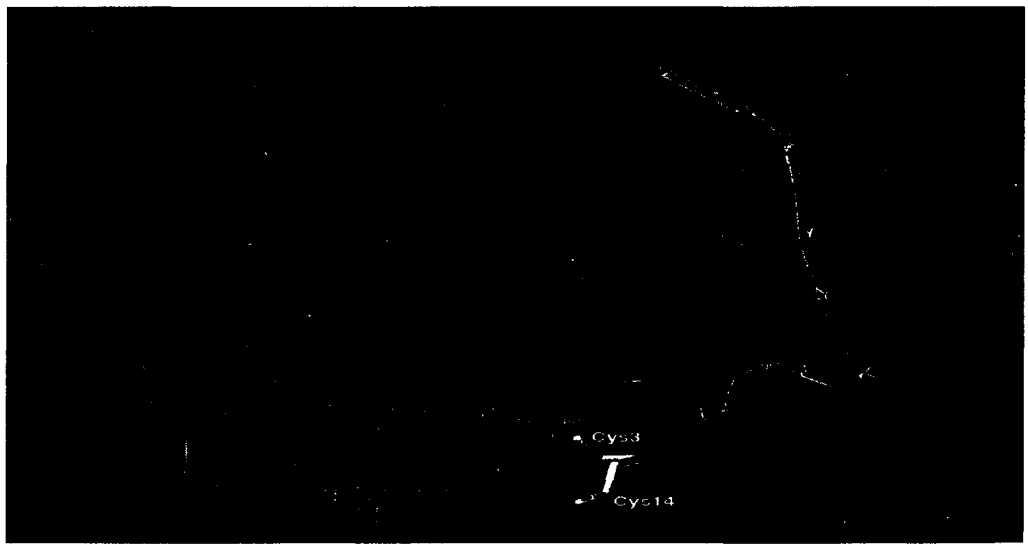


Fig.1 **Amino acid sequence and secondary structure of SST-14 (A) and SST-28 (B).** Cysteine residues represented in bold form a covalent interaction leading to the formation of a disulfide bond.

In clinical studies SRIF was shown to regulate not only the release of GH from the pituitary but also glucagon, prolactin, gastric acid, insulin and gastrin secretion (16,17) suggesting that SST plays a role not only in pituitary regulation but also affects other organs such as the pancreas and stomach. In view of the widespread effects and possible clinical significance of the hormone, several groups attempted to isolate the molecule from different species and examined organs other than the brain for SST content. Soon after it was realized that SST-14 was found to be widely distributed throughout the central nervous system (CNS) and peripheral tissues such as the gastrointestinal tract and the pancreas (18,19,20).

As early as 1974, Brazeau, in one of his original reports on the isolation of SST mentioned " *...it remained to be seen whether this peptide is the only one of a family of SRIFs; there is at least one other fraction of SRIF activity which is distinct from Somatostatin*" (21). Later, Arimura and colleagues demonstrated the presence of multiple immunoreactive forms of SST, thus paving the way for the search of the SST precursor (11). In 1976, Schally et al. isolated SST-14 from porcine hypothalamus and confirmed the structure to be identical to that of the ovine hypothalamus however, they discovered other immunologically active forms of SST as well (20). Similarly, other labs provided evidence for the heterogeneity of SST (19,22).

While looking at the SST characteristics in anglerfish (AF) pancreatic islets, Patel et al. not only attempted to study the localization and synthesis of SST-14 in pancreatic tissue but, the possible existence of a larger precursor peptide as well (23). In addition to observing the wide tissue distribution of SST, the molecule was further characterized in extracts of rat hypothalamus, cortex, stomach and pancreatic islets through fractionation

on a Shephadex G-25 column. Interestingly, a second peak was observed in addition to the one that corresponded to SST-14 supporting the notion that SST is most likely synthesized as part of a larger precursor.

Having reported the existence of higher molecular forms of SST which possess somatostatin immunoreactivity, the possibility that these substances could be oligomers of SST or SST bound to larger molecules remained questionable. The authenticity of a SST precursor was proven by Millar (24) and Noe et al. (25). The first group separated sheep hypothalamic extracts on a Sephadex G-25 column; the two eluted peaks were then lyophilized separately and treated with urea. After chromatography, the peptides maintained their characteristics observed prior to treatment negating the possibility that they were oligomeric species (24). The second group used pulse chase incubations followed by gel filtration, leading to the production of 8-15kDa pool identified as the precursor pool (25). This pool was then subjected to treatment with dissociative agents (urea, guanidine hydrochloride) and reducing agents (thioglycolic acid, thioglycol). There was no appreciable loss of radioactivity in any of these treatments providing support that SST is part of a larger precursor, with the additional observed peak not representing an oligomer of SST.

This novel peptide which cross-reacts with SST-14 antibody was found to be an NH_2-treminally extended form of SST and not the precursor (24,26). In 1980, six years after SST's original discovery, Pradayrol et al. were first to report the primary structure of the newly discovered peptide which they isolated from porcine intestinal tissue and called it somatostatin 28 (SST-28) Fig.1B (27). Other groups in that same year confirmed Paradoyl's discovery by isolating SST-28 from different tissues such as rat pancreatic

islets (28) and porcine hypothalamic tissue (29). The 1980s period was also known as the cloning era due to the emergence of the molecular biology field. Eventually SST was successfully fully cloned in human (30) and rat (31), and the full-length precursor was found to be composed of 116 a.a named preprosomatostatin (PPSST) in which the first 24 a.a represented the signal peptide sequence.

Studies on the biosynthesis and processing of PPSST were extensively characterized in our laboratory (Fig.2). Larger molecular forms of SST were further characterized in hypothalamic extracts and were shown to consist of two polypeptides with molecular weights of 4 and 24 kDa accounting for 5% and 35% of total tissue immunoreactivity (32a-c). Similar results were obtained using cultured hypothalamic neurons thus providing an *in vitro* system for investigating the biosynthesis and regulation of SST (33). Additionally, mammalian PPSST and its products demonstrated no binding to Concavalin A, Artorus Precatorius or agarose soybean lectin, indicating that they do not undergo any post-translational modifications such as glycosylation with the exception of endoproteolytic cleavage and disulfide bond formation (34). Having identified the cleavage products of PPSST, Galanopoulou AS et al. attempted to characterize the endoproteases responsible for PPSST processing and the significance of subcellular compartmentalization in the production of both SST-14 and SST-28 (35 a-c).

Since the discovery of the SST precursor attempts were carried out to identify additional bioactive domains most likely present within the 80 a.a preceding the C-terminal dodecapeptide. In 1987, seven years after the structure of SST-14 was identified, Benoit et al successfully isolated a third cleavage product of PPSST from the rat gastric antrum (36). It was identified as a 1 kDa decapeptide without any known biological

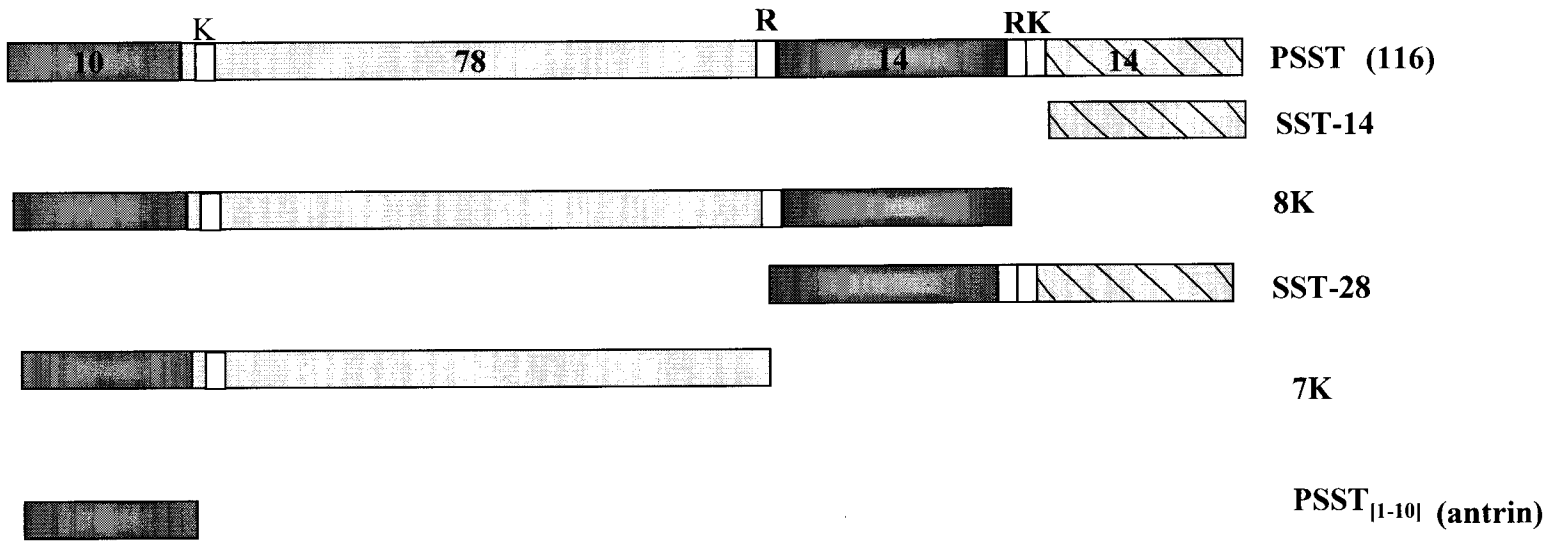


Fig.2 Diagram depicting mammalian PSST and its cleavage products.

activity attributed to it that is produced by cleavage at the NH_2-terminus of PSST (Fig.2). This cleavage product is generally referred to as antrin and also known as $PSST_{[1-10]}$ (36). Additionally, eleven years after the discovery of antrin, mRNA encoding the precursor of a new member of the SST family, whose distribution is primarily restricted to the cortical γ-aminobutyric acid (GABA) containing interneurons, was isolated from rat brain encoding 112 a.a and was appropriately called preprocortistatin (PPCST) (Fig.3) (37).

1.2 Evolution

SST is an ancient peptide that exists even in single-cell organisms such as *Tetrahymena pyriformis, Bacillus subtilis,* and *Plasmodium Falciparum* (38), suggesting its evolution prior to the appearance of cell-cell and nerve cell communication. Following the purification of SST-14 and SST-28 and the identification of their a.a sequence, a direct approach was undertaken by Hobart et al (39) and Goodman et al (40a-b) to characterize the complete SST precursor, as well as the SST mRNA, and gene. Much of the initial work on the characterization of the SST precursors was carried out in the fish pancreas which contains a high ratio of endocrine versus exocrine tissue thus providing a rich source of pure islets. Therefore using the endocrine portion of the AF pancreas two distinct mRNAs generating different SST precursors were surprisingly discovered: AF PPSST I (121amino acid) encoding AF SST-14 and AF PPSST II (125amino acid) producing AF SST-28 (Fig.4). The biologically active forms of the hormone were each found at the C-terminal end of the larger precursor.

Similarly, in the pancreas of channel catfish (CF), two predicted SST precursors were isolated: CF PPSST I (141 a.a), which gives rise to SST-14 (41, 42), and CF PPSST

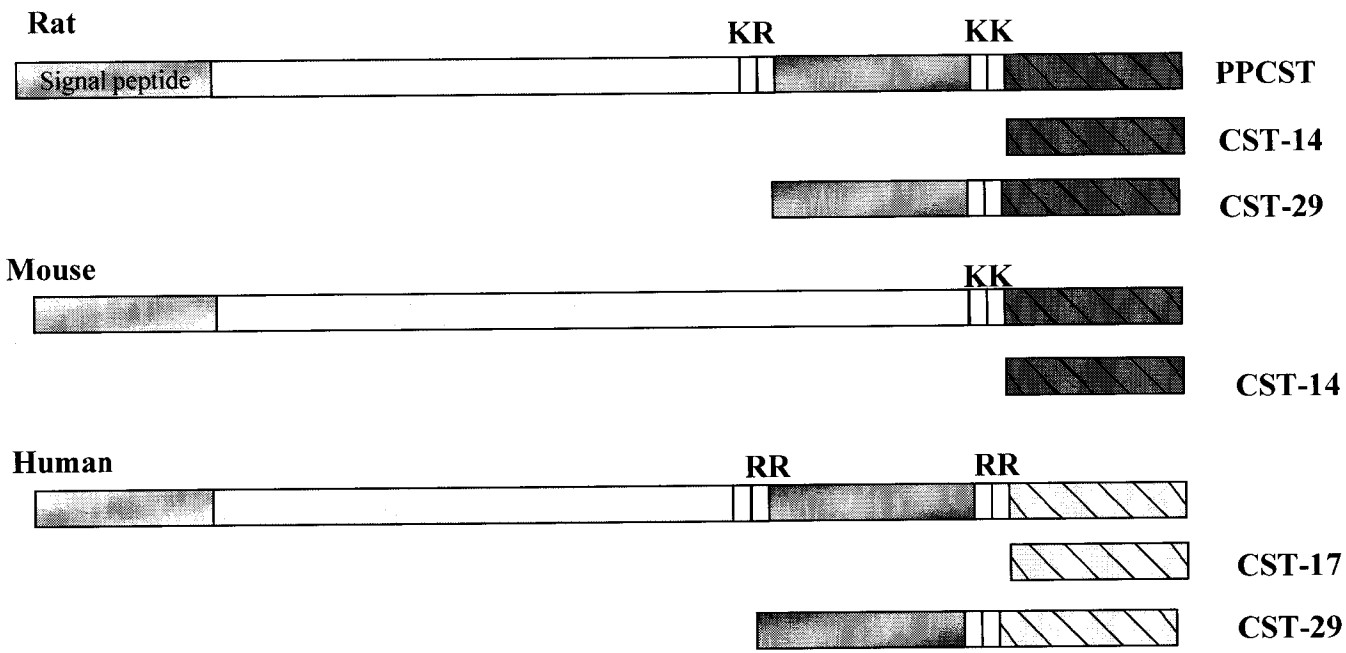


Fig.3 Schematic diagram illustrating the structure of rat, mouse and human PPCST. PPCST is synthesized as an inactive precursor containing a signal peptide sequence. The putative cleavage sites by processing enzymes have been indicated (KK, KR, and RR). Notice that in mouse there is no CST-29.

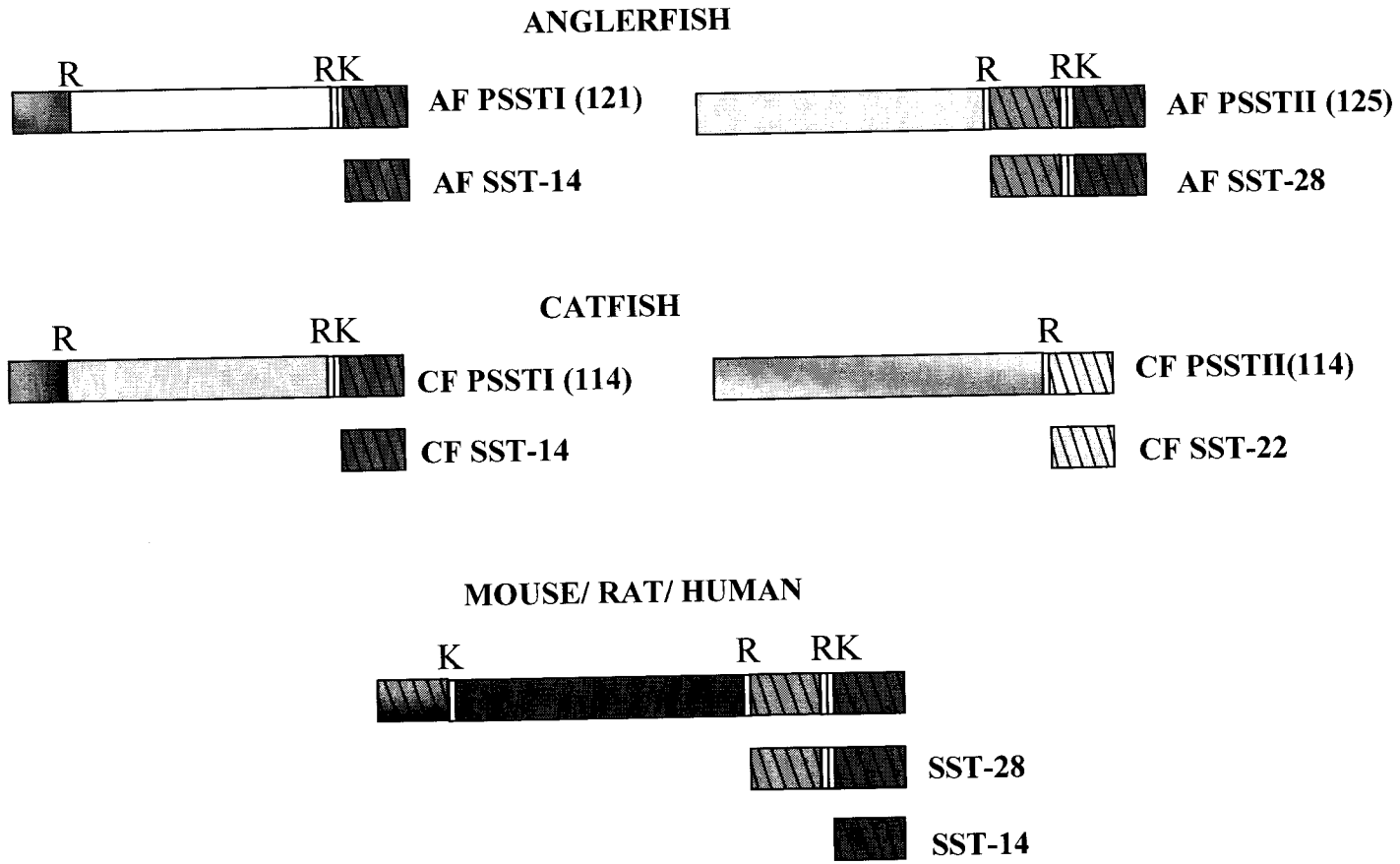


Fig.4 Schematic depiction of pathways comparing PSST processing in fish and mammals

II (105 a.a), which is processed to a homologue of SST-28, CF SST-22 (43). In rat (31, 44) and human (30) a single mRNA has been isolated which codes for both SST-14 and SST-28 suggesting that these molecules are derived from a common precursor encoding for 116 amino acid In addition, an antrin-like peptide has also been detected in the endocrine portion of the stomach of the CF, *Ictalurus nebulosus* (45).

Argos et al (46) and Su Chung et al (47) aligned the nucleotide precursor and amino acid sequence of a number of species such as rat, CF and AF. At the protein level sequences at the NH_2-terminal region of human, rat, AF PPSST I, and CF PPSSTI corresponding to antrin have been highly conserved across these species suggesting that it too might have biological activity, similar to SST-14 and SST-28, that is yet to be determined (Fig.5). On the other hand, sequences coding for SST-14 have been completely conserved along with the cleavage site containing the following sequence: RERK↓. Sequences upstream of SST-14 are less homologues; mammalian SST-28 shares 40% to 66% homology with its two fish counterparts. However, the a.a sequence from rat to humans is completely conserved throughout the entire protein with the exception of only 4 amino acid

The second SST related gene PPCST has been cloned from rat, mouse and human (48, 49). Interestingly, the mouse and rat PPCST are almost completely homologues at the protein level, when compared with the human sequence (Fig.6). In humans PPCST is cleaved at a dibasic RR site producing CST-17 whereas mouse and rat PPCST are cleaved at a dibasic KK site leading to the production of CST-14 (Fig.3). Alignment of the amino acid sequence between mouse, rat, and human PPCST reveals that the 14 a.a at

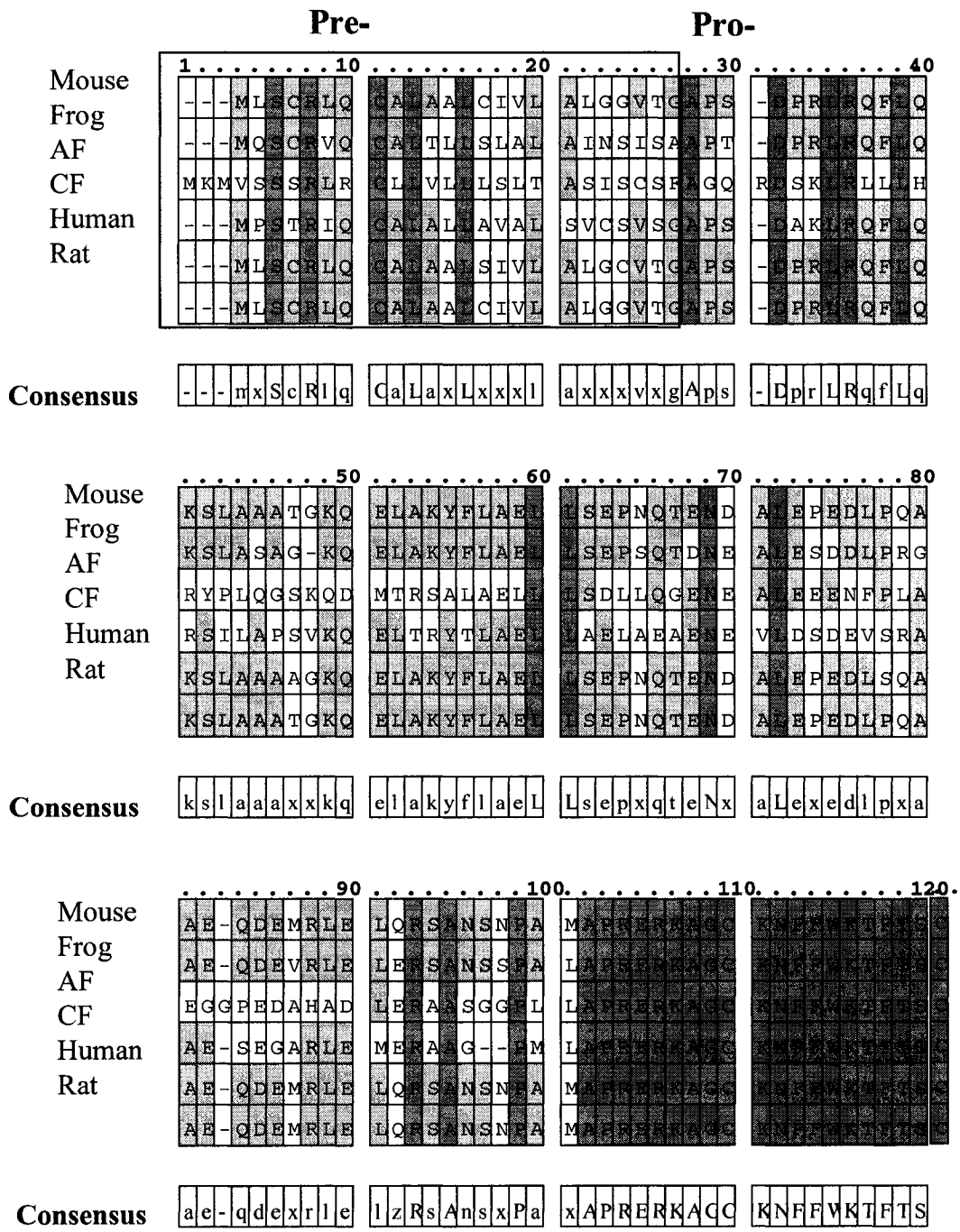


Fig.5 **Analysis of PPSST sequence homology across different species.** The boxed region represents the signal peptide that is the pre sequence in SST, usually cleaved early. This is followed by the pro region of SST. Certain sequences at the NH_2-terminus of PPSST are conserved throughout vertebrate evolution. However, sequences coding for SST-14 at the C-terminus are completely conserved.

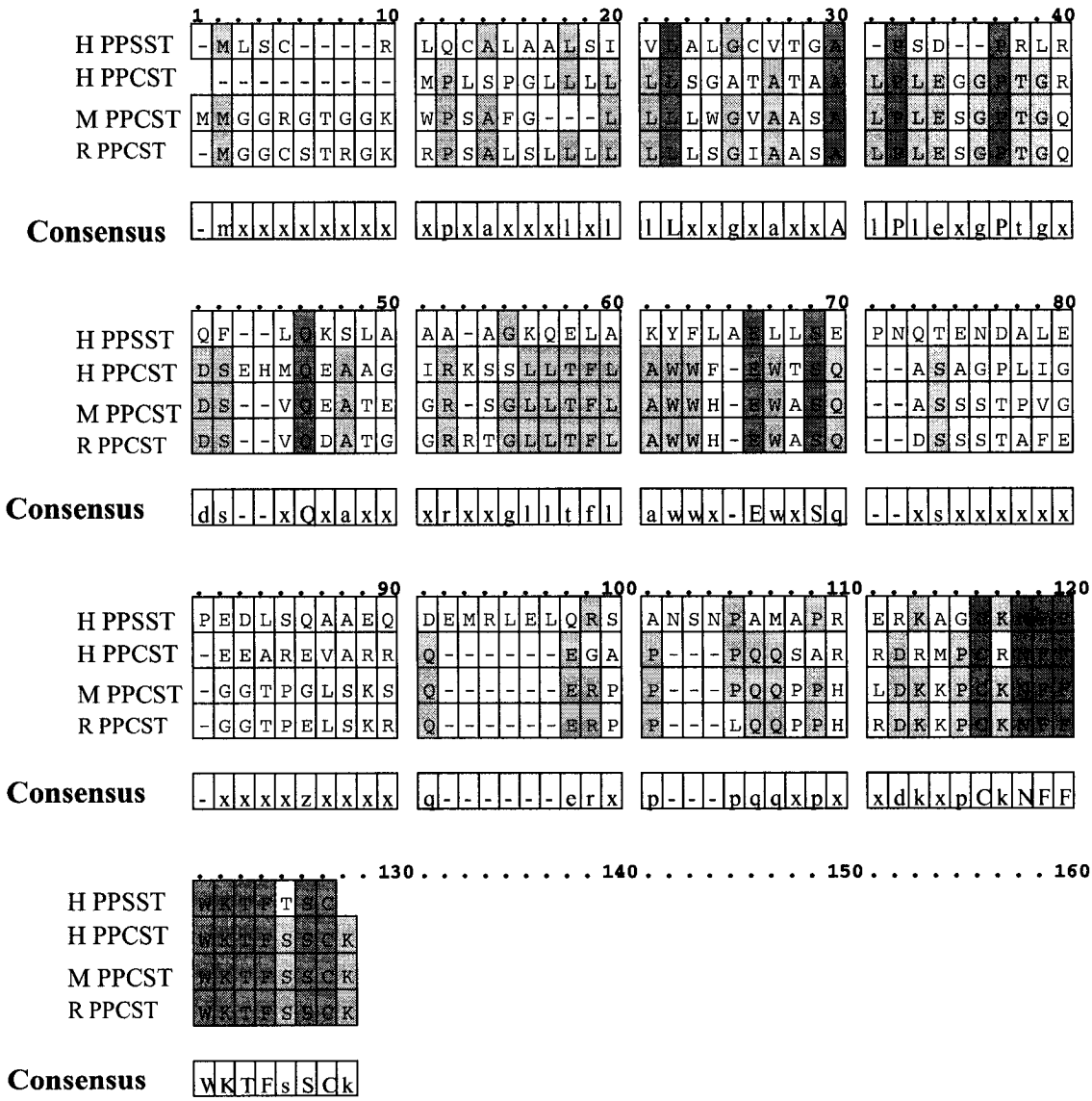


Fig.6 Alignment of human PPSST sequence with human (H), mouse (M) and rat (R) PPCST. Homologues Sequences between SST and CST are mainly located in the C-terminal region coding for CST-14.

the C-terminus are most similar to SST. This strongly suggests that CST-14 is probably the only active peptide derived from the precursor and that SST and CST are products of separate genes. Additionally, the mouse and rat PPCST sequence share 11 out of 14 residues with SST-14. In contrast, the human sequence shares 10 homologues residues with SST.

It has long been thought that in tetrapods, only a single gene encoding PPSST exists. Recently, another gene has been isolated from the frog *Rana ridibunda* that is only expressed in the brain (50). It encodes a 103 amino acid protein and is homologues to SST at the C-terminal region as well, with no appreciable sequence homology in the rest of the protein. This gene is believed to be the mammalian counterpart of CST due to its localization in the brain only, its homology to PPSST at its COOH-terminal end, and the presence of a proline residue at position 2, similar to PPCST.

1.3 Anatomical distribution

SST producing cells are not only localized to the hypothalamus, but are dispersed throughout the CNS, peripheral nervous system, endocrine pancreas, gut, and in small numbers in the thyroid, adrenals, submandibular glands, kidneys, prostate and placenta, and immune cells (14, 19, 51, 52). Nerve fibers containing SST have been detected in the heart as well. The typical morphological appearance of a SST cell is that of a neuron with multiple branching processes or a secretory cell often having short cytoplasmic extensions (53).

Within the CNS, the highest concentration of SST is encountered in the hypothalamus within the median eminence, followed by the periventricular, arcuate and

ventromedial nuclei (19). In extra hypothalamic regions of the brain the spinal cord contains the highest concentration of SST representing approximately 40% of that for the whole hypothalamus. This is followed by the cerebral cortex and the brain stem, which are around 25% that of the hypothalamic SST content (19, 33, 32a, 54). The olfactory lobe, cerebelllum, and pineal contain < 10% of the hypothalamic level. An interesting point is that technically the cerebral cortex contains the highest concentration of SST in the whole brain, whereas the hypothalamus makes up 7.3% of total SST due to its much smaller size. Similar to other neuropeptides, SST co-exists with a number of peptides and transmitters such as: noradrenaline, GABA, neuropeptide Y, and enkephalin (55).

In peripheral organs SST is present in high concentration in the stomach, pylorus, duodenum, jejunum, and pancreas and in small numbers in the thyroid, adrenals, submandibular glands, kidneys, prostate and placenta (54, 52). In the pancreas SST cells are virtually exclusively islet D cells, and account for 2-3% of total adult islet cell population (56). Gut SST cells are of two types: D cells located in the mucosal glands from the cardiac portion of the stomach to the rectum (with the highest concentration occurring in the antrum) and in neurons that populate both the submucous and myentric plexuses in all segments of the gastrointestinal tract (57).

Comparing the relative distribution of SST in both the CNS and peripheral tissue, the gut accounts for about 65% of total SST, the brain for 25%, the pancreas for 5% and the remaining organs account for 5% (19).

Both SST-14 and SST-28 are readily released from tissues and are detectable in the blood. The major source of circulating SST is from the gastro intestinal tract (58). Circulating SST is then inactivated rapidly by the liver and kidneys reaching a half-life of

2-3 minutes (51). It is also secreted into cerebrospinal fluid (CSF), most likely emanating from all parts of the brain (59). The concentration that it attains in CSF is usually twice that reached in the general circulation. Amniotic fluid is rich in SST as well (51).

PPSST processing is characterized by tissue specific variations whereby the relative proportions of SST-14 and SST-28 vary considerably in different tissues (58). SST-14 predominates in neural tissues, retina, peripheral nerves and is virtually the only form, in pancreas and stomach. The predominant form in the intestinal mucosa is SST-14. On the other hand SST-28 accounts for 20-30% of SST in the brain.

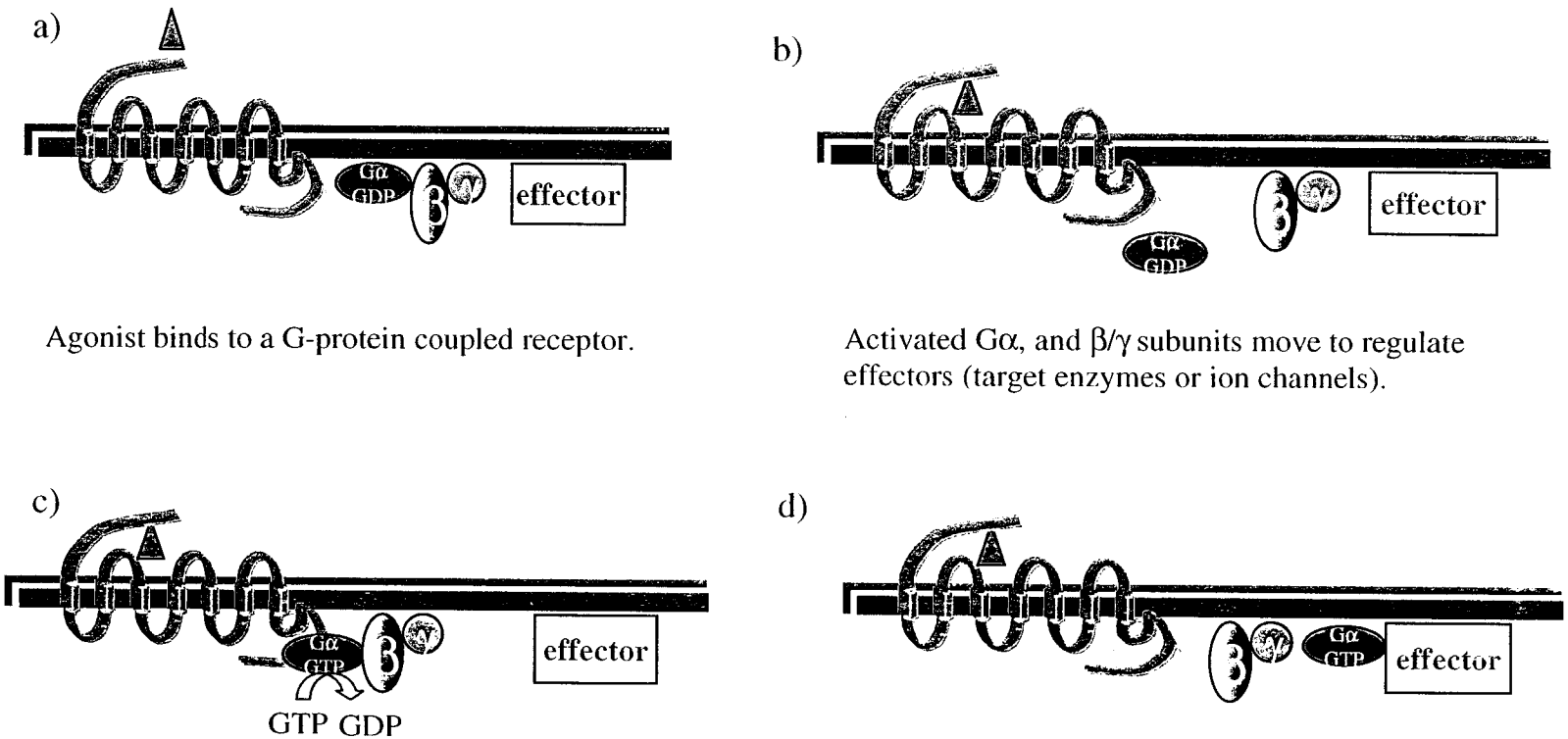
Having established the two SST bioactive peptides that are produced through cleavage at the C-terminal end of PPSST, another product isolated from the endocrine portion of the stomach corresponding to the first 10 a.a at the NH_2-terminus of mammalian PPSST was isolated in rat (36). The highest concentration of the decapeptide at the time was found in the gastric antrum hence the proposed name antrin. Antrin was later found not to be restricted to the gastric antrum only rather, the highest concentration was actually observed in the hypothalamus, followed by the cerebral cortex, the antrum, jejunal mucosa, and pancreas (60, 61). In general relative tissue amounts of antrin were proportional to that of SST-14 and SST-28 with the exception of the pancreas which contained the lowest concentration of $PSST_{[1-10]}$. Similar to SST-14 and SST-28, antrin undergoes secretion into the rat portal circulation as well (62).

Contrary to SST, CST is only localized in the brain and has not been detected in any peripheral tissues (37, 48). It is only found in GABAergic cortical interneurons in the cerebral cortex and in the hippocampus, but not in the hypothalamus.

1.4 Biological actions through receptor interaction

The diverse actions of SST are mediated through a family of heptahelical G-protein coupled receptors (GPCRs) that were first described in the rat pituitary tumor cell line GH4 cell line by Schonbrunn and Tashjian using whole cell binding analyses (63). Between 1992 and 1995 the structure of SST receptors (SSTRs) was elucidated through molecular cloning. Five individual subtypes were identified and found to be encoded by a family of five nonallelic genes located on separate chromosomes (64). All of the genes are intronless with the exception of SSTR2 which gives rise to splice variants SSTR2A and SSTR2B. There are thus six putative SSTR subtypes of closely related size. SSTRs have been localized in varying densities in the brain, gut, heart, lung, liver, spleen, placenta, pituitary, gut, endocrine and exocrine pancreas, adrenals, thyroid, kidneys, and immune cells (64, 65). Additionally, they have been localized to various cell lines such as mouse pituitary tumor cells (AtT-20), hamster insulinoma, islet tumor cells (Rin m5), human embryonic kidney cells (HEK 293) and mammalian breast cancer cells (MCF7).

All GPCRs share a common tertiary structure consisting of a single polypeptide chain that threads back and forth across the lipid bilayer seven times, with an extracellular N-terminus and a cytoplasmic C-terminus (66). Interestingly at current estimates they account for ~ 1% of the genes present in the mammalian genome (67). The general mechanism by which GPCRs transduce their signals is through interacting with heteromeric guanine-nucleotide-binding proteins (G proteins) (Fig.7), which then interact with effector systems (ion channels or target enzymes) regulating various intracellular processes through second messengers. In turn the signal is passed on by



Agonist binds to a G-protein coupled receptor.

Activated Gα, and β/γ subunits move to regulate effectors (target enzymes or ion channels).

G-protein complex is activated through a GDP to GTP switch in the Gα subunit

GPCR becomes directly coupled to effectors.

Fig.7 General cascade depicting the steps following ligand binding to the SSTRs.

altering the behaviour of selected intracellular proteins (68-70). The adenylate cyclase (AC)-cAMP-protein kinase A (PKA) pathway is one of the most widely studied intracellular effector (71, 72). There is a general agreement that all five SSTRs once activated are functionally coupled to inhibition of the adenylate cyclase pathway via a pertussis toxin (PTX) sensitive g-protein (Giα) leading to a decrease in intracellular cAMP (73). Additionally, in mammalian cells an elevation in cAMP turns on the gene that encodes for SST (73).

The second effector is the activation of K^+ channels that are directly and positively coupled to SSTRs, including the inward rectifier (Kir) (64). This results in hyperpolarization of the cell membrane leading to a reduction of intracellular Ca^{2+} concentrations due to inhibition of depolarization induced by the Ca^{+2} currents, the third effector, through voltage gated Ca^{2+} channels. SST may directly inhibit voltage-gated Ca^{2+} channels and the effect can be enhanced by the Ca^{2+} /calmodulin-regulated protein phosphatase calcineurin (74). This ultimately leads to a decrease in intracellular calcium influx. Additionally, SSTRs have been shown to be coupled to Na^+/H^+ exchanger in colon carcinoma cells (75).

Other signaling pathways that have been described include the regulation of another crucial enzyme - phospholipase C (PLC) (64). Once activated, PLC cleaves inositol phosholipids to two products: inositol triphosphate (IP_3) and diacylglycerol (DAG). At this step the signaling pathway splits into two branches. IP_3 is a small water-soluble molecule that leaves the plasma membrane and diffuses through the cytosol; there it releases Ca^{+2} from the endoplasmic reticulum (ER). DAG on the other hand activates serine/threonine protein kinase C (PKC) that phosphorylates selected proteins in target

cells. SSTR2 in particular has been shown to stimulate tyrosine PLC/IP3 production via pertussis toxin sensitive as well as insensitive mechanisms (76, 77).

Experiments have also suggested that stimulation of SSTRs leads to the activation of serine threonine phosphotyrosine phosphatases (PTPs) in different cell types via a PTX pathway which in turn dephosphorylates target proteins thereby inactivating them (76, 78). Recently particular interest has been devoted to the regulation of mitogen activated protein kinase (MAPK) pathways which might represent an important facet of SST signaling. MAPK phoshporylate many different proteins, including nuclear transcription factors involved in cell proliferation (79).

Therefore along with its wide anatomical distribution and its effects mediated through receptor interaction, generally SST mainly acts as an inhibitor on multiple targets including the brain, gut, pituitary, endocrine and exocrine pancreas, adrenals, thyroid and kidneys (64). In the pituitary SST inhibits both basal and stimulated release of growth hormone and thyrotropin, with no effects on prolactin, adrenocorticotropin, and gonadotropins. In pancreatic islets it inhibits the secretion of insulin, glucagon and somatostatin itself. In the gut it inhibits the secretion of gastrin, gut glucagon, vasoactive intestinal peptides, mortilin and secretin. The peptide impairs motility of the stomach, gallbladder, small intestine, reduces intestinal absorption of all classes of nutrients including water, reduces blood flow to the stomach, and inhibits cell proliferation in the stomach mucosa. Recently, SST has been shown to be responsible for accelerating the movement of cerebellar granule cell migration in the early phase of development and for terminating it in the late phase through altering intracellular Ca^{++} concentration and K^{+} channel activity (80).

At the cellular level the actions induced by SST can be divided into four mechanisms: regulation of neurotransmission, glandular secretion, smooth muscle contraction and cell proliferation (63). The profound ability of SST to inhibit cell secretion so widely can be explained through SSTR induced reductions of cAMP by inhibiting AC, a fall in Ca^{2+} influx due to its effects on K^{+} and Ca^{+2} ion channels, and stimulation of phosphatases such as calcineurin which inhibit exocytosis (Fig.8). On the other hand induction of PTPs dephosphorylates growth factor receptor kinases and inactivation of the MAPK signalling cascade leads to inhibition of cellular proliferation.

Regions within the SST sequence that are important for binding to SSTRs are the amino acid residues Phe^{7}, Trp^{8}, Lys^{9} and Thr^{10}, which comprise a B turn necessary for biological activity (Fig. 1A) (64). Thus for effective pharmacotherapy with SST, the general strategy for designing SST analogs has been to retain these crucial residues in order to have more selective actions and to attain greater metabolic stability than the naturally occurring peptide.

A SST knockout (KO) mouse has been created to further elucidate its role (81). These mice developed normally without any recognizable defects however, SST was shown to be important in regulating sexually differentiated GH secretion and action. Generally the episodic release of GH from pituitary somatotrophs results from the interplay between inhibitory SST and stimulatory GH-releasing hormone. The pulsatile GH release differs between males and females, and SST expression is greater in males than it is in females. Results from the KO experiments demonstrated that SST is a physiological determinant of masculinized GH secretory dynamics and sexually dimorphic patterns of somatic growth. Therefore effects of SST deficiency are more

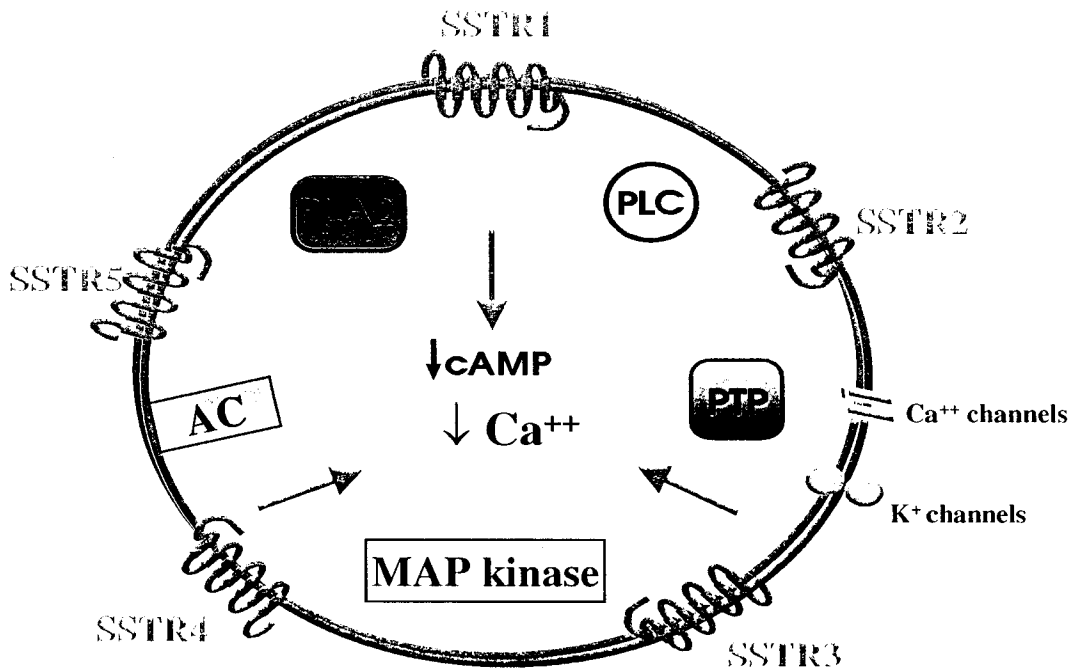


Fig.8 Schematic overview of the common signalling pathways downstream from GPCRs. Hormone binding to the receptor initiates a series of events ultimately leading to a decrease in cAMP and Ca^{++2}.

pronounced in males than in females due to normally greater expression of SST in the male hypothalamus. Additionally, the brain of SSTKO mice demonstrated upregulation of subtypes 1,2, and 5 mRNA expression and dowregulation of SSTR3 with no changes in SSTR4. Peripheral tissues displayed both subtype and tissue specific changes in SSTR mRNA expression.

