Université de Montréal

Mécanisme de ciblage des prohormones convertases vers les granules de sécrétion denses

par
Dimitrios Dikeakos

Département de Biochimie
Faculté de Médecine

Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de Ph.D.
en Biochimie

Juin, 2008

Université de Montréal
Faculté des études supérieures

Cette thèse intitulée:

Mécanisme de ciblage des prohormones convertases vers les granules de sécretion denses

présentée par :
Dimitrios Dikeakos

a été évaluée par un jury composé des personnes suivantes :

Guy Boileau, président-rapporteur
Timothy L. Reudelhuber, directeur de recherche
James G. Omichinski, co-directeur
Sylvie Mader, membre du jury
Geoff Hendy, examinateur externe
Serguei Chteinberg, représentant du doyen

Résumé

Les cellules endocrines et neuroendocrines contiennent des organelles spécialisées nommées les granules de sécrétion denses. Ces organelles renferment des protéines et des peptides qui sont sécrétées uniquement lorsque la cellule reçoit un stimulus physiologique. Le ciblage des protéines vers les granules de sécrétion est indispensable à la production de certaines hormones peptidiques tels l'insuline et le glucagon. Tandis que des études ont démontrés que les protéines contiennent des signaux pour être ciblés dans divers compartiments cellulaires, aucun signal canonique n'a été découvert pour le ciblage vers les granules. Nous avons déjà démontré que des hélices alpha associées à la membrane et situées dans la région C-terminale de la prohormone convertase PC1/3 jouent un rôle important dans la capacité de cette protéine d'être ciblée vers les granules.

Notre hypothèse est que les hélices alpha sont responsables de cibler les protéines dans les granules de sécrétion et que ce ciblage est médié par des interactions membranaires.

Le but de cette étude était de déterminer le mécanisme utilisé par les hélices alpha pour cibler des protéines vers les granules. Premièrement, nous avons déterminé les caractéristiques biophysiques d'hélices alpha aptes à cibler des protéines vers les granules en testant une série d'hélices synthétiques qui varient en termes de la composition des résidus, la charge, l'amphipathicité et l'hydrophobicité. Deuxièmement, nous avons testé si une hélice alpha était nécessaire pour le ciblage vers les granules des trois enzymes PC résidentes des granules (PC1/3, PC2 et PC5/6A). Nous avons également vérifié l'efficacité du ciblage vers les granules et comparé les différences entre les trois enzymes PC résidentes des granules. Troisièmement, nous avons résolu la structure tridimensionnelle d'un des domaines de ciblage de PC1/3. La fonction individuelle de chaque résidu a été déterminée par mutagénèse dirigée.

Nos résultats démontrent que la présence de résidus chargés (négativement ou positivement) et qui sont ségrégés d'une surface hydrophobe dans les hélices alpha jouent un rôle critique dans la fonction des hélices alpha de cibler des protéines vers les granules.

Des hélices alpha ont ciblée PC1/3, PC2 et PC5/6A vers les granules. Une analyse détaillée des structures prédites formées par les domaines de ciblages des enzymes PC démontre une corrélation entre la capacité des hélices de cibler une protéine vers les granules et la présence d'une surface hydrophobe. De plus, la détermination de la structure tridimensionnelle d'un des domaines de ciblage de PC1/3 ($PC1/3_{711-753}$) a nécessité la présence d'une micelle composée d'éléments membranaires. La structure et un essai fonctionnel ont démontré la nécessité de la Leucine 745 (L745) pour assurer le ciblage vers les granules.

En résumé, nos résultats démontrent la nécessité de résidus hydrophobes situés dans une hélice alpha pour cibler des protéines vers les granules de sécrétion et ce possiblement à l'aide d'interactions membranaires.

Mots-clés: granules de sécrétion, trafic de protéines, trafic membranaire, résonance magnétique nucléaire

Abstract

Endocrine and neuroendocrine cells contain specialized secretory organelles called dense core secretory granules (DCSGs). These organelles are the repository of proteins and peptides that are secreted in a regulated manner when the cell receives a physiological stimulus. The targeting of proteins to DCSGs is crucial for the generation of peptide hormones including insulin and adrenocorticotropic hormone. While previous work has demonstrated that proteins destined to a variety of cellular destinations contain targeting sequences, no single consensus sequence for secretory granule sorting signals has emerged. It has been previously shown that membrane associated alpha helical domains in the carboxy-terminal tail of the prohormone convertase PC1/3 play an important role in the ability of this region to direct DCSG targeting.

Our hypothesis is that alpha helices are responsible for redirecting proteins to DCSGs and that this targeting is mediated by protein-lipid interactions.