Models that describe the interaction of GPCRs with their G-proteins are generally based on the assumption that receptors exists as monomers and couple to G-proteins in a 1:1 ratio (67). Recent pharmacological, biochemical, and physical studies in our lab based on fluorescence resonance energy transfer (FRET) provided evidence that members of the SSTR family undergo agonist-dependent homo- and heterodimerization and that dimeric association alters SSTR functions (82a). SSTR5 forms heterodimers with SSTR1 but not with SSTR4 suggesting that heterodimerization is a specific process that is restricted to some but not all receptor combinations. Additionally, interaction between distantly related members of the GPCR family was studied for the first time in our lab (82b) in which SSTR5 was shown to dimerize with dopamine receptor 2 (D2R). Exposure to selective SSTR5 agonist or selective D2R agonist resulted in an increase in the level of dimers, which suggests that activation of one of the receptors in complex was sufficient to mediate dimerization. Ligand binding studies revealed that the SSTR5 and D2R in combination exhibit higher affinity for a combination of selective SSTR5 and D2R agonists compared with a single agonist alone. Furthermore, the combination of agonists resulted in an increase in the efficacy of signaling. The two receptors were also shown to colocalize through immunocytochemistry in the same neurons within the

cerebral cortex. Thus heterodimerization could lead to changes in receptor function through modulation of its properties.

With regard to the second SST related protein CST, synthetic CST has been shown to share several biological properties with SST. It is capable of interacting with all five SSTRs on GH4 pituitary cells, inhibits the accumulation of cAMP, and depresses neuronal activity (37, 48, 49). Nonetheless the effects of CST on cortical activity, locomoter behavior and sleep are unique and not present in SST, suggesting the esxitence of a separate CST receptor. Additionally CST, unlike SST, antagonizes the effects of acetylcholine on cortical excitability thereby causing synchronization of slow brain waves.

2. Protein transport in the early compartments of the secretory pathway

2.1 Overview

Mammalian cells contain up to 10,000 different kinds of proteins, in order for a cell to function properly each of its numerous proteins must be localized to the correct cellular membrane or compartment (eg. cytosol, mithochondria, membrane, vesicles). SSTRs must be properly delivered to the membrane for them to transduce specific intracellular signals, and SST must be properly packaged into vesicles to be released upon stimulation. The process of directing newly synthesized proteins to particular destinations inside the cell is referred to as protein sorting or targetting along the secretory pathway. This process occurs at several levels inside the cell and is critical for the organization and functioning of eukaryotic cells.

The secretory pathway is arguably the most important and the most complicated sorting pathway in the eukaryotic cell. During its passage from the ER the initial cellular compartment for proteins in the secretory pathway to its final destination, a protein travels through many organelles with different internal environments and is subject to a number of modifications (83). The initial sorting event occurs during growth of the polypeptide chain on cytosolic ribosomes. All proteins such as PPSST entering the secretory pathway contain a specific signal peptide sequence always at the NH_2-terminus that directs the ribosomes synthesizing them to the ER (84). Protein synthesis continues and is completed on ribosomes attached to the rough ER membrane (the presence of these bound ribosomes distinguishes the rough ER from smooth ER). Most proteins in the lumen of the ER are in transit, en route to other destinations. The completed protein then moves to the next compartment, the Golgi complex, if it does not contain an ER retention signal and is subsequently sorted to various destinations such as the plasma membrane, secretory vesicles, and lysosomes (83). Synthesis of all other encoded proteins is completed on free cytosolic ribosomes, and the completed polypeptide chain is released into the cytosol (85). These proteins remain in the cytosol unless they contain specific signals that direct them to the mitochondria, peroxisomes, or nucleus.

2.2 Birth of proteins entering the secretory pathway on ribosomes in the ER

Synthesis of most secretory proteins begins on free ribosomes in the cytosol (83). Ribosomes are composed of two subunits a large 50s subunit and a small 30s subunit. The direction of ribosomal protein synthesis was established by Howard Dintzis whereby incoming amino acid are added to a polypeptide C-terminus, that is polypepitde synthesis

proceeds from N-terminus to C-terminus (86). The presence of a 16- to 30- residue ER signal sequence at the NH_2-terminus directs the ribosome-polypeptide complex to the ER membrane and initiates transport of the growing protein chain across the ER membrane (84, 87). A signal peptide sequence is typically composed of one or more positively charged amino acids followed by a continuous stretch of 6-12 hydrophobic residues that is essential for its function; except for these features signal peptides have nothing else in common (Table 1). In general they are not found on completely formed proteins implying that signal peptides are cleaved from the protein by a *signal peptidase* localized to the ER lumen while it is still growing on ribosomes (87).

Two key proteins initiate the interaction of the signal sequence with the ER membrane: the **signal recognition particle (SRP)** and the **SRP receptor**. SRP is a cytosolic protein that transiently binds to the ER signal sequence in a nascent protein as soon as it emerges from the ribosome (88), to the large ribosomal subunit, and to the SRP receptor in the ER membrane also known as a *docking protein* (89). This causes a pause in protein synthesis giving the ribosome time to bind to the ER membrane before synthesis of the polypeptide chain is completed. SRP and its receptor only initiate the transfer of the nascent chain across the ER membrane, they then dissociate from the protein chain accompanied by GTP hydrolysis. The polypeptide is then transferred to a set of transmembrane proteins called translocon whereby the signal sequence binds to it. Once the nascent chain-translocon complex assemble and the translocon gates open, the elongating chain passes directly from the large ribosomal subunit passing through the translocon center. Two abundant ER membrane proteins that are part of the translocon

Table 1 **Comparison of signal peptide sequences in proteins entering the secretory pathway.**

Protein	Amino Acid Sequence
Preproalbumin	Met-Lys-Trp-Val-Thr-**Phe-Leu-Leu-Leu-Leu-Phe-Ile-Ser-Gly-Ser-Ala-Phe-Ser-** ↓ Arg...
Preprosomatostatin	Met-Leu-Ser-Cys-Arg-**Leu-Gln-Cys-Ala-Leu-Ala-Ala-Leu-Cys-Ile-Val-Leu-Ala-Leu-Gly-Val-Thr-Gly-** ↓ Ala...
Preprocarboxypeptidase E	Met-Ala-Gly-Arg-Gly-Gly-Arg-Val-**Leu-Leu-Ala-Leu-Cys-Ala-Ala-Leu-Val-Ala-Gly-Gly-Trp-Leu-Leu-Thr-Ala-**Glu-Ala- ↓ Gln...
Pre-IgG light chain	Met-Asp-Met-Arg-Ala-Pro-Ala-Gln-**Ile-Phe-Gly-Phe-Leu-Leu-Leu-Leu-Phe-**Pro-Gly-Thr-Arg-Cys- ↓ Asp

Hydrophobic residues are in boldface; arrows indicate the site of cleavage by signal peptidase.
Note: the signal peptide sequence in any precursor protein is referred to as pre-. Once the signal peptide is cleaved what remains is the pro-sequence

complex are important for protein translocation into the ER lumen. One of these proteins is called translocating chain associated membrane (TRAM) protein which binds to the ER signal sequence once it is handed over by the SRP to the translocon. TRAM spans the membrane at least eight times and is essential for protein translocation. The second protein is called Sec61p which attaches the large ribosomal subunit to the ER membrane. At this point both SRP and the SRP receptor are free to initiate insertion of other secretory proteins. The growing polypeptide chain continues to grow and the signal sequence gets cleaved off, and is rapidly degraded in the ER lumen. The polypeptide continues to elongate until translation is complete. The ribosomes are then released and the remaining C-terminus of the protein is drawn into the ER lumen, the translocon gates shut, and the protein fully folds (90-92). Throughout this process the growing chain is never exposed to the cytosol and only folds once it reaches ER lumen.

2.3 Post–translational modification and quality control in the ER

Newly synthesized proteins undergo a number of modifications in the ER which include: formation of disulfide bonds, proper folding, assembly into multimeric proteins which occurs exclusively in the ER, glycosylation and proteolytic cleavage which takes place in both the ER and Golgi. Disulfide bond (-S-S-) formation on cysteine residues in the same or different polypeptide chains help stabilize the tertiary structure of many proteins and are formed in the lumen of the ER and not in the cytosol. Thus soluble cytosolic proteins synthesized on free ribosomes lack disulfide bonds (93, 94).

In the complex formation of a protein there exists four levels of architecture known as primary, secondary, tertiary and quaternary. The primary structure is simply the

unique linear sequence of its amino acid The secondary structure involves coiling or folding of certain segments of a protein into regularly repeating structures consisting of either alpha (α) helix or Beta (β) pleated sheets resulting from the formation hydrogen bonds between the CO and NH groups of different residues. The tertiary structure is created through hydrophobic interactions between nonpolar side chains causing the protein to fold back on itself (93). In many cases amino acid portions of a protein are far apart and are brought closer together in the tertiary structure, which can be reinforced, through the formation of disulfide bonds in some cases. Often two or more folded structures will bind together to form quaternary structures, which is also known as multimeric because it is composed of several separate polypeptides chains or monomers.

Glycosylation is a process that involves the covalent addition of one or more carbohydrate chains to proteins and takes place in both the ER and Golgi. Oligosacharides transferred to the NH_2- side chain group of an asparagine amino acid in the protein are said to be N-linked oligosaceharides. The transfer is catalyzed by a membrane bound enzyme called oligosaccharyl transferase immediately after the protein emerges within the lumen of the ER (93, 94). Biosynthesis of N-linked oligosaccharides involves the *en bloc* transfer of preformed oligosacharides to the protein. The precursor oligosacharide is built up sugar by sugar prior to its transfer to a protein and is usually held by a lipid molecule called dolichol in the ER membrane. N-linked oligosaccharides are composed of N-acetylglucosamine, mannose, and glucose containing a total of 14 sugar residues approximately. N-linked oligosaccharides on glycoprotiens are eventually further trimmed and modified in the Golgi apparatus through the removal or addition of certain sugars one at a time. An important example of N-linked oligosaccharides are

lysosomal enzymes which become phosphorylated in the *cis*-Golgi producing a manose-6 phosphate. Less frequently, oligosaccharides are linked to the hydroxyl group on the side chain of serine, threonine, or hydroxylysine amino acid in which case they are known as O-linked oligosacharides and are formed in the Golgi (95). They are generally short, often containing one to four sugar residues and are added one at a time with each transfer catalyzed by a different glycosyltransferase enzyme.

Newly synthesized proteins fold into their proper conformation within a few minutes after their synthesis in the ER. The correct folding of proteins is a critical step in determining their subsequent fate. This process is accelerated by the presence of a number of proteins within the ER lumen referred to as molecular chaperones, originally studied in another context as heat shock proteins (Hsps) that are synthesized in the cell in response to stress (96). These proteins are also found in the cytosol and mithochondria. Two folding catalysts have been intimately tied to protein folding in cells: Hsp60 and Hsp70 also known as binding protein (BiP). ER chaperones tansiently bind to proteins and prevent them from forming aggregates or misfolding thereby enhancing their ability to fold into their proper confirmation (97). Additionally, Hsp70 has been shown to bind to exposed hydrophobic patches in the ER lumen of the nascent growing polypeptide while being translocated, thus providing a directionality to the process by sterically hindering the movement of the polypeptide backwards toward the cytoplasm (98).

Generally 30% of newly synthesized proteins from normal genes in various cell types never attain proper native structure due to their complex size and inherent difficulties in folding. The fate of misfolded proteins is basically degradation. Misfolded proteins mainly arise from a genetic mutation located in the gene that they are

synthesized from such as the well-studied cystic fibrosis transmembrane conductance regulator (97). Cells have therefore employed several intracellular proteolytic pathways for degrading misfolded or denatured proteins. One major intracellular pathway involves degradation by enzymes within lysosomes (99). These are membrane-limited organelles containing acidic hydrolases serving as the site for degradation of luminal and transmembrane proteins of the endocytic and secretory pathway. Distinct from the lysosomal pathway, are cytosolic mechanisms for degrading proteins through proteasomes (100). Proteasomes are large cylindrical multisubunit complexes (composed of two 19S subunits and a 20S subunit) found in the cytosol and the nucleus. Their role is to regulate the breakdown of cytosolic and nuclear proteins that have been altered.

In order for misfolded proteins retained in the ER to be degraded by proteasomes, they need to be translocated back into the cytosol followed by subsequent degradation (100, 101). Steps involved in the dislocation process first begin with the binding of ER chaperones such as BiP and calnexin to the misfolded protein thus preventing it from forming aggregates (97, 100, 101). Second, there should be a source of energy to power the translocation of the misfolded protein back to the cytosol. This can be provided by the 19S subunit of the proteasome and the cytoplasmic Hsp70 protein, both of which possess intrinsic unfolding ATPase activity. Third, dislocation in the ER involves a proteinaceous channel that allows transfer of the polypeptide across the lipid ER bilayer. The work of Wiertz et al. (1996) demonstrated that MHC class I chains targeted for destruction were transiently associated with the Sec61p complex, a pore forming subunit of the translocon (102). Therefore dislocation can be mediated by the same translocon which allowed initial entry of the polypeptide into the ER. However in the case of degradation, the

protein moves in the reverse direction by which it was initially inserted. Additionally, proteasomes are usually bound to the cytoplasmic face of the ER membrane thus allowing to degrade polypeptides cotranslocationally (101).

Protein degradation generally involves the covalent attachment of ubiquitin molecules to the protein targeted for destruction at a Lys residue (101). Uniquitin is a 76 a.a polypeptide expressed in all eukaryotic cells. The process requires the complex work of three classes of enzymes known as E1s (ubiquitin activating enzymes), E2s (ubiquitin-conjugating enzymes Ubcs), and E3s (ubiquitin-protein ligases). Fig. 9 is a schematic diagram depicting the general mechanism of an ER retained protein degradation.

Once protein folding is complete the next destination of a protein is the Golgi apparatus, except for those containing an ER retention signal (83, 101). Retention of soluble ER-resident proteins is mediated by a short, four amino acid sorting signal, identified as KDEL (Lys-Asp-Glu-Leu). The retention signal works not by anchoring resident proteins in the lumen of the ER but by the selective retrieval of ER-resident proteins after they have escaped in transport vesicles and have been delivered to the *cis* Golgi network. In the *cis* Golgi network a specific membrane-bound receptor protein binds to the ER retention signal and packages any proteins displaying the signal into special transport vesicles that return the protein to the ER.

2.4 Cargo (membrane and soluble) export from the ER

Once folded or assembled properly in the ER secretory and membrane proteins move to their next destination the Golgi apparatus. It was Palade in 1975 who originally proposed that intracellular protein transport between organelles in eukaryotic cells is mediated by

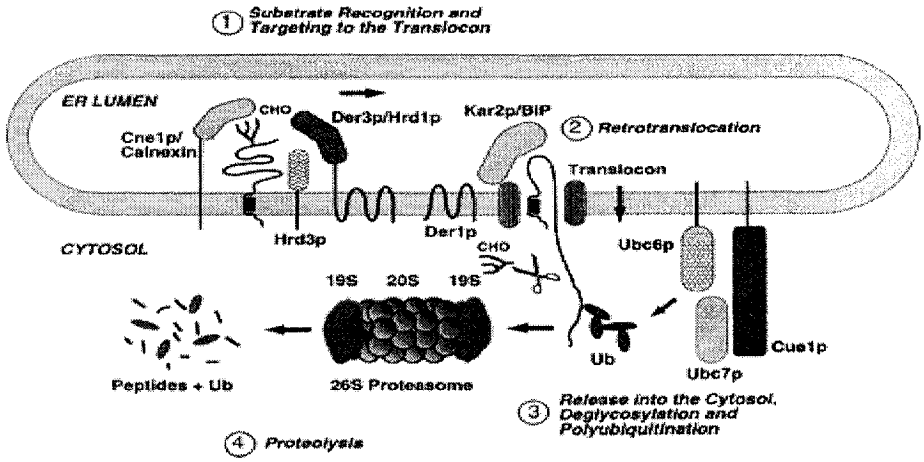


Fig. 9 Stepwise schematic of the ubiquitin-dependent protein degradation pathway.

Misfolded proteins are recognized by ER chaperones calnexin or BiP causing the substrate to be targeted to the translocon (step 1). The translocon is then opened and reprogrammed for retranslocation from the ER lumen back into the cytosol. Next the polypeptide chains are unfolded and forced through the translocon (step 2). The retranslocated polypeptide is released into the cytosol, deglycosylated and polyubiquitinated (step 3). The protein then binds to the proteasomal complex 26S and degraded to small peptide molecules (step 4).

From *Bonifacino JS. Ubiquitin and the control of protein fate in the secretory and endocytic pathways. Annual Reviews in Cellular and developmental Biology 1998; 14: 19-57.*

small transport 50-80-nm vesicles whereby, vesicles bud from a parent organelle and fuse with the membrane of a particular target organelle (103). Cells have a variety of coats, allowing different species of vesicles to depart from various subcellular locations. Up to date there are three different types of coated vesicles each composed of a number of subunits. First specialized coatomer-coated vesicles known as COPII allow exit of proteins from the ER Table 2 (104,105). These vesicles bud from specific ER regions known as transitional ER (tER) exit sites (106). Although COPII vesicles are intensively studied nothing is known about how vesicle budding is restricted to the tER sites. The COPII coat is a polymer formed by the ordered assembly of five cytosolic proteins (Fig. 10) which begins with the binding of a small GTPase Sar1p.GTP to the target membrane (107). Cells contain large families of regulatory GTP-binding proteins that have a covalently attached lipid group helping them bind to membranes (108). Two structurally distinct classes are recognized: monomeric GTP-binding proteins called monomeric GTPases consisting of a single polypepitde chain, and the already discussed trimeric GTP-binding proteins called G-proteins consisting of three different subunits that interact with GPCRs. Sar1p is a monomeric GTPase whereby the GDP-bound form of Sar1p is normally cytosolic. These GTPases act as binary switches for coat formation such that the GTP bound state is active (coats assemble) and the GDP state is inactive (coats disassemble) (109). The factors that influence the rate of guanine nucleotide exchange (GEFs) and hydrolysis [GTPase activating proteins (GAPS)] are therefore key regulatory components of coat formation. The GEF involved in activation of Sar1p is Sec12p, a transmembrane protein localized to the ER (110). Sar1p.GTP then recruits two large

Table 2. Coat proteins of COPII- and COPI-coated vesicles.

	Subunits			
Protein complex	**Mammals**	**Yeast**	**Size**	**Features**
COPII				
Sec13 complex	hSec13p	Sec13p	~34 kDa	WD-40 repeats
	hSec31p	Sec31p	~150 kDa	WD-40 repeats
Sec23 complex	hSec23p	Sec23p	~85 kDa	GAP for Sar1p
	hSec24p	Sec24p	~105 kDa	
Sar1	hSar1p	Sar1p	~21 kDa	Ras family of GTPases
COPI				
Coatomer	α-COP	Ret1p	~140 kDa	WD-40 repeats
	β-COP	Sec26p	~107 kDa	
	β'-COP	Sec27p	~102 kDa	WD-40 repeats
	γ-COP	Sec21p	~97 kDa	
	δ-COP	Ret2p	~57 kDa	
	ε-COP	Sec28p	~35 kDa	
	ζ-COP	Ret3p	~20 kDa	
ARF 1	ARF 1	yARF 1/2/3	~20 kDa	Ras family of GTPases

From *Wieland F, Harter C. Mechanisms of vesicle formation: insights from the COP system. Current Opinion in Cell Biology 1999; 11: 440-446.*

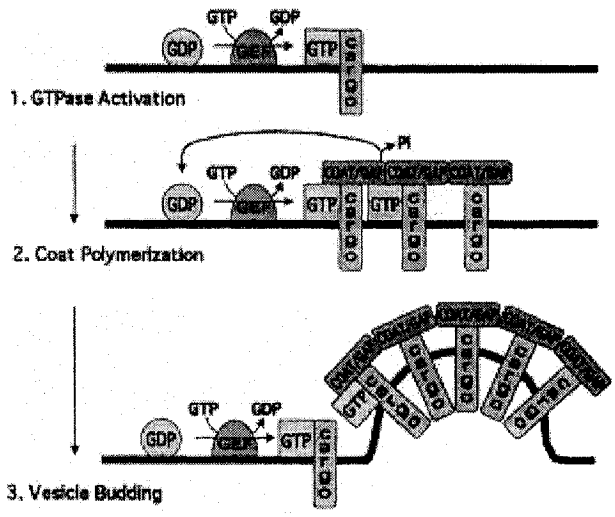


Fig. 10 General Model for COPII vesicle budding.

1. Small GTPases (Sar1P or ARF) are activated through GEFs and associate with vesicle cargo to initiate coat polymerization.
2. Subsequent binding of hetero-oligomeric protein complexes coat subunits bind to GTPase-cargo complexes.
3. Deformation of specialized portions of the donor membrane to form coated buds.

From *Barlowe C. Traffic COPs of the early secretory pathway. Taffic 2000 1: 371-377.*

complexes Sec23/24p heterodimer and Sec13/31p heterodimer which bind sequentially (107). Fusion of vesicles with their target membranes requires prior dissociation of the coat through GTP hydrolysis. This is achieved by GAP, in which case the Sec23 component of the Sec23/24p complex causes GTP hydrolysis of Sar1p (111). On the other hand Sec24p has no known homology to other GAP proteins, and has no effect on GAP activity of Sec23 (110) but is absolutely important for vesicle formation.

For a long period of time the transport of cargo proteins from the ER to the Golgi has been referred to as a default pathway not requiring any specialized signals whereby any protein entering the ER, once folded and assembled properly is automatically transported to the Golgi apparatus via a nonselective bulk flow mechanism (112,113). However, mounting evidence suggests that ER export infact requires selective incorporation of cargo into COPII vesicles. Using immunoelectron microscopy and an *in vitro* assay that reconstitutes ER-Golgi export of vesicular stomatitis virus glycoprotein (VSV-G), a type I transmembrane protein, Balch et al. in 1994 provided the first quantitative evidence demonstrating that a newly synthesized membrane protein is both sorted from ER resident proteins and concentrated during ER export (114). The next question to address is the mechanism by which cargo proteins are selected for export.

The role of certain COPII components in sorting membrane and soluble cargo proteins has been demonstrated in yeast (115). The authors postulated that if coat proteins are involved in the selection of molecules for transport then, at some point before the emergence of a bud the cargo molecules and vesicle proteins might interact directly or indirectly with certain COPII components. Using the glutathione-S-transferase (GST) pull down assay to test for COPII-cargo interactions, incubating microsomes with Sar1p-

GST and Sec23/24p resulted in greater recovery of both soluble and membrane cargo proteins versus incubations with Sar1p-GST alone or Sar1p-GST and Sec13/31p. Similar results have been demonstrated in mammals (116). Purification of functional coat complexes from rat liver cytosol revealed that Sar1p and the Sec23/24p complex are necessary and sufficient for the mobilization of VSV-G into vesicles while excluding ER resident proteins. However in order for the vesicles to bud subsequent addition of Sec13/31p is required. Having established the importance of certain coat components in cargo selection, a logical question to ask is whether or not there is a specific ER export signal in the cargo itself possibly dictating its ability to enter COPII coated vesicles.

With regard to membrane proteins two ER export motifs present in the cytoplasmic domain of membrane proteins have been characterized. An export signal involved in ER to Golgi transport was originally identified in VSV-G (117). Deletion of the entire cytoplasmic C-tail slowed down ER exit of VSV-G with two acidic residues Asp21 and Glu23 being critical in influencing the rate of transport. Additionally, the Asp21XGlu23 was able to confer export of a protein that is normally retained in the ER. A similar signal has also been demonstrated in another transmembrane protein, lysosomal acid phosphatase (117). A diacidic signal, Phe-Cys-Tyr-**Glu**-Asn-**Glu**-, in the C-tail of the Kir channel has also been implicated in ER export, with the flanking residues of the diacidic motif also being essential (118). Through measurements of single channel current traces, surface expression of Kir was significantly reduced upon C-terminal truncation of the ion channel due to disruption of forward trafficking. Using green fluorescent protein (GFP) to visualize intracellular protein movement, *wt* Kir was mainly localized to the Golgi membrane and cell surface. On the other hand, C-terminally

truncated Kir was primarily found in the ER co-localized with ER-resident protein BiP. Recently, another type of export motif consisting of hydrophobic residues with a PheXXXPheXXXPhe motif at the proximal C-terminus of D1R, a GPCR, highlights the importance of an ER export signal where all three hydrophobic residues are required for normal receptor transport (119). Functional consequences of mutating the three hydrophobic Phe residues independently were studied through examining ligand binding and cAMP production. Compared to *wt,* mutant constructs demonstrated negligible levels of binding at the cell surface and were incapable of triggering cAMP production in response to dopamine. Similarly ER exit of ER-Golgi intermediate compartment protein 53 (ERGIC-53), a type one transmembrane protein that recycles between the ER and Golgi, was dependent on two C-terminal phenylalanines (Phe-Phe) (120,121, 122) which were also found in the p24 family of transmembrane proteins (123). In general the most likely mechanism involved in packaging of transmembrane proteins into COPII vesicles is through direct interactions of the C-terminal sorting motif with the Sec23/24p coat complex (120, 121, 122).

Unlike membrane proteins which may possess sorting signals recognizing COPII coat subunits directly, the selection of soluble cargo like SST within the lumen of the ER requires the interaction of the cytosolic part of a transmembrane receptor with COPII coat components driving selective incorporation of cargo proteins into coated vesicles (124). At least two families of proteins might serve as transport receptors for ER-Golgi transport. Mammalian ERGIC-53 belongs to the lectin family of proteins and selectively binds to specific carbohydrate structures on proteins thus implicating it in guiding newly synthesized glycoproteins from ER to Golgi (124). Consistent with this idea some

glycoproteins such as blood coagulating factor V and VIII were inefficiently secreted when ERGIC-53 was nonfunctional (126). Through DNA sequence analysis it was demonstrated that the combined deficiency of the blood factors, an autosomal recessive bleeding disorder, resulted from a mutation in the gene coding for ERGIC-53. The interaction with the receptor was specific because blood levels of other plasma proteins in combined deficiency patients were normal.

Another type of vesicular transmembrane protein responsible for transport of lumenal cargo belongs to the p24 family (127). In yeast there are two p24 family members: Emp24p and Erv25p which have been localized to the ER COPII vesicles. Homologues of Emp24p and Erv25p have also been identified in mammals and designated p24 and p23 respectively (128). Like ERGIC-53, they contain a large lumenal and a short cytoplasmic domain interacting with coat components. The role of the p24 family in secretory protein transport was elucidated in yeast mutants lacking Emp24p (129). The defect lead to slow delivery to the Golgi of a subset of lumenal proteins such as periplasmic invertase. Other soluble proteins such as glycosylated pro α factor (gpαF) was not effected, reinforcing the fact that cargo selection is specific and not random. Recently the receptor responsible for proper packaging of gpαF was found to be ER-vesicle protein of 29 kD (Erv29p) (130). Using the yeast haploid strain lacking Erv29p, a reconstituted cell-free assay that measures transport of [^{35}S]gpαF to the Golgi complex was used. In membranes lacking Erv29p no transport of gpαF was detected due to a general decrease in its packaging into COPII vesicles. Additionally, packaging into COPII vesicles was reduced when membranes were incubated with antibody against Erv29.

To follow COPII vesicle movement in living cells from the ER, investigators constructed a recombinant gene encoding a chimeric protein consisting of green fluorescent protein (GFP) and VSV-G (131). By combining GFP technology with high-resolution electron microscopy it was demonstrated that upon export from the ER *en route* to the Golgi, COPII vesicles initial target is the ERGIC also known as vesicular tubular clusters (VTCs) due to its morphological appearance (132). A motor on the tip of the pre-Golgi compartment then appears to pull it along microtubules toward the Golgi eventually either maturing into or fusing with the *cis*-Golgi membranes (132,133).

2.5 ER-Golgi intermediate compartment (ERGIC)

Two opposing models exist that explain the ERGIC. One model suggests that the VTCs are a unique compartment containing a core of nonrecycling resident proteins distinct from those present in either the ER or Golgi. In this model COPII vesicles fuse with pre-existing tubular elements (106, 134). Alternatively, the second model argues that VTCs are a dynamic structure that continuously forms *de novo* from the fusion of ER-derived COPII vesicles that pinch off from tER (135). They would then move *en bloc* to the cis-Golgi compartment and fuse with it (106,134). Despite these two opposing models VTCs are still defined as a unique compartment however, their composition remains to be firmly established. It is clear that during export from the ER, resident ER proteins such as folding chaperones are efficiently excluded from COPII vesicles. Very few markers can be defined as residents of the ERGIC however, an important contribution to the identification of the ERGIC came with the discovery of the previously discussed 53 kDa type I membrane protein ERGIC-53 (136). It is a mannose selective-lectin (137) that is

not only required for the maintenance of appropriate levels of coagulating factors V and VIII in the blood (125) but is the most popular marker for the ERGIC to date that constitutively recycles between the ERGIC and ER (138). Once ERGIC-53 delivers its cargo to the intermediate compartment through COPII vesicles, it recycles back to the ER in COPI vesicles. Therefore, this stage of the secretory pathway is considered the first sorting station for forward (anterograde) and backward (retrograde) traffic (139,140).

2.6 COPI coated vesicles

Like its distant cousin COPII, COPI coat is a polymer formed by the ordered assembly of cytosolic proteins, which shape lipid membranes to produce transport vesicles Table 2 (141). Polymerization of the coatomer coat begins in the cytosol where a small GTP-binding protein known as adenosine diphosphate-ribosylation factor (ARF) 1 is recruited to the membrane releasing its bound GDP for GTP (142) Fig. 11. However, in contrast to COPII-coated vesicles, COPI coatomer complex is composed of seven conserved subunits that are recruited en bloc pre-assembled to the membrane (143). The coatomer then binds to ARF1.GTP through the β- and γ- COP subunit (144). Another component that is necessary for COPI vesicle budding belongs to the p24 protein family which binds via its C-terminal domain to certain coatomer components Fig. 12 (145). These type I vesicular transmembrane proteins are therefore not only involved in transport of soluble cargo proteins from the ER to the Golgi, but are necessary for COPI budding. *In vitro* reconstitution of COPI vesicles from chemically defined liposomes demonstrated that all that is needed to bud a COPI coated vesicle are the cytosolic ARF1.

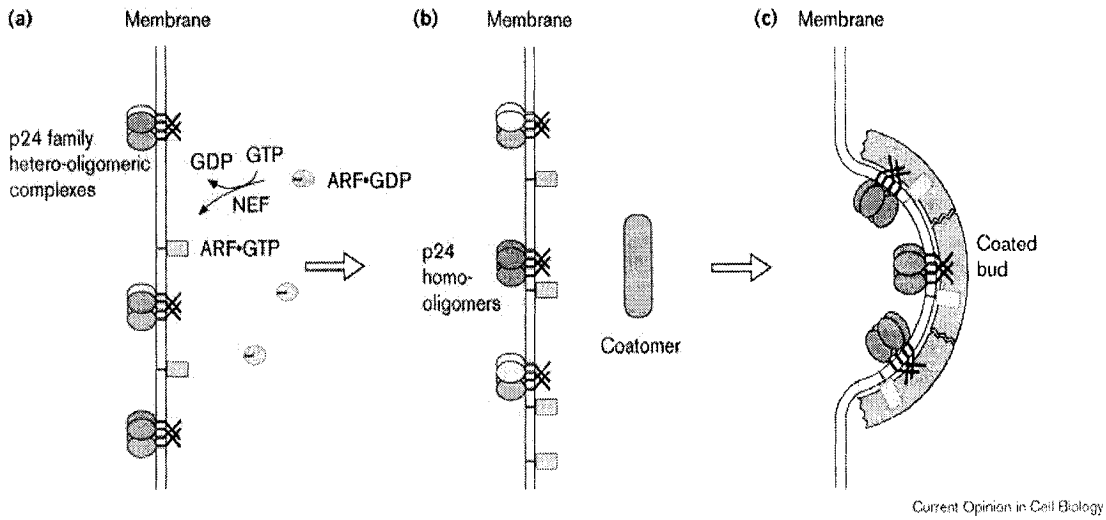


Fig. 11 Hypothetical model demonstrating the steps involved in polymerization of COPI-coated vesicles.

a) Following formation of ARF-GTP in the cytosol, it is then recruited to the membrane.

b) ARF.GTP might induce dissociation of hetero-oligomeric complexes of p24 proteins resulting in the formation of homo-oligomers. This represents high affinity binding sites for coatomer subunits.

c) Coatomer interaction with p24-oligomers induces a conformational change and polymerization of the complex shaping the membrane into a coated bud.

From *Weiland F, Harter C. Mechanisms of vesicle function: insights from the COP system. Current Opinion in Cell Biology 1999; 11:440-446.*

coatomer and cytoplasmic domains of putative cargo/receptors (p24 proteins) in the presence of GTP (146). Therefore a bivalent interaction of coatomer with membrane bound ARF1.GTP and cytoplasmic tails of p24 forming a trimeric complex is required for coat polymerization. Similar to COPII, fusion of vesicles with their target membrane requires prior dissociation of the coat (147). When the GTP bound to the ARF1 is hydrolyzed to GDP via ARFGAP, the coat depolymerizes releasing the transport vesicle for fusion with target membrane (148).

Recently it has been proposed that proteins do not only necessarily move in the forward direction but, there needs to be a way to redistribute them back through the secretory pathway otherwise the cell would expand indefinitely (149). Additionally, integral membrane and soluble ER proteins escape to other compartments in departing transport vesicles reinforcing the need for their selective retrieval since their retention is not completely sufficient. In 1994, Pierre Cosson and Froncois Letourneur were first to implicate the role of COPI vesicles in retrogade transport (150). They found that particular components of the COPI coat interacted with a dilysine motif KKXX in the C-tail of an escaped type I transmembrane ER resident protein suggesting a role for the coatomer in its retrieval back to the ER Table 3. Similarly, once ERGIC-53 and P24 deliver their cargo to the ERGIC in COPII vesicles, their recycling back to the ER in COPI coated vesicles is also mediated by a dilysine ER targeting signal that binds COPI components (145,151,152). The requirement of a dilysine motif in the C-tail of resident ER membrane proteins was originally reported for E19, a protein encoded by adenovirus 3 (153). Transplantation of the E19 cytoplasmic domain sequence onto either the T-cell

Table 3. Membrane proteins with potential endoplasmic reticulum localization motifs

	COOH-terminal localization	Subcellular localization	Protein type
Carboxy-terminal di-lysine motifs			
3-Hydroxy-3-methylglutaryl-coenzyme A reductase	LQGACT**KK**TA(i)	ER	III
53-kDa Sarcoplasmic protein	ETPKN**RYKK**H (i)	ER	I
C-8 Sterol isomerase (ERG2)	GKNLLQN**KK**F	ER	I
Calnexin	SPRNR**KPR**RE	ER	I
Ceramide UDP-galactosyltransferase	GHIKHE**KK**VK	ER ?	I
Emp47	RQEII**KTK**LL (ii)	Golgi	I
ERGIC-53	QQEAAA**KK**FF (iii)	ERGIC	I
Glucose transporter type 7	SDQVK**KM**K**N**D	ER	III
Glucose-6-phosphatase	VLGQPH**KK**SL	ER	III
Glycerol uptake/efflux facilitator protein	SHYGNA**KK**VT	?	III
Glycoprotein 25L	KNFFIA**KK**LV	ER	I
GPI:protein transamidase (GAAI)	VVVRS**KE**K**Q**S (iv)	ER	III
High affinity Ig receptor α subunit	QKTGKG**KKK**G (v)	Cell Surface	I

Modified from *Teasdale RD, Jackson MR. Signal mediated sorting of membrane proteins between the endoplasmic reticulum and the Golgi apparatus. Annual reviews in Cell and Developmental Biology. 1996; 12:27-54.*

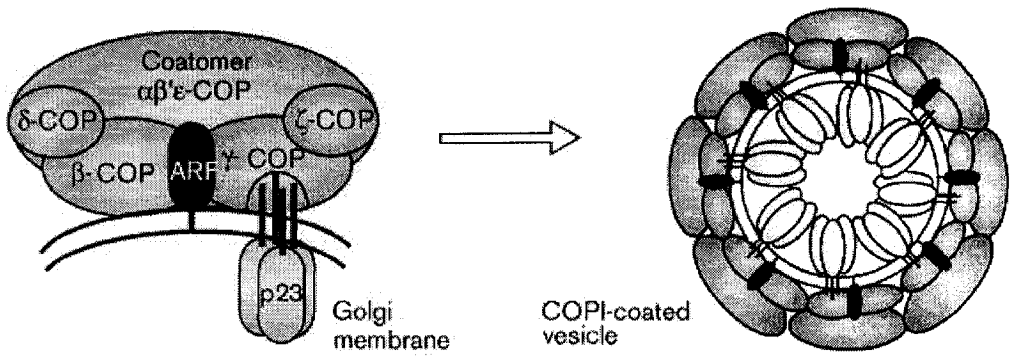


Fig. 12 Model for COPI-coated vesicle formation. A trimeric complex consisting of ARF.GTP, 7 coatomer subunits and a tetramer of cytoplasmic tails of p24 members is postulated to provide the molecular basis for vesicle budding.

From *Weiland F, Harter C. Mechanisms of vesicle function: insights from the COP system. Current Opinion in Cell Biology 1999; 11:440-446.*

surface receptor glycoproteins CD4 or CD8 resulted in efficient targeting of the chimeric proteins to the ER. The di-lysine motif appears to be conserved across eukaryotic species and mutagenesis studies demonstrated remarkable conservation of the functional motif between yeast and humans (154). As more sequences of the ER resident membrane proteins are determined the cytoplasmically located dilysine motif has become a common feature for retrograde movement although some substitutions by arginine residues are permitted Table 3 (155).

Protein recycling in the early secretory pathway was initially described for soluble ER resident proteins possessing a C-terminal K/HDEL signal (156). In animal cells the motif encodes a KDEL motif, whereas in *Saccharomyces cerevisiae* it codes for HDEL. By comparing the amino acid sequence of three lumenal ER residents proteins known as glucose regulated protein 78 (grp78), grp94 and disulphide isomerase, it was noted that they share a C-terminal sequence KDEL (156). Deletion of the grp78 C-terminal sequence caused it to be secreted into the medium with loss of retention in the ER once transfected into COS-cells. On the other hand the addition of the KDEL sequence C-terminal to the lysozyme protein, a protein that is constitutively secreted from the cell, lead to its accumulation in the ER rather than secretion. The exact mechanism or receptor responsible for retrieval of ER resident proteins was originally discovered in yeast and found to be the product of the ER retention defective 2 (ERD2) gene. The gene codes for the HDEL receptor and interacts with soluble ER proteins bearing the HDEL sequence at their C-terminus (157). That same year (1990) the KDEL receptor was cloned in humans and was found to be similar in protein sequence, size and properties to the ERD2 gene product (158). The receptor is a transmembrane protein that spans the membrane seven

times and is predominantly localized to the ERGIC and *cis*-Golgi compartment although, its capacity to retrieve escaped K/HDEL proteins extends all through the Golgi (159, 160). Upon ligand binding in the Golgi apparatus, the receptor-ligand complex undergoes a conformational change that triggers retrogade transport in COPI coated vesicles back to the ER (161). The ionic conditions in the ER dissociate the resident ER protein/receptor complex allowing the receptor to return back to the ERGIC/*cis* Golgi for reuse (162).

In 1997 Orci et al elegantly demonstrated through electron microscopy and biochemistry that COPI vesicles are not only responsible for redistributing proteins backwards in the secretory pathway but are also capable of transporting cargo in the anterograde (forward) direction (163). Double-labeling studies were performed to determine whether proinsulin, a prohormone that undergoes forward transport across the Golgi stacks, and KDEL receptors were present in the same or different populations of COPI containing transport vesicles. They were unable to see any significant co-localization concluding that KDEL and pro-insulin are found in distinct populations of COPI coated vesicles, and that these vesicles have the ability to carry cargo in both directions.

3. The Golgi apparatus

3.1 General aspects

The Golgi was discovered by the Italian biologist Camillo Golgi in 1898 over 100 years ago consisting of flattened membranous sacs (164). Generally proteins exported from the ERGIC enter the first Golgi compartment, the *cis*-Golgi. They then move to the next compartment the *medial* Golgi and, finally to the *trans* compartment. At this level

proteins are segregated into different transport vesicles and dispatched to their final destinations - the plasma membrane, lysosomes, or specialized storage vesicles (165, 166). Additionally, the Golgi compartment houses a large number of enzymes thus playing a major role in modifying glycosylation patterns on proteins and processing (167).

3.2 Glycosylation

N-linked oligosacharides arise when blocks of 14 residues are added co-translationally to newly synthesized polypeptides in the ER (167). These glycans are then subjected to extensive modifications as they mature and move through the ER and Golgi. In the Golgi glycoprotein glycan chains undergo trimming of mannoses by glycosidases and in many cases new sugars including N-acetylglucosamine, galactose and sialic acid are added during the terminal glycosylation procedure to produce complex N-linked glycans through glycosyltransferase enzymes (168). However, it is not only the N-linked oligosacharide chains on proteins that are altered but, O-linked glycosylation responsible for the production of proteoglycans are modified as well (169).

3.3 Protein transport across the Golgi stacks

With regard to protein transport across the stacks in his 1975 monograph Whaley wrote" No cellular organelle has been subject of as many, as long lasting or as diverse polemics as the Golgi apparatus" (170). This statement is just as true in 2002. The challenges surrounding the Golgi don't concern what it does but how it does it, particularly in the matter concerning protein transport across the Golgi stacks. In 1968

early morphologists proposed that Golgi compartments are transitory dynamic structures where cisternae move progressively across the stack from the *cis* to *trans* face (171). However, the cisternal progression model was disregarded mainly because it failed to explain the presence of vesicles associated with the rims of the Golgi cisternae (172). The second problem was that secretory cargo appeared to move through the Golgi stacks in minutes, whereas resident Golgi proteins often have a half-life of many hours. If the stacks simply progressively mature in a conveyor belt fashion, then resident Golgi proteins should be swept out at the same rate as secretory proteins. Such considerations led to the vesicular traffic model, which envisions the Golgi as a stable compartment. In this model different resident Golgi proteins are anchored in distinct compartments and proteins are transported through the stacks by secretory vesicles (173-175). However, the main problem with the vesicular model is that it works perfectly for the transport of small membrane and soluble proteins but, fails to explain the process by which large multimeric protein complexes such as algal scales and procollagen (Pc) are transported (176, 177) These cargo are more than 20 times the size of the 50-70 nm storage vesicles, making them too large to be packaged.

The best evidence to date for the cisternal progression model was a paper published in 1998 by Bonfani et al. Fig. 13 (178). Pc is a long 300nm rod like protein rich in hydroxyproline and proline residues which are important for proper folding through hydroxylation in the ER. In order to monitor the transport of supramolecular collagen aggregates already present in the Golgi of chicken fibroblast, cells were treated with 2,2'-dipyridyl (DPD), an iron chelator that selectively and reversibly inhibits the hydroxylation of prolines. Treating cells with DPD blocks arrival of new fibers from the

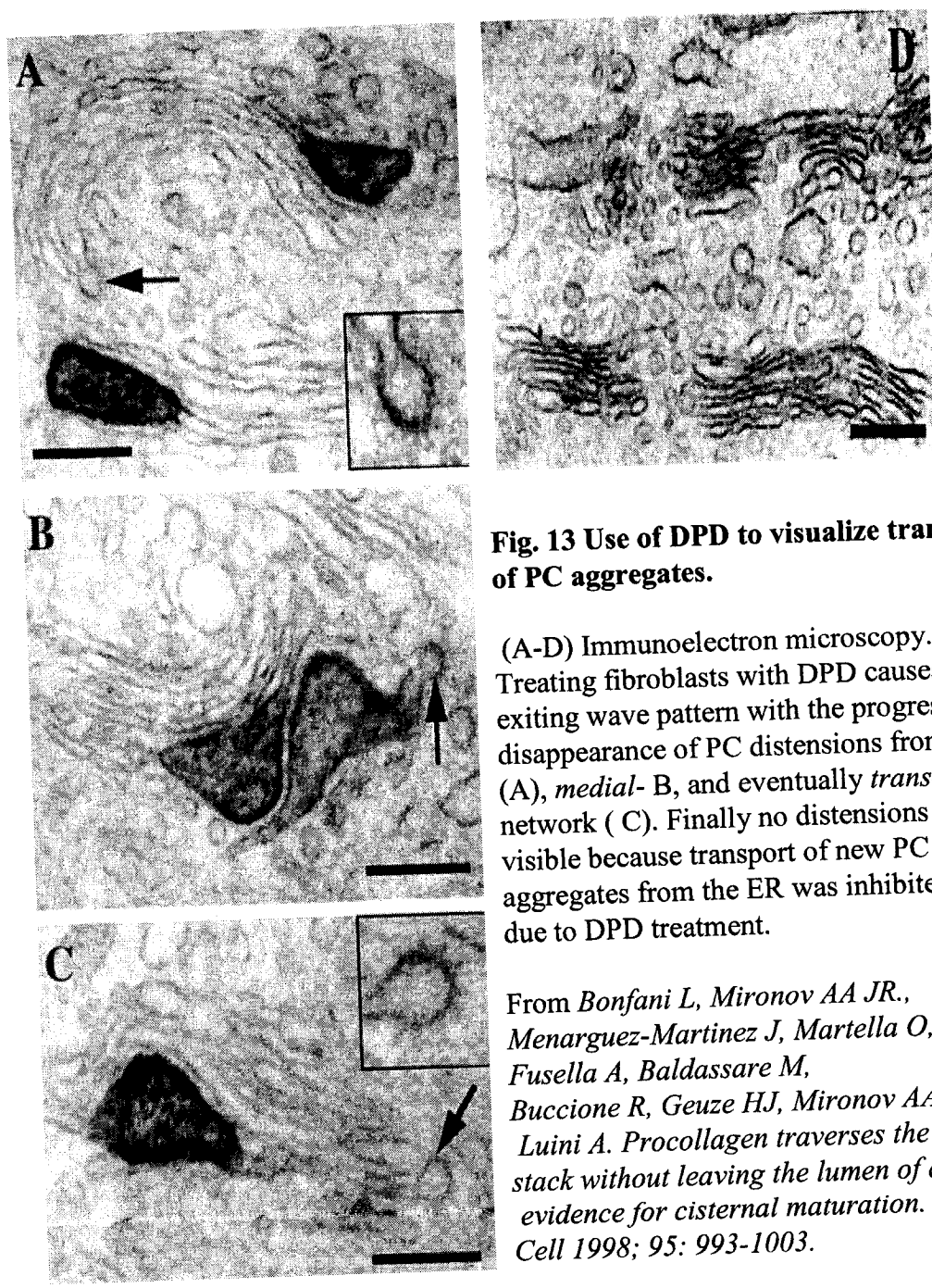


Fig. 13 Use of DPD to visualize transport of PC aggregates.

(A-D) Immunoelectron microscopy. Treating fibroblasts with DPD causes an exiting wave pattern with the progressive disappearance of PC distensions from *cis*- (A), *medial*- B, and eventually *trans*-Golgi network (C). Finally no distensions were visible because transport of new PC aggregates from the ER was inhibited due to DPD treatment.

From *Bonfani L, Mironov AA JR., Menarguez-Martinez J, Martella O, Fusella A, Baldassare M, Buccione R, Geuze HJ, Mironov AA, Luini A. Procollagen traverses the Golgi stack without leaving the lumen of cisternae: evidence for cisternal maturation. Cell 1998; 95: 993-1003.*

ER. Interestingly, Pc fibers were shown to pass through the Golgi in an "exiting wave" pattern; in which case they first disappeared from the cis, then medial, and finally the trans compartment. The size and thickness of the Pc-containing distensions did not change across the stacks implying that aggregates moved en-bloc rather than disassembly, followed by forward transport. Most importantly through three-dimensional reconstitutions of Golgi stacks based on consecutive serial sectioning of a large number of stained and immunolabelled cells, they were able to demonstrate that Pc remained within the lumen of the Golgi throughout the transport process. Later, the same group demonstrated that the cisternal progression model is a universal model which can be applied for the majority of secretory proteins including small diffusible cargo such as VSV-G as well (179). The rate of VSV-G and Pc transport across the stacks was shown to be similar, implying a common method of protein transport for both large and small protein molecules.

Such conflicting results produced by the two models can be accommodated by a unified model incorporating both cisternal progression and vesicular transport Fig. 14 (170, 180-182). In this model cargo proteins move forward through the Golgi by cisternal progression, while resident Golgi proteins must stay behind. COPI vesicles are then responsible for the retrograde transport of resident Golgi proteins which carry them backwards one cisternae at a time. Such a process maintains the polarized distribution of Golgi enzymes thereby conserving the identity of the compartment.

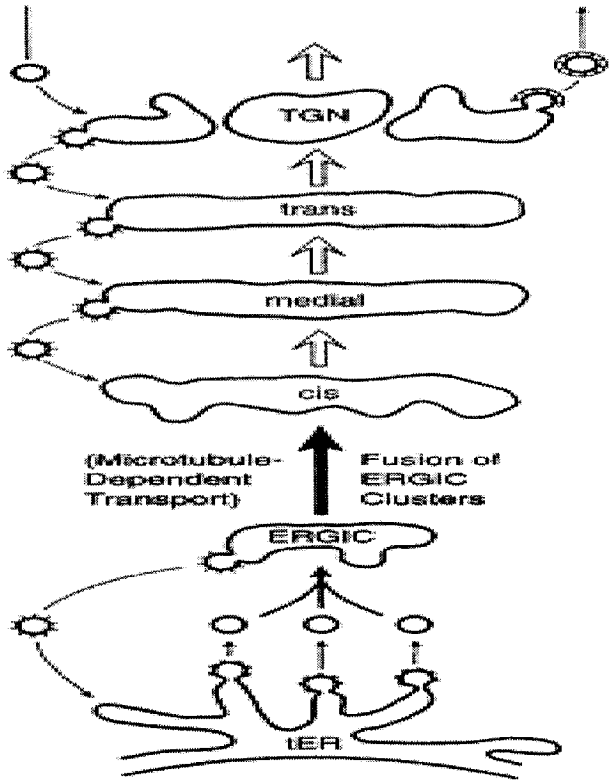


Fig. 14 Summary of protein transport. Once synthesized proteins are transported via COPII vesicles that mature into or fuse with the ERGIC. Selected components are retrieved and are transported back to the ER in COPI vesicles. ERGIC clusters then travel along microtubules and fuse with one another producing the *cis*-Golgi compartment. The cisternae progress in a conveyor belt fashion, while COPI coated vesicles move in the retrograde dicrection carrying resident Golgi proteins. Ultimately the *trans*-Golgi undergoes terminal maturation producing secretory vesicles.

From *Glick BS, Malhorta V. The curious status of the Golgi apparatus. Cell 1998; 95:883-889.*

4. The constitutive and regulated secretory pathway

4.1 General features

The Golgi compartment has always been assumed to be the sorting organelle in the secretory pathway. By following the paths of two proteins with different destinations through immunocytochemistry, Orci et al. directly demonstrated that the *trans*- most cisternae of the Golgi is the compartment implicated in segregating proteins into either the constitutive secretory pathway (CSP) or the regulated secretory pathway Fig. 15 (RSP) (183). Therefore once proteins reach the *trans*-Golgi (TGN) they are sorted to two different pathways causing secretory cells to fall into two categories either constitutive or regulated Table 4 (184).

In eukaryotes constitutive secretory cells are very common and include liver, fibroblast, and muscle cells. In this pathway the only way of effecting the level of protein secretion is through altering the rate of protein synthesis. Antibody secreting lymphocytes are also good examples of a constitutive secretory cell (185). They do not contain a large intracellular pool of antibodies because their transport vesicles do not concentrate the antibodies intracellulary rather, they release their content after synthesis through a passive bulk flow mechanism. Generally such transport vesicles have a short half-life making them very difficult to be detected under the microscope. Examples of cell lines displaying a constitutive pattern of release include COS-, HEK-293-, and CHO-cells Table 4.

Table 4 Examples of Regulated and Constitutive proteins and cell types

Constitutive Secretory Pathway	
Protein Type	**Example**
Serum Proteins	Albumin Immunoglobulins
Extracellular Matrix proteins	Collagen Fibronectins Proteoglycans
Cell type	Liver Fibroblasts Lymphocytes
Cell lines	CHO- HEK-293 COS-
Regulated Secretory Pathway	
Peptide hormones	Insulin SST Glucagon Pro-opiomelanocortin (POMC) Pro-renin
Enzymes	Trypsin Amylase Proproconvertase (PC) 1 & 2
Granin family	Chromogranin A (CGA) Chromogranin B (CGB)
Cell type	Pancreatic β-islet cells Mammary glands Pituitary gland
Cell Lines	Mouse pituitary tumour (AtT-20) RIN 5F Mouse neuroblastoma (Neuro-2a) Rat pheochromocytoma (PC12) GH3 cells (pituitary cells)

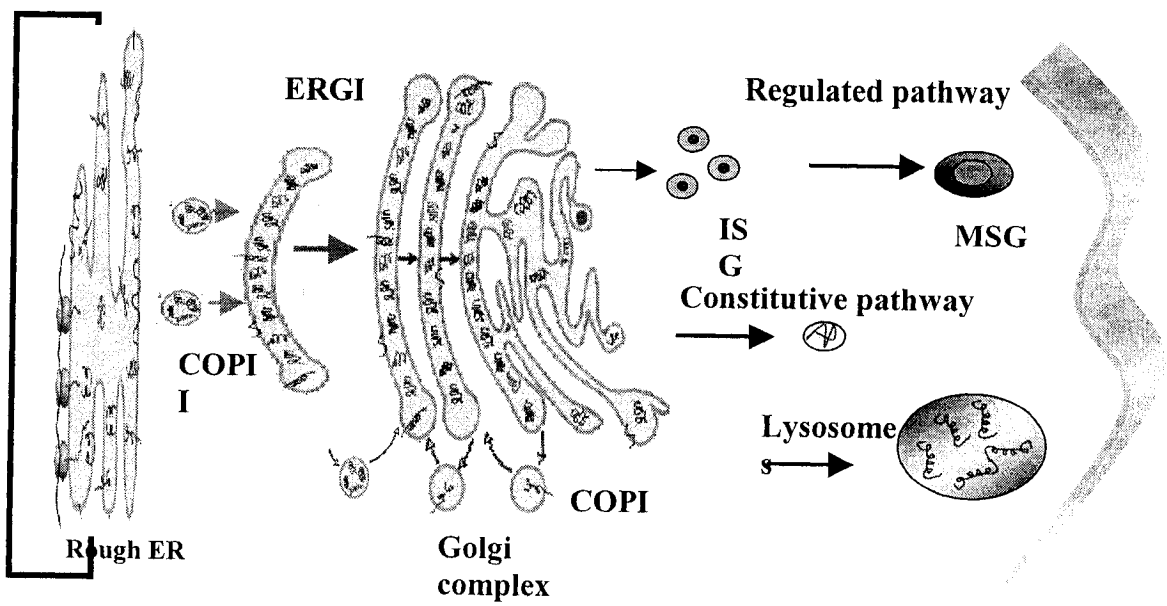


Fig. 15 Compartmental organization of the secretory pathway.

In addition to the CSP that is required and present in all cells, specialized sceretory cells contain a second pathway by which soluble proteins are initially stored in. (184). This is the RSP mainly found in endocrine and neuroendocrine cells specialized for secreting products such as hormones like SST, neurotransmitters, or certain enzymes rapidly on demand. Rapid transient release of cell content is achieved by storing large amounts of protein in specialized regulated secretory vesicles (RSVs) that have a long half-life making it possible to be stained for and visualized under the microscope. In general RSVs are much larger in size than those found in the CSP ranging from 50 to a 100 nm in diameter and, only fuse with the membrane and release their cell content upon presence of an extracellular stimulus. In endocrine cells, the secreted protein can be concentrated up to 200 fold giving such cells an electron dense core under the electron microscope (186). Such morphological features provide these cells a distinctive characteristic allowing them to be distinguished from constitutive secretory cells. For example pancreatic β-islet and pituitary cells (Table 4) are regulated secretory cells that store newly synthesized insulin and SST in specialized RSVs secreting them only in response to a stimulus in the blood (187, 188). However, it is well known that these and other regulated secretory cells simultaneously utilize both secretory pathways (189,190) thus raising the question of how within a single cell are the two different types of secretory proteins, all of which are soluble in the lumen of the TGN, sorted to the correct pathway?

Therefore in a cell capable of regulated secretion at least two different classes of proteins exist which need to be separated prior to leaving the TGN- those destined for RSPs, and those destined for immediate delivery to the cell surface ie. the CSP Fig. 15 (189). Another difference between CSVs and RSVs is that RSVs acquire a clathrin coat

imparting curvature to the carrier vesicle-membrane leading to its budding from the TGN (187, 188, 191). Clathrin is the third type of coat protein in addition to the previously mentioned COPI and COPII.

4.2 Budding of clathrin coated vesicles (CCVs) from the TGN

The major protein component of CCVs (also referred to as RSVs) is clathrin itself, a protein complex that has been highly conserved in evolution. Clathrin was originally identified in 1975 by having a clathrate or basket-like appearance hence the name clathrin (192). It is composed of three identical heavy- (193) and three light chains (194) forming a three legged triskelion (three legged pinwheel) Fig. 16. Additionally, between the fibrous clathrin and the membrane lie the adaptor proteins (APs) also known as assembly proteins (195, 196). Adaptors are involved in nucleating clathrin onto cellular membranes of the TGN and the plasma membrane.

There are two types of clathrin interacting assembly particles of which AP1 triggers CCV formation at the TGN, and the AP2 complex is responsible for clathrin dependent sorting at the level of the plasma membrane. AP1 is a heteromeric complex composed of four subunits: γ, $\beta 1$, $\mu 1A$, and $\sigma 1$ (197). Induction of clathrin assembly is believed to be mediated through the $\beta 1$ subunit (198) where as the $\mu 1A$ subunit recognizes sequence motifs in the cytoplamic domains of transmembrane proteins/receptors sequestered into CCVs. On the one hand adaptors interact with clathrin, but on the other hand they interact with the cytoplasmic domain of transmembrane proteins leading to their incorporation into the vesicles. In turn this leads

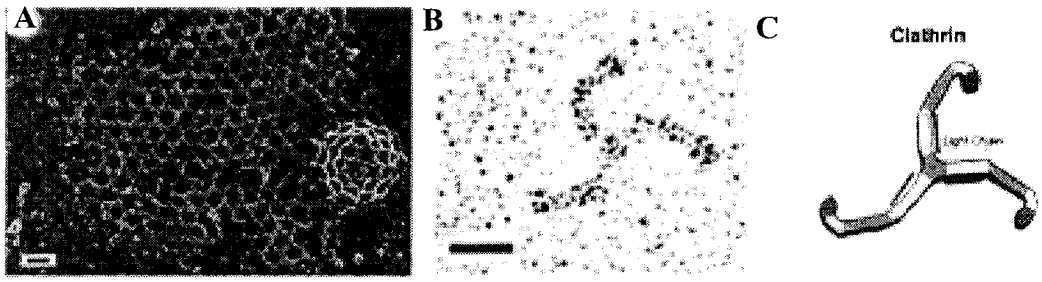


Fig. 16 (A-B) A clathrin triskelion purified from bovine brain CCVs.
C) Representation of a basic clathrin molecule composed of three heavy chains and three light chains.

Modified from *Brodsky FM, Hill BL, Acton SL, Nathke I, Wong DH, et al. Clathrin light chains: arrays of protein motifs that regulate coated vesicle dynamics. Trends in Biological Science 1991; 16: 208-13.*

to the capturing of soluble cargo proteins such as SST that putatively interact with its corresponding receptor inside the vesicles. In this way polymerization of clathrin provides the organizing function for protein sorting whereby a selected set of soluble cargo proteins bound to specific membrane receptors are incorporated into the lumen of each newly formed CCV. Although, there is no direct experimental evidence as of yet implicating clathrins' role in sorting of SST to the RSP.

Two main classes of sorting signals in the cytosolic tail of transmembrane proteins have been identified for targetting at the level of the TGN and plasma membrane: a tyrosine-based signal which has been extensively characterized (199, 200) or a dileucine motif (201, 202). Both classes of sorting signals interact with adaptor complexes AP-1 and AP-2. The two clathrin coated vesicle populations AP1 and AP2 are both assembled from a single pool of cytosolic clathrin trimers, but each can be distinguished by their respective adaptor proteins (203). It is postulated that one way to achieve the spatial separation whereby AP-1 is targeted to the TGN and AP-2 to the plasma membrane within a cell is through the recruitment of a small GTP-binding protein ARF to the TGN (203, 204). Therefore a general model for clathrin coat assembly at the TGN begins with the interaction of cytosolic GDP.ARF1 with GEF leading to a GDP-GTP exchange and association of the GTP.ARF with the membrane through an exposed myristic acid moiety. The membrane bound ARF.GTP then associates with the membrane binding site for AP-1 (a putative docking protein) allowing it to interact with the membrane with high affinity. This then initiates clathrin coat assembly. Upon stimulation of GTP hydrolysis, ARF.GDP is released from the docking site resulting in lowered binding affinity for AP-1.

Golgi-localized γ-ear-containing ADP-ribosylation factor-binding protein (GGA) is the most recent addition to the collection of coat proteins from the TGN. They have been shown to localize mainly to TGN membrane in mammalian cells and to interact with ARFs (205). The implication of GGA in sorting of receptor proteins from the TGN was demonstrated for sorting of manose-6-phosphate receptor (M6PR) into CCVs. Using the yeast two-hybrid system and GST pull down assay the C-tail of M6PR was found to interact with GGA through a leucine motif. GGA is then responsible for recruiting clathrin onto Golgi membranes by interacting with ARF.GTP Fig. 17 (206, 207, 208). Such findings indicate that GGA appears to fascilitate a similar pathway to AP-1/clathrin complex suggesting the possible existence of two classes of clathrin coated vesicles budding from the TGN. GGA, clathrin and AP-1 distribution was studied in the same cell using a novel triple labeling procedure to compare their localization (209). The results demonstrated structures that are positive for both clathrin and AP-1, and for both clathrin and GGA thus confirming the existence of two different populations of RSVs emerging from the TGN. A possible explanation for having two different types of CCVs is that they can select for different types of cargo receptors at the TGN.

4.3 Specialized domains in proproteins (soluble) implicated in their sorting to the RSP

It is believed that one of the requirements for a soluble protein such as SST to enter the RSP is some sort of a signal most likely present within its amino acid sequence. The need to postulate the presence of a sorting signal to carry proteins into RSVs came

from investigations demonstrating that not all proteins are detected in secretory granules (210,

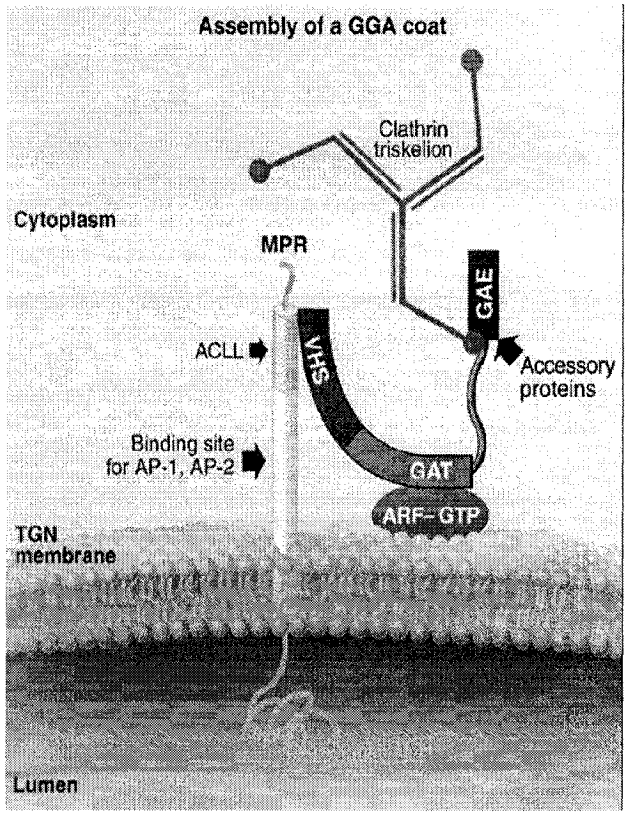


Fig. 17 Model for assembly of GGA-containing coat. ARF.GTP attaches to Golgi membranes, which is followed with the binding of GGA through its GAT domain. Clathrin would then be recruited to the membrane bound complex and attaches to GGA through its GAE domain.

From *Tooze SA. GGAs tie up loose ends. Science 2001; 292: 1663-1665.*

211). If within a cell there is more than one type of secretory pathway, a soluble protein in the lumen of the TGN may need to be assigned a route in order to enter RSVs. For example, AtT-20 cells endogenously express pro-opiomelanocortin (POMC) precursor hormone that is packaged into RSVs. Subsequently it is processed into adenocorticotropin (ACTH) (212). Once cells are stimulated with the secretagogue 8-bromo-cAMP, mature ACTH is released into the culture medium. In contrast, endogenously expressed laminin protein does not enter a storage form and is transported to the cell surface more rapidly by entering the CSP (213). Its secretion from the cell is constitutive and does not require the presence of secretagogues. Similarly when human proinsulin (a hormone having a similar travel objective to that of POMC) was heterologously stably expressed in AtT-20 cells, it was properly packaged into RSVs and was released into the medium upon stimulation in a similar fashion to that of POMC (214). Therefore segregation of a subclass of secretory macromolecules into secretory granules, despite the existence of another potential secretory pathway, suggests that these molecules have specific functions related to regulated hormone secretion or storage. It is possible then to assume that all peptide pro-hormones share some common property that targets them to the secretory granules.

In 1980 Blobel suggested that the determinants for intracellular protein sorting reside in discrete segments for each polypeptide, which can be permanent or transient features of a protein (215). These segments are characterized by their redundancy, shared by many structurally otherwise different proteins whose common denominator is an identical route within a cell. Later, amino acid analysis of 15 prohormones, including

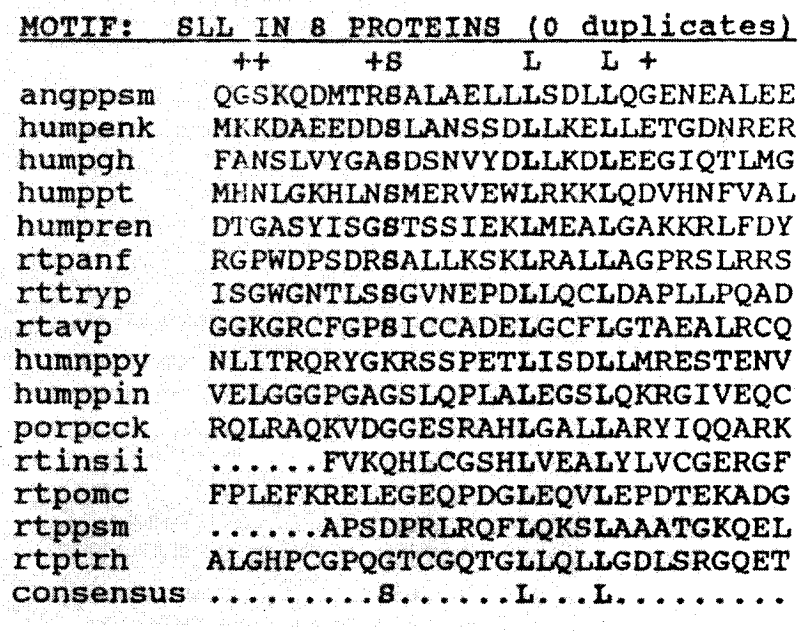


Fig. 18 Alignment of pro-proteins shown to be targeted towards the RSP in AtT-20 cells.

One striking feature among these pro-hormones is the presence of a common motif composed of two leucine residues separated by three amino acid

From *Kizer JS, Tropsha A. A motif found in propeptides that may target them to secretory vesicles. Biochemical and Biophysical Research Communication 1991; 174: 586-592.*

SST that have been shown to be correctly sorted into the RSP in AtT-20 cells, lead to the discovery of a motif consisting of two leucine residues separated by three amino acid that is shared by all of them Fig. 18 (216). Performance of a Chou-Fasman plot for secondary structure prediction in that region indicated a high probability of an α-helical structure formation for all 15 propeptides. Such a motif was experimentally proven to be important in targeting protein convertase 1 (PC1), a protease whose activity is largely confined to dense core RSVs in neuroendocrine cells, to the RSP (217). Analysis of the C-terminal domain of PC1 through PCR mutagenesis and pulse chase studies, revealed a sorting domain composed of an α-helical structure containing two leucine residues by being important for sorting.

A sorting signal may also exist for prohormones without requiring two leucine residues rather, the sorting signals can be composed of a disulfide bonded loop (218). It has been demonstrated that deletion of the 13 residues (Cys^{8} to Cys^{20}) at the NH_2-terminus of POMC causes missorting of the prohormone to the CSP. The investigators report that the sorting motif for POMC resides in a unique 13-amino acid amphipathic loop structure, stabilized by one disulfide bridge formed by the two cysteine residues. The requirement for the integrity of the disulfide-bridge in sorting to RSP has also been demonstrated for chromogranin B (CGB) (219, 220). Chanat et al. demonstrated that treatment of PC12 cells with dithiothreitol (DTT), a membrane thiol reducing agent known to prevent disulfide bond formation, resulted in constitutive secretion of CGB. The effect of DTT was reversed upon its removal from the feeding medium thus, allowing CGB to re-enter the RSP. Additionally, deletion of the 22 a.a stretch containing

the disulfide-bonded loop in human CGB resulted in its constitutive release in PC12 cells (221).

In the case of SST, it is well established that it undergoes regulated release. Among the intracellular mediators known to modulate SST secretion include ions, cAMP, and activators of protein kinase-C (15, 16, 51). Little is known however about the exact sorting motif for targetting of PSST to the RSP. Its sorting information is believed to reside in the first 78 amino acid The importance of this region was addressed by studying the differences in targeting capabilities of AFPPSST I and AFPPSST II (222). Heterologues expression of both precursors independently in AtT-20 cells and RIN 5F cells resulted in regulated release of the PPSST I precursor product and constitutive release of PPSST II product. The major differences between the two AF PPSST precursors lies in their pro-region. Therefore, the pro-region was tested for its targeting function to the RSP by creating a fusion gene containing the leader sequence and, the NH_2-terminal 54 a.a of rPPSST linked to the COOH-terminal 48 a.a of AFPPSST II. Transfected RIN 5F cells secreted the processed product upon stimulation with cAMP demonstrating that, the N-terminal sequences of PPSST rerouted the hybrid protein to the RSP. Later, experiments in which the first 82 amino acid containing the pro-region of PPSST were fused to α globin protein, a protein that does not undergo regulated release, resulted in its successful targeting to the RSP (223). Additionally, construction of mutant precursors of rPPSST that either lack, or have replaced portions of the pro-region revealed that the NH_2-terminus not only contains signals for regulated secretion but that there could be multiple signals each of which could independently cause sorting to two different pools of RSVs (224). Such a conclusion was due to differences in cellular

stimulation produced by treating the cells with two different secretogogues. Nevertheless, no specific region or sequence within the pro-region of PSST has yet been identified as carrying sorting information for SST.

4.4 Search for a sorting component in the TGN

Although the RSP sorting mechanism is not completely understood, it most likely involves an interaction of the soluble protein with a component of the TGN membrane. A sorting signal motif alone is not sufficient to cause transport of pro-proteins to the RSP. Two main models exist for sorting at the TGN. One is called the aggregation model, involving the preferential tendency of proteins to self-aggregate by excluding other proteins from entering and being packaged into RSVs (210, 211). The second model implicates a specific TGN sorting receptor which would bind to a specific region in the soluble protein leading to its packaging into CCVs.

In 1988, the first sorting receptor was described by using affinity chromatography to isolate proteins capable of binding to regulated secretory hormones (211). A set of proteins with sizes in the 25-kDa range were purified from dog pancreatic tissue, mainly localized to the TGN with capabilities of binding to two regulated secretory proteins insulin and growth hormone but, not to immunoglobulin that is secreted via the constitutive route. However, these proteins were later found to only cross-link with prohormones destined for the RSP but were no longer considered a true sorting receptor (225). More recently, it has been suggested that the membrane bound form of carboxypeptidase E (CPE), an enzyme involved in trimming prohormones at basic residues, is a sorting receptor in endocrine cells (226). The enzyme is present exclusively

in the TGN and in RSVs of neural and endocrine cells. Within the secretory pathway, CPE is first synthesized as an inactive membrane bound 55 kDa form that attaches to the membrane of the TGN through its 22 C-terminal residues by adopting an α-helical structure that fully spans the membrane (227). The tail is then cleaved off proteolytically to yield the enzymatically more active 53kDa soluble form involved in processing of prohormones (228, 229). Therefore, CPE has two main roles within the RSP: 1) the soluble form functions as a processing enzyme within secretory granules; 2) the membrane bound form serves as a sorting receptor by targeting prohormones to the RSP, presumably operating at the TGN.

The importance of CPE for regulated secretion was originally discovered by studying the binding capacity of the iodinated form of the POMC sorting signal and to RSVs and, Golgi-enriched membranes (226). CPE was then evaluated functionally as a sorting receptor through CPE antisense studies in Neuro 2a cell lines. POMC was missorted to the CSP in antisense transfected cells as monitored by secretion and immunofluorescence studies. Similarly *in vivo* experiments in the CPE^{fat} mice expressing a mutation in the CPE gene (230), POMC was found to be released constitutively without displaying any inhibitory control by dopamine; a neurotransmitter that regulates POMC release in the pituitary (231).

The ability of CPE to sort other prohormones was analyzed by carrying out molecular modeling studies indicating that, proenkephalin and proinsulin have similar putative sorting signal motifs as POMC (232, 233). Although, no direct experimental evidence for a sorting domain has been elucidated in either prohormone. By using antisense strategy, both prohormones were found in a perinuclear area surrounding the

Golgi in CPE depleted cells. Additionally, no response was detected when cells were stimulated with either K^+ or Ca^{++} depolarizing buffer. Interestingly, in the same study, chromogranin A (CGA) was correctly sorted to the RSP in cells downregulated in CPE expression thus, implying a requirement of an alternate sorting receptor other than CPE for its targeting to RSP. CGA is a major soluble protein of sceretory vesicles and known to be present in virtually all neurons and endocrine cells, mainly bound to other proteins, hormones, and metal ions in the secretory pathway (234). Later, the finding that CPE is a sorting receptor was challenged by other investigators demonstrating that proinsulin release was stimulated upon secretogogue application in primary β-islet cells isolated from CPE-deficient Cpe^{fat} mice (235). Such an observation implied that proinsulin was properly sorted to RSVs, with CPE having an unlikely effect on its targeting.

A third potential receptor implicated in protein sorting is the intraluminal inositol 1,4,5-triphosphate receptor (IP3R); a receptor involved in calcium regulation and is expressed in both the TGN and RSVs (236). In an attempt to isolate a potential trans membrane protein capable of binding to CGA by coulumn chromatography, a 260 kDa protein was isolated corresponding to IP3R. The IP3R appeared to bind CGA and CGB in a pH dependent manner preferentially at pH 5.5 consistent with the pH levels present in the TGN lumen and vesicles (236, 237). Later it was demonstrated that CGA and IP3R physically interacted with each other as demonstrated through co-immunoprecipitation experiments (238). Since both CGA and CGB are both major soluble proteins in RSVs generally found in association with Ca^{2+}, the role of IP3R in their targeting to the RSP is likely but not definitive. The ability to bind soluble proteins is in itself not sufficient to prove IP3Rs involvement in sorting of chromogranins. Unfortunately, no direct evidence

as of yet has demonstrated the role of IP3R in targeting of CGA towards the RSP. Protein-protein interaction has been the sole mechanism used to describe the process whereby proteins enter the RSP from the TGN. However, recently there has been increasing evidence suggesting that lipids are important in regulated targetting of proteins as well (239-242).

In the fluid lipid bilayer, different lipid species are distributed assymetrically over an exoplasmic and cytoplasmic leaflet resulting from preferential packaging of sphingolipids and cholesterol into moving platforms or rafts. Specific protein species are then selectively incorporated or excluded by attaching to the bilayer or the lipid raft Fig. 19 (239). Lipid rafts are known for being insoluble in the detergent triton thus, making it a popular technique used to analyze for raft association with certain proteins. They are mainly known for apical sorting of membrane proteins in polarized epithelial and hepatic cells and, delivery of cholesterol and sphingolipids to the cell surface (240, 241).

Experiments investigating the role of lipid rafts in regulated secretion were originally demonstrated by examining the effects of altering intracellular cholesterol levels on secretory granule biogenesis in AtT-20 cells (242). Cholesterol depletion was shown to block the formation of secretory granules containing POMC. However, the inhibition was reversible; granule formation was restored by re-addition of cholesterol. Additionally, raising intracellular cholesterol levels resulted in an increase RSV biogenesis as judged by monitoring enhanced packaging of CGB into TGN-derived vesicles. Similar experiments have shown that sphingolipid depletion resulted in the missorting of PC2, a protease of the regulated secretory pathway involved in intracellular maturation of prohormones (243). Using TGN/granule enriched membranes isolated from

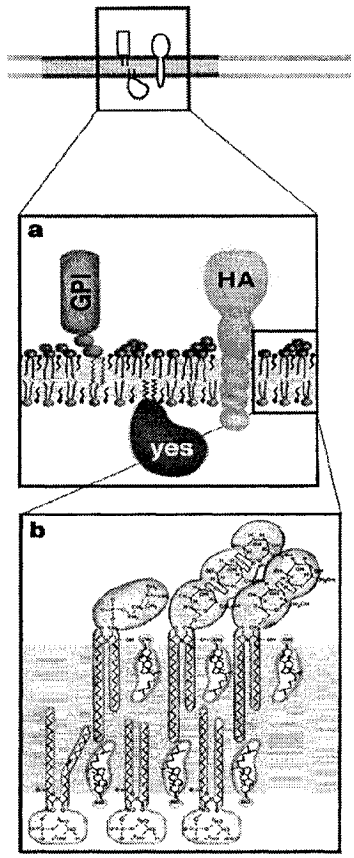


Fig.19. General diagram explaining the organization of rafts in the fluid membrane.

a) Rafts contain proteins that selectively attach to the exoplasmic leaflet. Others can also bind to the cytoplasmic side of the lipid microdomain.
b) The lipid bilayer is assymetrical with shpingomyelin and glycoshpingo-lipids in the exoplasmic side, and cholesterol present in both leaflets.

From *Simons K, Ikonen E. Functional rafts in cell membranes. Nature 1997; 387: 569-572.*

AtT-20 cells, *in vitro* synthesized PC2 bound to the membrane in a pH-dependent manner preferentially at pH 5.5. The solubility of PC2 was later determined by western-blot analysis following membrane extraction with triton. Most of the PC2 remained attached to the membrane pellet implying its insolubility in triton. The role of lipid rafts in directly sorting PC2 was assessed by studying the intracellular distribution of PC2 in AtT-20 cells upon treatment with the sphingolipid-inhibitor fumonisin. PC2 demonstrated a perinuclear pattern of localization, with its absence in both the cytosol and at the tips of the cell. Therefore, it is plausible that reduction of sphingolipid content in the TGN blocked PC2 interaction with lipid rafts thus, preventing its entry into RSVs.

Recently, the role of lipid rafts has also been shown to play an important in the ability of CPE to carry out its role as a sorting receptor for POMC in AtT-20 cells (244). Membrane CPE has been shown to be insoluble in triton`, in accordance with the criteria by which rafts are identified, suggesting that it may interact with detergent resistant-membranes. To determine the kinetic stage at which CPE acquires its lubrol insolubitity, pulse-chase experiments followed by western-blots demonstrated that CPE associates with detergent-insoluble membranes in the TGN and, immnature secretory granules. Membrane cholesterol was required for POMC binding to CPE particularly since, cholesterol depletion of the extracted membranes followed by a binding assay resulted in a dose dependent decrease in POMC binding. Additionally, the in *vitro* results were correlated with in *vivo* biological function of CPE by carrying out immunocytochemistry and secretion studies for POMC in cholesterol depleted AtT-20 cells. Punctate staining of

POMC was completely abolished and the cells were not responsive to stimulation demonstrating that CPE's association with lipid-rafts is critical for its sorting function.

4.5 Possible role of aggregation in protein sorting to the RSP

The appearance detected with the electron microscope of dense material in the *trans*-Golgi lumen and in secretory granules of regulated secretory cells appears as an insoluble mass of proteins generally referred to as, aggregates Fig. 20. This morphological observation is due to a characteristic trait of the RSP whereby, secretory proteins start to become concentrated and condensed at the TGN and later in granules leading to the formation of aggregates from which constitutively secreted proteins are excluded (183, 184, 189). The fact that aggregates start forming at the TGN raises the possibility that aggregation is an effective sorting mechanism and a factor in RSVs formation. This lead to consider an alternative transport model, the aggregation model, for regulated sorting at the TGN.

Aggregation of proteins inevitably will segregate them from others that do not aggregate but remain in solution. The main cause of aggregation is believed to be due to changes in the lumenal milieu within the TGN and granules as compared to that of the ER. Two key parameters are involved in influencing the aggregational properties of propeptides within the secretory pathway: the pH and Ca^{+2} concentrations (245-247). Generally, organelles of the secretory pathway from ER to Golgi to RSVs tend to become increasingly acidic with increasing high levels of Ca^{2+} as well (190, 248-250). The TGN pH has been shown to be around 6.4 with a 10mM Ca^{2+} concentration whereas the granules are more acidic (pH 5.2-5.5) containing 20-50 mM concentration of Ca^{+2}.

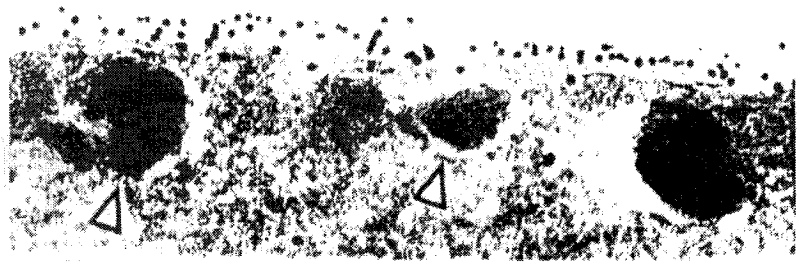


Fig. 20 Electron microscope image of the TGN in AtT-20 cells. The condensation of proteins commences in the trans-most Golgi cisternae leading to the formation of aggregates represented by the arrowheads.

From *Orci L, Ravazzola M, Amherdt M, Perrelet A, Powell SK, Quinn DL, Moore HP. The trans-most Golgi cisternae of the golgi complex: a compartment for sorting of secretory and plasma membrane proteins. Cell 51: 1039-1051.*

Support for the aggregation model came from studies demonstrating that several regulated secretory proteins aggregate under acidic pH and high calcium-concentration. The most widely studied protein family that has been used as a paradigm for the aggregation-mediated sorting model are the granin family of proteins which includes CGB (secretogranin I), CGA, and secretogranin II (SGII) (245, 246, 251). The low pH/calcium induced aggregation is thought to be mediated by certain structural features among all granins particularly an abundance of acidic residues distributed through out the polypeptide chain. Aggregation, was a selective process in that immunoglobulin, a secretory protein that is not packaged into secretory granules, did not form aggregates under similar conditions (251). Additionally, other regulated secretory proteins have been shown to undergo aggregation such as PC2 and POMC (247, 252). Incubation of PC2 transfected oocyte extracts at pH 5.5 with Ca^{+2} lead to its sedimentation, whereas at pH 7.0 PC2 was localized exclusively to the supernatant (247). Similarly, it has been demonstrated that insulin forms an insoluble crystalline structure in granules sedimenting rapidly once granule content were isolated (253, 254).

Even in the event that it wasn't possible to demonstrate the ability of proteins to self-aggregate, this would not rule out the aggregation model. Regulated secretory proteins may thus require an interaction with another lumenal TGN protein, which in itself is able to aggregate and associate with the membrane. Recently, there has been mounting evidence pointing to the existence of aggregational chaperones whose role is to aid in the aggregational process leading proteins into RSVs. The granin family of proteins have taken the spotlight for such a process. This is supported by their widespread occurrence in secretory granules of endocrine and neuronal cells and, their capability of

co-aggregating with certain peptide hormones (255-257). Therefore, granins are not only one of the major soluble proteins packaged into RSVs in neuroendocrine cells but, may also have a central role in packaging other proteins into the RSP. Overexpression of CGB in AtT-20 cells lead to a 3-fold increase in POMC storage which was demonstrated by pulse-chase studies followed with immunoprecipitation (258). The increase in storage correlated with a basal decrease in the amount of POMC secreted into the medium of the cells. Additionally the higher expression levels of CGB correlated with the amount of intracellular POMC ie. the higher the CGB expression, the more POMC remained stored in granules. These observations lead investigators to conclude that CGB at the TGN promotes sorting of POMC into RSVs. Other groups analyzed the effect of CGA depletion using RNA- antisense strategy on dense-core granule formation in PC12 cells (259). Using electron microscopy there was a general decrease in the quantity of RSVs as compared to *wt* PC12 cells Fig. 21. POMC secretion in these cells displayed high levels of basal release in the absence of stimulation, with no significant increase in secretion upon secretogogue application. Additionally, transfection of CGA in a pituitary cell line (6T3) lacking the regulated secretory pathway restored granule formation due to the punctate staining pattern observed for both CGA and POMC, and restoration of POMC regulated release. Thus, CGA was demonstrated to act as a regulatory switch with the ability to regulate secretory granule formation.

The role of both CGB and CGA as helper proteins in the RSP of neuroendocrine cells can easily be compared with that of 7B2. 7B2 is a protein that associates with the precursor form of PC2 during intracellular transport from the ER to the Golgi preventing its premature activation and thus premature cleavage of precursor prohormones such as

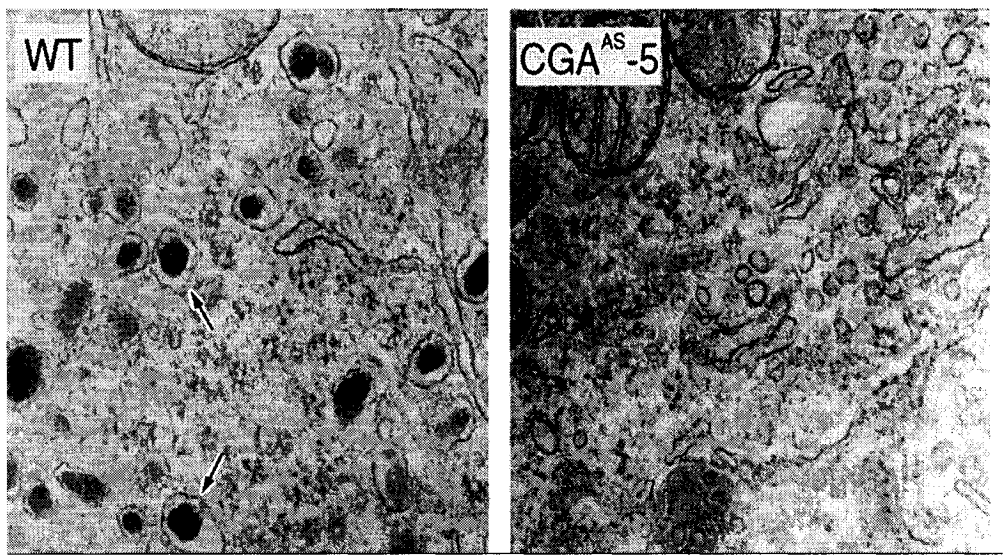


Fig. 21 Electron microscope image of CGA-depleted PC12 cells.
Electron micrograph of *wt* PC12 cells left, and CGA-depleted right. Dense core secretory granules are abundant in *wt* cells, but very scarce in CGA-depleted cells.

From *Kim T, Tao-Cheng JH, Elden LE, Loh YP. Chrmogranin A, an on/off switch controlling dense-core secretory granule biogenesis. Cell 2001; 106: 499-509.*

PSST (260, 261). 7B2 is packaged into RSVs along with PC2 and, with the assumption that 7B2 is only expressed in cells containing PC2. However, additional roles for 7B2 were highlighted in the finding that 7B2 is found in brain areas lacking PC2 expression, while the converse has never been observed (262). Indeed, recent data has demonstrated that 7B2 null mice exhibited marked increases in plasma circulating levels of ACTH released from the pituitary compared to *wt* mice (263). Generally, the release of ACTH is under the control of certain hypothalamic hormones. Such a finding signals an important additional role for 7B2, not related to control of PC2-mediated effects but, to the regulation of pituitary hormone secretion such as ACTH.

Other helper proteins reported to aid in regulated targeting that are not themselves sorted to the RSP include the His-tagged secreted alkaline phosphatase (SEAP-His) (264). Alkaline phosphatase is in fact a marker for the CSP but, once tagged with hexameric histidine (His) peptide it attains the ability to cause calcium induced aggregation of regulated secretory proteins such as proenkephalin. Additionally, basal secretion of CGA was reduced in cells expressing His^6-SEAP; consistent with reduced release of basal CGA while stimulated secretion was increased. A key point is that without employing the hexameric His tag, SEAP has no effects on enhancing regulated release of CGA or enhancing the aggregational properties of proenkephalin. In this case however the chaperone (His^6-SEAP) itself does not co-aggregate with regulated secretory proteins or, become sorted into the RSP and its secretion remained constitutive despite the modification.

Aggregation alone, however, has been proven not to be sufficient for sorting. For example, certain modifications on CGB such as reduction of the disulfide bonds by DDT

treatment affected its sorting capabilities whereas its ability to aggregate at the TGN was not hindered (265). Therefore, a combination of both models is more plausible whereby protein aggregates bind to a specific receptor at the TGN leading to efficient packaging into RSVs.

5. Proportion convertases (PCs)

5.1 Cleavage at basic residues

a) General features

Post-translational modification of one form or another is central to the synthesis of fully active forms of almost all proteins. Such modifications can include phosphorylation, sulphation and glycosylation. Another fundamental example is protein processing whereby biologically active proteins and peptides passing through the secretory pathway are often generated via intracellular proteolysis of inactive precursors in appropriate cellular compartments (266-268). In 1967, Steiner provided the first direct evidence for the processing of a higher molecular-mass precursor, proinsulin, which was biologically cleaved into a smaller form, insulin (269). Since that time it has become apparent that the vast majority of regulatory peptides are derived from higher molecular precursors.

Generally, proteases are divided into two categories: a) *exopeptidases* that cleave bonds at the amino- (aminopeptidases) or carboxy- (carboxypeptisases) terminal end of the protein; b) *endopeptidases* cleave internal bonds within the substrates (270). PC's belong to the endopeptidase family, cleaving substrates at single or paired basic residues. The substrate precursors can be neural and peptide prohormones such as PSST,

proteolytic enzymes, growth factors, numerous type-I membrane bound proteins such as receptors and cell signaling molecules (266-268). Progress towards the identification of the processing enzymes responsible for cleaving precursors at basic residues began in 1984 with the successful cloning of the first authentic PC. The protein isolated encoded a TGN localized enzyme named Kex2, or kexin, responsible for cleaving a hormone in yeast known as alpha-mating factor at a Lys-Arg site that is implicated in promoting yeast mating (271). Six years later, in 1990, the cloning of the mammalian convertase counterpart began and continues with the discovery of a family of proteolytic enzymes that are homologous to the yeast processing protease kexin (268, 272, 273). Additionally, due to the homology of the PC catalytic domain to that of bacterial subtilisin convertases, PCs are often referred to as subtilisin/kexin-like calcium- dependent serine proteinases.

Contrary to yeast which contain a single gene encoding kexin, in mammals there are seven known mammalian PCs which have been molecularly characterized to date Fig. 22. The seven mammalian PCs include PC1 (also called PC3), PC2, furin (also called PACE), PACE4, PC4, PC5 (also called PC6), and finally PC7 (266-268, 274). Certain PCs exhibit additional protein-isoforms produced through alternative splicing of a single precursor mRNA and include PACE4 (274, 275), PC4 (276), PC5 (277) and PC7 (274). Additionally, mammals contain both Golgi-localized enzymes as well as nonanchored prohormone convertase. Those that are membrane anchored include furin, PACE4, PC5B and PC7 that are type-I-membrane-associated enzymes with a transmembrane sequence close to their C-terminus thus allowing them to cycle between the TGN and the cell surface (266-268, 274, 275, 278).

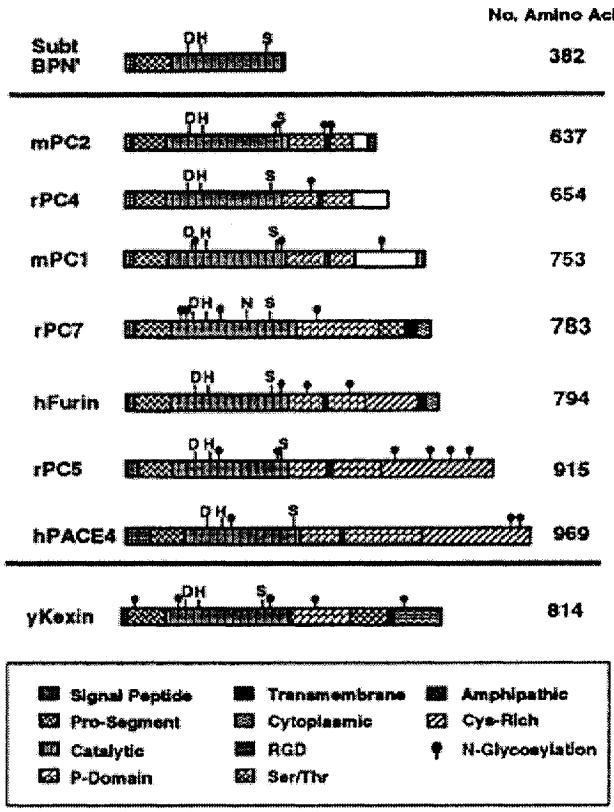


Fig. 22 schematic representation of the seven mammalian PC family and comparison with ancestral bacterial subtilisin and yeast-kexin.

From *Seidah NG, Chretien M. Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. Brain Research 1999; 848: 45-62.*

Each enzyme exhibits both unique and conserved structural motifs with all members containing a signal peptide, a pro-segment, a catalytic subunit and a P-domain Fig. 22. The catalytic subunit contains the conserved catalytic triad Asp/His/**Ser**, hence the name serine proteinases whereby the enzyme binds to its substrate through the Ser residue (266-268, 270). Homology among PCs is considered highest in the catalytic domain whereas the C-terminal sequences following the P-domain vary between each member. The P-domain is critical for convertase folding in the ER and may influence the marked calcium dependency and more acidic pH optima required for some PCs especially, PC1 and PC2 (266-268, 279). PCs specialize in cleaving substrates at basic residues with the general classical motif (Arg/Lys-(Xn)-Arg↓ where n = 0, 2, 4, or 6 and X is any a.a except for Cys and rarely Pro. However, upstream basic residues at the P4 and P6 position also contribute to substrate recognition (280, 281). In most cases endoproteolytic cleavage by PCs is always followed by exoproteolytic removal of the exposed C-terminal basic residues by a carboxypeptidase, such as the already described CPE enzyme (282).

PCs are synthesized as inactive precursors otherwise, they will cleave everything in sight. The general autoactivation steps required for a PC to become capable of cleaving substrates requires a series of properly timed and organized events (266-268). Following translation of the nascent chain and translocation into the ER lumen, the enzyme folds properly followed by autocatalytic cleavage of the NH_2-terminal propeptide. The propeptide remains associated with the catalytic domain through non-covalent interactions acting as a potent inhibitor thus, preventing random cleavage of substrates. Once the cleaved inactive proenzyme reaches the TGN, the non-covalently attached NH_2-terminal segment detaches. This event is due to the calcium-enriched environment along

with the more acidic versus neutral pH conditions encountered earlier in the ER lumen. A second cleavage within the prodomain then precludes further inhibitory interactions, resulting in full activation of the PC. Such a scheme applies to all PCs with the exception of PC2 that is more unique and somewhat different (283).

Tissue distribution and subcellular localization of the PCs is diverse and, can be subdivided into four categories. Furin, PC5B, and PC7 are found in varying amounts in wide variety of tissues, generally retained in the TGN by virtue of their transmembrane domain providing them with a strategic location to cleave substrates entering the CSP *en route* to the cell surface (1,2,3,8,9,12). PC1 and PC2 are the major forms expressed only, in the neuroendocrine system and brain acting on precursors entering the RSP (266-267). The short soluble isoform of PC5 (PC5A) also cleaves precursors entering the RSP but, contrary to PC1 and PC2, it has a broader tissue distribution in both endocrine and non-endocrine cells (283). PACE4 is expressed in both endocrine and non-endocrine cells conceivably processing precursors in both the CSP and RSP. Finally, PC4 mRNA expression is exclusive to the testis (285).

b) Furin

Furin, the first discovered mammalian subtilisin is encoded by the *fur* gene (286). The partial sequence of human furin was originally obtained in 1986 (286), and later completed in 1989 (287) with its expression being ubiquitous and widespread in tissues and cell lines (286-288). Using *insitu* hybridization furin expression has been localized to the brain as well in peripheral tissues such as the liver, gut, teeth, bone and lachrymal gland cleaving substrates in the CSP (289). The importance of furin was particularly

highlighted in *fur* knockout mice which die early in embryonic development between 10 to 12 days (290).

With regard to enzyme activation, furin is a type-I transmembrane protein by virtue of its C-terminal transmembrane domain that is first synthesized as a prepro-enzyme in the ER (291). Following removal of the signal peptide, a second cleavage takes place soon after depositing the molecule into the RER, approximately 10 minutes later, at an NH_2-terminal -Arg-Thr-Lys-Arg- site through autocatalytic cleavage Fig. 23. Propepitde cleavage is a necessary step for the eventual activation of the enzyme and for subsequent export of the membrane bound enzyme into the TGN (292, 293). In the neutral pH and low free Ca^{+2} environment of the ER the cleaved profragment remains noncovalently associated with furin preventing its ability to cleave substrates. The complex is then transported to the Golgi. The mildly acidic environment of the Golgi and higher Ca^{+2} concentrations induces a second cleavage of the profragment which binds less tightly to furin leading to its dissociation and, eventual activation of furin. Such a process occurs early in the Golgi prior to entry into the TGN (292-294). Very little is known about the fate of the pro-segment once it detaches from furin. Pulse-chase studies following monensin treatment, a drug that inhibits any forward transport from the *cis*- and *medial* Golgi cisternae to the TGN, demonstrated that furin was fully active and capable of cleaving substrates despite its inability to enter the TGN (293). Such data imply that the enzyme becomes active very early in the Golgi. Once furin reaches the TGN it mainly concentrates there but does cycle between this compartment, the plasma membrane and endosomes. Sorting signals responsible for furins routing within the cell

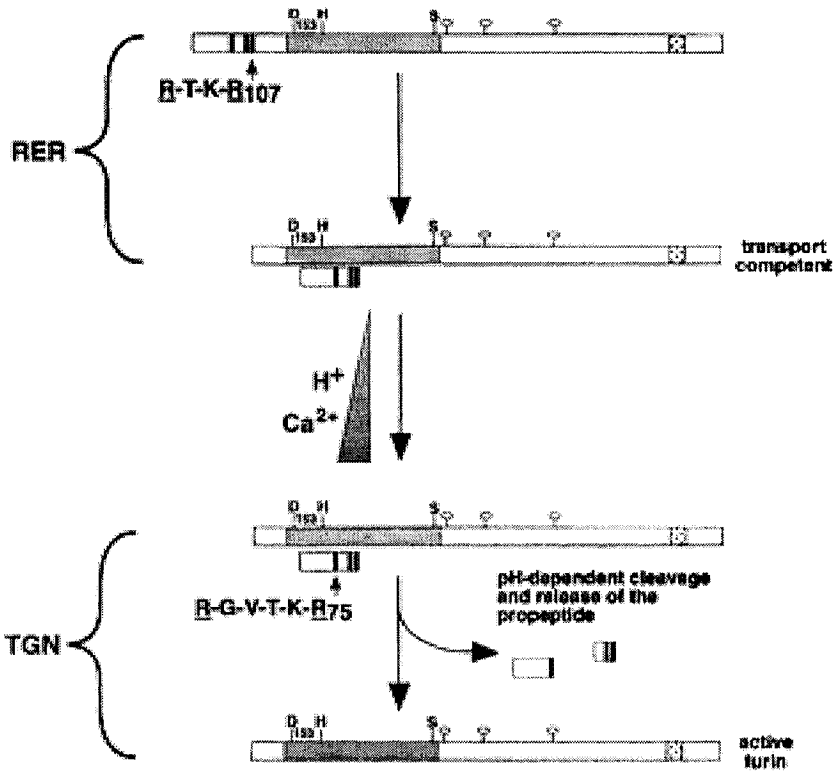


Fig. 23 Model for furin activation.

From *Anderson ED, VanSlyke JK, Thulin CD, Jean F, Thomas G. Activation of furin endoprotease is a multiple-step process: requirements for acidification and internal propeptide cleavage. EMBO Journal 1997; 16: 1508-1518.*

reside in its cytosolic tail, which have been extensively analyzed and used as a model for study of protein trafficking within the secretory pathway (295, 296).

In the TGN, a tyrosine based motif along with the phosphorylated acidic cluster in the C-tail of furin promotes recruitment of AP1 specific assembly proteins onto Golgi membranes (297). Furin then enters CCVs. Once furin is in a post-TGN endosomal compartment, it is either shuttled to the cell surface/early endosomes or is retrieved back to the TGN (298). At the level of the cell surface a tyrosine-based motif is responsible for its internalization through packaging into CCVs (296). In endosomes, a novel cytosolic protein known as phosphofurin acidic cluster sorting protein (PACS) directly interacts with the phosphorylated acidic cluster in the C-tail of furin connecting the endoprotease to the clathrin sorting machinery thereby directing furin back to the TGN (298).

Compilation of studies examining the processing of various precursors and their mutants revealed the cleavage specificity of furin to be comprised of an -Arg-X-Lys/Arg-Arg- motif with the catalytic domain being responsible for defining its substrate preference (299, 300). Furins localization in the TGN allows it to be present in a great location to process many precursors, both soluble and membrane bound, moving through the secretory pathway particularly the CSP. Examples of substrates that it encounters along the secretory pathway include growth factors and their receptors (301, 302), prohormones such as PSST (35b), bacterial toxins and envelope glycoproteins of many viruses including that of the HIV and the Ebola virus (268, 303).

c) PC1 and PC2

PC2 cloning was demonstrated simultaneously by two groups in 1990 through screening a human insulinoma (304) and mouse pituitary cDNA library (305). A year later in 1991 full length PC1 was cloned from rat pancreatic tissue (306). Northern blot analyses and *insitu* hybridization studies demonstrated that contrary to furin, PC1 and PC2 expression is restricted to endocrine and neuroendocrine cells (307). They are both soluble enzymes, lacking a transmembrane domain, and are mainly localized to regulated secretory granules cleaving substrates at dibasic cleavage sites. Similar to furin, a number of steps are required for zymogen activation. However, the process is somewhat different for both enzymes especially PC2.

PC1 is synthesized in the ER as a prepro-enzyme that undergoes two cleavage steps within the ER. The first step involves removal of the signal peptide followed by an autocatalytic intermolecular processing of its N-terminal prosegment producing an 87-kDa-enzyme Fig. 24 (308). The cleaved product then enters the Golgi and passes through its compartments until it reaches the TGN. Due to a decrease in the pH gradient encountered the TGN, PC1 becomes partially active capable of cleaving only certain substrates (309). The 87-kDa form is then targeted into RSVs by virtue of certain sequences present in the C-tail of the enzyme. Once in granules, PC1 is further shortened autocatalytically at an Arg-Arg site by removing 135 amino acid at the C-terminus, producing a 66-kDa form (309). Full activation of PC1 only takes place within RSVs and studies employing temperature block and BFA have supported this idea (310, 311). In general, depending on the substrate, the two forms (87- and 66- kDa) are both active

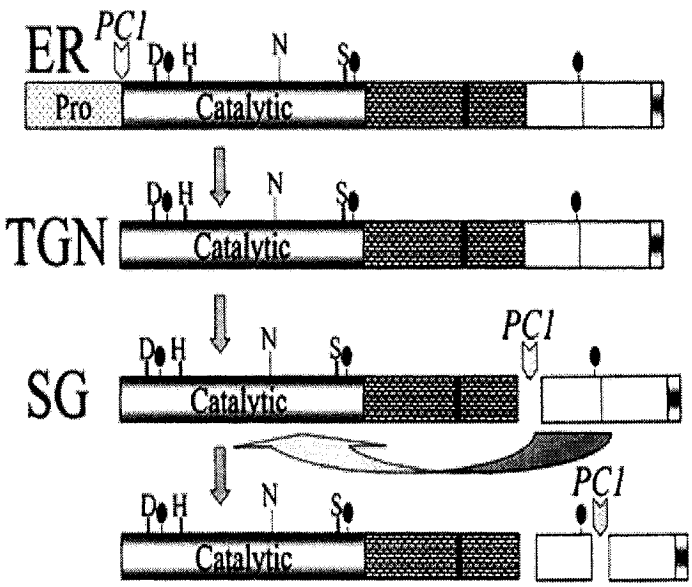


Fig. 24 Diagram illustrating the steps involved in the activation of PC1 as it moves along the secretory pathway.

From Seidah NG, Benjannet S, Hamelin J, Mamarbach AM, Basak A, Marcinkiewicz J, Mbikay M, Chretien M, Marcinkiewcz M. The subtilisin/kexin family of precursor converatses: emphasis on PC1, PC2,/7B2, POMC and the novel enzyme SKI-1. Annals of the New York Academy of Science 1999; 885: 57-74.

but removal of the carboxyl-terminal tail dramatically increases PC1 activity towards prohormone substrates (309). For example PC1 was capable of cleaving PSST in cells devoid in secretory granules, clearly demonstrating that PC1 is partially active prior to entry into RSVs (35a). However co-transfection of prorenin, a regulated secretory enzyme, and PC1 in CHO cells did not yield mature renin (312). Therefore, prorenin conversion does not occur in constitutive cells devoid of secretory granules. This suggests that in the context of the TGN the 87-kDa form of PC1 may have enough affinity for certain substrates. In contrast substrates such as prorenin require the removal of the C-terminal tail of PC1 within granules hence, limiting the activity of the processing protease to particular compartments depending on the substrates.

Comparatively, pro-PC2 zymogen activation is different from other mammalian subtilisin/kexin convertases studied so far in two respects. First pro-PC2 is slowly transported from the ER to the TGN, the compartment where its conversion into PC2 begins and continues in secretory granules as opposed to the ER (313, 314). Therefore compared to other PCs its activation is delayed to post-Golgi compartments. Second of all PCs only PC2 requires a specific binding protein known as 7B2.

d) The PC2 chaperone: 7B2

The polypeptide 7B2 was initially isolated from porcine and human pituitary glands with its sequence being highly conserved throughout evolution suggesting an important biological role for the protein (307, 315). Similar to PC2, it is widely expressed in most endocrine and neural cells mainly localized in RSVs (316). It is first synthesized as a 25-29 kDa precursor-protein, which is cleaved in the TGN by a furin-like enzyme at

its carboxy terminal end to an 18-21 kDa form (317). Following processing, 7B2 is packaged into RSVs (318). Initial reports relating 7B2 to PC2 were carried out in 1989 and 1992 through physiological manipulation of POMC synthesis, that is high in animals adapted to a black background and, low in white-adapted animals in the amphibian intermediate pituitary of *Xenopus laevis* gland (319, 320). By studying genes associated with POMC gene expression, differential hybridization techniques were applied involving screening of a pituitary cDNA library for 7B2, POMC and PC2 mRNA. 7B2 mRNA expression was shown to be tightly regulated to that of POMC and PC2, suggesting a functional role for it in regulating pituitary prohormone maturation.

A momentous breakthrough in the study of 7B2 function came about in reports demonstrating a tight inhibitory effect of of 7B2 on PC2 enzyme activity in *in vitro* studies (321). Additionally, by visually examining the 7B2 sequence for presence of functional domains highlighted two important regions. One region revealed that the C-terminus was homologous to members of the inhibitory protein family (321). Later, studies by Braks et al. demonstrated that the other region displayed remarkable amino acid sequence similarities to a chaperone protein further extending the roles of this molecule (322). Pulse-chase studies demonstrated that proPC2 is bound to pro7B2 in the early compartments of the secretory pathway, with the interaction being a transient one. The authors at the time proposed that the physiological role of 7B2 is mainly a chaperone holding proPC2 in an inactive state in the ER-Golgi region, allowing a switch to take place only once in the TGN/granules. Soon it became evident that 7B2 is required for the production of active PC2 (323). Overexpression of rat 7B2 in various neuroendocrine cell lines, greatly facilitated the kinetic maturation of proPC2, both in AtT-20 and in Rin5f

cells. Eventually the dynamic interaction between these two neuroendocrine molecules was further characterized in numerous other reports with a general model proposed, explaining the complex interaction of PC2-7B2 (324, 325).

Pro7B2 binds to Pro-PC2 in the ER due to the relative alkaline conditions of this compartment facilitating pro-PC2 migration to the Golgi. In the TGN, pro-7B2 is cleaved into an N- (21 kDa) and C- (31 a.a) terminal fragment remaining attached to pro-PC2. This is then followed with autocatalytic cleavage of pro-PC2 prodomain as the complex is transported into granules. In the acidic conditions of RSVs, PC2 further cleaves the C-terminal fragment of 7B2 at a Lys-Lys↓ site, two residues intimately tied with the inhibitory effect on PC2, producing two fragments (325). In the presence of CPE, the lysine extended fragment is trimmed off producing a fragment lacking any inhibitory action. All 7B2 fragments eventually dissociate from the enzyme along with the pro PC2 piece allowing the enzyme to become fully active in vesicles.

e) PC1, PC2, 7B2 and disease

The important roles of PC1, PC2, and 7B2 have recently been exemplified by the clinical characterization of gene mutations and from gene KO technology. Additionally such experiments should make it possible to delineate the developmental roles of these enzymes and to ascertain the extent of functional redundancy between the PC gene products. For example, an animal model for PC1 deficiency has not been yet generated but recent studies from a woman with severe early childhood obesity was pointed out with elevated plasma levels of proinsulin and POMC (326). The study eventually lead to the identification of a genetic defect in the PC1 gene whereby a change in a single base

(GGG→AGG) leads to a compound heterozygote for mutations in PC1 (327). The translated enzyme is unable to undergo antocatalytic activation causing its retention in the ER and ultimate degradation.

On the other hand PC2 null-mice have been created. Effected mice are still able to reproduce but have elevated levels of proinsulin, PSST, and proglucagon (328). Finally, 7B2 null mice display the most severe phenotypic changes, which include no demonstrable PC2 activity and deficiency in processing islet prohormones which include proinsulin, proglucagon, and proenkephalin (263). Surprisingly, these mice have elevated plasma levels of ACTH that is normally cleaved by PC1 and not PC2. These mice then go on to develop severe pituitary Cushings disease surviving at most 9 weeks after birth. Interestingly, given the almost-universal requirement for post-translational processing of prohormones it is remarkable that neither PC1 nor PC2 deficiency displayed very severe phenotypic changes compared to that of furin, whose role could have been compensated by PC7, and 7B2.

f) C-terminal processing of SST

Apart from disulfide bond formation, endoproteolytic cleavage, a key step in the proteolytic maturation of PSST, is the second type of post-translational modification to occur in the case of mammalian PSST. Processing of mammalian PSST principally occurs at the C-terminal end generating the two bioactive from SST-14 and SST-28 Fig. 2 (35 a-c, 51, 52, 58). SST-14 is produced through dibasic cleavage at an Arg-Lys residue, whereas endoproteolysis at a monobasic Arg site generates SST-28 and a 7-kDa peptide. The putative cleavage points were predicted by studying PSST sequences

isolated from AF pancreatic tissue (39). The sequences predicted that SST-14, occupying the C-terminal end of the precursor, is most likely produced by cleavage at an Arg-Lys doublet, which can be found at position –2, -1 from the NH_2-terminus of the SST-14 sequence. SST-28 on the other hand is produced at a putative Arg residue.

Initial work towards the elucidation of the specific proteases involved in PSST processing began in 1984 through the creation of a synthetic peptide containing the necessary predicted cleavage sites (329). Extracts of rat hypothalamic tissue were incubated with the peptide, thus representing the source of enzyme. The enzyme isolated was capable of cleaving the synthetic peptide and SST-28 to SST-14 *in vitro*. Later, in 1987, direct biochemical evidence demonstrated that two different types of endopeptidases (330a) mediate cleavage to either SST-14 or SST-28. The authors examined the processing of AF PPSST I and AF PPSST II to SST-14 and SST-28 by developing a special assay which allows rapid determination of PPSST converting activity. Two separate enzymes were isolated: one responsible for SST-14 production and the other for SST-28. Additionally, the enzyme responsible for SST-14 production was capable of processing proinsulin to mature insulin.

In 1991 following identification of multiple mammalian cDNAs coding for PCs which include furin, PC1 and PC2, opportunities were created allowing the isolation and identification of enzymes capable of cleaving PSST at basic sites. Through Edman degradation the partial sequence of an enzyme purified from AF islets with capability in cleaving PSST to SST-14 demonstrated homology to both human and mouse PC2 of more than 64% and 57% respectively (330b). Such was the first reported finding associating PSST processing with the subtilisin/kexin family. Direct studies towards the

elucidation of specific PC enzymes involved in PSST processing were later carried out in our laboratory.

The role of PC1 and PC2 in SST-14 production was characterized through heterologues processing of rat PPSST in both endocrine AtT-20 cells and, constitutive COS-7 cells using cDNA transfection experiments (35a). PPSST transfected COS-7 cells processed PSST mainly to SST-28 and to small amounts of SST-14. However, processing was considered inefficient due to the presence of high concentrations of unprocessed precursor. Since PC1 and PC2 are both not expressed in COS-7 cells, contransfection experiments of COS-7 cells with PSST and either, PC1 or PC2 were used to study their effects on SST processing. Co-transfected cells with PC1 and PPSST displayed significant increases in SST-14 production with no changes in SST-28 levels compared to cells mono-transfected with PSST alone. PC2 on the other hand, had no effects on PSST processing in this system. The inability of PC2 to cleave PSST in a constitutive secretory cell was postulated, at the time, to be due to the relatively different environmental conditions encountered by PC2 in a constitutive secretory cell compared to that of a regulated one. Comparatively, in AtT-20 cells, PPSST was almost completely processed, mainly to SST-14. Such results can be attributed to the much higher levels of endogenous PC1 protein expression in AtT-20 cells compared to COS-7 cells. Additionally, expression of PC1 mRNA in rat brain by insitu hybridization correlates with the regional distribution of SST-14 especially in areas rich in PC1 expression (331).

The role of PC2 in PPSST processing was revisited again in co-transfection experiments of PPSST and PC2 in a regulated secretory cell line: GH3 cells (35c). These cells express furin but very low levels of PC2. In cells co-transfected with PC2 and

PPSST, a marked increase in SST-14 levels was detected thus qualifying PC2 as a putative SST-14 convertase which appears to require the milieu of a regulated secretory cell. Additionally, the role of PC2 as an authentic SST-14 convertase was further strengthened in experiments demonstrating the complete absence of SST-14 production in extracts derived from PC2-/- mice (328).

Furins role in PSST processing stemmed from structure function studies revealing its substrate specificity to be composed of the following motif: R-X-R/K-R↓ (299, 332). Additionally, an Arg at position -1 is essential, with at least two out of three basic residues required at position -2, -4, -6. Such a specificity profile demonstrated considerable overlap with that for processing at the monobasic Arg cleavage site predicted for SST-28 containing the following sequence: **R**-L-E-L-Q-**R.** Indirect evidence leading towards speculations of PPSST furin cleavage resulting in SST-28 production was demonstrated through heterologues processing of rat PSST in COS-7, PC12, AtT-20, and GH3; all of which express furin (35a). More direct evidence for furin's role was provided by overexpressing the enzyme in COS-7 cells in a dose dependent manner using vaccinia virus infection experiments (35b). A dose dependent increase in SST-28 production was observed through HPLC experiments, which correlated with the levels of furin expression.

Overall, one cannot preclude the existence of additional enzymes capable of both SST-14 and SSt-28 generation.

Recently, it has been demonstrated that the presence of basic amino acid although necessary but, may not be sufficient for recognition by the PC endoproteases. Secondary structure motifs surrounding the cleavage site such as β-turns or α-helical structures have

been shown to play important roless by discriminating between functional dibasic residues and those, which are not, cleaved (333, 334). In the case of PPSST the region occupying SST-28 (1-12), the NH_2-terminal sequence of SST-28, separating the two C-terminal basic cleavage sites has been shown to play a key role in prohormone processing as predicted through secondary structure predictions (335). The SST-28 (1-12) segment contains a PAMAP motif with two β-turns positioned upstream and downstream of it. Generally proline residues play special roles in protein structure. Based on that, a number of mutations were created to evaluate the role of the proline motif in influencing PSST dibasic processing. The peptide sequence PAMAP itself forms a helical motif where replacement of either proline residue abolished cleavage specificity of the precursor at either basic processing sites. Hence, both the global conformation along with the basic residues are important for PPSST recognition by PCs.

Untimately, defining the cellular co-localization of each putative processing enzyme (furin, PC1, and PC2) along with SST is necessary in order to ascribe a more definitive role for these enzymes. However, no one has yet looked at SST-28 or SST-14 production along with PCs in particular cells.

As for processing at the NH2-terminus, cleavage has always been assumed to take place at a monobasic Lys^{13} residue (36, 60-61), however an enzyme implicated in such a process event is yet to be identified.

g) Substrate Cleavage and Compartmentalization

Although significant progress has been made in our understanding of the mechanisms involved in PC activation with their structural characterization and

intracellular distribution being well documented, many questions still remain particularly concerning the intracellular compartments in which processing is initiated. Generally, proprotein processing leads to the generation of at least two smaller peptides produced through cleavage of a single precursor, giving rise to functional diversity in hormonal production. Additionally, in many instances multiple enzymes can act on a single precursor. The effect of compartmentalization (TGN *vs* granules) of two precursor proteins, proinsulin and POMC, will be discussed in the following section in addition to PPSST.

Originally it was proposed that proteolytic processing can only occur once the precursor is packaged into granules. By employing a combination of immunoelectron microscopy techniques and drug treatment with antimycin, a drug responsible for arresting protein exit from the Golgi, it was demonstrated that proinsulin cleavage is initiated in the acidic clathrin-coated vesicles that bud from the TGN (253). Furthermore, using the pH probe 3-(2,4-Dinitroanilino)-3' amino-N-methyldipropyl-amine (DAMP) that accumulates in mature granules, there was a direct relationship between the number of DAMP- and insulin specific immunoreactive sites. Similarly, inhibition of the ATP-dependent pump, implicated in maintaining the acidity of the granule milieu, blocked insulin conversion (336, 337, 338). On the other hand, morphological data from a number of other laboratories provided evidence that prohormone endoproteolytic processing can also be initiated in the TGN (339-341).

POMC is a multifunctional precursor once cleaved gives rise to a host of biologically active peptides which include ACTH, β-lipotropin (β-LPH), β-endorphin (β-End), and α-melanotropin (αMSH). In order to identify the intracellular compartments

involved in POMC cleavage, specific affinity-purified antisera that only recognize the processed products of POMC in transfected AtT-20 cells were used. Both immunofluorescence and electron microscopy techniques demonstrated that POMC cleavage products were localized, not only throughout the cytoplasm of the cell but, were also evident in the *trans* most cisternae of the Golgi (341). Cleavage of POMC by PC1 and PC2 at dibasic cleavage sites was originally demonstrated by their co-transfection with mouse POMC in the constitutively secreting African green monkey kidney epithelial cell line BSC-40 and in endocrine cell line rat PC12 and AtT-20 using recombinant vaccinia virus vectors (339, 340). POMC products were efficiently produced in both cell types, constitutive and regulated. Therefore unlike insulin, POMC can be correctly processed in cells devoid of secretory granules implying that its processing is not restricted to secretory granules and can begin in the TGN.

PSST is C-terminally cleaved by two enzymes producing SST-14 that is cleaved by PC1 and PC2 and, SST-28 produced through furin cleavage (35a-c). Based on the enzymes acting on PSST one would expect that SST-28 production takes place in the Golgi, whereas the cleavage compartment of SST-14 is questionable. First attempts to characterize the subcellular compartment implicated in SST-28 generation were done using AF PPSST II cDNA (342). By using a combination of immunoflueorescence and immunogold staining, SST-28 was clearly observed in the TGN network of the cells in addition to, granules. Later, studies aimed at identifying the subcellular compartments in which cleavage at both, the monobasic Arg and dibasic Arg-Lys takes place, were demosntrated in subcellular fractionation experiments of rat cortical brain (343). Three main secretory compartments were isolated which corresponded to the ER, Golgi and

granules. Immunocytochemsitry, electron microscopy and HPLC results all demonstrated that both SST-14 and SST-28 were enriched in the Golgi fraction. Together with the use of temperature block and monensin treatment, known to inhibit forward transport from the Golgi, both cleavages were not inhibited. Therefore, similar to POMC and unlike insulin, SST processing including SST-14 production can take place in the TGN. For example Xu & Shields used a permeabilized cell system derived from rat anterior pituitary GH3 cells infected with PPSST to study its processing (344). Temperature block, followed by pulse-chase studies demonstrated efficient processing of PPSST to SST-14 and SST-28 in the TGN. Finally, our lab has provided several lines of evidence regarding the compartment in which PPSST is processed.

The kinetics of PPSST processing were originally studied using rat hypothalamic and cortical neurons. Pulse-chase studies demonstrated that PPSST processing begins as early as 5 minutes after pulse labeling (33, 34) implying that, its cleavage can take place within the TGN. Additionally, co-expression experiments of PC1 and rPPSST in the constitutive COS-7 cells resulted in significant production of SST-14 detected in cell extracts with no net change in the amount of ST-28 production. In another approach, poorly granulated GH4 cells co-transfected with PC2 and rPPSST were used to study the effect of hormone-induced granulation in improving SST-14 production (35c). Hormonal treatment failed to improve SST-14 production thus, excluding a significant role of secretion granules in PPSST maturation. Further evidence excluding the role of granules was obtained from studies using constitutive rat islet SST producing tumor cell line 1027B2 that endogenously express furin, PC1 and PC2 (345). Morphologically, based on

electron microscopy, these cells lack granules however, HPLC analysis demonstrated that they are capable of processing PPSST to SST-14 and SST-28.

h) Possible role of cleavage site in prohormone targeting

A few studies in the literature have concentrated on the importance of the cleavage site itself in influencing the direction that a prohormone will take i.e. regulated or constitutive. In order to shed light on this hypothesis Brakch and colleagues stably expressed PPSST and various mutant constructs in AtT-20 cells, followed by HPLC and RIA in order to determine the pattern of PPSST processing (346). Mutating the monobasic Arg responsible for SST-28 production did not effect regulated release of SST-14 upon secretegogue treatment. On the other hand, mutating the two dibasic cleavage site resulted in inhibition of SST-14 production and more importantly SST was not released upon stimulation. Processing of PSST to SST-28 was not effected since it takes place within the CSP. Such results inidcate that the dibasic cleavage site is important in targeting of PSST to the RSP.

Another prohormone shown to require the dibasic cleavage site for proper targeting is prorenin (347). Prorenin, a regulated secretory protein, is an aspartyl protease critical in the regulation of the cardiovascular system. Similarly, AtT-20 cells were stably transfected with *wt* or mutated forms of human prorenin. Pulse chase studies were performed to follow the fate of the labeled proteins in the secretory pathway. In a mutant construct whereby the dibasic cleavage site was changed to a furin consensus site, renin cleavage was not inhibited however the hormone was no longer released upon stimulation. Such results imply that premature cleavage of the renin prosegment, most

likely by furin in the Golgi, prevented the sorting of renin to the RSP. In the case whereby the basic cleavage site was changed to an Ala residue, prorenin cleavage was completely blocked and the precursor was released into the medium constitutively. Interestingly, the importance of protease cleavage sites in the sorting of proteins has also been demonstrated for proteins that normally enter the CSP such as the case of nerve growth factor (NGF).

Pro-NGF is a neurotrophin that usually enters the CSP and is cleaved by furin (348). Using vaccinia virus expression system, pro-NGF processing was monitored in AtT-20 cells infected in the presence or absence of virally introduced α_1-ATPDX, an α_1-anti-trypsin derivative that selectively interferes with furin's ability to process precursor proteins within the TGN (349). Blocking furins activity caused pro-NGF to enter the RSP; due to the conditioned pattern of secretion and a positive response to cellular depolarization. Furthermore, pro-NGF's immunoreactivity accumulated in punctate vesicles within the cytoplasm and the tips of cell processes. Such observations suggest that inhibiting furin cleavage targets some of the pro-NGF to the RSP where, its release can be promoted by extracellular cues. Identical results have been demonstrated for another neurotrophin whose targeting becomes shifted from constitutive to regulated once furin cleavage is inhibited (350).

Collectively, one can conclude that sensitivity to furin-mediated cleavage particularly in the TGN is an important factor in determining whether a protein is sorted to the constitutive vs RSP.

5.2 Cleavage at hydrophobic residues

a) Molecular cloning of enzyme(s) with hydrophobic specificity

Having discussed substrates cleaved at basic sites along with their respective enzymes, a number of precursors are also cleaved at non-basic sites such as C-terminal to Ala, Ser, Thr, Met, Val and Leu (268, 283). The identification of the enzymes responsible for such processing is required particularly, since such cleavage is responsible for the production of sterol regulatory element binding protein (SREBP) involved regulating cholesterol metabolism (351) and, the Alzheimer's amyloidogenic peptides Aβ40, -42, -43 (352). The road towards the discovery of proteases responsible in cleaving substrates at hydrophobic residues was lead by two groups: Goldstein and Brown and, Seidah and colleagues. The first group began their search in 1996, with their active work on the SREBP molecule (351).

SREBP protein forms a hairpin loop structure that is embedded within the membrane and is composed of three segments Fig. 25 (351). First the NH_2-terminal segment which projects into the cytosol second, the middle segment which forms a helical hairpin membrane anchor consisting of two transmembrane helices separated by a short loop that projects into the lumen of the ER and nuclear envelope. Third the carboxy terminal segment which projects into the cytosol as well. Physiologically, when cells are depleted of cholesterol, two cleavages occur within the SREBP molecule. The first cleavage requires the clipping of the short loop near an Arg residue known as site-1, breaking the attachment between the two transmembrane sequences separating SREBP. The resulting two pieces, N- and C-terminal intermediates, remain membrane bound (351). This cleavage is accelerated in sterol-depleted cells and it is reduced when sterols

accumulate thus, making the site-1 protease as the target of feedback regulation. The second cleavage (site-2) follows automatically and is not regulated but, is an unusual intramembranous one involving the N-terminal segment whereby a portion of it is released into the cytosol, leaving the membrane with the other portion still attached. It then travels to the nucleus where it acts as a transcription factor activating a host of genes which encode enzymes required for biosynthesis and uptake of cholesterol and unsaturated fatty acids (351). In view of the regulatory role of the site-1 protease, further knowledge regarding its structure, identity and substrate specificity is desirable.

In order to identify the exact position of site-1 cleavage a combination of *in vitro* mutagenesis of SREBP transfected into HEK-293 cells, immunoprecipation, and radiochemical Edman degradation were carried out (353). Sequencing results of the C-terminal fragments strongly suggested that site-1 cleavage occurs C-terminal to a hydrophobic Leu residue. In order to determine the a.a(s). that are critical for cleavage, a systematic series of point mutations were created in which Ala residues were individually substituted for most of the a.a(s). in the lumenal loop separating the two membrane spanning sequences. Only two single substitutions reduced cleavage dramatically: the Arg519Ala and the Leu522Ala. Hence, the Arg at the P4 position and Leu at the P1 are the only two residues in that region which cannot be substituted, with a general recognition sequence being composed of an RXXL motif. Therefore, this type of enzyme displays different substrate specificity to that of the already identified eukaryotic PCs, due to its hydrophobic specificity.

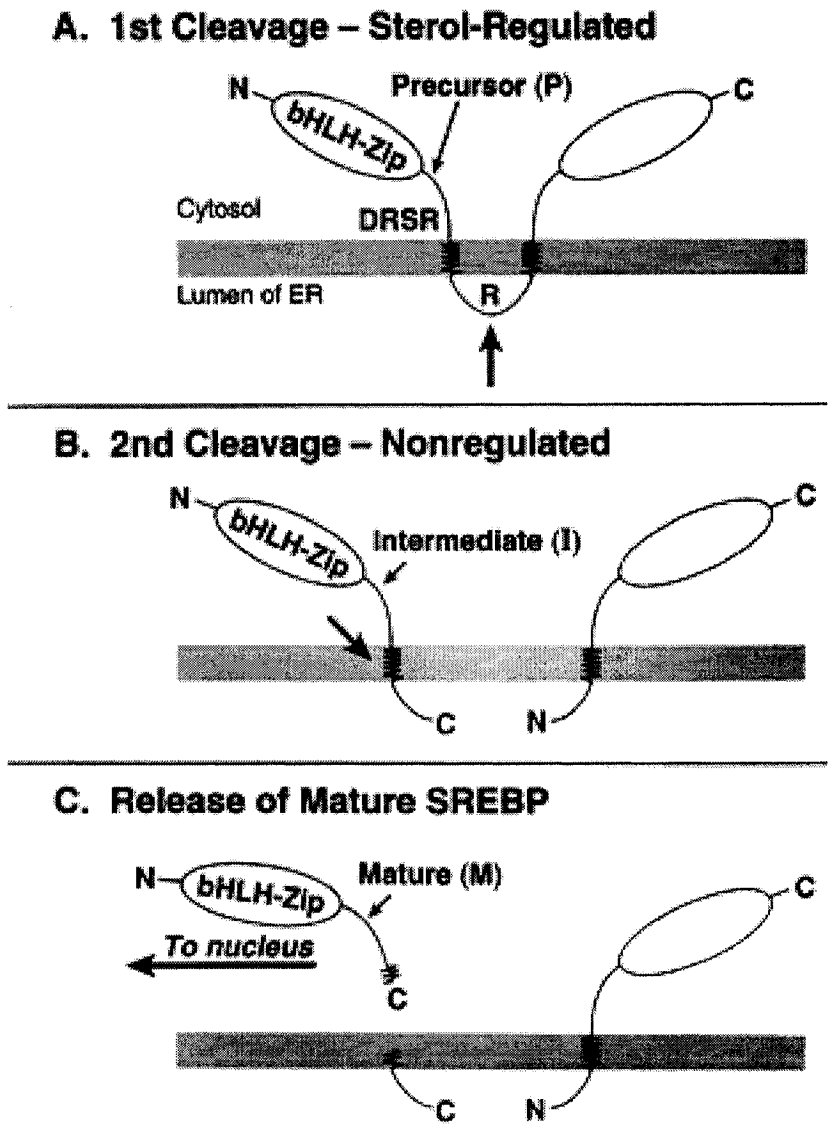


Fig. 25 Model of the two-step proteolytic cleavage of membrane-bound SREBP.

From *Sakai J, Duncan EA, Rawson RB, Hua X, Brwon MS, Goldstein JL. Sterol-regulated release of SREBP-2 from cell membranes requires two sequential cleavages, one within a transmembrane segment. Cell 1996; 85: 1037-1046.*

So far, nothing has been published regarding the actual structure or properties of this novel enzyme until, in 1998, the lab of Goldstein and Brown successfully cloned the protease and named it subtilase-1 P (S1P) (354). Database searches revealed that S1P has feature characteristics of the serine/protease superfamily such as the catalytic triad consisting of an Asp acid, His and a Ser residue. Other notable features include the presence of a membrane-spanning domain similar to that found in furin determined.

Similarly Seidah and colleagues later, independently successfully cloned the same enzyme using a different approach (353). In their case the substrate used was a 32 kDa protein pro-brain-derived neurotrophic factor (pro-BDNF), usually cleaved at two different sites. A cleavage at the NH_2-terminus of the molecule produces a 28 kDa protein that is not biologically active, whereas a second cleavage at the C-terminus by furin or PC1 produces the biologically active form of pro-BDNF (14 kDa) (268).

Employing reverse transcriptase strategy and using degenerate oligoneucleotides Seidah and colleagues cloned a type I transmembrane bound enzyme from rat PC12 cells, mouse AtT-20 cells and in human tissue which they named subtilisn-kexin-isozyme I (SKI-1) (355). The enzyme was found to be 100% identical to S1P. Phylogenetic structural characteristics of the predicted amino acid sequence revealed that SKI-1 is a serine protease thus making it the eight member of the PC family. In order to confirm that SKI-1 is infact a proteolytic enzyme they co-expressed it with various substrates in COS-7 cells which included: POMC, HIV gp160, and pro-BDNF. Upon co-expression, results revealed that, it was only in the case of pro-BDNF that there was a net increase in the level of secreted 28 kDa BDNF without any significant alterations in the amount of the 14 kDa form. To examine whether the 28 kDa product is produced from cleavage at basic

residues or at an alternative site, N-terminal microsequencing of the 28 kDa product demonstrated that it is generated by a unique cleavage site at a hydrophobic Thr residue in the **RGLT↓** sequence of pro-BDNF.

Since the discovery of SKI-1/ S1P, two other eukaryotic convertases of similar nature were discovered in the malaria parasite: *Plasmodium falciparum* (P*f*-SUB-1) that is soluble (356) and membrane bound P*f*-SUB-2 (357). These enzymes are beleived to play a crucial role in erythrocyte invasion by cleaving a malaria surface protein at a **Leu↓Asn** site, making them an attractive potential target for new approaches in antimalarial therapy).

b) Characteristic features of SKI-1/S1P

Tissue distribution of SKI-1/S1P mRNA in adult male rats revealed that the enzyme is very widely expressed, if not ubiquitous (355). For example it is found in skin, muscle, bone, brain, peripheral nervous system, liver, spleen, retina, thyroid and adrenal gland, kidney and intestine. Similar to other subtilases, which possess a prodomain, that acts as an intramolecular chaperone and a highly potent inhibitor, activation of SKI-1/S1P requires the release of the pro-segment in an organelle specific manner. Following synthesis in the ER, autocatalytic processing takes place at various hydrophobic sites leading to the production of an active enzyme (359). Employing a combination of mass spectrometry and synthetic peptide digestion it was demonstrated that the enzyme undergoes autocatalytic activation through sequential cleavage at two sites: **R**SL**K↓** and **R**TL**L↓**. Additionally in the case of human SKI-1/S1P a third cleavage site exists at an

RLVF↓ (359, 360). While most of the autoprocessing is catalyzed by SKI-1/S1P, the participation of other proteases cannot be excluded.

The intracellular compartment where activation takes place was determined through pulse chase experiments in the presence or absence of BFA. All processed forms of SKI-1/S1P peaks were detectable despite the treatment; strongly implying that zymogen processing to its active form occurs early along the secretory pathway either in the ER or *cis*-Golgi (355, 359). Hence unlike the already described PCs, SKI-1/S1P doesn't require the acidic environment found in the later compartments of the secretory pathway. The full length pro-segment of SKI-1 has been proven to be inhibitory, and remains attached noncovalently in a complex with the enzyme however it is much less potent compared to that for PC1, PC2 and furin (359).

Membrane bound SKI-1/S1P can also be secreted into the medium as an active soluble enzyme (355, 360). Similar to other cell surface proteins that undergo constitutive or regulated release, such a process is referred to as ectodomain shedding. The shedding process of any transmembrane protein requires its cleavage in a region close to the transmembrane domain. However, little is known about the identity of the protease, its mode of activation and the structural determinants involved in the process. In the case of SKI-1/S1P the intracellular site whereby the shedding reaction takes place was determined by creating a construct of SKI-1/S1P containing an ER retention signal followed with transfection into HEK-293 cells. Immunoprecipitation experiments of contents present in the medium of the cells, demonstrated that SKI-1/S1P was undetectable. Similarly BFA treatment blocked the shedding process (360). Therefore

the, majority of SKI-1/S1P shedding occurs following transport from the ER/cis Golgi compartments.

With regard to pH dependency, incubating cells at various pH values lead to successful cleavage of pro-BDNF implying a wide pH-dependence profile revealing activity at both acidic and neutral pH values ranging from pH 5.5 to pH 7.3 (355, 359). Such results are in accordance with the ability of SKI-1/S1P to cleave substrates early along the secretory pathway in the ER as opposed to the Golgi. Interestingly, while looking at the pH preference for SREBP cleavage, the data revealed that the pH optimum is actually 6.5. This suggests that SREBP, contrary to pro-BDNF, might be cleaved outside of the ER perhaps in the Golgi. Therefore, the pH optimum for SKI-1/S1P activity appears to be dependent on the substrate employed. Indeed, there is now cellular evidence suggesting that SREBP requires vesicular transport out of the ER to the Golgi prior to cleavage by SKI-1/S1P (361, 362). The key to such regulated processing is an additional player called the SREBP cleavage-activating protein (SCAP) which forms a complex with SREBP in the ER. Studies on the glycosylation patterns of SCAP have shown that it moves from ER to Golgi when cells are grown in the absence of sterols. Sterol depletion is basically sensed by SCAP which then escorts SREBP, in a complex, to the *cis*-Golgi compartment where it encounters SKI-1/S1P. It is only in this compartment that S1P/SKI-1 cleaves SREBP (362).

In addition to its ability to act at various pH ranges, analysis of the inhibitory profile of the enzyme revealed that Ca^{+2} chelators and serine protease inhibitors blocks its activity (353, 357). This reinforces its relationship to the serine enzyme clan of subtilases that are Ca^{+2} dependent with SKI-1/S1P being most efficient at 2-3 mM Ca^{2+}.

C) Substrate and cleavage specificity of SKI-1

As previously discussed SKI-1/S1P cleaves SREBP at an RSVL↓ (354) and pro-BDNF at an RGLT↓ motif (355). The requirement for an Arg residue at the P4 position being critical whereas specificities at the P1 position are more relaxed. Additional information regarding the substrate preferences of SKI-1/S1P cleavage was obtained through a combination of Ala scanning mutagenesis and mass spectrometric analysis of fluorometric substrates. The collective data demonstrated that cleavage C-terminal to an Arg, Ala, or Phe residue reduces cleavage but does not abolish it completely (354, 359). However, changes in the P1 position to glutamic acid, proline or valine blocks cleavage completely. Substituting the Arg at the P4 position on the other hand is unacceptable with the exception of Lys which only produces a partial loss in cleavage (354). It can therefore be summarized that SKI-1/S1P cleaves precursors exhibiting the consensus motif R/K-X-X-Z↓ where Z is preferentially Leu and Thr but excludes Val, Pro or Glu. Having identified such a new subtilase with two identified substrates for it, the search for more is inevitable.

The ER transmembrane protein ATF6 is a transcription factor that plays an important role in UPR in the ER (363). When the UPR accumulates in the ER, ATF6 is cleaved proteolytically releasing the NH_2-terminus which then translocates into the nucleus. There, it activates transcription of various genes encoding chaperones responsible for restoring the folding of proteins in the ER lumen (364). Such a scenario is very similar to that of SREBP activation. A possible relationship between the processing of ATF6 and SREBP was suggested by the observation of an RXXL sequence in the lumen of ATF6 at a site that is close to the transmembrane domain (365). To test the

hypothesis that SKI-1/S1P participates in ATF6 processing a series of experiments were done in *wt* CHO and mutant cells lacking S1P activity. A significant decrease in ATF6 processing was detected in mutant cells with the defect being reversed upon reintroduction of SKI-1/S1p into them. Additionally, mutating either the R or L residue independently to Ala or Pro respectively lead to severely reduced amounts of ATF6 detected in the nuclear extracts. Such data further implicates SKI-1/S1P in ATF6 processing. Recently SKI-1/S1P has also been shown to be responsible for the processing of the Lassa virus glycoprotein at an RXXL site as well (366). The processing appears to be critical for the release of infectious viral particles. Once patients are infected with the virus they develop hemorrhagic fever and in some cases it might lead to death. Therefore the role of this novel protease expanded beyond the regulation of lipid metabolism and is now implicated in the mechanism by which the ER stress response is triggered in animal cells and in the pathology of Lassa virus infection.

6. Specific aims

At the time when I first started working on my project in September of 1997 in Fraser laboratories, known for work on SST and its receptors, attempts to characterize targeting motifs and specific docking receptors in the RSP were in their initial phases. Following the detailed analysis of the amino acid sequences of 15 propeptides that have been experimentally shown to be correctly sorted to secretory granules in AtT-20 cells, Kizer and Trophsha in 1991 identified a striking feature common among them (216). A motif that is shared between the 15 propeptides was identified to be composed of an amphipathic helical sorting sequence containing two leucine residues separated by three amino acids. In parallel the essential role of a disulphide bonded-loop in the sorting of granins and POMC was clearly elucidated (218-221). Additionally, in 1997 in the laboratory of Loh YP the membrane bound form of CPE was characterized as a sorting receptor in the TGN, making this the first report of its kind (226). As for prohormone processing, the existence of PCs capable of cleaving at hydrophobic residues was initiated in the laboratories of Goldstein and Brown with the successful cloning of human S1P in 1998 (354). This was then followed by a similar finding lead by Seidah and colleagues in 1999 paving the way to search for new substrates cleaved by this novel enzyme (355). With regard to the PPSST molecule, molecular forms of PPSST cleavage especially, at the C-terminus were well characterized and plenty of information regarding their biological roles existed (35 a-c, 51, 52, 58. 330-335). However, the curious status of the NH_2-terminus remained a mystery.

The NH_2-terminal region of PSST is highly conserved throughout vertebrate evolution (from catfish to man) stressing its importance inside the cell. What has been

confirmed is that a processing event takes place within that region resulting in a 10 a.a peptide with initial cleavage postulated at a monobasic Lys^{13} residue followed by carboxyppetidase trimming (36, 60-61). It is well documented that all SST producing cells cleave antrin and secrete it in response to extracellular stimulation. Therefore, its localization is not restricted to the gastric portion of the endocrine stomach. Such a processing occurrs at variable efficiencies in both regulated and constitutive cell lines. A physiological role for the NH_2-terminus was originally addressed by Sevarino et al in 1989 who in an attempt to study the differences in targeting capabilities of AFPPSST I and AFPPSST II (222).

And so, this is where I come in. In my PhD project I mainly focused on the aminotrminal terminus of PSST. Hence, in the next three chapters the PPSST NH_2-terminal region will be extensively analyzed and dissected with the following three main questions asked: 1) Does the NH_2-terminus play a role in targeting PPSST to the RSP? 2) Is PPSST cleaved at the Lys^{13} site mediating antrin production, and by the action of which enzyme? 3) What is the identity of the putative receptor, most likely in the TGN, involved in properly targeting PPSST to the RSP?

CHAPTER B

A CONSERVED α-HELIX AT THE AMINO TERMINAL OF PROSOMATOSTATIN SERVES AS A SORTING SIGNAL FOR THE REGULATED SECRETORY PATHWAY

SMMARY

Mammalian prosomatostatin (PSST) contains the bioactive peptides SST-14 and SST-28 at the C-terminal end of the molecule and a putative sorting signal in the propeptide segment for targeting the precursor to the regulated secretory pathway. The N-terminal segment of PSST consists of an amphipathic α-helix which has been totally conserved throughout vertebrate evolution. We have analysed 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 analysing basal and stimulated release of SST-14 LI, and by immunocytochemistry for staining of SST-14 LI in punctate granules. Deletion of the $PSST_{[3-15]}$ segment blocked regulated secretion and rerouted PSST for constitutive secretion as unprocessed precursor. Alanine scanning mutagenesis identified the region Pro^5 to Gln^{12} as being important in precursor targetting with Leu^7 and Leu^{11} being critical. Molecular modelling 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 C-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. Additionally in an attempt to test the ability of PSST[1-19] to act as a heterologues sorting signal for directing green and red fluorescent protein to the regulated secretory pathway (RSP), they were both found capable of entering the RSP once allowed to enter the endoplasmic reticulum.

INTRODUCTION

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) which 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 ER 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 proneuropeptide precursors in the TGN into either the CSP or RSP (4-6). Sorting is an active process which requires some form of recognition of the secretory protein (2,4-6). It is one step in a multi-step 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 inter-dependent 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 which aggregate easily are not targetted into the RSP and modifications on proteins such as chromogranin B and IGF-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 chloroquine and bafilomycin A1 is without effect on the ability of these hormones to aggregate in secretion granules in GH_4C_1 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 which 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 which 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 mistargetting to the CSP (16-22). A sorting sequence domain has been described in the prosegment of POMC, enkephalin, SST, chromogranins, and PC1 (18-23). The most compelling arguments for a specific sequence sorting motif have come from studies of POMC and prosomatostatin (PSST) (18,19,21,24-28). In the case of POMC, structure-function and molecular modelling studies have identified a sorting signal motif in the N-terminal segment made up of a disulphide bond constrained amphipathic hairpin loop which binds to a sorting receptor

identified as membrane-associated carboxypeptidase E (CPE) (Fig.1) (21, 25). Molecular modelling has revealed a similar putative sorting motif in two other precursors pro-enkephalin and proinsulin (25). Mutation of the binding site on CPE or *in vitro* antisense depletion of CPE or genetic obliteration of CPE in the CPE^{fat} mouse lead to missorting of POMC, pro-enkephalin, and proinsulin (25,26,29). Not all secretory proteins, however, are recognized for sorting by CPE. For instance, chromogranin A which possesses a RSP sorting domain similar to that in POMC does not use CPE as a sorting receptor, suggesting the existence of other sorting receptors (26). PSST is another well characterized precursor that has been suggested to harbor a sorting signal (18,19,28). Mammalian PSST is processed post-translationally at C-terminal dibasic and monobasic sites to yield SST-14 and SST-28 respectively (30,31). In addition, cleavage at an unknown site at the N-terminal region has been implicated in generating the decapeptide $PSST_{[1-10]}$ without any known biological activity (32). The $PSST_{[1-10]}$ sequence is conserved throughout vertebrate evolution (33) and deletion of this region results in missorting of the mutant precursor (28). (Fig.1) A comparison of the amino acid sequence and secondary structure of the PSST N-terminal segment with that of 14 other prohormones that have been shown experimentally to be sorted to secretory vesicles in AtT-20 cells has identified a common motif consisting of a degenerate amphipathic α-helix (34). This consensus sorting sequence in the case of PSST lies within residues 3-15 and differs from the disulphide bond containing hairpin loop structure in POMC, pro-enkephalin, and proinsulin (Fig.1). In the present study, we have analysed the $PSST_{[3-15]}$

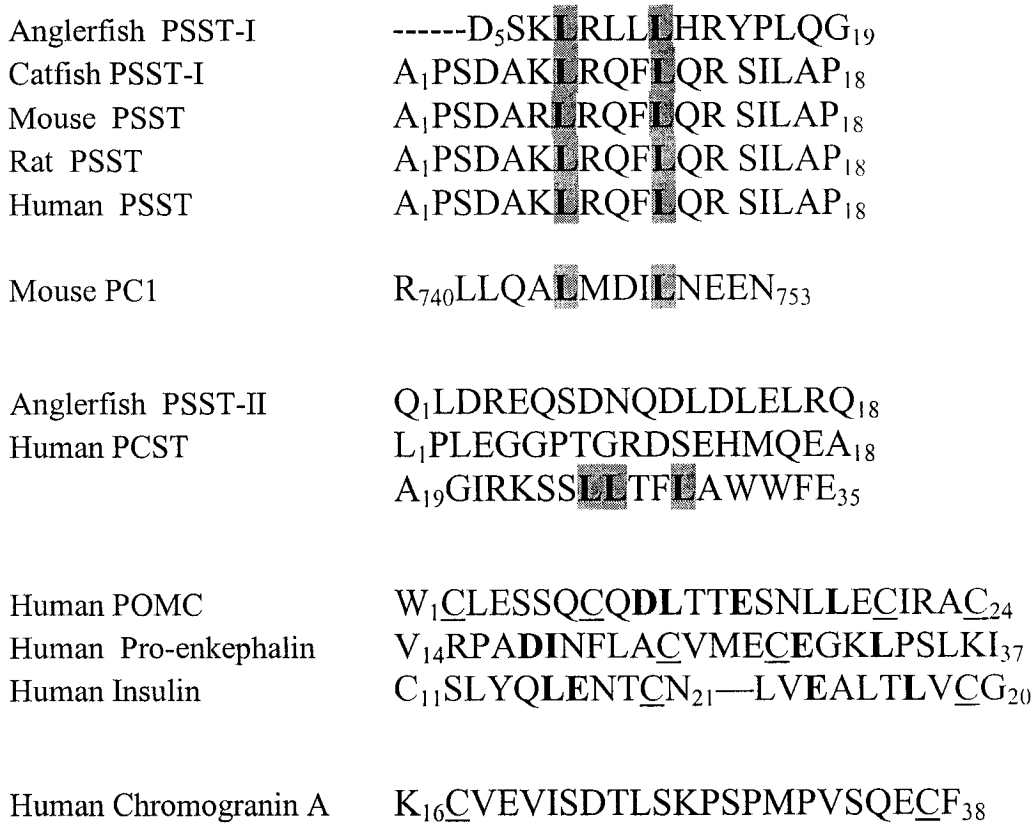


FIGURE 1. Comparison of the N-terminal PSST sequences of anglerfish I, catfish I, mouse, rat and human precursors. The bolded leucine residues separated by three amino acids are highly conserved hydrophobic residues predicted to play a crucial role in the formation of an α-helix. For comparison are shown sequences of human PCST which features a leucine containing α-helix not at the N-terminus but further downstream in the molecule, anglerfish PSSTII N-terminal sequence (which does not feature an α-helix), and human PC1 (which displays an α-helix comparable to that in mammalian PSST. Also compared is the disulphide bond constrained amphipathic hairpin loop motif in POMC, proenkephalin, and insulin postulated to act as a sorting signal for binding to carboxypeptidase E. Chromogranin A which also contains this motif, however, does not interact with CPE.

segment as a sorting signal by alanine scanning and deletional mutagenesis. We show that Leu^{7} and Leu^{11} which form part of a contiguous hydrophobic patch on the surface of the α-helix are critical for sorting function and that C-terminal processing of PSST to SST-14 and SST-28 can occur constitutively but is relatively inefficient in the absence of correct precursor targetting to the RSP. We have also used PPSST fusions with green fluorescent protein (GFP) and the recently cloned red fluorescent protein (DsRed) to explore routing of the chimeric proteins in AtT-20 cells. Surprisingly, appending the SST signal peptide was sufficient to yield GFP and DsRed storage in RSVs making the technique unsuitable to study intracellular protein transport in cell lines containing regulated secretory machinery.

EXPERIMENTAL PROCEDURES

Materials: Synthetic peptides were obtained as follows: SST-14, SST-28 (Bachem Marina del Rey, CA); Tyr^{0} SST-14 (Peninsula, Belmonte, CA); acetonitrile and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Montreal, Quebec); heptafluoro-butyric acid (HFBA) was obtained from Pierce Chemicals (Rockford, IL); pepstatin-A, tetradecanoylphorbolacetate (TPA) and phenylmethylsulphonyl fluoride (PMSF) were from Sigma (St. Louis, MO). Forskolin (FSK) was purchased from Calbiochem; Dulbecco's Modified Eagle's Medium and fetal bovine serum were purchased from Gibco-BRL (Gaithersburg, MD). 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 preproSST (rPPSST) was constructed in the expression vector pTEJ8. Using rPPSST as template a

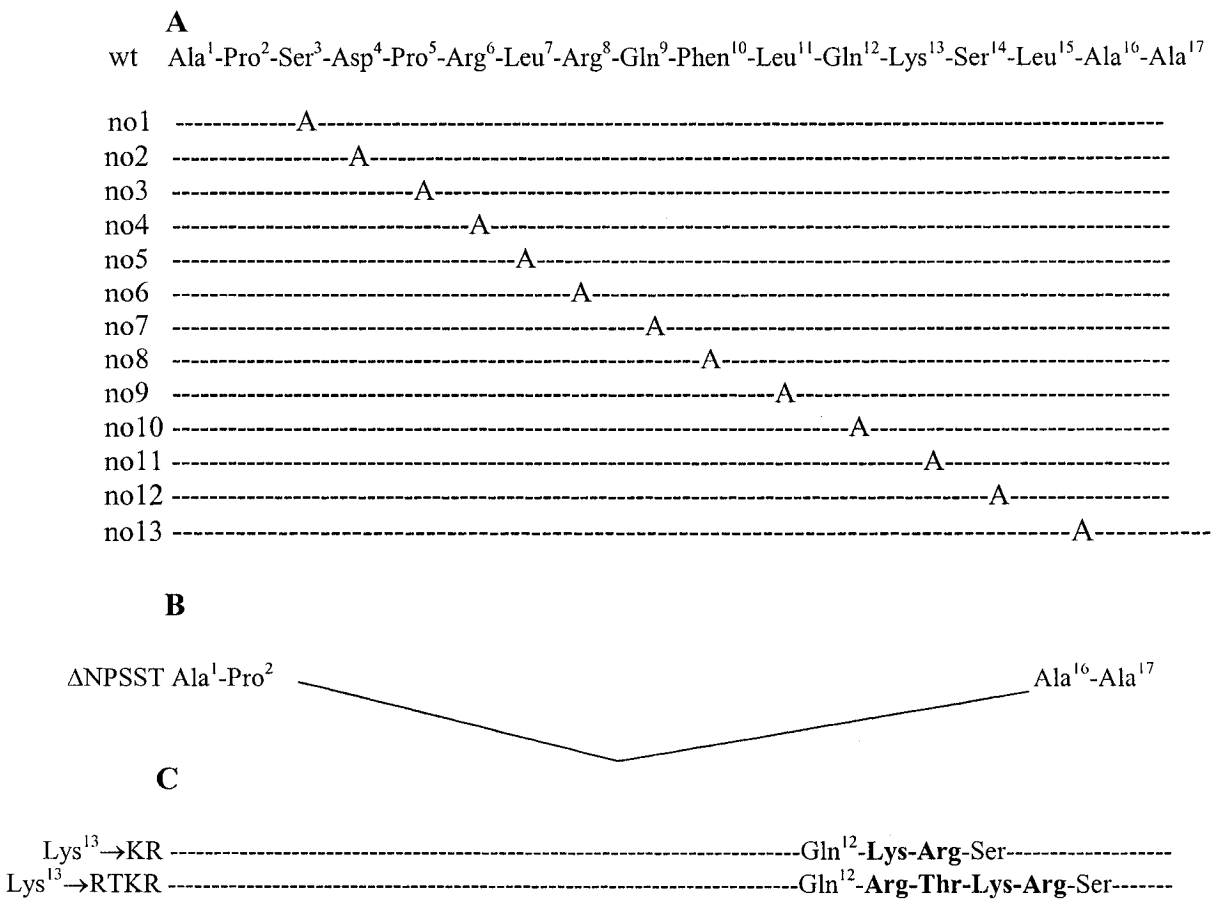


FIGURE 2: Schematic illustration of the alanine substitution PSST N-terminal mutants (A), $PSST_{[3-15]}$ deletion mutant, ΔN PSST (B), and mutants substituting Lys^{13} for KR and Lys^{13} for RTKR (C).

series of mutants were created by the PCR overlap extension technique (35) (Fig.2): (i) alanine scanning mutagenesis substituting Ala for each of the 13 residues from Ser^3 to Leu^{15} in PSST; (ii) NH_2-terminal deletion mutant deleting residues 3-15 of PSST (ΔNPSST); (iii) insertional mutants substituting Lys^{13} with KR or RTKR. To construct the mutants two fragments were created separately which included a 5' fragment containing the desired mutation using primer A and a reverse primer, and a 3' fragment using primer B and a forward primer where the forward primer and reverse primers are mirror images of each other. The fragments were then ligated in a third PCR reaction to generate the full-length mutant PPSST cDNA. For example, to create Ser^3 to Ala, primers A and 2 were used to synthesize the 5' fragment of PPSST and primers B and 1 were used to generate the 3' fragment. Primer A was designed to contain *Hind III* endonuclease restriction site, Kozak consensus sequence, and initiation codon. Primer B contained 3' flanking sequence and stop codon followed by an Eco R1 restriction site.

Primer A 5'-ATT CATA AGC TTG CCG CCA CCA TGC TGT CCT GCC GT - 3' (forward)

Primer B 5'-TAG TAG ATG AAT TCC TAA CAG GAT GTG GAA TGT - 3' (reverse)

The following forward and reverse primers bind to the same region of rPPSST cDNA and were designed to contain the desired mutation:

Ser^3 5'-ACCGGGGCGCCCGCGGACCCCAGA - 3' (forward)

5'-TCTGGGGTCCGCGGGCGCCCCGGT - 3' (reverse) (nt 66-90)

Asp^4 5'-ACCGGGGCGGCCCTCGGCCCCCAGACTCCGTCA - 3' (forward)

5'-TGACGGAGTCTGGGGGGCCGAGGGCGCCCCGGT - 3' (reverse) (nt 66-98)

Pro^5	5'-GCGCCCTCGGACGCCAGACTCC GTCA-3' (forward)
	5'-TGACGGAGTCTGGCGTCCGAGGGCGC -3' (reverse) (nt 72-98)
Arg^6	5'-TCGGACCCCGCACTCCGTCAGTTTCT -3' (forward)
	5'-AGAAACCTGACGAGTGCGGGTCCGA -3' (reverse) (nt 78-104)
Leu^7	5'-CGGACCCCAGAGCTCGTCAGTTTCTG -3' (forward)
	5'-CAGAAACTGACGAGCTCTGGGGTCCG -3' (reverse) (nt 90-116)
Arg^8	5'-ACCCCAGACTCGCTCAGTTTCTGCA -3' (forward)
	5'-TGCAGAAACTGAGCGAGTCTGGGGT -3' (reverse) (nt 83-108)
Gln^9	5'-ACCCCAGACTCCGTGCGTTTCTGCAGAA -3' (forward)
	5'-TTCTGCAGAAACGCACGGAGTCTGGGGT -3' (reverse) (nt 83-111)
Phe^5	5'-AGACTCCGTCAGGCTCTGCAGAAGT -3' (forward)
	5'-ACTTCTGCAGAGCCTGACGGAGTCT -3' (reverse) (nt 88-113)
Leu^{11}	5'-CTCCGTCAGTTTGCGCAGAAGTCTCTG -3' (forward)
	5'-CAGAGACTTCTGCGCAAACTGACGGAG -3' (reverse) (nt 91-118)
Gln^{12}	5'-TCAGTTTCTGGCGAAGTCTCTGGCGGCT -3' (forward)
	5'-AGCCGCCAGAGACTTCGCCAGAAACTGA -3' (reverse) (nt 96-124)
Lys^{13}	5'-CAGTTTCTGCAGGCCTCTCTGGCGGCT -3' (forward)
	5'-AGC CGC CAG AGA GGC CTG CAG AAA CTG -3' (reverse) (nt 96-123)
Ser^{14}	5'-AGTTTCTGCAGAAGGCTCTGGCGGCTGCCA -3' (forward)
	5'-TGGCAGCCGCCAGAGCCTTCTGCAGAAACT -3' (reverse) (nt 98-127)

Leu^{15} 5'-CTGCAGAAGTCTGCAGCGGCTGCCACC -3' (forward)

5'-GGTGGCAGC CGC TGCAGACTTCTGCAG -3' (reverse) (nt 103-138)

$Lys^{13}\rightarrow$ KR 5'-CAGTTTCTGCAGAAGAGGTCTCTGGCGGCT -3' (forward)

5'-AGCCGCCAGAGACCTCTTCTGCAGAAACTG -3' (reverse) (nt 96-120)

$Lys^{13}\rightarrow$RTKR 5'-CAGTTTCTGCAGAGGACAAAGAGGTCTCTGGCGGCT -3' (forward)

5'-AGCCGCCAGAGACCTCTTTGTCCTCTGCAGAAACTG -3' (reverse) (nt 96-120)

ΔNPSST 5'-GGTGTCACCGGGGCGCCCGCGGCTGCCACCGGGAA-3' (forward)

5'-TTCCCGGTGGCAGCCGCGGGCGCCCCGGTGACACC-3' (reverse) (nt 60-134, segment with deletion of nt 79-117)

PCR was carried out with 50 ng of PPSST cDNA in 100 μl containing 20 mM Tris-HCl, 200 μM dNTPs, 1.5 mM $MgCl_2$, 6% DMSO, and 2 units of *p.f.u.* (Stratagene) using the following conditions: denaturation at 94° C for 80 s, anealing at 59° C for 50 s, and extension at 72° C for 60 s for 25 cycles followed by extension at 72° C for 10 min. PCR products were separated by agarose gel electrophoresis, the amplified bands electroeluted and purified. Fragments B-1 and A-2 were then fused in a ligation reaction using flanking primer pair A and B. After PCR ligation, the products were digested to completion with *EcoR1* and *Hind III* and the purified fragments were subcloned into

Hind III-EcoR1 multiple cloning sites of pTEJ8. All recombinant plasmid constructions 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 transfected.

GFP and DsRed constructs: Using rPPSST in PTJE8 as a template, GFP cloned in pEGFP-N1 vector (Clontech laboratories, California USA) and DsRed cloned in pDsRed2-N1 vector (Clontech laboratories, California, USA) were each independently fused at their NH_2-terminus to the signal peptide of SST along with the first 19 amino acids of the PPSST sequence comprising Ala^1 to Thr^{19} residues. The insert was cloned in frame in the multiple cloning sites present in either vector. Two primers were used a forward primer was designed to contain an *EcoR 1* (underlined) endonuclease restriction site, Kozak consensus sequence, and initiation codon (bold). The reverse primer contained 3' flanking sequence of PSST and a BamH 1 (underlined) restriction site.

Forward primer: 5'-ATTCATGAATTCGCCGCCACC**ATG**CTGTCCTGCCGT-3'

Reverse primer: 5'- TGATGGATCCCCGGTGGCAGCC-3'

As a control, GFP and DsRed were fused to the signal peptide of PPSST alone using similar primers as above but a different template (the ΔNPSST cDNA).

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 Ser-X-tend in an atmosphere of 5% CO_2 and 95% air in a humidified incubator at 37° C. Cells were plated in 100 x 20 mm Petri dishes and transfected at 50% confluency with 3-

5 μg of the appropriate plasmid construct by lipofectamine (Gibco-BRL) 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 6 well plastic Petri dishes and grown to 80-90% confluency 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 5 wells were incubated with DMEM-1% bovine serum albumin containing PMSF and pepstatin-A, 20 μg/ml each. To study regulated secretion, cells were incubated with FSK 20 μm or TPA 10^{-7} M for 4 h. Media were then harvested, centrifuged at 1000 x g for 5 min to remove detached cells and the supernatant acidified to pH 4.8 with 1 M acetic acid and stored at -20° C pending radioimmunoassay (RIA) analysis of SST-14 LI. Attached cells were extracted by scraping into 1 M acetic acid containing PMSF and pepstatin-A (20 μg/ml each) on ice. The cell suspension was further extracted by sonication followed by centrifugation at 5000 g for 30 min. The supernatant was stored at -20° C for RIA and HPLC analysis.

HPLC: Pooled acidified secretion media and cell extracts were diluted 1:7 with 0.1% TFA and concentrated using Waters Sep-Pak C18 cartridges. The adsorbed peptides were analysed 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 0.2% HFBA 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 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), [^{125}I] Tyr^{0} SST-14 radioligand, synthetic SST-14 standards and a 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 forms was characterized by fluorescence immunocytochemistry (36). Stably transfected AtT-20 cells were plated at 1.25 x 10^{5} cells/well in 24 well plates coated with 50 mg/ml polyornithine. On day 3 at ~ 60-70% confluency, cells were washed twice in PBS, and fixed in 2% paraformaldehyde (in 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 1:1000) for 8-12 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 fluoroscein (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.

Cells transfected with the fluorescent constructs were simply fixed in 4% paraformaldehyde as described above, mounted on coverslips and viewed under regular light microscopy containing green and blue filters.

Secondary Structure Prediction and Model Building: The secondary structure of rPSST (residues Ala^{1}-Cys^{92}) 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 α-helical region Pro^{5}-Thr^{19} was constructed from standard geometries using the BIOPOLYMER module in SYBYL 6.6 molecular modeling software (Tripos Inc., St. Louis, MO). N- and C-termini were blocked with acetyl and methylamino groups respectively. Structural refinement was carried out by energy minimization using AMBER 4.1 all-atom force field (38) and a distance dependent (4R) dielectric constant.

Statistical Analysis: Results are expressed as mean ± SE. Statistical analysis was carried out by one way Anova followed by Dunnet's significance test. Significance was indicated by p value of < 0.05.

RESULTS

Basal and Stimulated Release of wt PPSST

AtT-20 cells expressing wt PSST released total SST-14 LI at a low basal rate of 0.54 ± 0.08 ng/ml/4h representing 8.3% of total cell content (Fig. 3a). FSK stimulated

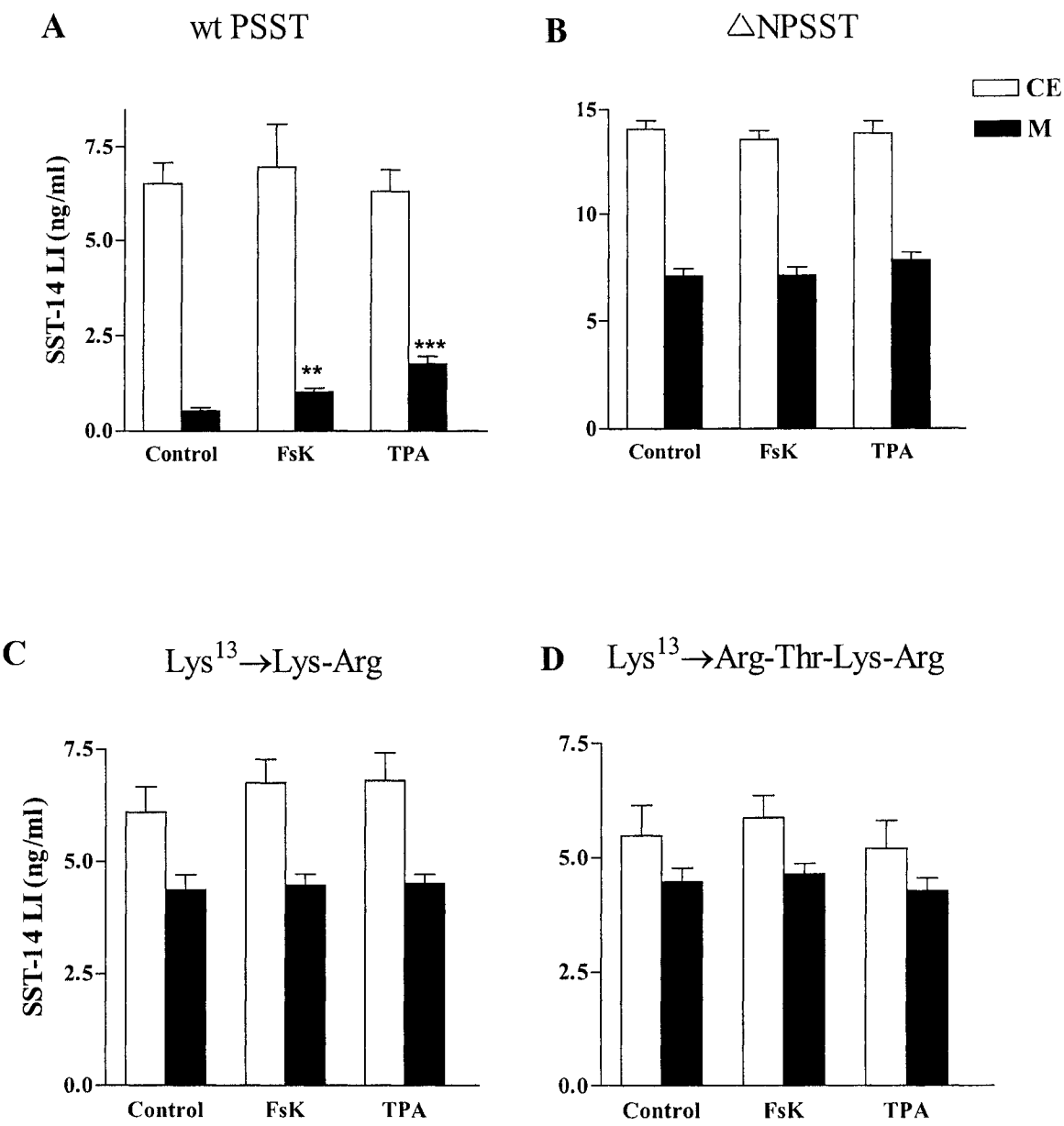


FIGURE 3: Comparison of basal, FSK, and TPA stimulated secretion of SST-14 LI from AtT-20 cells stably expressing (A) wt PSST, (B) ΔN PSST, (C) Lys^{13} to Lys-Arg mutant, (D) Lys^{13} to Arg-Thr-Lys-Arg mutant. Transfected cells were incubated for 4 h with control media or media containing FSK 20 μM or TPA 100 nM. At the end of the incubation, cell extracts (CE) and media (M) were separately assayed for SST-14 LI. Mean ± SE of five measurements from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control.

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 PSST 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 PSST sorting to the RSP.

ΔNPSST, KR and RTKR Substitution Mutants

To assess the sorting function of the NH_2 terminal domain of PSST, 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 N-terminal PSST cleavage endogenously by the prohormone convertases furin or PC1/PC2 respectively. AtT-20 cells stably expressing the ΔNPSST mutant released SST-14 LI at a high basal rate (7.1 $\pm$ 0.33 ng/ml/4 h) representing approximately 50% of total cell content (Fig. 3B). Release was unresponsive to FSK or TPA stimulation during a 4-hour incubation (7.12 $\pm$ 0.38 and 7.83 $\pm$ 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. 3C, D). These results were correlated with immunocytochemistry. Contrary to wt PSST 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

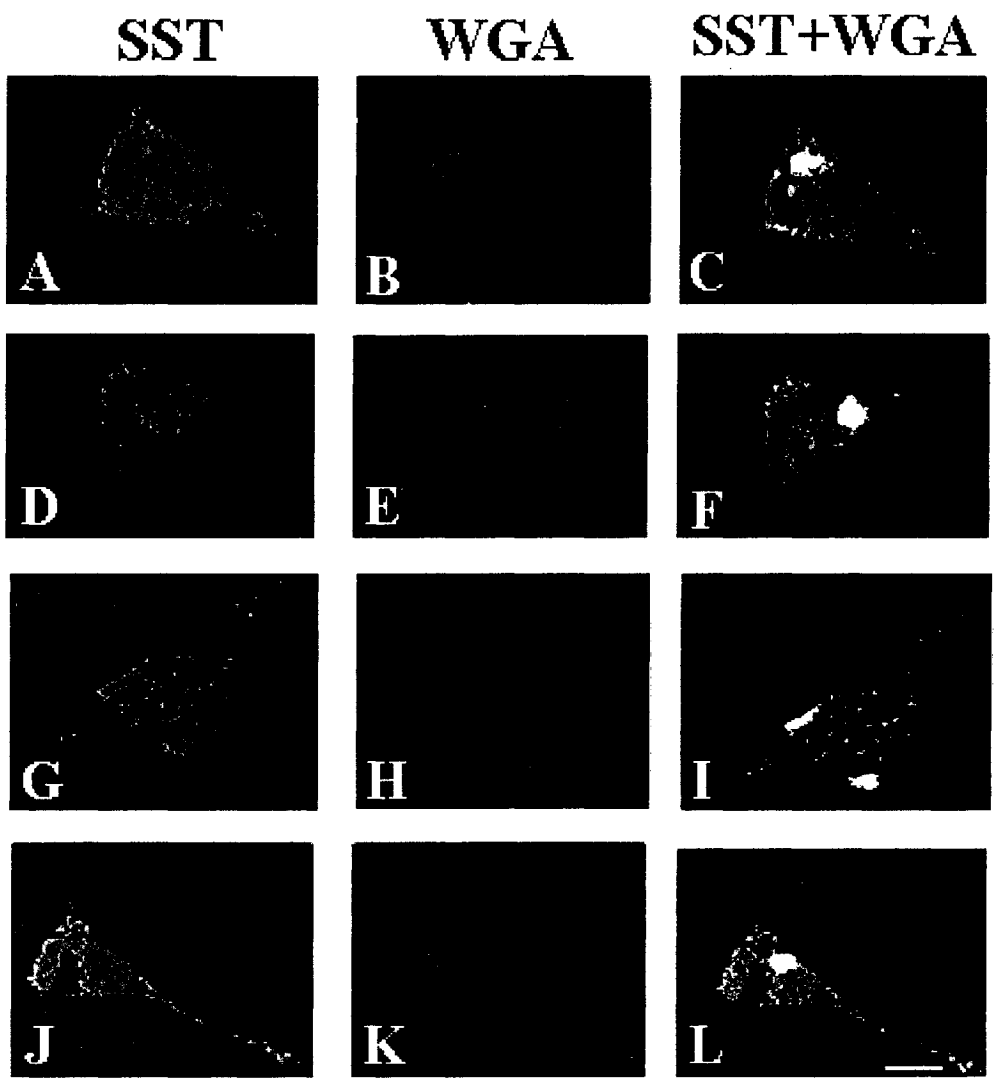


FIGURE 4: Immunofluorescence localization of SST-14 LI in AtT 20 cells expressing wt PPSST(A,B,C), and PPSST mutants Lys^{13} to KR (D,E,F), Leu^{7} to Ala (G,H,I), Lys^{13} to Ala (J,K,L). The left hand panels show SST-14 LI identified by Cy3 red fluorescence. The middle panels show wheat germ agglutinin (WGA) localized as green fluorescence in the Golgi, and the right hand panels show the cellular colocalization. Note the presence of SST-14 LI throughout the cytoplasm in panels A-C and J-L. SST-14 LI is absent in cytoplamic granules in D-F, and G-I and colocalized with WGA in the Golgi region. Similar localization was seen in the case of the $\Delta PSST_{[3\text{-}15]}$ and Leu^{13} to Ala mutants (not shown). Scale bar = 25 μM.

WGA and corresponded to the TGN (Fig. 4, D-F). Similar results were obtained with ΔNPSST and RTKR mutants. Constitutive secretion, absence of secretagogue responsiveness, and lack of SST-14 LI staining in punctate granules suggest that the $PSST_{[1-15]}$ domain harbors important information that is essential for sorting PSST correctly to the RSP.

Alanine Substitution Mutants

Having found that the amino terminal 3-15 domain of PSST 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 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 to that of wt PSST (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 to 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 to the amounts found with the Lys^{13} to KR and Lys^{13} to RTKR substitutions. These results were correlated with the ability of the AtT-20 cell transfectants to respond to stimulation with FSK (20 μM) or TPA (100 μM) for 4 h (Table 1). The Ser^3 Asp^4 Lys^{13} Ser^{14} and Leu^{15} mutants all displayed increased release of SST-14 LI in response to both FSK and TPA. Like wt PSST, the Lys^{13} Ser^{14} and Leu^{15} mutants showed a 2-fold increase in secretion in response to FSK whereas the Ser^3 and Asp^4 mutants exhibited somewhat reduced 1.6 - 1.7 fold

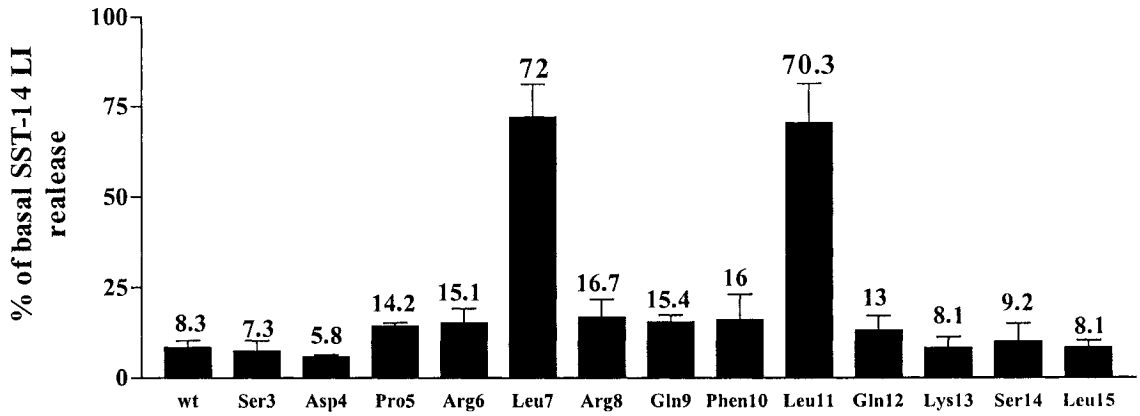


FIGURE 5: Percent basal SST-14 LI released into the medium of AtT-20 cells transfected with Ala substitution mutants of the $PSST_{[3-15]}$ region. Mean values in each case are shown as numbers above the bars. Mean $\pm$ SEM. n = 5. Representative of three independent experiments.

Table 1: Comparison of basal, FsK and TPA stimulated secretion of SST-14 LI from AtT-20 cells stably transfected with wt and Ala substitution mutant.

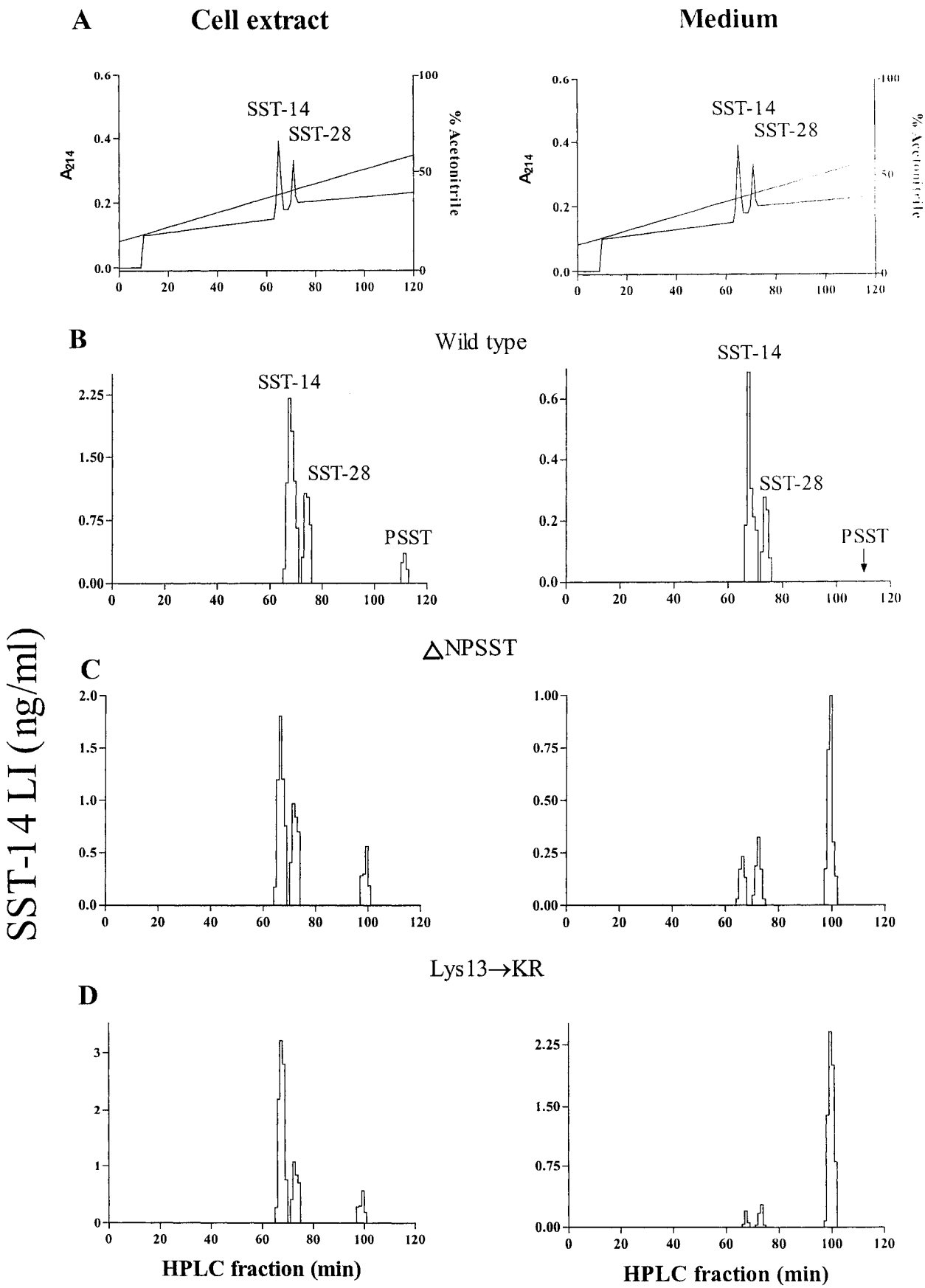
	Amount of SST-14 LI released/4 hrs (ng/ml)		
	Control	**FsK**	**TPA**
wt	0.54 ± 0.08	1.02 ± 0.10**	1.76 ± 0.20***
Ser^{3}	1.01 ± 0.11	1.66 ± 0.10*	2.15 ± 0.31**
Asp^{4}	0.33 ± 0.08	0.57 ± 0.05*	0.78 ± 0.09**
Pro^{5}	0.81 ± 0.23	0.77 ± 0.09	0.79 ± 0.10
Arg^{6}	1.51 ± 0.75	1.63 ± 0.67	1.54 ± 0.88
Leu^{7}	3.40 ± 0.20	3.72 ± 0.11	3.65 ± 0.08
Arg^{8}	1.02 ± 0.20	1.34 ± 0.11	0.97 ± 0.23
Gln^{9}	0.95 ± 0.40	0.88 ± 0.31	0.80 ± 0.21
Phe^{10}	1.57 ± 0.43	1.67 ± 0.25	1.47 ± 0.32
Leu^{11}	4.40 ± 0.07	4.30 ± 0.06	4.19 ± 0.10
Gln^{12}	0.71 ± 0.31	0.84 ± 0.15	0.79 ± 0.23
Lys^{13}	0.62 ± 0.07	1.20 ± 0.20**	2.20 ± 0.10***
Ser^{14}	0.35 ± 0.03	0.72 ± 0.10**	0.69 ± 0.03**
Leu^{15}	0.99 ± 0.15	2.12 ± 0.18**	2.58 ± 0.30***

stimulation. Both wt and the five responsive mutants displayed differentially greater sensitivity to TPA compared to FSK stimulation. Thus TPA induced a 3-fold increase in SST-14 LI release from wt PSST and a 2-4 fold increase in the case of the Ser^{3} Asp^{4} Lys^{13} Ser^{14} and Leu^{15} mutants. In contrast, FSK produced an approximate doubling of SST-14 LI release from wt and the five responsive mutants. Ala substitution of the 8 amino acid residues from Pro^{5} to Gln^{12} rendered all of these mutants totally unresponsive to both FSK and TPA stimulation (Table 1). The ability of the mutants to respond to secretagogues was correlated with the granular morphology of the cells. Figure 4 depicts the subcellular distribution of immunofluorescent SST-14 LI in representative point mutants. As examples of mutants displaying high basal secretion and loss of regulated secretion, the Leu^{7} point mutant showed immunofluorescent SST-14 localized in a perinuclear area which overlapped the distribution of WGA staining (Fig. 4, G-I). Unlike wt PSST cells, SST-14 LI was not identified in the cell body of the two mutants. As an example of a point mutant which continued to display regulated secretion, the Lys^{13} mutant displayed a punctate pattern of staining throughout the cytoplasm similar to wt PSST implying proper PSST targetting to secretory granules (Fig. 4, J-L).

Effect of N-Terminal PSST Mutations on C-Terminal Processing to SST-14 and SST-28

To characterize the products of PSST processing, cell extracts and media from AtT-20 cell transfectants were fractionated by HPLC followed by RIA of the eluting fractions (Fig. 6). The elution positions of the peaks obtained were compared with those of synthetic SST-14 and SST-28 or of purified PSST chromatographed under identical

conditions. Table 2 compares the percent of SST-14, SST-28, and unprocessed PSST derived from HPLC chromatograms. Extracts of cells of wt transfectants displayed 3 peaks coeluting with synthetic SST-14 (retention time 67 min), 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: SST-28 was obtained in FSK and TPA stimulated release medium (data not shown). The Leu^{15} to Ala mutant displayed comparable HPLC profiles to wt PSST in both cell extracts and media. Thus PSST 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 diversion of PSST from the regulated to the CSP (ΔNPSST, Lys^{13} to KR, Lys^{13} to RTKR, Leu^{7} to Ala, Leu^{11} to Ala) displayed a different HPLC profile of SST-14, SST-28, and unprocessed PSST (Fig. 6C-F, Table 2). In the case of the Leu^{7} to Ala mutant, despite the missorting of 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


A
Cell extract
Medium
SST-14
SST-28
A_{214}
% Acetonitrile
B
Wild type
PSST
C
ΔNPSST
D
Lys13→KR
SST-14 LI (ng/ml)
HPLC fraction (min)

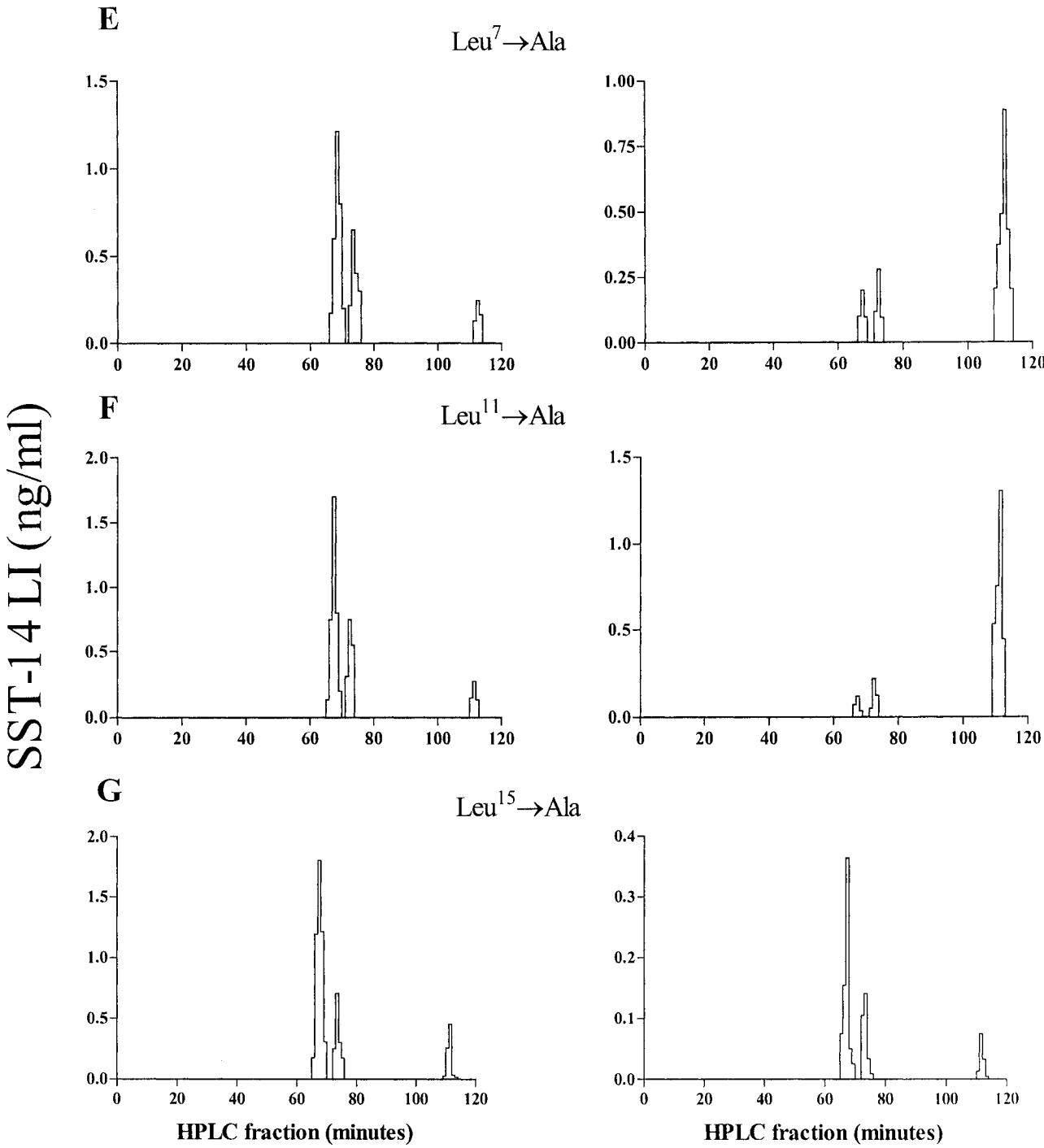


FIGURE 6: HPLC profile of SST-14 LI in cell extracts (left panels) and secretion media (right panels) from AtT-20 cells transfected with wt (B), ΔN PSST (C), Lys^{13} to KR mutant (D), Leu^{7} to Ala mutant (E), Leu^{11} to Ala mutant (F), Leu^{15} to Ala mutant (G). Panel A illustrates the elution position of synthetic SST-14 (retention time 67 min) and SST-28 (retention time 73 min) detected by absorbance at 214 nM. The elution position of PSST (111 min) is indicated. Profiles representative of four experiments.

Table 2. Comparison of the % 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

	% SST-14	% SST-28	% PSST
Wt	65 ± 5	28 ± 7	7 ± 2
Δ NPSST	61 ± 2	26 ± 3	13 ± 3
Lys^{13}/KR	62 ± 5	25 ± 6	13 ± 6
Lys^{13}/RTKR	62 ± 11	31 ± 5	7 ± 1
Ser^{3}/A	70 ± 8	23 ± 4	7 ± 1
Leu^{7}/A	58.6 ± 6	30.8 ± 3	10.6 ± 5
Leu^{11}/A	62.2 ± 7	28 ± 8	9 ± 2.2
Lys^{13}/A	68.3 ± 10	22 ± 5	9.7 ± 4
Leu^{15}/ A	67 ± 8	24 ± 3	9 ± 2

Medium

	% SST-14	% SST-28	% PSST
Wt	70 ± 9	30 ± 3	0
Δ NPSST	22 ± 3	27 ± 2	51 ± 4
Lys^{13}/KR	20 ± 3	22 ± 5	58 ± 9
Lys^{13}/RTKR	16 ± 1	20 ± 4	64 ± 5
Ser^{3}/A	68 ± 5	25 ± 3	7 ± 3
Leu^{7}/A	14 ± 3	18 ± 5	68 ± 7
Leu^{11}/A	10 ± 2	17 ± 5	73 ± 11
Lys^{13}/A	68 ± 5	28 ± 6	4 ± 1
Leu^{15}/ A	65 ± 6	26 ± 6	9 ± 5

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 Leu^{11} to Ala mutant and the ΔNPSST, Lys^{13} to KR, and Lys^{13} 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 which fails to be targetted to the RSP can still be processed to SST-14 and SST-28 in TGN compartments. However, PSST targetting is critical for efficient processing of the releaseable pool of SST-14 and SST-28.

Molecular Modeling of rPSST

We constructed a structural model of the Pro^5 to Thr^{19} sequence of rPSST based on the 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 which 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 the point mutants created for this study which 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

A

```
Rat PSST 1 APSDPRLRQFLQKSLAAATGKQELAKYFLAELLSEPNQTENDALEPEDLPQAAEQDEMRLELQRSANSNPAMAPRERKAGCKNFFWKTFTSC
Cons ssp         hhhhhhhhhhhhhhhhhhhh hhhhhhhhhhhhhhhhhh          h          hhhhhhhhhhhhhhhhhhhh          hhhhhh          bbbbbb
```

B

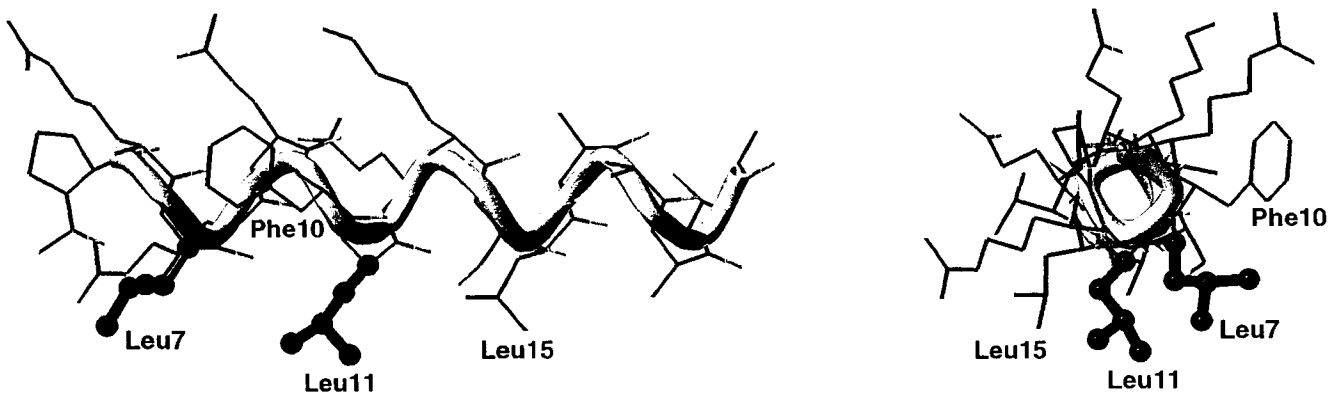


FIGURE 7: (A) Consensus secondary structure prediction for rPSST Ala^{1} to Cys^{92} sequence. h, denotes α-helix; b, denotes β strand. Leu^{7} and Leu^{11} residues are bolded.

(B) Structural model of rPSST Pro^{5} to Thr^{19} sequence. Two orthogonal views are shown. Residues forming the hydrophobic face of the α-helix are labelled. The side chains of Leu^{7} and Leu^{11} are shown in ball and stick representations.

attributed to the removal of critical side chains from the hydrophobic surface which likely forms a binding interface for the propeptide rather than to global conformational changes introduced by the mutations.

The signal sequence of PPSST is sufficient for GFP and DsRed to localize to secretory granules

In order to study whether the signal peptide and the amphipathic α-helix alone in SST are both suficient to reroute a non regulated secretory protein to the RSP, using GFP and DsRed we established stable AtT-20 cell lines transfected with the following constructs: GFP, DsRed, SST signal peptide fused to GFP and/or DsRed, and SST signal peptide with the first 19 amino acids of PSST fused to GFP and/or DsRed. Cells transfected with GFP (fig.8A) or DsRed (fig.8D) alone demonstrated no preferential localization with the fluorescence distribution dispersed throughout the cytosol and nucleus. Fluorescence results of cells transfected with chimeric proteins to the SST signal peptide and the α-helix displayed proper localization to granules (fig. 8B & E). Surprisinlgy in cells transfected with the signal peptide of SST alone fused to either GFP or DsRed, the fluorescence accumulated in the TGN areas and the tips of the cellular processes where secretory granules accumulate and also in vesicular structures distributed throughout the cell (fig. 8C & F). The signal peptide is present in every protein entering the secretory pathway (40) and it is required for entry into the ER where it is usually cleaved during protein translation. The ability of either fluorescence molecule to be properly targeted to the RSP implies that each contains inherent ability to be targeted once allowed to enter the secretory pathway.

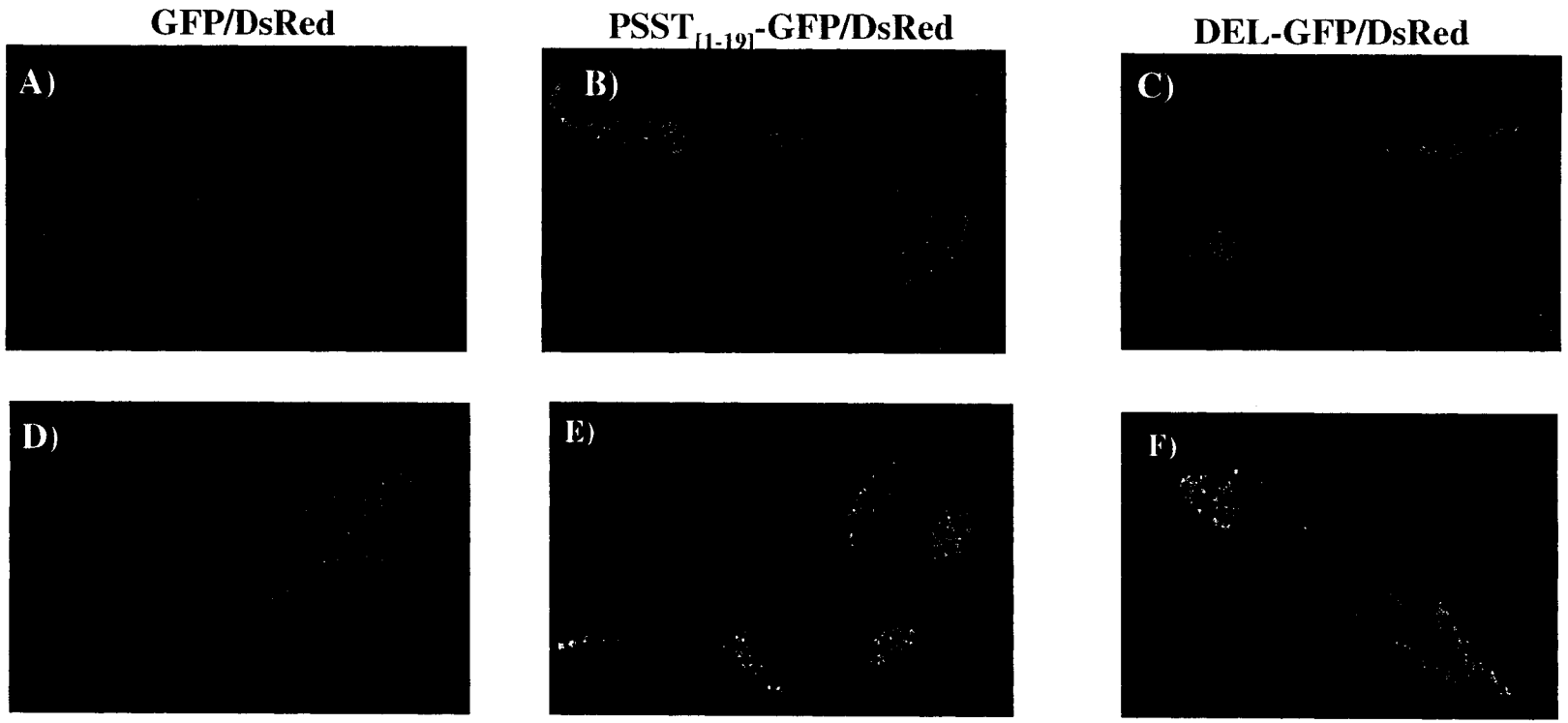


Fig.8 AtT-20 cells transfected with GFP/DsRed and in fusion with SST. Cells expressing GFP or DsRed (A&D respectively), GFP or DsRed fused to the signal peptide and first 19 amino acids in PSST (B&E respectively), GFP or DsRed fused to the signal peptide of PSST (C&F). Cells were visualized using regular light microscopy with intrinsic fluorescence.

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 N-terminal α-helix does not impair the ability of PSST to be processed at the C-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.

Several previous studies have shown that the prosegment of PSST harbors a sorting signal (18,19,28). For instance, a SST fusion protein consisting of the signal peptide and proregion of anglerfish (af) PSST1 fused to α-globin is sorted to the RSP in transfected GH_3 cells, whereas the α-globin gene joined to the β-lactamase signal peptide is degraded in the secretory compartment (18). af PSSTI transfected in Rin5F cells is directed to the RSP whereas af PSSTII is mainly targetted to the CSP (19). A fusion protein comprising the first 54 residues of rPSST and the last 48 amino acids of af PSSTII is correctly targetted to the RSP (19). Deletion of the $rPSST_{[1-10]}$ 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 N-terminal sequences of rPSST (and probably af PSSTI but not af PSSTII) contain intracellular targetting information (19). Molecular modelling of rPSST reveals an α-helix at residues 5-19 with the side chains of residues Leu^{7} , Phe^{10}, Leu^{11} and Leu^{15} 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 procortistatin (PCST) (where it is located not at the N-terminus but further downstream at residues 19 to 35) but is not present in af PSSTII consistent with the targetting data obtained for this precursor experimentally (Fig.1). The secondary structure predictions of a dozen other prohormones that are known to be targetted to the RSP also reveal a common amphipathic α-helix similar to that in PSST which qualifies as a putative sorting signal (34). We have analysed the PSST N-terminal α-helix as a sorting signal by detailed mutagenesis. Deletion of the $PSST_{[3-15]}$ segment blocked regulated secretion of SST-14 LI in response to both TPA and FSK and rerouted PSST for constitutive secretion as unprocessed precursor. Similar results were obtained with two other mutants in which the Lys^{13} residue was substituted with RTKR, a classic furin motif, or the dibasic motif KR to enhance N-terminal PSST cleavage endogenously by furin or PC1/PC2 respectively. Analysis of N-terminal processing by N-terminal specific RIA confirmed a 2 and 3 fold increase in cleavage of a $PSST_{[1-10]}$ like product from these mutant precursors (data not shown). An additional possibility for the missorting is that insertion of extra basic residues at Lys^{13} disrupted the α-helix. Thus removal of the PSST N-terminal α-helix by mutagenesis, or endogenously by endoproteolysis both resulted in precursor missorting. The complete abrogation of TPA and FSK stimulatory responses by the three deletion mutants differs from the results of Sevarino et al who found that deletion of the $rPSST_{[1-10]}$ segment induced only partial loss of regulated secretory responses to TPA but not FSK (28). Since the N-terminal domain of PSST is crucial for precursor targetting to secretory granules, it is surprising to find that the precursor is normally processed at the N-terminus to generate $PSST_{[1-10]}$. The site of processing has

been postulated to be Lys^{13} although this region does not qualify as a substrate for monobasic cleavage by a prohormone convertase (PC)-like enzyme (32,41). Recent studies suggest that N-terminal processing of PSST is effected by the novel protease subtilisin-kexin isoenzyme SKI-1 which cleaves at Leu^{11} (41,42). The biological significance of PSST N-terminal processing is unclear although it is known that $PSST_{[1-10]}$ cleavage is relatively inefficient compared to that of SST-14 and SST-28, and is highly tissue-specific with moderate production of the peptide in stomach and brain, and virtually none in islet cells or intestinal mucosa. Even in antral D-cells, the site of maximum $PSST_{[1-10]}$ synthesis, only a small subpopulation of secretory granules (30% in rat, 10% in human) contain the peptide (42). Our finding that deletion of the $PSST_{[3-15]}$ domain results in missorting of the precursor suggests that endogenous N-terminal PSST processing must be a late event, distal to the TGN sorting process, and its function may be to target the precursor to a subpopulation of secretory granules. Alanine scanning mutagenesis identified the region Pro^5 to Gln^{12} as being important in precursor targetting with Leu^7 and Leu^{11} being critical. These results complement the modelling data and suggest that these two residues located in close proximity on the hydrophobic surface of the α-helix may provide a binding interface for interaction with a putative sorting receptor. Recently, an amphipathic α-helix in the C-terminal segment of PC1 with critical leucine residues at Leu^{745} and Leu^{749} has also been reported to mediate targetting of the convertase to the RSP (22). Unlike PSST, the C-terminal region of PC1 contains two segments of ~ 40 residues, each of which can independently target the convertase to the RSP and which both harbor α-helices. These results provide direct evidence that an α-helix in PSST and PC1 mediates the targetting of the two proproteins to the RSP, and

suggests 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 disulphide 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 Cys^{8}/Cys^{20} residues that form a disulphide bridge (21). Molecular modelling has revealed a similar putative disulphide 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 and acts as a low affinity, high capacity sorting signal receptor (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 or *in vitro* antisense depletion of CPE, or genetic ablation of CPE in the CPE^{fat} mouse all lead to missorting of POMC, proenkephalin, and proinsulin (25,26). Other studies, however, have found that proinsulin is sorted to the RSP in pancreatic islets from CPE deficient fat mice as well as in cell lines derived from pancreatic β cells of these mice (45,46). Additionally, chromogranin A which possesses a POMC-like sorting signal does not use CPE as a sorting receptor (26). Thus, not all sorting signals are recognized by CPE suggesting the existence of other sorting receptors.

Whether there is a putative receptor that interacts with the α-helical sorting signal that we have identified in PSST and which is common to a number of other neuroendocrine precursors remains to be determined.

Processing of prohormones at basic residues is effected by a family of subtilisin related mammalian Ca^{2+}-dependent serine proteinases known as PCs with seven current members furin, PACE4, PC1, PC2, PC4, PC5A/B, and PC7 (41). Furin, PC5B and PC7 are membrane bound and along with PACE4 process proteins in the CSP whereas PC1, PC2, and PC5A process neuroendocrine precursors that are targetted to secretory granules. The cellular compartment in which cleavage occurs is controversial. Proteolytic processing of several hormone precursors, e.g. proinsulin and propresophysin, occurs largely or exclusively in secretory granules. Immunogold labelling studies have shown that proinsulin cleavage is a post-Golgi event initiated in acidic clathrin-coated immature secretory vesicles and completed in mature uncoated granules (47). Several recent studies, however, have demonstrated that limited to extensive proteolytic cleavage of some hormone precursors can also occur proximally in the TGN (48-51). This comes as no surprise since the converting enzymes already exist in an active form in this compartment (furin, PC1, PC5, PC7, PACE4) and the weakly acidic (pH ~ 6.5) milieu would favor proprotein processing (31,41). Conversion of SST-14 from PSST is mediated by either PC1 or PC2 (31). Although both convertases are present in secretory granules, PC1 also exists in an active form in the TGN and is, therefore, capable of SST-14 conversion in this compartment whereas PC2 is optimally active in secretory granules (22,41). Monobasic cleavage of SST-28 is effected by furin and/or PACE4 (52,53). Blockade of PSST targetting to secretory granules by the N-terminal deletion and Leu^7

and Leu^{11} PSST mutations led to an escape of large quantities of 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/PACE4 (for SST-28). These results are consistent with previous studies which have shown significant processing of PSST in the absence of secretory granules in TGN compartments (49,51). Overall, this means that the N-terminal PSST conformation does not influence enzyme recognition and PSST cleavage at the C-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.

We then decided to test the ability of the PSST[1-19] to act as a heterologous sorting signal for redirecting proteins normally destined for the CSP and for targeting nonsecretory proteins to the RSP. Based on the mutagenesis studies, which have shown this segment at the NH_2-terminus of PSST to be the critical sorting signal, hybrid genes containing rPSST signal sequence and residues 1-19 of the pro sequence were fused in frame to the NH_2-terminus of GFP and DsRed. Fluorescent proteins have been extensively used in cell-biological applications particularly, GFP and DsRed isolated from jellyfish have revolutionized our ability to study protein localization in living cells (54,55). The technique is particularly attractive because unlike other commonly used reporter systems it is noninvasive to the cells and detection of either protein does not require an additional substrate; as the protein can be easily monitored using simple light microscopy containing appropriate filters. Virtually any protein can be tagged and the

resulting chimera often retains parent-protein tagging and function when expressed in cells. Unfortunately, the results presented in this study demonstrate that both proteins once attached to only the signal peptide of SST are capable of directing themselves to the RSP. These findings make it necessary to be careful not to casually assume the lack of targeting information contained in any protein prior to carrying out proper tests. Hence, GFP and DsRed should no longer be considered as passive reporter molecules to be used for studies aimed at studying protein trafficking in the secretory pathway in particular endocrine cells. Similarly, other groups have demonstrated the inherent ability of GFP to enter the RSP (56-58).

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CHAPTER C

Prosomatostatin is Proteolytically Processed at the Amino Terminal Segment by the Subtilase SKI-1.

Abstract

Processing of prohormones to generate active products typically occurs at basic residues via cleavage by proprotein convertases. A less common type of cleavage is mediated at hydrophobic residues (L, V, F, N) or small amino acids residues (A, T, S). Efforts to identify the proteinases responsible for processing of precursors at hydrophobic amino acids has led to the recent cloning of a new type-I membrane bound subtilase called SKI-1. The NH_2-terminal region of prosomatostatin, previously shown to contain a sorting signal for the regulated secretory pathway, is processed to generate $PSST_{[1\text{-}10]}$ (antrin). The exact cleavage mechanism is unknown, but has been assumed to involve monobasic processing at Lys^{13} followed by carboxypeptidase trimming. We find that K13A mutation did not block antrin production. Since the prosomatostatin sequence R^{8}-Q^{9}-F^{10}-$L^{11}\downarrow$ qualifies as a potential SKI-1 substrate, using a vaccinia virus expression system together with HPLC and radioimmunoassay we find that overexpression of recombinant human SKI-1 in COS-1 and HEK-293 cells significantly increased the amount of $PSST_{[1\text{-}10]}$. Additionally in CHO cells lacking SKI-1 there was a significant reduction in antrin production, that was increased upon SKI-1 expression. Mutagenesis studies show that efficient processing of PSST to antrin requires the **RXRXXL** motif. However such NH_2-terminal cleavage is not a prerequisite for the formation of SST-14 and SST-28.

Introduction

Biologically active proteins and peptides are produced via intracellular limited proteolysis of inactive precursors. Most often such processing occurs at either monobasic or dibasic residues as a result of cleavage by mammalian serine proteinases related to bacterial subtilisin and yeast kexin. These enzymes known as proprotein convertases (PCs), cleave a variety of precursors at a general consensus **(R/K)**-$(Xaa)_n$-**(K/R)**↓ sequence, where Xaa is any amino acid except Cys and n = 0,2,4 or 6. Such cleavage type is involved in processing of many growth factors and their receptors, most polypeptide hormones and neuropeptide precursors, surface and secretory glycoproteins. However, a number of precursors are also cleaved at non-basic sites, C-terminal to Ala, Ser, Thr, Met, Val and Leu. This type of cellular processing has been implicated in the generation of bioactive proteins and peptides such as the site 1 cleavage of sterol regulatory element binding proteins (SREBPs) involved in the regulation of cholesterol and fatty acid metabolism, and the production of the Alzheimer's amyloidogenic peptides Aβ 40 and 42 (1, 2).

There are presently seven known mammalian PCs that have been identified comprising PC1 (PC3), PC2, furin (PACE), PC4, PC5 (PC6), PACE4, and PC7. Tissue distribution analyses of these enzymes indicate that PC1 and PC2 are expressed mainly in neural and endocrine tissues cleaving precursors entering the regulated secretory pathway (RSP), PC4 is exclusively in reproductive germ cells, and PC5 and PACE4 to varying degrees in many tissue types. Furin and PC7 are both type I membrane proteins residing in the *trans*-Golgi network (TGN), they display ubiquitous tissue distribution and process precursors within the constitutive secretory pathway (CSP). Each of the above kexin-like

subtilases exhibit both unique and conserved structural motifs, with all members containing a signal peptide, a pro-segment, a catalytic subunit, and a P-domain. Proteases are usually synthesized as inactive zymogens, that are activated following autocatalytic excision of the NH_2-terminal prosegment that is thought to act as an intramolecular chaperone and as a specific inhibitor of the parent protease (1, 2). Enzyme activation is an event taking place in the TGN or secretory granules resulting in the disposal of the prosegment. Such regulation plays an important role in determining the site and cellular location at which biologically active products are derived from inactive precursor proteins (1-4).

Less commonly than cleavage at basic residues bioactive products can also be produced by limited proteolysis at hydrophobic and small amino acid residues. Efforts to identify such proteinases led to the recent cloning of a new type-I membrane bound subtilase called subtilisin-kexin-isozyme 1 (SKI-1) or site-1 protease (S1P), whose amino acid sequence is highly conserved among rodent and human species (5, 6). It is the first known mammalian subtilisin/kexin-like enzyme capable of cleaving proproteins at nonbasic residues. It is a Ca^{2+} –dependent subtilase that is widely expressed with possible substrate cleavage specificity C-terminal to Thr, Leu, Phe and Lys residues. Mutational analysis has demonstrated that the presence of an Arg at the P4 substrate position is very critical for cleavage (5-9). In addition to autocatalytic processing (8, 9), the first two major substrates identified for SKI-1 are SREBP cleavage at an **R**SV**L**↓ within the early compartments of the Golgi (6) and the 32-kDa human pro-brain-derived neurotrophic factor (hpro-BDNF) at an **R**GL**T**↓ cleavage site generating a 28-kDa form in the endoplasmic reticulum (ER)/early Golgi (5). Recently, two other substrates have now

been added to the list: ATF6 cleavage at an **RSVL**↓ site (10), a transcription factor that plays a central role in the unfolded protein response also called the ER stress response, and the surface fusion glycoprotein (GP) of the Lassa virus at an **R**RL**L**↓ site producing two fragments implicated in disease development (11) (Table 1).

Mammalian prosomatostatin (PSST) consists of a 10 kDa precursor which is cleaved endoproteolytically to yield several mature products (Fig.1). Processing occurs principally at the C-terminal segment of the molecule to produce two bioactive products (12,13). A dibasic cleavage site at an **RERK**↓ mediated by PC1/PC2 leads to the production of SST-14 and furin/PACE4 cleaves at a monobasic site **R**LELQ**R**↓ generating SST-28 (13, 14). In addition, cleavage at a monobasic Lys^{13} residue at the NH_2-terminal region has been implicated in generating the decapeptide $PSST_{[1-10]}$ also known as antrin (15). $PSST_{[1-10]}$ is called antrin because it was originally isolated in the gastric antrum (15) although all somatostatin (SST) producing tissues have subsequently been shown to be rich in antrin (16,17). The NH_2-terminus of PSST is highly conserved through out vertebrate evolution suggesting an important biological role for it (16, 17). Recent studies from our laboratories have demonstrated that the $PSST_{[5-19]}$ comprises an amphipathic α helix which plays an important role in targeting the precursor to the regulated RSP (18).

Little is currently known about the steps involved in NH_2-terminal PSST maturation namely, the enzyme and subcellular compartmentalization of the processing event. The current study was designed to test the hypothesis that SKI-1 participates in the processing of antrin. Since cleavage at the NH_2-terminus of PSST has never been shown to be mediated at the monobasic Lys^{13} residue, in this study we find that Lys^{13} →Ala

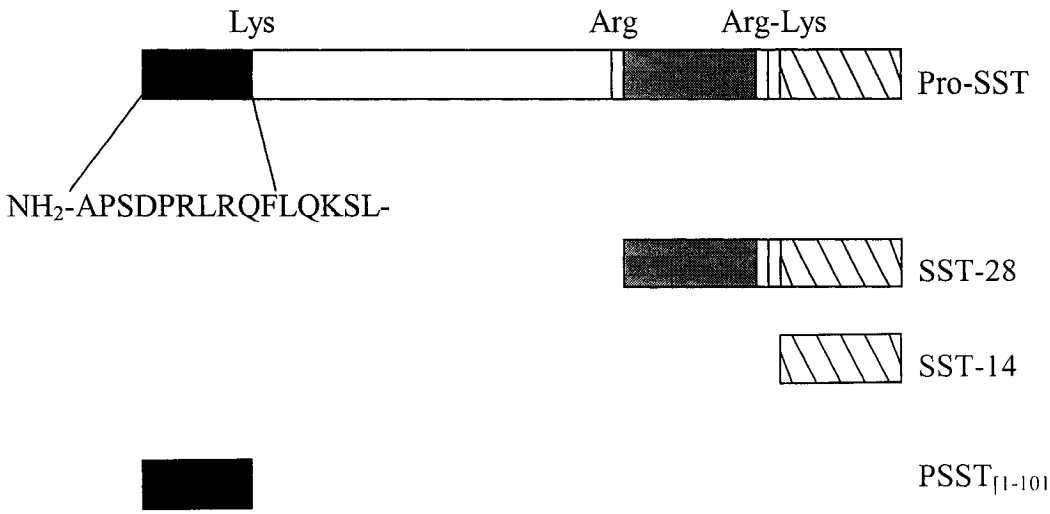


FIGURE 1: Schematic illustration of PSST and its cleavage products with the amino acid sequence of antrin represented in bold.

Table 1 Processing of substrates by SKI-1/S1P

Precursor protein	**Cleavage site sequence** P8-P7-P6-P5-P4-P3-P2-P1↓
(h)proSKI-1/S1P	
site B	R -K- V-F- **R**-S-**L** -K↓
site B'	V- T- P-Q- **R**-K-**V**- F↓
site C	R- H- S-S- **R**-R-**L**- L↓
hProBDNF	K -A- G-S- **R**-G-**L**- T↓
hSREBP-2	S- G- S-G- **R**-S-**V**- L↓
LassaV-gp	I- Y- I-S- **R**-R-**L**- L↓
hATF6	A- N- Q-R- **R**-H-**L**- L↓
rPSST?	D- P- **R**-L- **R**-Q-**F**- L↓

TABLE 1: Comparison of amino acid sequences of the already identified four SKI-1 substrates with NH_2-terminal PSST. The sequences are aligned without introduction of gaps. Amino acids that potentially play the same role in proteolytic cleavage of PSST are in bold highlighting the hydrophobic Leu residue at the potential P1 position of PSST and, the basic Arg at P4 with an additional Arg at P6.

mutation did not block $PSST_{[1-10]}$ conversion. Scanning the amino acid sequence at the NH_2-terminus of PSST, we find a region containing the following sequence: -R8-Q9-F10-L11- which qualifies as a SKI-1 substrate (Table 1). Using a vaccinia virus (vv) expression system together with RP-HPLC and radioimmunoassay (RIA) we find that overexpression of recombinant human SKI-1 with rat preprosomatostatin (rPPSST) significantly increased the amount of $PSST_{[1-10]}$ generated. Additionally, in CHO cells lacking SKI-1 (19) there was a significant reduction in antrin production, with the defect being corrected upon reintroduction of the enzyme into the cell. Site directed mutagenesis suggest that SKI-1 participates in antrin production requiring the RXRXXL motif. Furthermore, antrin production is not an obligatory step in the C-terminal conversion of PSST to the biologically active forms SST-14 and SST-28.

EXPERIMENTAL PROCEDURES

Materials: Synthetic peptides were obtained as follows: SST-14, SST-28 (Bachem Marina del Rey, CA); Tyr^0 SST-14 (Peninsula, Belmonte, CA); acetonitrile and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Montreal, Quebec); heptafluoro-butyric acid (HFBA) was obtained from Pierce Chemicals (Rockford, IL); pepstatin-A, and phenylmethylsulphonyl fluoride (PMSF) were from Sigma (St. Louis, MO). Dulbecco's Modified Eagle's Medium and fetal bovine serum were purchased from Gibco-BRL (Gaithersburg, MD). 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 PPSST cDNAs: cDNA for wild type rPPSST were constructed in the expression vector pTEJ8. Using rPPSST as template, a series of point mutants were created by the PCR overlap extension technique. To construct the mutants two fragments were created separately which included a 5' fragment containing the desired mutation using primer A and a reverse primer, and a 3' fragment using primer B and a forward primer where the forward primer and reverse primers are mirror images of each other. The fragments were then ligated in a third PCR reaction to generate the full-length mutant PPSST cDNA. Primer A was designed to contain *Hind III* endonuclease restriction site, Kozak consensus sequence, and initiation codon. Primer B contained 3' flanking sequence and stop codon followed by an Eco R1 restriction site.

Primer A 5'-ATT CAT AAG CTT GCC GCC ACC ATG CTG TCC TGC CGT - 3' (forward)

Primer B 5'-TAG TAG ATG AAT TCC TAA CAG GAT GTG GAA TGT - 3' (reverse)

PCR was carried out with 50 ng of PPSST cDNA in 100 µl containing 20 mM Tris-HCl, 200 µM dNTPs, 1.5 mM $MgCl_2$, 6% DMSO, and 2 units of *p.f.u.* (Stratagene) using the following conditions: denaturation at 94° C for 80 s, annealing at 59° C for 50 s, and extension at 72° C for 60 s for 25 cycles followed by extension at 72° C for 10 min. PCR products were separated by agarose gel electrophoresis, the amplified bands electroeluted and purified. Fragments B-1 and A-2 were then fused in a ligation reaction using flanking primer pair A and B. After PCR ligation, the products were digested to completion with *EcoR1* and *Hind III* and the purified fragments were subcloned into *Hind III-EcoR1* multiple cloning sites of pTEJ8. All recombinant plasmid constructions

were verified by sequencing of double stranded DNA (University Core DNA Service, University of Calgary, Alberta, Canada) and at least two separate clones of each mutant were independently transfected. The following forward and reverse primers bind to the same region of rPPSST cDNA and were designed to contain the desired mutation.

1) Point mutations to Ala residues:

Arg^6	5'-TCGGACCCCGCACTCCGTCAGTTTCT -3' (forward)
	5'-AGAAACCTGACGAGTGCGGGTCCGA -3' (reverse) (nt 78-104)
Arg^8	5'-ACCCCAGACTCGCTCAGTTTCTGCA -3' (forward)
	5'-TGCAGAAACTGAGCGAGTCTGGGGT -3' (reverse) (nt 83-108)
Arg^6-Arg^8	5'-TCGGACCCCGCACTCGCTCAGTTTCTGCA-3' (forward)
	5'-TGCAGAAACTGAGCGAGTGCGGGGTCCGA-3'(reverse) (nt 78-117)
Leu^{11}	5'-CTCCGTCAGTTTGCGCAGAAGTCTCTG -3' (forward)
	5'-CAGAGACTTCTGCGCAAACTGACGGAG -3' (reverse) (nt 91-118)
Lys^{13}	5'-CAGTTTCTGCAGGCCTCTCTGGCGGCT -3' (forward)
	5'-AGCCGCCAGAGAGGCCTGCAGAAACTG -3' (reverse) (nt 96-123)

Point mutation to Pro residue

Leu^{11}	5'-ACTCCGTCAGTTTCCGCAGAAGTCTCT-3' (forward)
	5'-AGAGACTTCTGCGGAAACTGACGGAGT-3' (reverse) (nt 87-115)

Cell Culture, Transfection, and analyses of Stable AtT-20 cells: AtT-20 mouse anterior pituitary cells were cultured in Dulbecco's Modified Eagle's Medium with 5% fetal bovine serum supplemented with Ser-X-tend in an atmosphere of 5% CO_2 and 95% air in a humidified incubator at 37° C. Cells were plated in 100 x 20 mm Petri dishes and transfected at 50% confluency with 3-5 μg of the appropriate plasmid construct by lipofectamine (Gibco-BRL) and stable G418 (0.861 mg/ml) resistant nonclonally selected

cells were propagated for study. Stably transfected AtT-20 cells were grown to 80-90% confluency after which they were prepared for studies of SST-14 like immunoreactivity (SST-14LI) or $PSST_{[1-10]}LI$. Attached cells were extracted by scraping into 1 M acetic acid containing PMSF and pepstatin-A (20 μg/ml each) on ice. The cell suspension was further extracted by sonication followed by centrifugation at 5000×g for 30 min. The supernatant was stored at -20° C for RIA and RP-HPLC analysis.

Transient transfections in CHO cells: CHO-K1, or mutant CHO cells [SRD-12(-)] cells were plated on 150mm dishes and grown as monolayers. At 80% confluency the cells were transiently transfected with 3μg each of rPPSST, human α_1-antitrypsin portland ($h\alpha_1$.PDX), with or without hSKI-1, by using Lipofectamine (GIBCO). On the third day the cells were prepared for study by removing the feeding medium and replacing it with DMEM/F12, 1% bovine serum albumin together with protease inhibitors PMSF and pepstatin A (20 μg/ml each). The next day media was harvested, centrifuged at 1,000xg for 6 min to remove detached cells and the supernatant acidified to pH 4.8 with 1M acetic acid containing PMSF and pepstatin A (20 μg/ml each).

Vaccinia Virus infections of COS-1 and HEK-293 cells: COS-1 or HEK-293 cells were plated in 100mm diameter plastic petri dishes and grown as monolayers. After 1-2 days the cells were infected with either vv: rPPSST+ $h\alpha_1$PDX or vv: rPPSST+ $h\alpha_1$PDX+ hSKI-1. 22 hrs after infection media were replaced with secretion medium containing DMEM with 1%BSA, and enzyme inhibitors (PMSF and pepstatin A, 20 μg/ml each).

Following an overnight incubation, media and cells were collected as previously described (13, 14).

RP-HPLC: Pooled acidified secretion media and cell extracts were diluted 1:7 with 0.1% TFA 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 (13, 14). The column was eluted at room temperature (21° C) at 1 ml/min with 12-55% acetonitrile and 0.2% HFBA 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 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.

Somatostatin Radioimmunoassay: Two separate RIAs directed against the C- and NH_2-terminal regions of PSST were employed as follows: I) RIA for SST-14 LI was performed using a rabbit anti-SST antibody (R149), [^{125}I] Tyr^{0} SST-14 radioligand, synthetic SST-14 standards and a bovine serum albumin-coated charcoal separation method. 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. RIA for $PSST_{[1-10]}$ LI was measured using R203 antibody, [^{125}I] Tyr^{10} $PSST_{[1-10]}$ ligand, and $PSST_{[1-10]}$ standards. This assay detects $PSST_{[1-10]}$, PSST, and all its COOH-terminally extended forms such as PSST.

Secondary Structure Prediction and Model Building: The secondary structure of hpro-BDNF (residues Pro^{1}-Pro^{69}) was predicted with the NPS @ consensus secondary structure prediction algorithm (20) 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 α-helical region Leu^{47} to Asp^{64} was constructed from standard geometries using the BIOPOLYMER module in SYBYL 6.6 molecular modeling software (Tripos Inc., St. Louis, MO). N- and C-termini were blocked with acetyl and methylamino groups respectively. Structural refinement was carried out by energy minimization using AMBER 4.1 all-atom force field (21) and a distance dependent (4R) dielectric constant. A similar approach was carried out for rPPSST (18).

Statistical Analysis: Results are expressed as mean ± SE. Statistical analysis was carried out by one way Anova followed by Dunnet's significance test. Significance was indicated by p value of < 0.05.

Results

$PSST_{[1-10]}$ cleavage is not mediated by the classical Precursor convertases

Because PCs can only cleave at either mono or dibasic cleavage sites (1-4), cleavage at the NH_2-terminal region of PSST has always been assumed to occur at the Lys^{13} residue followed by carboxypeptidase trimming leading to the production of $PSST_{[1-10]}$ (15-17). To check this theory a mutation was created in which the Lys^{13} residue was changed to an Ala whereby, cleavage by PCs should be blocked because they are unable to cleave C-

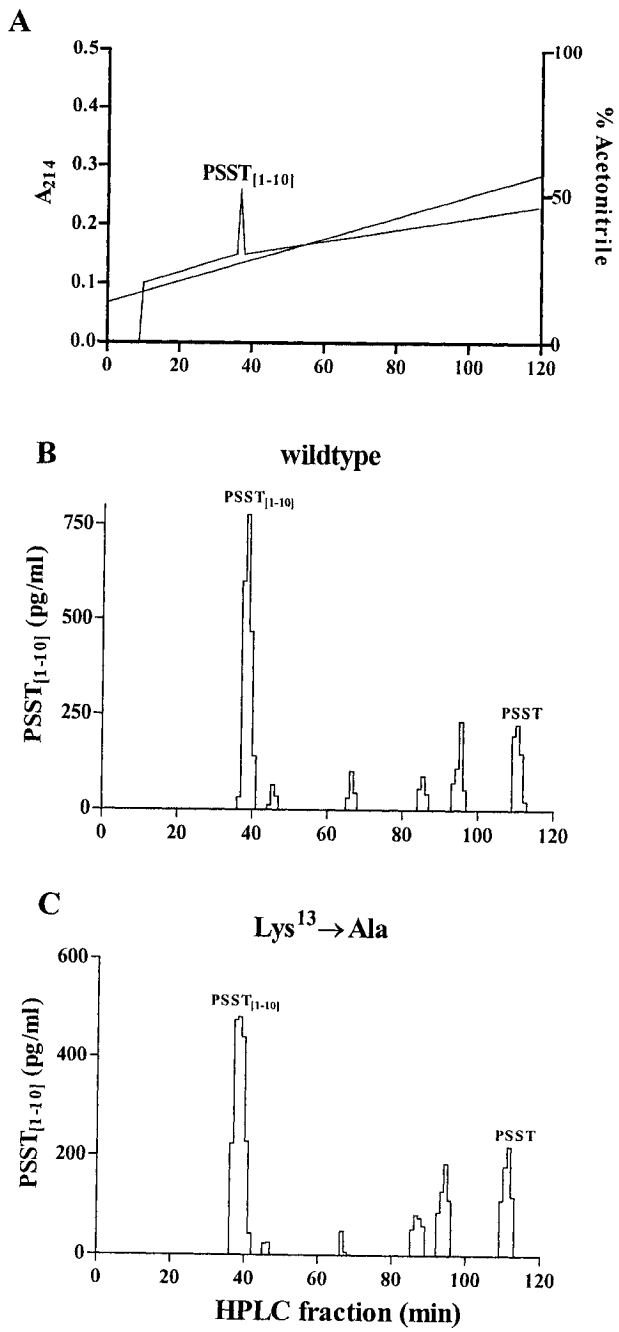


FIGURE 2: HPLC profile of cell extracts from AtT-20 cells transfected with WT (B), and Lys^{13} to Ala mutant (C). Panel A illustrates the elution position of synthetic $PSST_{[1-10]}$ (retention time 38 min) detected by absorbance at 214nM. Profiles are representative of four experiments.

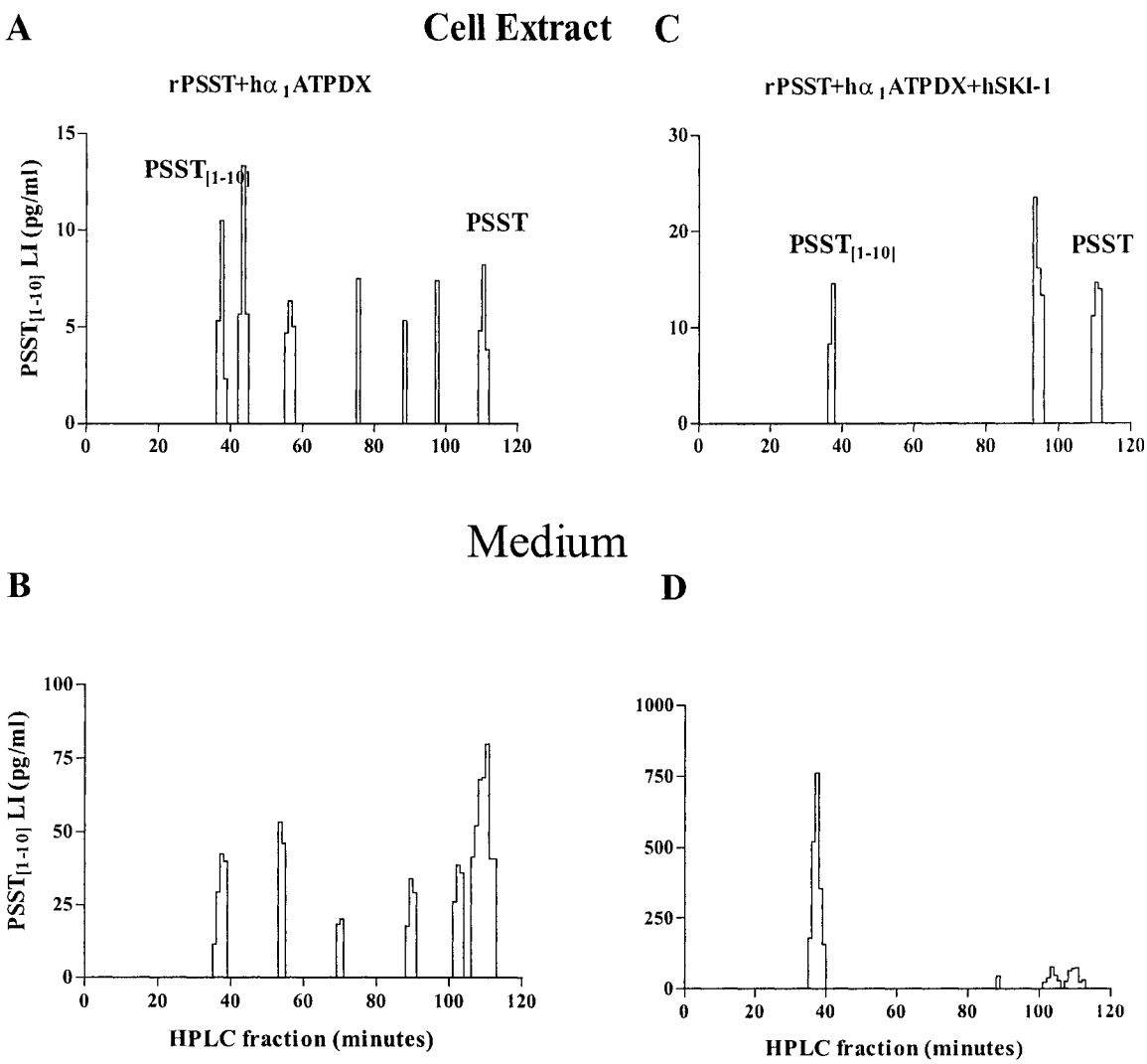
terminal to residues that are not basic (1, 2). Wildype (WT) PPSST and K13A mutant construct were separately stably transfected into AtT-20 cells. In order to characterize PSST processing to $PSST_{[1-10]}$, RP-HPLC eluates of the extracts from the transfected cells were analyzed by RIA using the R203 antibody. A 1-kDa peptide co-eluting with synthetic $PSST_{[1-10]}$ (Fig. 2A) was the dominant intracellular processed product in cells transfected with WT PPSST (representing 46 ± 6 % of total cellular $PSST_{[1-10]}$ LI) (Fig. 2B). Several other HPLC peaks were obtained and were shown to correspond to $PSST_{[1-10]}$ LI pepitdes as previously described which include the SST precursor (13, 16). Interestingly, $PSST_{[1-10]}$ cleavage was not blocked when cells were transfected with the K13A mutant; antrin was efficiently produced and represented 48 ± 4 % of $PSST_{[1-10]}$ LI (Fig. 2C). This implies that antrin is not produced through cleavage at a basic residue by the classical PCs, i.e., furin, PC1 or PC2.

Possible role of the mammalian proteinase subtilisin kexin isozyme in antrin conversion

Table 1 compares the amino acid sequences of hpro-BDNF, hATF6, hSREBP-2, and Lassa virus GP with rPSST suggesting a possible relation between certain SKI-1 substrates and PSST. Since the NH_2-terminal PSST region contains the $-\mathbf{R}^{8}$-Q^{9}-F^{10}-$\mathbf{L}^{11}\downarrow$ sequence which qualifies as a potential SKI-1 substrate, we investigated directly the ability of SKI-1 to cleave PSST to $PSST_{[1-10]}$. HEK-293 cells and COS-1 cells were co-infected with vv:rPPSST, hα1-PDX and either without (control cells) or with recombinant hSKI-1. Benjannet et al have previously demonstrated that hα1-PDX is a potent inhibitor of all tested PCs within the CSP (22). Therefore, cells were co-infected

with the recombinant inhibitor to exclude any possible role of PCs. i.e. furin or PC1/PC2 in antrin production. Cell extracts and media were analyzed by RP-HPLC followed by NH_2-terminal $PSST_{[1-10]}$ RIA. Analysis of the NH_2-terminal processing pattern in control HEK-293 cells infected with vv:rPPSST + hα1-PDX revealed a small 1 kDa peak intracellularly co-eluting with $PSST_{[1-10]}$ accounting for 19% of total immunoreactivity (Fig. 3A). This finding shows that despite inhibition of processing by PCs, $PSST_{[1-10]}$ was not affected thus representing basal antrin production in these cells. This is expected since SKI-1 is ubiquitously expressed in all tissues and cell lines (2, 5). In the medium, the cells secreted antrin (~16%) in small amounts with the full-length precursor PSST representing the major form (Fig. 3B). On the other hand, overexpression of SKI-1 in HEK-293 cells produced a significant increase in antrin conversion as judged by the increase in the ratio of antrin to precursor in both, cell extracts and medium (Fig. 3C and D). Significant amounts of antrin were especially detected in the medium accounting for ~79% of $PSST_{[1-10]}$ LI (Fig. 3D). Therefore SKI-1 overexpression increased $PSST_{[1-10]}$ conversion more than 5-fold, from ~16±3% to 79±7% of total immunoreactivity observed in the medium of HEK-293 cells and relatively similar results were observed in infected COS-1 cells (increase from 14±3% to 36±4 of total immunoreactivity) (Fig. 4).

Given the ubiquitous distribution of SKI-1, the availability of a cell line lacking this enzyme presented an ideal system for further authenticating a functional role for it as a $PSST_{[1-10]}$ convertase. We therefore performed a series of experiments in WT and mutant CHO cell line lacking SKI-1 (SRD-12B)(-) (19) to further determine whether the processing of PSST requires the same enzyme responsible for pro-BDNF, SREBP and ATF6 processing. SRD-12B(-) cells were capable of cleaving transfected PSST but the



FIGUE 3: HPLC analysis of amino terminal processing of rPSST in HEK-293 cells. Cell extracts (upper pannel) and secretion media (lower pannel) infected with either rPPSST+hα1-PDX (A&B) or with rPPSST+ hα1-PDX +hSKI-1 (C&D) collected following an overnight incubation were assayed for amino terminal $PSST_{[1-10]}$. Representative of three experiments.

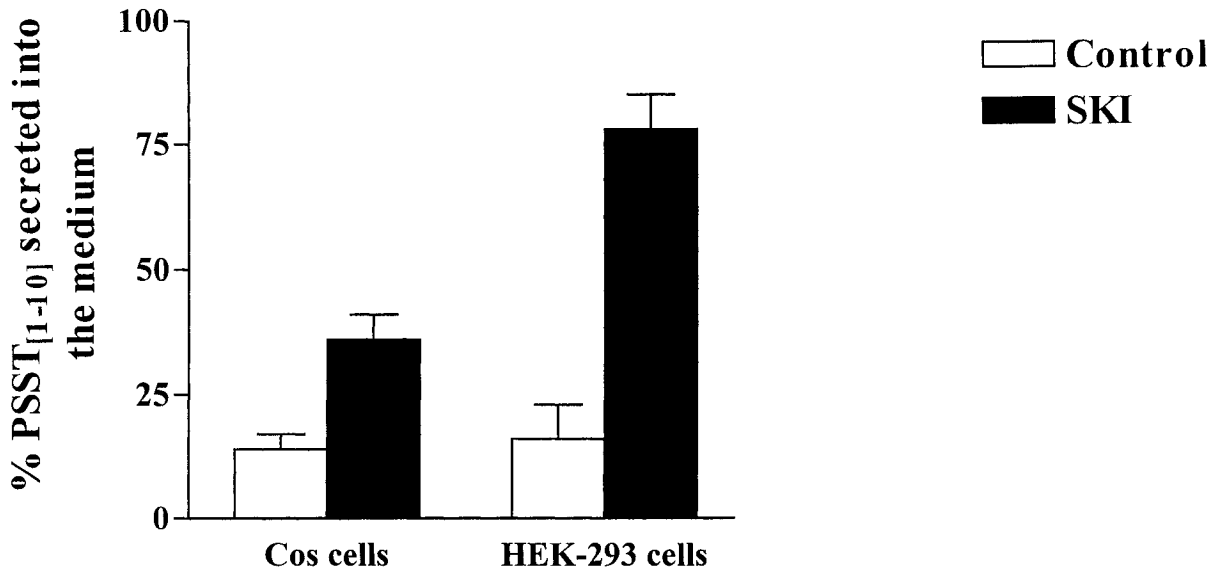


FIGURE 4: Comparison of NH_2-terminal proteolytic processing of PSST in COS-1 and HEK-293 cells. Media of control cells and those overexpressing hSKI-1 were harvested as described in the experimental procedure and analyzed by HPLC followed with $PSST_{[1-10]}$ RIA. Representative of three experiments.

efficiency of processing was decreased as judged by the significant reduction in antrin compared with that in WT CHO cells (Fig. 5). To determine whether the protease-deficient cell line could produce antrin again, hSKI-1 was reintroduced into the cells SRD-12B(+). We notice that the defect was corrected by transiently co-transfecting the cells with a cDNA encoding hSKI-1 along with rPPSST.

Identification of cleavage site and specific sequence requirement for PSST recognition by SKI-1

If some of the PSST processing is mediated by SKI-1, then processing should be reduced when the amino acids at the recognition sites are replaced (5-11). We therefore produced a series of mutations in which alanine residues were individually substituted in place of residues that could be potentially important (Fig. 6). Mutants were independently, stably transfected into AtT-20 cells. Three alanine substitutions, the R6A at the P6 position, R8A at the P4 position and the R6A+R8A double mutant, reduced cleavage dramatically. The L11A mutant did not produce any significant decrease in antrin production consistent with previous data demonstrating a much more relaxed substrate specificity at the P1 position compared to P4, with the hydrophobic Phe residue at the P2 that is still intact (9). To further dissect the requirement for leucine at the P1 position, a mutation was created by changing it to a Pro residue. Such a mutation resulted in a significant decrease in the amount of antrin conversion. These results provide strong support for the hypothesis that SKI-1 participates in PSST processing most likely cleaving C-terminal to a Leu^{11} residue. With regard to residue specificity, SKI-1 accepts an Ala at the P1 position whereas substitutions of either the Arg^{6} or Arg^{8} severely disrupted $PSST_{[1-10]}$ production. As

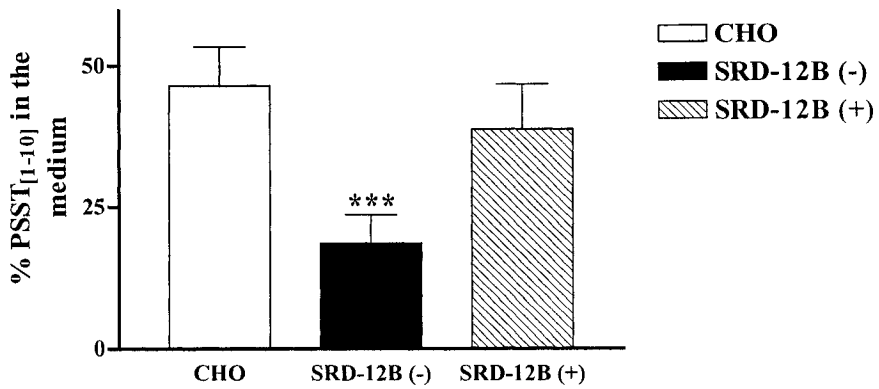


FIGURE 5: Comparison of $PSST_{[1-10]}$ production in WT CHO cells, SRD-12B(-) [mutant CHO cells lacking SKI-1 activity], and SRD-12B (+) cells [mutant CHO cells re-transfected with hSKI-1]. All three cell types were also transiently transfected with rPPSST and hα1-PDX. After transient transfection the cells were incubated overnight and then, the media were analyzed by HPLC followed with $PSST_{[1-10]}$ RIA. Mean ± SE from three independent experiments. *** $p < 0.001$ vs control.

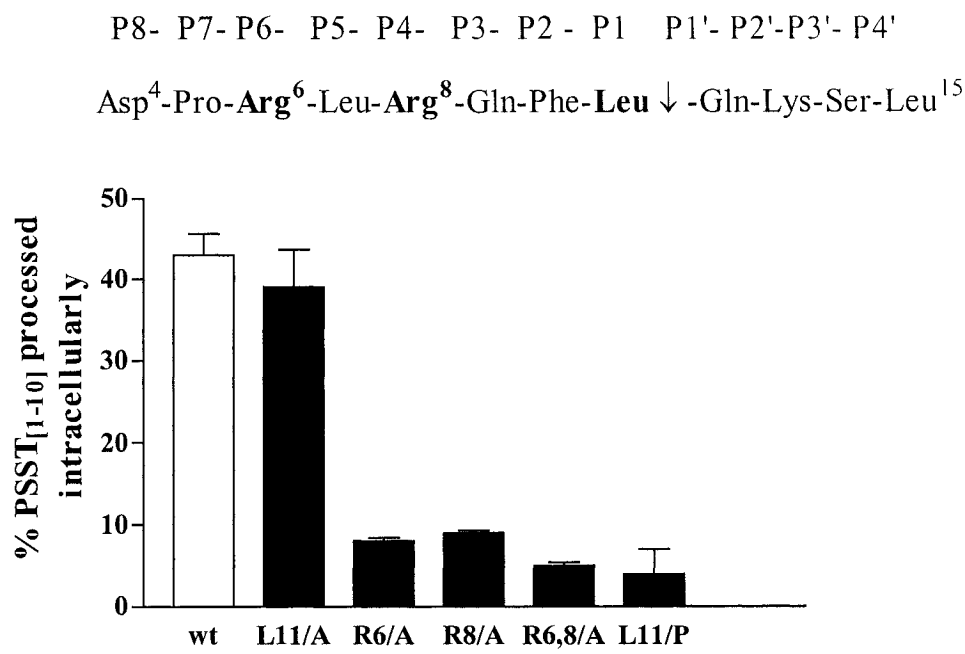


FIGURE 6: Mutational analysis of the N-terminal PSST processing site for cleavage specificity by SKI-1. Wild type or mutant rPPSST was co-infected with hSKI-1 in AtT-20 cells. The proposed consensus motif for SKI-1 (Arg/Lys)-X-hydrophobic-(L, T) identifies Arg^{6}, Arg^{8}, Phe^{10}, Leu^{11} in PSST as residues important for SKI-1 recognition. Mutating Lys^{13} to Ala has no effect on $PSST_{[1-10]}$ processing. Likewise, mutating Leu^{11} (a critical residue) to Ala does not affect processing. However, substituting Leu^{11} with Pro and substituting either of the two arginines at positions 6 and 8 or a double Arg^{6}, Arg^{8} mutation all result in marked inhibition of $PSST_{[1-10]}$ conversion.

such, this is the first report for SKI-1 requiring an Arg at the P6 position even when the Arg at P4 is still present.

Inhibiting antrin cleavage doe not impair SST-14 and SST-28 production

One of the possible roles of antrin is its masking effect of the PC mediated cleavages(s); cleavage at the NH_2-terminus of PSST might be a prerequisite for the production of SST-14 and/or SST-28. To characterize the products of PSST processing, cell extract and media of transfected AtT-20 cells were fractionated by RP-HPLC followed by RIA of the eluting fractions. The elution positions of the peaks obtained were compared with those of synthetic SST-14 and SST-28 chromatographed under identical conditions and monitored by UV (Fig. 7A). Figure 7 illustrates RP-HPLC profiles of SST-14 LI forms in cell extract of WT and two mutants that displayed inhibition of antrin cleavage: L11Pand R6/R8A. In cell extract of WT transfectants three peptide peaks were observed the first two of which co-eluted with synthetic SST-14 (retention time 67 min) representing 65% of total cellular SST-14 LI, and SST-28 (retention time 73 min) comprising of 28% of total cellular SST-14 LI. The third remaining peak (retention time 111 min) represented the full-length precursor with only 7% of total immunoreactivity. Looking at the two representative SST precursor mutants they were also capable of cleaving PSST to its biologically active forms implying that the ability of PCs to cleave PSST at its C-terminus is independent of NH_2-terminal cleavage by SKI-1 (Figs. 7C and D).

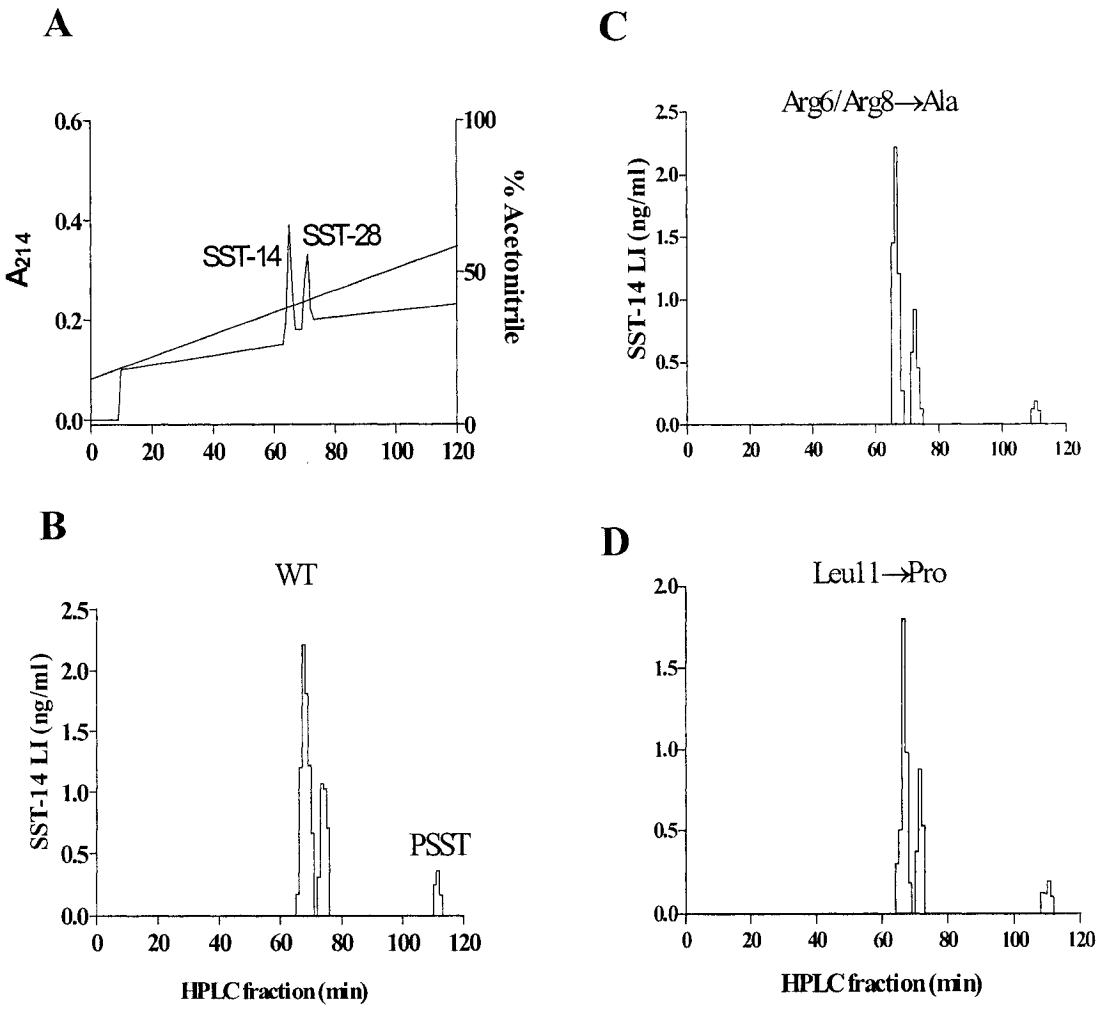


FIGURE 7: HPLC profiles of SST-14 LI in cell extracts from AtT-20 cells transfected with WT (B), Arg6/Arg8 to Ala mutant (C) and Leu11 to pro mutant (D). Pannel A illustrates the elution positions of synthetic SST-14 (retention time 67 min) and SST-28 (retention time 73 min) detected by absorbance at 214nM. The elution position of PSST (111 min is indicated). Profiles are representatives of four experiments.

Discussion

In this study we show that $PSST_{[1-10]}$ is generated through a distinct processing pathway different from that of the well characterized PCs responsible for the generation of SST-14 and SST-28. Antrin production was not blocked by hα1-PDX, an inhibitor of PCs, or by mutating the putative monobasic Lys^{13} cleavage. Scanning the NH_2-terminal sequence of PSST highlighted an -**R**^{6}-L^{7}–**R**^{8}-Q^{9}-F^{10}-**L**^{11}↓ motif which qualifies as a potential SKI-1 substrate. Using a vaccinia virus expression we directly tested the ability of SKI-1 to cleave PSST by over expressing it in both HEK-293 cells and COS-1 cells. We find that overexpression of hSKI-1 in both cell lines significantly increased the amount of $PSST_{[1-10]}$ secreted into the medium. On the other hand in cells lacking SKI-1 there was a significant reduction in antrin production. Additionally, alanine-scanning mutagenesis revealed that efficient processing of PSST to antrin requires at least two arginines in the **RXRXFL** recognition motif. Processing of PSST at the C-terminus to SST-14 and SST-28 does not appear to require initial processing of PSST to $PSST_{[1-10]}$.

Earlier work postulated that antrin is synthesized by initial cleavage of PSST at the -Lys^{13} ↓ Ser^{14} peptide bond to generate $PSST_{[1-13]}$, which would then be C-terminally trimmed through the action of a carboxypeptidase to $PSST_{[1-10]}$ (15-17). Such a processing could conceivably be generated by a furin-like activity. However, these types of convertases preferentially cleave substrates having a monobasic Arg as opposed to Lys at the P1 position (23, 24). To test this hypothesis expression of furin was accomplished using a vv recombinant expression to induce graded increase in doses of furin expression in COS-7 cells (25). The protease demonstrated dose dependent ability to cleave SST-28 but was unable to effect monobasic cleavage of rPSST to $PSST_{[1-10]}$, suggesting the

possible existence of another monobasic convertase with preference for Lys rather that Arg at the P1 position to account for antrin production. Similarly when LoVo cells, a human adenocarcinoma cell line expressing an inactive form of furin resulting from a point mutation (26), were infected with rPPSST to study the possible role of furin in amino terminal processing, antrin was still detected in these cells and represented 15% of total immunoreactivity intracellularly (25). Additionally, co-infection of furin in these cells along with rPPSST did not alter the pattern of antrin production thereby excluding furin as the endopeptidase implicated in this putative monobasic cleavage. Therefore in this study we decided to carry out a more stringent test for the role of the furin-like PCs in the processing antrin at the postulated Lys^{13} cleavage site by replacing it with an Ala residue. $PSST_{[1-10]}$ cleavage was not effected by the mutation suggesting the role of another class of enzymes, other than the already characterized basic-amino acid specific PCs with a likely non-basic cleavage specificity.

Monitoring the biosynthesis and the post-translational processing of the SST precursor to $PSST_{[1-10]}$ by using vv expression system in both HEK-293 and COS cells overexpressing hSKI-I resulted in enhanced production of antrin particularly detected in the medium. On the other hand in mutant CHO cells lacking SKI-1 [SRD-12(-)] reduced but detectable levels of antrin were generated with reversal of the defect upon transfection of WT hSKI-1. Similar results were observed when ATF6 was transfected into SKI-1 deficient CHO cells, implying that the requirement for the enzyme is partial (10). Since cleavage was not completely abolished in SRD-12(-) cells, the possible existence of another enzyme in addition to SKI-1 capable of cleaving PSST at

hydrophobic residues remains open. Currently we are in the process of identifying such an enzyme.

The conlusions reached from the overexpression studies are supported in the mutagenesis studies performed in AtT-20 cells. With regard to the cleavage specificity, it appears that SKI-1 cleaves PSST C-terminal to a Leu residue, and the presence of an Ala at this position does not hinder the processing ability of SKI-1. However, a Pro at the P1 position significantly decreased the amount of antrin production. Surprisingly, both the arginines at the P4 and P6 positions are equally important for PSST cleavage. This finding is novel because it reports for the first time the importance of an Arg residue at the P6 position in addition to the well characterized P4 (5-11). Such results predict that SKI-1 participates in PSST cleavage at a motif containing a P6 and P4 Arg, P2 Phe and P1 Leu residue. Like the combination of PCs and carboxypeptidases E and D (27), a specific carboxypeptidase is then required to trim out the newly exposed C-terminal Leu residue. Recently, additional carboxypeptidases have been discovered in the human genome with cleavage specificity at hydrophobic Leu residue (28). A logical question to ask next is: what is the physiological significance of antrin processing?

PSST processing at its C-terminus is mediated by PC1/PC2 to produce SST-14 and, SST-28 results though the action of furin/PACE4 (13, 14). A possible role of antrin is its potential inhibitory activity towards furin/PC1/PC2, i.e. its presence may prevent the formation of SST-14 and SST-28. To test this hypothesis the products of C-terminal PSST processing from cell extract and media of transfected AtT-20 cells with mutant PPSST cDNA in which L11P and R6/R8A were fractionated by RP-HPLC followed by SST-14 LI RIA of the eluting fractions. In the case of the two mutants they were both

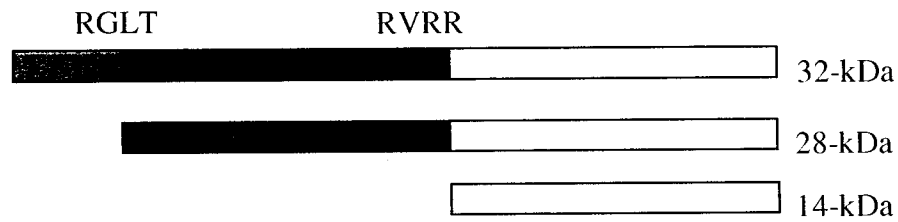
capable of cleaving PSST to its biologically active forms without displaying any changes in the pattern of precursor processing compared to WT, despite dramatic reductions in antrin production. This implies that the generation of SST-14 and SST-28 doesn't require initial processing of PSST to $PSST_{[1-10]}$ and that the NH_2-terminal segment does not seem to play a role in regulating the extent of C-terminal processing. Once secreted from the cell SST-14 and SST-28 actions are mediated by a family of G-protein coupled receptors (GPCRs) with five subtypes SSTR1-SSTR5 characterized to date (30). Since antrin is released into the medium and its secretion can be stimulated through forskolin treatment (16), it might play a biological role through the activation of GPCRs. However, experiments addressing such a question haven't been extensively characterized. On the other hand we have recently demonstrated a clear role for the PSST NH_2-terminus in sorting to the RSP (18).

Secretory proteins are synthesized as precursors on ribosomes in the ER. Once folded properly they are transported to the Golgi apparatus passing through its compartments until they reach the TGN. Therein, secretory proteins are either targeted to the CSP that transports proteins to the cell surface by bulk flow or the RSP; which delivers secretory proteins in response to specific stimulus (31,32). Sorting to the RSP is an active process and one of the basic requirements for such a process is believed to include a sorting motif (33, 34). The sorting motif resides in discrete segments for each polypeptide which can be permanent or transient features of a protein (35). These segments are characterized by their redundancy, shared by many structurally otherwise different proteins whose common denominator is an identical route within a cell. Amino acid analysis of 15 prohormones including PSST that have been shown to be correctly

sorted into the RSP in AtT-20 cells lead to the discovery for the first time of a motif consisting two leucine residues separated by three amino acids that is shared by them all (36). In the case of PSST it is well established that the prohormone undergoes regulated release and several studies have shown that the prosegment of PSST harbors a sorting signal (37, 38). Molecular modelling of rPPSST revealed an α-helix at residues 5-19, containing antrin, with the side chains of residues **Leu**^{7}, Phe^{10}, **Leu**^{11} and Leu^{15} forming a contiguous hydrophobic patch on the helix surface The participation of the $PSST_{[1-19]}$ stretch in sorting of SST directly to the RSP along with the importance of the two Leu residues was characterized by constructing deletion, insertional and point mutations (18). A similar motif was experimentally proven to be important for targeting PC1, a protease whose activity is largely confined to dense core RSVs in neuroendocrine cells, to the RSP as well (39).

Interestingly, pro-BDNF and PPSST are the only two proneuropeptides described to date that are cleaved by SKI-1. Like all precursor proteins, pro-BDNF is synthesized as an inactive preproprotein that is cleaved at the NH_2-terminus in the ER by a signal peptidase producing pro-BDNF producing, a 32 kDa protein. Subsequently a second cleavage at the NH_2-terminus as well is mediated by SKI-1 in the ER/cis-Golgi compartment producing a 28 kDa protein (2, 5, 40) (Fig. 8A). On the other hand the biologically active form is produced through C-terminal processing believed to be mediated by either furin and PC1 with the generation of mature BDNF not requiring initial processing of pro-BDNF to the 28-kDa form as well (2). The biological role of the 28-kDa is not yet known but proper sorting towards the RSP may lie within the pro-domain. Such a domain hasn't yet been extensively investigated in the case of pro-BDNF

A)



B) P[1]MKEANIRGQGGLAYPGVRTHGTLESVNGPKAGSRGLTSLADTFEHVIEELLDEDQKVRP[69]

hhhhhhhhhhhhhhhhhhhhhhh

C)

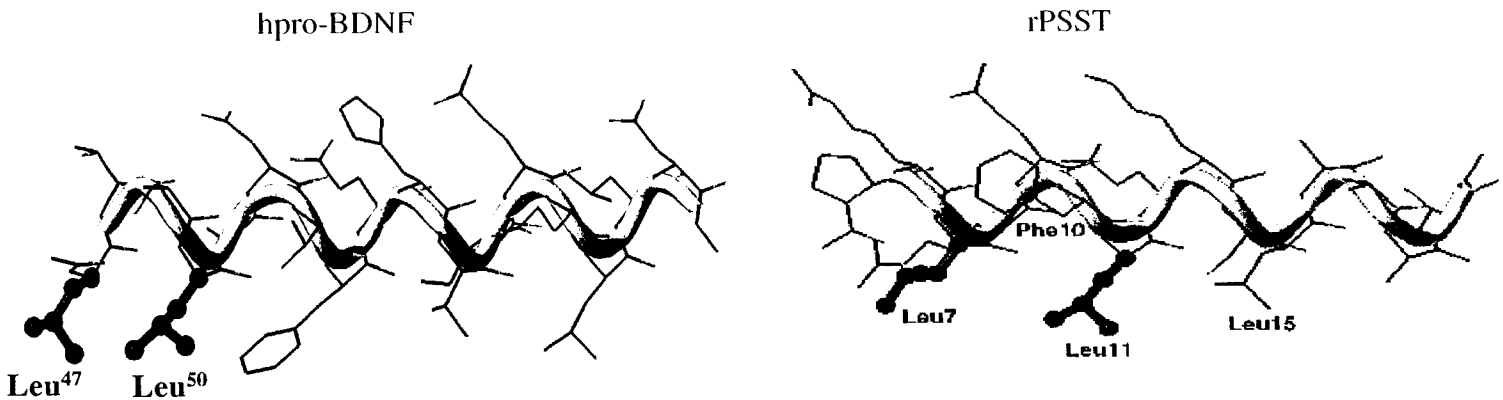


FIGURE 8: A) Schematic diagram illustrating the cleavage products of hpro-BDNF, highlighting the similarities between it and PSST. B) Consensus secondary structure prediction for hpro-BDNF Pro^{1} to Pro^{69}. h denotes α-helix. Leu residues possibly implicated in targeting are represented in bold. C) Structural model comparing $rPSST_{[5-19]}$ (left) and hpro-BDNF (right) both forming an amphipathic helical structure at the NH_2-terminus of the molecule from.

but similar to PSST, molecular modeling of pro-BDNF NH_2-terminus reveals an α-helix at residues 47-64 with two hydrophobic $Leu^{47}$and Leu^{50} residues facing the same side of the helix surface (Figs. 8B and 8C).

A definite conclusion however is that the compartment in which antrin is produced must be very critical, otherwise loss of the targeting segment early along the secretory pathway will lead to failure in proper sorting. Therefore regulation of targeting to the RSP in the case of PSST may also be regulated by amino terminal cleavage, in addition to its role as a sorting motif. For instance premature antrin processing prior to entry into the Golgi can influence proper PSST targeting. To replicate such a situation we have previously created a deletion mutant in which the Ser^3 to Leu^{15} residues were removed followed by stable transfection into AtT-20 cells (18). The resulting mutation diverted PSST and its processed forms to the CSP observed by the absence of secretagogue responsiveness with lack of SST-14 LI staining in punctate granules. Overall, the current data expands the substrate specifity for SKI-1 to include the NH_2-terminus of PSST. Surprisingly we found that PSST NH_2-terminus cleavage does not take place at a basic site, but most likely at the -$\mathbf{R}^6$-L^7–$\mathbf{R}^8$-Q^9-$\mathbf{F}^{10}$-$\mathbf{L}^{11}$↓ recognition motif with the additional importance of an Arg at the P6 position. SKI-1/S1P partially participates in antrin production along with other convertases that are yet to be determined. Once translated rPPSST passes through an orchestrated series of events that are important for its proper targeting to the RSP. Additionally processing to the mature forms of PSST is independent of NH_2-terminal processing.

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CHAPTER D

A yeast two-hybrid system used to identify a potential interacting receptor for the regulated secretory pathway.

Abstract

Generally neuroendocrine cells contain two pathways the regulated secretory pathway (RSP) and the constitutive secretory pathway (CSP). The CSP is used for membrane renewal and passive secretion of proteins that are not responsive to secretagogues. The RSP is used to store prosomatostatin (PSST) and other proteins in secretory granules to be subsequently released in response to appropriate stimuli. Both pathways initially meet in the rough endoplasmic reticulum (ER) where proteins are synthesized and eventually transported to the *trans*-Golgi (TGN) compartment. Here proteins segregate to different pools of vesicles by as yet a poorly defined mechanism. One theory postulates the possible existence of a sorting receptor for the RSP that interacts with regulated secretory proteins. The membrane bound form of carboxypeptidase E (CPE) has been shown to exhibit characteristics of a sorting receptor and is implicated in sorting of a number of neuroendocrine hormones. Sorting of PSST to the RSP in neuroendocrine cells and its retention in secretory vesicles has been shown to require an amphipathic α-helix composed of two critical leucine residues. In the following study we used the sorting motif in PSST as a bait to screen a human fetal brain library in a yeast two-hybrid assay to isolate possible interacting proteins implicated in somatostatin targeting. 34 positive clones were recovered, 10 of which have similar sequences implying a very strong interaction. The isolated clone appears to be involved in protein folding within the ER. Interestingly, we also find that CPE interacts with the PSST sorting signal in a yeast two-hybrid assay.

Introduction

Most biological processes involve protein-protein interactions mapping promise to reveal many aspects of the complex regulatory network underlying cellular function. Therefore, a common focus among molecular and cellular biologists is the identification of proteins that interact with each other. The recent availability of the complete human genome (1) allows us to list the genes and encoded proteins responsible for executing the genetic program. However in order to understand the cellular machinery, simply listing the proteins is not enough at all, the dynamic interactions between them needs to be delineated, thus making the post-genome era of biology even more exciting.

Traditionally protein interactions have been studied individually through biochemical and biophysical techniques such as western blots, crosslinking, co-fractionation by chromatography and co-immunoprecipitation techniques (2-4) making large-scale screenings difficult and laborious. However, several methodologies have been developed for detecting large-scale interactions, among which include phage display technology (5) and the yeast two-hybrid screening assay originally discovered in 1989 (6) from which one can select a 'molecular needle in a haystack'.

The yeast two-hybrid screening has a proven track record in identifying candidate interacting partners in any given proteome. It is a powerful method for isolating novel proteins that bind to one's favorite protein for the purpose of learning more regarding its cellular function. Since the description of the system more than 3000 articles have been published describing molecular interactions characterized using this experimental approach (7). Successful utilization of the technique included protein interactions involved in the regulation of telomere structure, identification of substrate-kinase interactions, and deciphering certain steps implicated in intracellular signaling (7, 8). Therefore a variety of proteins have been found suitable to study in the yeast two-hybrid

system. However there are proteins found not to be amenable which include transcription factors, extracellular proteins, and membrane associated or transmembrane proteins (7, 8, 9).

Following the introduction of the technique over a decade ago, it has undergone some modifications. For instance, a one-hybrid system was introduced for the detection of protein-DNA interactions (10, 11). Additionally, the three-hybrid system was adopted for the elucidation of RNA-protein interactions which play a pivotal role in cellular processes such as translation, mRNA processing and infection by RNA viruses (12).

The original yeast two-hybrid developed by Stanley Fields, including the preceding modifications of the concept all rely on transcriptional activation-based reporter system (6, 7-9, 10-12). It is basically a yeast-based genetic assay to detect interactions *in vivo* focusing around the concept of restoring transcriptional activation to indicate interaction between proteins. Central to this technique is the fact that transcriptional activators are composed of two physically discrete domains: the DNA-binding domain (BD) and the activation domain (AD) that directs the RNA polymerase II complex to transcribe the gene downstream of the DNA binding site. Two major observations set the stage for the development of the technique. The first was the demonstration that it is possible to generate a hybrid protein that functions as a transcriptional activator by combining domains from two separate proteins (13). The second important observation was the realization that transcriptional activators do not necessarily have to bind to DNA to function rather; they can activate transcription indirectly through interaction with other proteins that bind to DNA (6, 7). Hence by taking advantage of the GAL4 transcription factor (TF) of the yeast *Sacharomyces cerevisiae* required for the expression of genes encoding enzymes implicated galactose utilization, two fusion proteins were created. One protein was fused to the BD of GAL4

and the other to the AD of GAL4. The two hybrids were then co-transformed into a yeast strain harboring appropriate reporter genes containing upstream binding sites. When the fusion proteins are brought into close proximity to each other and if they functionally complemented each other, transcriptional activation of downstream reporter genes takes place, thus implying the occurrence of an interaction (6-12) (Fig.1).

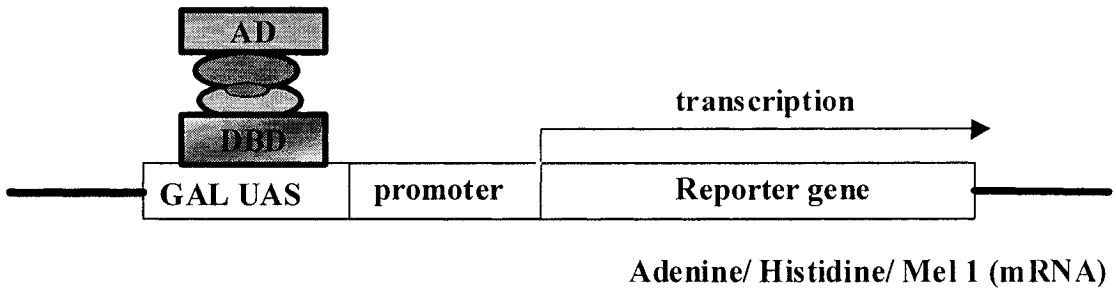


Fig.1 Model of transcriptional activation by reconstitution of GAL4 activity.

Recently, a bacterial equivalent of the two-hybrid system has been reported in *Escherichia coli* providing an alternative approach to screen complex libraries of proteins (14). Additionally, Ray et al. adapted a two-hybrid approach for imaging protein-protein interaction in living mice detected through the activation of the firefly luciferase reporter (15).

Using the yeast-two hybrid system I attempted to characterize candidate proteins (preys) that interact with the prosomatostatin (PSST) sorting signal (the bait) in order to isolate the long sought putative sorting receptor for the regulated secretory pathway (RSP) (16, 17). The TGN acts as the primary operator for protein sorting in the biosynthetic transport pathway. It has been demonstrated that carboxypeptidase E (CPE) controls secretory protein entry into granules in a manner that is completely independent of its enzymatic activity (18, 19). However such a finding is not without problems, in

particular, the original reports indicating an abundance of pro-insulin rich secretory granules in pancreatic β-cells of CPE^{fat} mice (20, 21).

Materials and Methods

For this study, I used the MATCHMAKER two-hybrid system (Clontech, Palo Alto, CA) which I set up in the lab.

Strain and growth media. Yeast strains used are AH109 and Y187. Growth media used were yeast extract, peptone, supplemented with 0.2% adenine hemisulfate and 2% glucose or, synthetic dropout medium containing the appropriate carbon source and amino acids.

Constructing the bait, transforming yeast, and assessing bait suitability. Two baits were constructed. cDNA for wild type rat preproSST (rPPSST) was constructed in the expression vector pTEJ8. Using rPPSST as template two baits were created by PCR and cloned in the pGBKT7 vector provided by Clontech containing the GAL4 DB. To construct them, two primers were used: a forward and reverse primer. The forward primer was designed to contain EcoR1 (underlined) endonuclease restriction site. The reverse primer contained two stop codons (bold) followed by a BAMH1 restriction site (underlined).

The following are the forward and two reverse primers that bind to rPPSST cDNA for the two baits:

Forward primer 5'-GATCGAATTCGCGCCCTCGGACCCCAGAC-3'

Reverse primer 5'-GATCGGATCC**TCACTA**GGCAGCCGCCAGAGACTTCT-3' PSST[1-19]

Reverse primer 5'-GATCGGATCC**TCACTA**CTCAGACAGCAGTTCTGCCAAG-3' PSST[1-35]

PCR was carried out with 50 ng of PPSST cDNA in 100 μl containing 20 mM Tris-HCl, 200 μM dNTPs, 1.5 mM $MgCl_2$, 6% DMSO, and 2 units of *p.f.u.* (Stratagene) using the following conditions: denaturation at 94° C for 80 s, annealing at 59° C for 50 s, and extension at 72° C for 60 s for 25 cycles followed by extension at 72° C for 10 min. PCR products were separated by agarose gel electrophoresis, the amplified bands electroeluted and purified. After PCR the products were digested to completion with *EcoR 1* and *BamH 1* and the purified fragments were subcloned *EcoR1-BamH 1* multiple cloning sites of pGBKT7 containing the tryptophan (Trp) nutritional marker. Both recombinant plasmids constructed were verified by sequencing of double stranded DNA (University Core DNA Service, University of Calgary, Alberta, Canada).

The bait was then transformed into an appropriate yeast strain AH109 that is a MAT **a** reporter strain containing three reporters (protocol provided by Clontech): HIS3 (coding for histidine), ADE (coding for adenine) and MEL1 (coding for α-galactosidase enzyme). The MEL1 gene product once transcribed produces an enzyme that is secreted into the medium and cleaves X-α-galactose causing yeast colonies to change color to blue (22). The use of three reporters, each under the control of distinct Gal4 upstream activating sequences is designed to help eliminate false positives - a common problem with the assay (7, 8, 9, 23). To select for properly transformed yeast, colonies were plated on 10-cm plates containing complete medium lacking tryptophan with 2% glucose provided as the carbon source. Plates were then placed at 30°C until colonies appeared. This usually takes 2-3 days. Colonies were then selected at random from each transformed plate. A master plate was eventually prepared and kept for up to a month at 4°C for later use.

The bait is then tested for toxicity effects and inherent ability to activate gene transcription. Checking for toxicity was done by comparing the growth rate in liquid culture of cells transformed with the vector/bait *vs* vector alone. If the bait strain culture grew noticeably slower, it implied that the bait must be toxic for the yeast. Checking for transcriptional activity of the bait was done by growing the transformed yeast onto various plates lacking specific nutritional markers with the following combinations each supplemented with 2% glucose:

-Trp/His, -Ade/-Trp, -Trp/X-α-gal.

200 μl of X-α-gal is spread on the medium at 2mg/ml and the plates were allowed to dry for three hours.

Construction of the prey. In parallel, a specific prey is constructed and a pretransformed library is purchased as a fusion to the AD of GAL4 in pACT2 vector coding for the leucine (Leu) nutritional marker. Using mouse preproCPE cDNA cloned in pcDNA 3.1, a prey was constructed of CPE from residues Leu^{16} to Phe^{434} thus excluding the signal peptide and the pro-region. The PCR conditions were the same as previously described. The forward primer contained the Nco 1 (underlined) and the reverse primer Xho 1 (underlined) restriction site with the following sequence:

Forward primer: 5'- GATCCCATGGAGCTCCAGCAAGAGGAC-3'

Reverse primer: 5'- GATCCTCGAGTTATTAAAAATTCAAAGTTTCTG-3'

CPE cloned in pACT2 is then transformed into Y187, a MAT α strain that is compatible for mating with AH109. CPE was also tested for its toxicity and transcriptional effects with a master plate prepared for later use. The pretransformed library used was a human fetal brain matchmaker cDNA library that is cloned in the Xho 1/EcoR 1 cloning site (Clontech).

Screening for interacting proteins. The two baits transformed in AH109 were mated with Y187 containing CPE using a small scale yeast-mating technique provided by Clontech. Incubated -Trp/-Leu/His/-Ade plates were allowed to grow for one weak at 30°C. Diploid colonies were then transformed onto higher stringency selection medium -Trp/-Leu/His/-Ade spread with X-α-Gal to confirm for true positive interactions.

Large scale yeast-mating was preformed for AH109 containing PSST[1-35] and the pretransformed human fetal brain library following protocol provided by Clontech. The cDNA library contains 1.0 X 10^6 independent clones. The mating culture was plated on 120 plates that are 10 cm in diameter wit the following nutritional composition: -Trp/-Leu/His/-Ade. Plates were stored at 30°C for four weeks. Colonies were then streaked on higher stringency selection containing X-α-Gal.

Confirmation of interaction. The positive colonies were further tested to confirm possible interactions of bait and prey.

a) Retest the phenotype: The initial diploid colonies resulting from the mating may contain more than one AD/library plasmid, therefore to eliminate such a possibility the positive clones are restreaked in the following order :

   -Leu/-Trp/X-α-Gal 3 times

   -Leu/-Trp/-Ade/-His/X-α-Gal 3 times

   A master plate is created for each true positive clone, sealed with parafilm and stored at 4°C for up to 4 weeks.

b) Isolate plasmid containing AD/library inserts from yeast following the Clontech protocol. DNA isolated is then introduced into DH5-α *E.*coli cells (Clontech) by electroporation.

c) Clustering of isolates and reducing the number of clones to process: AD/library inserts is amplified through PCR using two primers which anneal to specific regions within the pATC2 vector:

Forward primer: 5'-CTATCTATTCGATGATGAAGAT-3'

Reverse primer: 5'-TGCACAGTTGAAGTGAACTT-3'

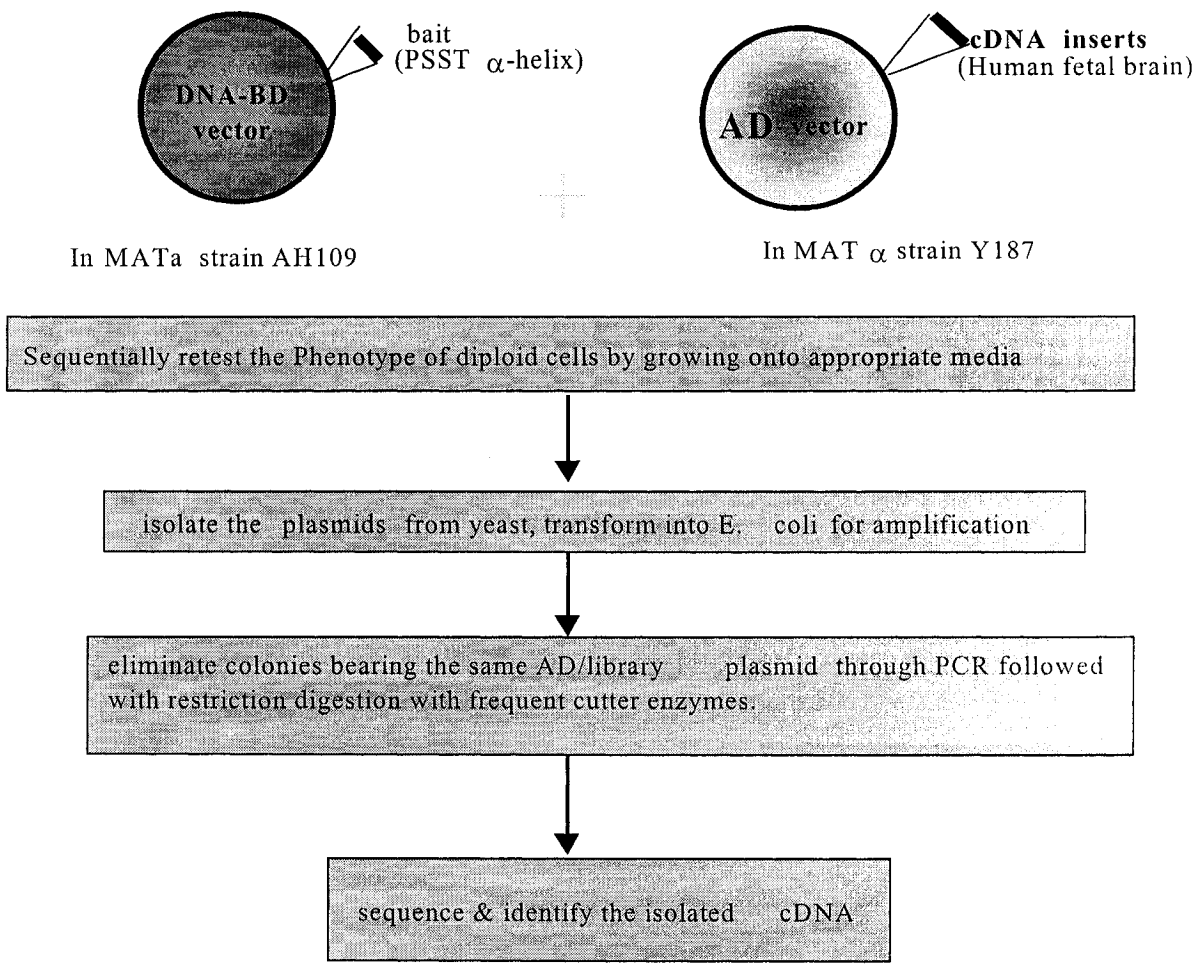
The PCR product is then digested by two frequent-cutter enzymes restriction enzyme Alu 1 and Hae 111. Fragment sizes are analyzed by agarose gel electrophoresis. The colonies are sorted and duplicates eliminated. A representative of each type of insert is sent for sequencing (University Core DNA Service, University of Calgary, Alberta, Canada). Glycerol stocks of each unique type is prepared and aliquots stored at -80°C.

d) Retest protein interaction in yeast: The DNA-DB/bait is transformed into Y187, and the DNA-AD/prey is transformed into AH109. The protein interaction is retested by performing small scale yeast-mating as previously described. The product is plated on -Trp/-Leu/-His/-Ade/X-α-GAL.

Analytical/bioinformatic methods. Sequences received from Calgary were then analyzed using GeneRunner to verify the presence of an open reading frame fused with the GAL4 AD. The resulting sequence was compared to those in GenBank databases at both DNA and protein level. At the DNA level, a standard nucleotide blast search for nearly short sequence matches were done using nonredundant (nr), Expressed Sequence Tag (EST) and High Throuput Genome Sequence (htgs) database. A protein and human genome blast were also performed. ExPASy proteomic tools at us.expasy.org/tools were used to predict many aspects of the translated open reading frame such as transmembrane helices and topology, protein sorting signals and localization sites and, secondary structure predictions.

Fig. 2 is a proposed scheme for verifying protein-protein interactions used in the screening.

Fig.2 Analysis & Verification of Putative Positive Clones



Results/Discussion

Sorting of true positive clones phenotypically.

Once the bait was confirmed not to be intrinsically transcriptionally active, AH109 haploid yeast cells transformed with the first 35 a.a in PSST (used as a bait), were mated with Y187 haploid cells containing a cDNA library isolated from the human fetal brain. Choosing the appropriate bait and library are both very critical for proper selection of interacting proteins (7, 9, 23). The first 35 amino acids in PSST were chosen as the bait because, they contain the sorting signal responsible for targeting PSST to the RSP. Somatostatin is a prohormone that is expressed in both the brain and peripheral tissues where it undergoes regulated secretion by entering the RSP (24, 25). Therefore, choosing a library containing cDNA isolated from brain tissue should appropriately contain a putative sorting receptor. In parallel small-scale yeast mating was also performed for pGBKT7-53 (DB/vector containing p53 fusion protein) propagated in AH109 which was mated with Y187 propagating pTD-1 (a vector encoding AD/SV40 large T antigen). This was chosen as a positive standard control for mating since p53 and SV40 large T antigen are well known to positively interact with each other (26). Following overnight mating, the resulting mixture from large scale mating was plated on 120 agar plates with medium containing the following specificities: Trp/-Leu/His/-Ade. Plates were allowed to grow for 4 weeks resulting in a total of 42 doubly transformed cells which were sequentially numbered. The colonies were then replicated on higher stringency selection medium (Trp/-Leu/His/-Ade/X-α-GAL) to eliminate false positives and to confirm the interaction between bait and prey which resulted in 36 blue colonies. A similar procedure was followed for the controls. Further screening was then carried out to retest the phenotype and to eliminate clones containing more than one AD/library plasmid. At the end of the screen 34 clones were phenotypically positives.

Generally the yeast two-hybrid system has a reputation for producing a significant number of false positives which require cumbersome analysis to separate the true interactions from the false positives (9, 10). There are many stages at which false positives may be isolated and they can include proteins that interact with any bait making it a non-specific and random interaction. Another source of false positives may be mutations or other random events of unknown nature. When mutations happen early during the propagation of haploids or diploids, more false positives may result than at later stages. Another limitation is the possibility of bridging effects (i.e. endogenous proteins can act as bridging factors and therefore imply a direct interaction although, only an indirect interaction takes place). For example, the HIV-encoded protein Rev was first demonstrated to interact with a yeast nuclear protein. Later, the interaction was shown to be mediated by a nuclear export factor that associates with both the yeast and HIV protein (27). Most false positives in conventional library screens cannot be identified without requiring additional experiments. An additional point to address is the possibility of missing an interaction ie. false negatives (7, 28). Reasons for missing an interaction are not clear but may include sequestration of the bait by interacting with endogenous yeast-proteins, steric constraints on bait interaction properties once fused to another protein, or the bait can even misfold. Additionally, in some cases the interaction between bait and prey can be a transient one hence not very strong, making it difficult to be detected in a yeast-two hybrid assay. Other possibilities leading to either false positives or negatives includes changes in folding and stability of the fusion protein once constructed which, could affect its transcriptional activation properties.

PCR amplification, enzyme digestion and DNA sequencing.

Once the positive clones were isolated, their DNA purified, the encoded pACT2 plasmids containing AD/prey from the 34 clones was subjected to PCR amplification. Fig.3 Represents PCR amplification of purified DNA from the 34 clones detected in the yeast two-hybrid screen. Some of the clones appear to have been isolated more than once. In order to confirm such an observation, the DNA was subjected to digestion by Alu 1 and Hae 111 and sent for sequencing using the forward PCR primer used to amplify the original insert. Using Gene runner 3.0 presence of an open reading frame was verified fused to the GAL 4 AD. Clones appearing more than once are the following, with each group representing a different insert:

3 ,6, 14, 19, 20, 30, 32, 40 and 41. (total of 10 clones represented in bold in Fig. 3)

17 and 21.

8 and 9.

35 and 37.

A large enough open reading frame resulting in translation of sufficient amino acids (> 20) fused to the AD was mainly detected for clones represented in bold (Fig.3) appearing 10 consecutive times. We therefore decided to focus on that clone which we will call 3 in the rest of the text particularly since, the interaction appears to be specific and not random. The others, encoded fusions of irrelevant small peptides.

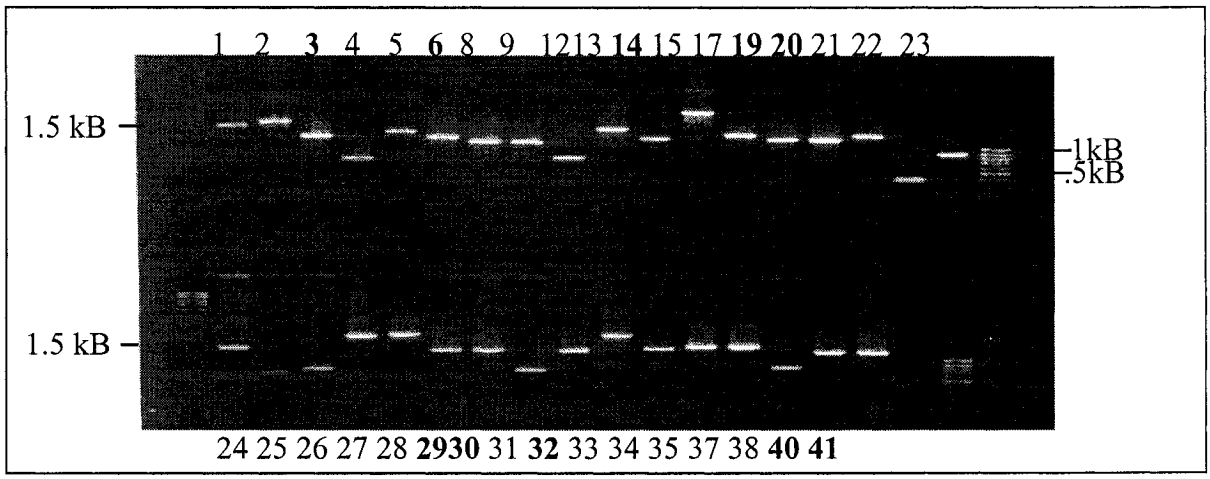


Fig.3 PCR amplification of purified DNA from 34 clones detected by two hybrid screen.

The interaction between the isolated clone 3 and PSST[35] was confirmed by repeating the transformation of PSST[35]/AH109 with clone3/Y187 and by retransforming PSST[35] into Y187, and clone 3 in AH109 followed by small scale yeast-mating. This procedure is carried out to confirm the interaction in multiple yeast strains. Two controls were used a positive one as previously described (Fig. 4A) and a negative control (Fig. 4B). The negative control used was PSST[35]/AH109 mated with Y187/p53 (nothing has been published to date implicating an interaction between the cytosolic protein p53 and SST that is stored intracellularly and is never exposed to the cytosol inside the cell). Blue colonies appeared when clone 3/Y187 was mated with PSST[35]/AH109 (Fig. 4C). Similarly, when the yeast strains were switched blue colonies appeared on Trp/-Leu/His/-Ade/X-α-GAL following a 5 day incubation period (Fig. 4D).

Computer sequence analysis

Protein and DNA blast searches were performed but did not yield any putative identity for the clone however, at the DNA level there were a few leads to work with. First,

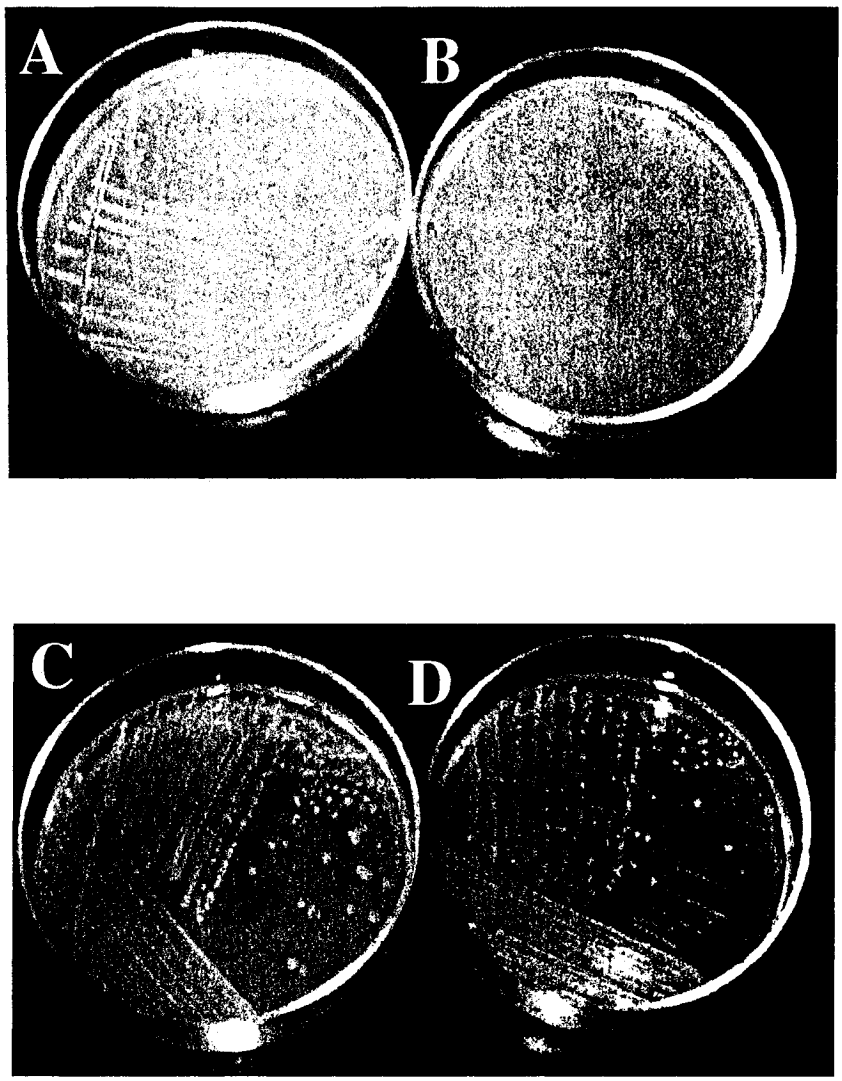


Fig. 4 Mated colonies grown on Trp/-Leu/His/-Ade/X-α-GAL.
A) positive control, B) negative control, C) PSST[35]/AH109 crossed with clone 3/Y187 and D) PSST[35]/Y187 crossed with clone 3/AH109.

blasting the sequence against the human genome demonstrated a homology region corresponding to a segment on chromosome 7 that is close to the centromere. Second nr, EST, and htgs blast search results interestingly demonstrated that the sequence is more or less present in humans and not in any other species such as mouse. We decided to focus our attention on a sequence result from the nr blast-search, which produced significant homology to our clone (99%). The sequence is named Homo sapiens clone 124-1V1 published by Benedetti et al, with the gene bank number AF290475.1 and composed of an mRNA sequence (29).

Clone 124-1V1 was originally identified in an attempt to isolate new genes induced during the unfolded protein response, a condition that can be induced by various treatments such as dithiothretiol (DTT) or by expressing mutant secretory proteins that do not fold properly thus, accumulate in the ER (30, 31). By treating human myelomonocytic U937 cells with DTT two bands were strongly up-regulated, one of which corresponded to 124-1V1. The differential expression of these two bands was then analyzed in two other cell lines: COS-7 and HEK-393 cells. In the case of the 124-1V1 band, no signal was detected in COS-7 cells (a monkey cell line) consistent with the results from the DNA blast performed by our lab. The authors postulated that such an observation could either be due to a lack of expression of this transcript in these cells or differences in the sequences between monkeys and humans. The authors then attempted to characterize the sequence of both bands by screening a cDNA library and using mRNA fingerprinting. In the case of the 124-1V1, no significant open reading frame was found in the coding strand, however two long open reading frames were present in the minus strand. Consistent with our results, the genomic clone for the fragment was also located on chromosome 7 however, the complete sequence of the clone remains to be determined. The authors published their work in 2000 at which point the genome project

was not complete. However, since then the human map has been completed providing clues with regard to the sequence. Attempts to contact and gain more information from the authors regarding the sequence have failed.

In any yeast two-hybrid one isolates a large number of clones and it is always difficult to focus on a single lead hoping that it is the correct one. We focused our attention on a single clone appearing 10 times in our screen because; it implied a strong interaction that seemed to be specific. Blast search results could not reveal an identity for it but clone 3 most likely appears to be involved in some aspects of protein folding. Currently we are attempting to study its distribution in various cell lines HEK-293, COS-7, CHO, and AtT-20 by Northern Blot analysis to confirm the hypothesis that it is only expressed in humans. Eventually, we will work on cloning the full length mRNA through a combination of RACE-PCR technique and screening a human cDNA library. Interaction of clone 3 with PSST will be confirmed by additional tests such as co-imunoprecipitation and colocalization through immunocytochemistry as well.

CPE phetonypically interacts with PSST

The importance of CPE in sorting prohomrones was originally demonstrated through chemical cross-linking experiments performed in order to identify the sorting receptor that interacts with POMC (18). Additionally in mutant mice lacking CPE, POMC was missorted to the CSP (19). To further substantiate CPE as a sorting receptor its depletion in Neuro 2A cells by antisnese RNA resulted in constitutive release not only of POMC but, of insulin and pro-enkephalin. The role of CPE as a sorting receptor was mainly challenged in a study demonstrating that pro-insulin targeting to the RSP in CPE^{fat} mice was not hindered thus making its role as a sorting receptor tentative (20). Despite the fact that we did not isolate CPE in the large scale screening, we attempted to

use the yeast-two hybrid small scale mating technique to test for an interaction between PSST[35] and CPE. Two sets of controls, as mentioned earlier, were mated in parallel (Fig. 5A and B). The resulting mated mixture following was plated on Trp/-Leu/His/-Ade, and incubated for one week at 30°C. Surprisingly, an interaction must have occurred between CPE and PSST[35], since colonies were observed after 5 days. No growth was observed on the negative control plates. In order to further confirm the interaction, colonies were re-streaked on Trp/-Leu/His/-Ade/X-α-GAL (Fig. 5C). Similar to positive control, diploid cells resulting from mating of yeast strain containing PSST[35] and CPE were blue. In order to narrow down the interacting region at the NH2-terminus of PSST, we used the PSST[1-19] as a bait. Interestingly, the results were positive (Fig. 5D). Therefore, in a yeast two-hybrid technique the NH_2-terminus of PSST, containing the amphipathic α-helix alone, interacts with CPE. The role of CPE as a sorting receptor needs to be confirmed by using antisense strategy to monitor the secretory profile of PSST into the medium. Down regulating CPE expression should inhibit any response upon addition of extracellular stimulation, with PSST being primarily localized to TGN. Molecular modeling of CPE can then be carried out to provide a ligand binding pocket for PSST which can be compared to that of POMC (32). A cell membrane assay can then be established to demonstrate specific binding of PSST to CPE. Additionally fluorescence resonance energy transfer (FRET), a technique well established in our lab, can be used to confirm an interaction between CPE and the NH2-terminus of PSST (33).

Conclusions

The yeast two-hybrid system can be used to study interactions between known partners as well as to screen expression libraries for novel interacting proteins. The work presented here started with a combination of both aims because (i) of already published

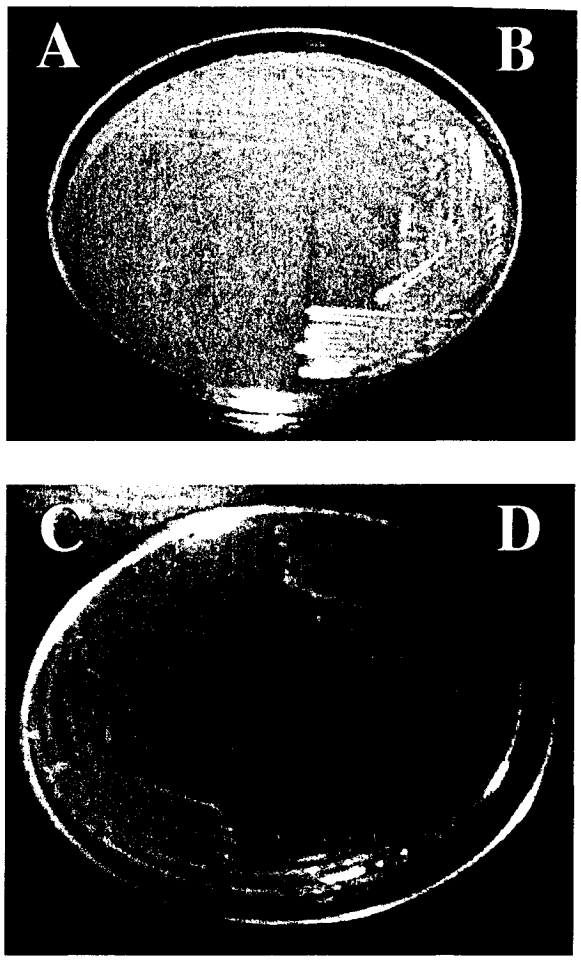


Fig. 5 Results of small scale yeast-mating.
A) Negative control, B) Positive control,
C) PSST[35] mated with CPE
D) PSST[19] mated with CPE.

data demonstrated a role for CPE in targeting of a number of prohormones and (ii) we were engaged in screening a human fetal brain cDNA library with PSST bait plasmids. The screening produced a candidate interacting protein that has been reported elsewhere to be involved in protein folding.

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CHAPTER E

DISCUSSION

DISCUSSION

Having addressed the three questions proposed in the introduction surrounding the status of the NH_2-terminus, significant progress has been made elucidating its functional role in targeting of PSST to the RSP. The identity of the enzyme implicated in antrin production along with the cleavage site was characterized as well. Additionally, as proteins function exclusively by means of interaction with other molecules, the systematic and comprehensive analysis of protein-protein interactions with the NH_2-terminus was carried out by using the yeast-two hybrid technique as a way to provide us with valuable information for better understanding its function.

Taking a trip along the RSP is a special privilege used by certain peptide hormones and neuropeptides. Once folded properly in the ER, prohormones are given the passport to exit from this quality control compartment with their ultimate destination the secretory granules that bud from the TGN releasing their content in a stimuli dependent manner. These granules play several essential roles in animal physiology related to communication, digestion and defense. This path, the RSP, is not present in all cell types rather only in professional protein secreting neuroendocrine cells. One of the basic requirements for such a process is believed to include a sorting motif. Using the PPSST molecule as a model, in chapter B, I mainly focused on the targeting role of the NH_2-terminus to the RSP. Previous experiments have already shown that heterologous expression of AF PPSST in AtT-20 cells resulted in regulated release of the PPSST I precursor product, which is exclusively SST-14, a peptide identical to rSST-14 (222). However, AtT-20 cells transfected with PPSST II gene resulted in constitutive release of SST-28, the cleaved product of PPSST II. Missorting of PPSST II precursor was

corrected when a hybrid protein containing the leader sequence and a portion of the pro-region of rPPSST was fused to the C-terminus of PPSST II. Later, experiments in which the first 82 amino acids of the pro region of PPSST were fused to α globin protein, a protein that does not undergo regulated release, resulted in successful targeting to the RSP in GH3 cells (223). Nevertheless, no specific region or sequence of this broader area has yet been identified as carrying sorting information. Through molecular modeling of rPPSST, an α-helix at residues 5-19 with the side chains of residues Leu^{7}, Phe^{10}, Leu^{11} and Leu^{15} forming a contiguous hydrophobic patch on the helix surface was revealed. This domain is highly conserved in all known vertebrate PPSST molecules as well as in the SST-related precursor PPCST (where it is located not at the N-terminus but further downstream at residues 19 to 35). Additionally, the secondary structure predictions of a dozen other prohormones that are known to be targeted to the RSP also revealed a common amphipathic α-helix similar to that in PSST which qualifies as a putative sorting signal (216). The participation of the $PSST_{[1-19]}$ stretch in sorting of SST to the RSP was therefore studied by constructing deletion, insertional and point mutations. Making a deletion mutant in which the Ser^{3} to Leu^{15} residues were removed caused PPSST to enter the CSP. Similar results were observed in the case of two other mutants in which the Lys^{13} residue was substituted with RTKR, a classic furin motif, or the dibasic motif KR to enhance N-terminal PSST cleavage endogenously by furin or PC1/PC2 respectively. Enhancing cleavage might have caused detachment of the targeting motif thereby hindering the ability of PPSST to be properly guided in the cell. Another possibility for the missorting is that insertion of extra basic residues at Lys^{13} disrupted the α-helix. Thus removal of the PPSST N-terminal α-helix by mutagenesis, or endogenously by

endoproteolysis both resulted in precursor missorting. Having identified the importance of the NH_2-terminus in sorting, detailed alanine scanning mutagenesis identified the region Pro^5 to Gln^{12} as being important in precursor targeting with Leu^7 and Leu^{11} being critical. These results complement the modelling data and suggest that these two residues are located in close proximity on the same hydrophobic surface of the α-helix providing a potential binding interface for interaction with a putative sorting receptor.

In secretory cells, peptide hormone production is a multistep process involving translation of mRNA encoding large molecular weights forms followed by processing. One of the ways the cell increases diversity of its products resides in its ability to generate multiple peptides via site-specific proteolysis of a single parent precursor. The detailed knowledge of PPSSTs simple structure and of its processing fragments made it a particularly interesting model to study the formation of multiple hormone products by differential processing of a single polyfunctional precursor. In order for PSST to exert its biological activity it must undergo processing at two different basic cleavage sites, within the C-terminus, producing SST-28 and SST-14. Knowledge surrounding the PCs implicated in such processing has been extensively characterized (35a-c, 329-334) however; the question of where proteolytic processing of prohormones takes place is a long-standing one. Furin and/or PACE4 effect monobasic cleavage of SST-28, whereas SST-14 is produced through cleavage by PC1and/or PC2 (35a-c). Based on the enzymes acting on PSST one would expect that SST-28 production takes place in the Golgi because furin is fully active in the TGN, whereas the cleavage compartment of SST-14 is questionable. The two organelles in question are the TGN or secretory granules.

Earlier morphological studies have implicated immature secretory granules as the site of prohormone processing (253, 336) however, subsequent evidence has suggested that it can also occur earlier along the secretory pathway within the TGN (339-341). Pulse chase experiments in hypothalamic neurons suggested that production of SST-14 was an early event, appearing as early as 15 or 20 min of pulse (33, 34). Similarly, studying the HPLC profiles of COS-7 cells transfected with rPPSST and PC1 demonstrated a significant increase in SST-14 processing compared to cells transfected with PPSST alone (35a). Additionally SST transfected $1027B_2$ cells, a cell line derived from rat islet D-cells that lost its ability to concentrate SST intracellularly in vesicles, retained their ability to process rPPSST to SST-14 and SST-28 (345). In chapter B, I attempted to elucidate the intracellular compartment in which PPSST is processed to SST-14 and SST-28 by characterizing the effects of precursor mistargeting on C-terminal processing. Blockade of PSST targeting to secretory granules by the N-terminal deletion or through alanine point mutations resulted in an escape of large quantities of 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. Such results confirmed previous predictions and indirectly indicated that PPSST processing can be initiated in the TGN and is not restricted to granules.

The best *in vivo* example demonstrating the ability of PPSST to be cleaved in the absence of granule requirement stems from its expression in immune cells, well known not to contain a RSP or even specialized secretory granules (367, 368). The SST content in lymphoid organs (spleen and thymus) of male rats was studied by using an anti-SST antibody recognizing both peptide forms SST-14 and SST-28. mRNA and sequential

immunostaining of rat spleen using SST antibody and subsequently a monoclonal antibody against a rat B-cell surface antigen revealed the presence of SST immunoreactivity in some, but not all, B cells. Sequential immunostaining of rat thymus revealed the presence of SST immunoreactivity in a small population of T lymphocytes in the medulla. Additionally activated macrophages isolated from granulomas of *Schistosoma mansoni*-infected mice (granulomas are the sites of intense inflammatory reactions; they encompass the parasite eggs and infiltrated lymphocytes, neutrophils and macrophages) produced SST-14 (368). Therefore, the role of secretory granules is puzzling, is it possible that they are merely a storage deposit for stored hormone that release their content upon cell stimulation?

In addition to C-terminal processing of the SST precursor, another product originally isolated from the endocrine portion of the stomach corresponding to the first 10 a.a. of PSST was isolated from rat in 1987 (36). The highest concentration of the decapeptide at the time was found in the gastric antrum thus the proposed name antrin. Additionally, processing of prohormones was believed to take place at basic residues only, hence a misinterpretation resulted in the general proposed mechanism by which antrin was produced (36, 60-62). Scanning the NH_2-terminus for basic residues, $PSST_{[1-10]}$ would be produced by initial cleavage of PSST at the -Phe^{10}-Leu^{11}-Gln^{12}-**Lys^{13}** ↓ **Ser^{14}** peptide bond to generate $PSST_{[1-13]}$. Following the action of the PC endopeptidase, a carboxypeptidase would then be required to trim out the C-terminal residues from the processing intermediate resulting in $PSST_{[1-10]}$. Despite the general acceptance of this model there are a few problems with it. First, the carboxypeptidase would be capable of removing the C-terminal basic Lys^{13} residue however, there is nothing published in the

literature describing a carboxypeptidase capable of removing the Gln^{12} residue. Second, generally PCs prefer to cleave substrates at a monobasic Arg residue as opposed to Lys residue (268); there is no substrate published to date that is produced through processing C-terminal to a monobasic Lys↓.

Previously we have attempted to characterize the enzyme responsible for antrin production. Furin represented the most likely candidate due to its preferential cleavage of substrates C-terminal to monobasic residues (35b). However our attempts failed and, the other PCs (PC1, PC2, PC4, and PC5) were not required as well since $PSST_{[1-10]}$ is produced in ample amounts in LoVo cells which are deficient in all of these enzymes. In chapter C, I decided to carry out a more stringent approach to test the requirement of the Lys^{13} cleavage site by mutating it to an Ala residue. Surprisingly, $PSST_{[1-10]}$ cleavage was not blocked and such a mutation produced no significant changes in antrin processing compared to *wt* cells. At the same time a new enzyme was cloned that is capable of cleaving C-terminal to hydrophobic residues and was called SKI-1/S1P making it the first and only enzyme up to date with such specificity (354, 355). This enzyme became an attractive candidate for cleaving the NH_2-terminus of PSST since, the consensus sequence in that region was found to be homologues to the recognition motif of the recently discovered protease.

Overexpression experiments of SKI-1/S1P in HEK-293 and COS-cells significantly increased the amount of antrin detected in the medium, making it a likely candidate enzyme. Additionally, detailed mutagenesis experiments demonstrated that efficient processing of PSST to antrin required at least two Args in the **RXRXPL** recognition motif. PSST represents the first substrate that requires both Args at the P4

and P6 position for proper processing. In contrast SREBP, pro-BDNF, ATF6 and the Lassa virus GP only require an Arg at the P4 position (354, 355, 365, 366). Interestingly in mutant CHO cells, lacking SKI-1, reduced but detectable levels of antrin were generated with reversal of the defect upon transfection of WT hSKI-1. Therefore the requirement for SKI-1/S1P is not absolute, with the possibility of another enzyme cleaving at hydrophobic residues highly likely. The need for the identification of more proteinases responsible for such processing is important, especially since they could play major roles in regulating neurodegenerative disorders such as Alzheimers disease.

Cleavage at the NH_2-terminus has been documented to take place in higher species such as humans, mouse and rat. Interestingly in lower species such AF which code for two SST cDNAs, the AF PPSST I cDNA resulting in SST-14 production contains the NH_2-terminal cleavage site for antrin that is absent in AF PPSST II cDNA, responsible for AF SST-28 production (39, 40a-b). With regard to the second SST related gene PPCST that has been cloned from rat, mouse, and human, details of its processing have only focused around the C-terminus with dibasic cleavage resulting in one or two biologically active products depending on the species (37, 48, 49). Nothing has been published yet addressing any NH_2-terminal cleavage products. So, Why does the cell have the need to cleave PSST to $PSST_{[1-10]}$.

Once processed our lab has demonstrated earlier that it is released from the cell into the medium and its secretion can be stimulated through forskolin treatment (60-62). In the medium it might play a biological role through the activation of GPCRs however, such studies haven't been carried out. A possible role of antrin could be its potential inhibitory effects towards furin/PC1/PC2 whereby its presence may prevent the formation

of SST-14 and SST-28. Such inhibitory effect of prosegments has been well documented for PCs making it a way for the cell to regulate biological activities of proteins (291, 292, 308, 325). We tested such a hypothesis by monitoring the products of C-terminal PSST processing from cell extracts and media of transfected AtT-20 cells with either two mutants of PPSST cDNA which displayed severe inhibition in antrin processing; L11P and R6/R8A. Results demonstrated that antrin cleavage did not represent an obligatory step in the generation of SST-14 and SST-28 making the pro-segment in PSST an unlikely inhibitor for PCs. So, how can one link the processing event at the NH_2-terminus to the targeting role for PSST!

Interestingly pro-BDNF and PPSST are the only two proneuropeptides cleaved by SKI-1, whereas the other substrates cleaved by SKI-1 do not enter the RSP. Pro-BDNF is a neurotrophin that is synthesized as a high molecular weight precursor containing a pro-domain (369). It is stored within dense core secretory vesicles and is released from the cell in response to extracellular stimuli. A definite conclusion however is that the compartment in which antrin is produced must be very critical; otherwise loss of the targeting segment early along the secretory pathway will lead to failure in proper sorting. Therefore regulation of targeting to the RSP in the case of PSST may also be regulated by amino terminal cleavage, in addition to its role as a sorting motif. For instance premature antrin processing prior to entry into the Golgi can influence proper PSST targeting. To replicate such a situation we created a deletion mutant in which the Ser^3 to Leu^{15} residues were removed followed by stable transfection into AtT-20 cells. The resulting mutation diverted PSST and its processed forms to the CSP observed by the absence of secretagogue responsiveness with lack of SST-14 LI staining in punctate granules.

It still remains unclear how prohormone processing and their enzymes are packaged and sorted at the level of the TGN. Association of proteins with a receptor is likely to be one of several mechanisms whereby proteins are targeted to dense core granules, however the search for such a receptor has been very frustrating. As protein interactions are an essential part of all life, we attempted to use the yeast two-hybrid technique to isolate a putative sorting receptor. With the completion of the human genome, dramatic changes have taken place facilitating the screening process. Our screening resulted in the isolation of a candidate interacting protein that has been reported elsewhere to be involved in protein folding with the name 124-1 (370). The full-length cDNA of the isolated clone has not been reported yet however, we are in the process of cloning it. Ultimately the biological significance of such an interaction needs to be confirmed and possible implications in SST folding need to be tested. Having been unsuccessful in isolating a potential receptor through the yeast two-hybrid screening process, I then attempted to directly test whether or not the SST sorting motif interacts with CPE.

CPE has been reported as a sorting receptor in the TGN that is exclusively present in the Golgi and secretory granules of neuroendocrine cells (226). Surprisingly, CPE was found to interact with the PSST sorting signal in a yeast two-hybrid assay. More work is obviously required to confirm the role of CPE in targeting of PSST however, is a sorting receptor sufficient by itself to sort such concentrated amounts of hormones to the RSP? If CPE is actually a sorting receptor it would certainly have to display remarkable properties one of which is the binding stoichiometry. Prohormones are known to be remarkably concentrated in granules reaching 60mM for certain prohormones thus

requiring large numbers of receptor molecules per unit membrane. Is there enough receptor in the TGN to carry out such a role? Such concentrations are required for the quantal release of large amounts of protein for each single exocytic event; a hallmark for the RSP. Even if each receptor was able to bind many cargo molecules it will also need to have broad specificity to accommodate all types of hormones entering the RSP. Therefore, since no receptor is sufficiently abundant to sort regulated secretory proteins with a 1:1 stoichiometry, peptide hormones can take advantage of their tendency to self-aggregate.

Self-organization or aggregation at the level of the TGN is another alternative to sorting (183, 184, 189). In brief, it is proposed that within the lumen of the TGN, proteins destined for the RSP aggregate in the face of mild acidity and high calcium concentrations encountered in this compartment. In an attempt to test the requirement for the amphipathic SST α-helix alone in targeting to the RSP, we created chimeric constructs of the fluorescent proteins GFP and DsRed fused with the SST signal peptide and the sorting motif. Generally, once GFP and DsRed are introduced into cells they display no preferential localization to any compartment making them an attractive technology to study intracellular protein localization (371). Surprisingly, in cells transfected with the signal peptide of SST alone fused to either GFP or DsRed the fluorescence accumulated in the TGN areas and the tips of the cellular processes where secretory granules accumulate and in vesicular structures distributed throughout the cell. Therefore, once allowed to enter the secretory pathway both molecules are capable to enter the RSP without requiring help from any sorting motif of another protein in endocrine cells. I then attempted test the ability of the PSST sorting signal to target

insulin like growth factor protein-1 (IGF-1) that is mainly synthesized in liver hepatocytes which do not contain any regulated sorting machinery (372). Similar to GFP/DsRed, once transfected into AtT-20 cells, IGF-1 was capable of entering the RSP without requiring any aid from the PSST sorting signal (data not shown). The presence of disulfide bonds in IGF-1 can explain its regulated release in endocrine cell lines. In the case of GFP, the lab of Gorr SU attributed its sorting ability to the formation of oligomers (a form of aggregation) through disulphide bond formation within the secretory pathway that alters the intracellular sorting of such proteins (373). Additionally, the role of aggregation through disulfide bond formation has been shown to be involved in the sorting of the granin family of proteins and POMC.

In light of the foregoing discussion, regulated secretory proteins may require a combination of both models whereby they associate with the membrane TGN receptor in aggregates.

And so progress has been made regarding certain sorting and processing aspects in this efficient and highly regulated factory we call a cell with some surprises discovered along the way however, much is still left unanswered. Does sorting for the RSP really only begin at the level of the TGN? Protein transport from the ER to the Golgi has been shown to be a regulated process requiring receptors and sorting motifs for soluble proteins. Additionally, it has been demonstrated that the Golgi is not a static, rather dynamic structure. If the Golgi cisternae are continuously moving forward from the cis- to trans-face then proteins should have an idea of where to localize along the Golgi membrane prior to entry into the TGN.

Beyond the pure biochemical and basic research interest in defining the specific intracellular events implicated in processing and sorting of PSST, combining the knowledge gained from the processing and targeting aspects to treatments of various diseases would be the ultimate satisfaction of any scientist. Such an approach was elegantly attempted by Rivera and colleagues in 2000 who used the ER as the storage compartment for genetically engineered secretory proteins such as insulin and growth hormone (374). The rationale for their approach was to develop a mechanism capable of faster delivery of such prohomones in a brief pulse that lasts ~4 hrs as opposed to a much slower response. They created a fusion protein that is linked to the therapeutic hormone insulin which aggregates and is retained in the ER, possibly because sorting motifs essential for ER export are masked. Aggregates of the recombinant protein dissolve upon binding a small, membrane-permeable drug (that can be added exogenously to cells), and export of the protein begins to the Golgi. The recombinant protein is designed to contain a furin cleavage site separating insulin from the protein that it is attached to. Therefore once in the Golgi, the recombinant protein is cleaved by furin releasing mature insulin that is then rapidly secreted. This led to a striking temporal control of serum glucose levels that mimicked the natural response to elevated blood sugar in a mouse model of hyperglycemia. The Rivera study clearly demonstrated that the compartments of the secretory pathway and their regulated sorting sites can be adapted to achieve rapid and efficient secretion of engineered therapeutic proteins. Such a technology may provide a simple platform from which to regulate secretion of therapeutic proteins in any type of cell.

CHAPTER F

CONCLUSIONS AND CLAIMS FOR ORIGINALITY

Conclusions and claims for originality:

1- The amino terminal region of PSST forms an amphipathic α-helix with the region Pro^{5} up to and including Gln^{12} being critical for precursor targeting to the RSP. Two residues are of particular importance, Leu^{7} and Leu^{11}.

2- Blocking targeting of PSST to the RSP does not impair the ability of PCs, most likely furin and PC1, to cleave the precursor to SST-14 and SST-28.

3- Other labs and I, at the same time, have demonstrated that fluorescence proteins such as GFP and DsRed contain inherent ability to be properly targeted to the RSP once allowed to enter the secretory pathway through attaching a signal peptide at their amino terminus. Therefore, they can not be used to study intracellular protein trafficking in cell lines containing regulated secretory machinery.

4- Contrary to previous belief, aminoterminal cleavage of PSST is not mediated by a PC with basic cleavage specificity. Additionally, processing does not take place C-terminal to a monobasic Lys^{13} residues.

5- Vaccinia Virus infections of COS-1 and HEK-293 cells demonstrated that SKI-1 participates in antrin production thus, making SST the fifth substrate described to date for SKI-1.

6- In mutant CHO cells expressing a defective SKI-1 enzyme, antrin production was not inhibited but markedly reduced. Therefore the possible existence of another enzyme, in addition to SKI-1, capable of cleaving PSST at hydrophobic residues remains open.

7- Mutagenesis studies show that efficient processing of PSST to antrin requires the **RXRXPL** motif. Such is the first report for SKI-1 requiring an Arg at the P6 position even when the Arg at P4 is still present.

8- HPLC results demonstrate that inhibiting antrin cleavage does not impair SST-14 and SST-28 production thus, amino terminal processing is not a prerequisite for C-terminal cleavage by furin, PC1, and PC2.

9- In a yeast two-hybrid screening assay the SST sorting motif strongly interacts with both clone 124-1V1 implicated in protein folding and, CPE.

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