The goal of this study was to determine the mechanism used by alpha helices to direct sorting of proteins to DCSGs. First, we determined the biophysical characteristics of sorting helices by testing a series of engineered alpha helices that vary in residue composition, charge, amphipathicity and hydrophobicity. Second, we tested whether an alpha helix was necessary for the DCSG targeting of the three DCSG granule resident PC enzymes (PC1/3, PC2 and PC5/6A). We also assessed the efficiency of entry into granules and compared differences between the three granule-resident PC enzymes. Lastly, we solved the three-dimensional solution structure of one of the PC1/3 helical DCSG-sorting domains. The function of the individual amino acids making up this DCSG-sorting domain was tested by site-directed mutagenesis.

Our results demonstrate that the presence of charged (either positive or negative) amino acids, spatially segregated from a hydrophobic patch in the alpha helices of secretory

proteins plays a critical role in the efficiency of alpha helices to direct secretory granule sorting. Alpha helices were also critical to target PC1/3, PC2 and PC5/6A to the regulated secretory pathway. Analysis of the predicted structures formed by these three granule sorting helices showed a correlation between their granule sorting efficiency and the clustering of hydrophobic amino acids in their granule targeting helices. Moreover, the determination of the three-dimensional solution structure of one of the DCSG-sorting domains of PC1/3: PC1/3 $_{711\text{-}753}$ required the presence of a micelle. The structure in conjunction with a functional DCSG-sorting assay revealed the importance of leucine 745 (L745) located within the alpha helix in mediating DCSG sorting.

In summary, our results demonstrate the requirement of hydrophobic residues situated in alpha helices to direct proteins to DCSGs possibly through membrane interactions.

Keywords: secretory granules, prohormone convertases, protein trafficking, membrane trafficking, nuclear magnetic resonance

Table of contents

List of tables

List of figures

List of abbreviations

ACTH: adrenocorticoptropic hormone

ACTH: adrenocorticoptropic hormone

Alpha-SNAP: alpha soluble NSF attachment protein

ANF: Atrial natriuretic factor

AP-1: adaptor protein-1

AP-2: adaptor protein-2

BDNF: Brain-derived neurotropic factor

C-ter: C-terminal domain

Cer: ceramide

CgA: chromogranin A

CgB: chromogranin B

CHAPS: 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate

COP-II: coat protein complex-II

COS: CV-1 in origin

COSY: correlation spectroscopy

CPE: carboxypeptidase E

CT-HSQC: constant time HSQC

DAG: diacylglycerol

DCSG dense core secretory granules

Dhc: dehydrocholesterol

DPC: dodecyl phosphocholine

EIF4e: eukaryotic translation initiation factor 4E

ER: endoplasmic reticulum

GFP: green fluorescent protein

GH4: growth hormone 4 cells

GHRH: growth hormone receptor hormone

GlcCer: glucosylceramide

GST: glutathione-S-transferase

H-ATPase: proton adenosine triphosphate -ase

HDEL: mammalian endoplasmic reticulum retention signal (histidine-aspartic acid-glutamic acid-leucine)

HMQC: heteronuclear multiple quantum coherence-total correlation

HSQC: Heteronuclear Single Quantum Coherence

HPLC: high pressure liquid chromatography

INS-1: insulinoma-1

ISGs: immature secretory granules

KDEL: mammalian endoplasmic reticulum retention signal (lysine-aspartic acid-glutamic acid-leucine)

NMR: nuclear magnetic resonance

NOEs Nuclear Overhauser Effects

NOESY: Nuclear Overhauser Effect Spectroscopy

NSF: N-ethylmaleimide sensitive fusion protein

PA: phosphatidic acid

PAM: Petidyl-α-amidating monooxygenase

PBS: phosphate buffered saline

PC: proprotein convertases

PC12: pheochromocytoma 12 cells

PE: phosphotidylethanolamine

PG: phosphatidylglycerol

PI: phosphatidylinositol

PM: plasma membrane

PN-1: protease nexin-1

POMC: proopiomelanocorticotropin

Pro-SAAS: non acronymic

PS: phosphatidylserine

PTB: polypyrimidine binding protein

PtdCho: phosphatidylcholine

RER: rough endoplasmic reticulum

REST: RE-1 silencing transcription factor

RMSD: random mean standard deviation

SEAP: Secreted alkaline phosphatase

SgII: secretogranin II

SgIII: secretogranin III

SM: sphingomyelin

SNARES: N-ethylmaleimide sensitive fusion protein attachment receptors

Syt IV: synatoptotagmin IV

SEM: standard error on mean

T-SNARE: target N-ethylmaleimide sensitive fusion protein attachment receptors

TAD: torsion angle dynamics

TGN: trans Golgi network

Type I membrane protein:

> a single transmembrane stretch of hydrophobic residues, with the portion of the polypeptide on the NH2-terminal side of the TM domain exposed on the exterior side of the membrane and the COOH-terminal portion exposed on the cytoplasmic side

V-SNARE: vesicle N-ethylmaleimide sensitive fusion protein attachment receptors

VAMP-2: vesicle-associated membrane protein-2

To Genny

Acknowledgements

First and foremost, I am indebted to my supervisor Timothy Reudelhuber. He inspired me from the beginning and constantly pushed me to become a rigorous scientist. I owe him the passion I now have for research. His supervision style was very simple: make sure everything is perfect and then I will guide you. In return, I received training on the bench, manuscript preparation, poster presentation and everything else all my fellow graduate students from neighboring labs never claim to have received.

My co-supervisor, Jim Omichinski also gratefully accepted to have a biochemist without any structural biology experience such as myself attempt an NMR project. Jim's daily advice and the nightly emails to boost my confidence when all experiments failed permitted me to complete the structural portion of my research project. Without a doubt Tim and Jim will be lifelong friends a true testament to the respect I have for my supervisors.

The help received from the entire lab is also greatly appreciated. Marie-Josée Lacombe (Forskolin) never refused to make a construct or to complete a pulse-chase assay. Her precision is something I will never equal. In the end, it was always best to just leave some things to Marie-Jo. Her friendship also was golden during my years at the lab and I will never forget the first day she brought me up to the lab. Chantal Mercure: experience and knowledge. Need I say more? She basically knows everything there is to know about the lab. Our discussions around the table in the office rivaled great political debates and some days we could have solved the whole world's problems over tea and an espresso. Nadheige Lochard, a graduate student was always there for me. We constantly pushed each other to excel in science and in the end I hope to have become half as good a scientist as her. Through the good or the bad and everything in between Nadheige was always there for

me. Matei Mireuta was my partner in crime. Summer student extraordinaire Matei never needed to be told things twice. Science related things that are. As much as we worked, we had great times. Dismantling an FPLC in the cold room until it worked, nothing really stopped us. I will never see picture framing in the same way after having worked with Matei. During the last years of my thesis, I met a 'spectacular postdoc' Paola Di Lello (Pat). She basically had the patience to train me as a spectroscopist even though on some days I could not even pick out alanines and isoleucines. I owe her a lot as Paola donated (charity time) many hours at the expense of her own project to make sure I got something out of mine. Paola is a true sister that I can never ever forget and I respect her immensely.

While the lab was great, on harder days it is my wife and love of my life Genny that kept me going. Recently she gave me the greatest gift of all: our son Elias. I adore you guys and apologize for not spending more time at home. Genny was there since the beginning and she deserves a medal for putting up with it all.

My mom, dad and sister were also always supportive of my career choices and never doubted any of my moves. I basically have the best wife and family in the world and am not afraid to say that.

I also thank both the FRSQ and University of Montreal for funding and all of the support staff at the IRCM. Many collaborators made my project much easier and I would like to thank Nabil Seidah, Claude Lazure, Pascale Legault, Michel Chrétien, Ajoy Basak, Gaétan Thibault, Christian Deschepper, Dror Warshawski, Alexandre Arnold and Isabelle Marcotte for excellent science.

Chapter 1

INTRODUCTION

The intracellular sorting of peptide hormone precursors to dense core secretory granules (DCSGs) is essential for their bioactivation. Despite the fundamental importance of this cellular process, the nature of the sorting signal(s) for entry of proteins into DCSGs remains a source of vigorous debate. In this chapter, I will highlight recent discoveries that are consistent with a model in which several protein domains, acting in cell-specific fashion and at different steps in the sorting process, act in concert to regulate the entry of proteins into DCSGs.

1.1 Protein codes: The advent of the signal sequence

The accurate sorting of proteins to their cellular destinations is of fundamental importance in biology and must occur with high precision in the context of a highly concentrated and extremely complex mixture of proteins. The identification of the “codes” carried by proteins that ensure their proper intracellular sorting has been a topic of intense and fruitful research for more than 40 years. As a result, most introductory textbooks now include descriptions of the canonical signals, which direct the sorting of proteins to specific cellular destinations (Summarized in Table 1.1). Thus, proteins are imported in the secretory pathway based on the presence of an N-terminal cleavable signal peptide containing 7-12 hydrophobic amino acids (*1*). Mitochondrial-destined proteins, contain an N-terminal code termed the "presequence" consisting of 20-50 residues enriched in positively charged (lysine or arginine), hydroxylated (tyrosine, threonine or serine) and hydrophilic sequences (*2*). The mitochondrial "presequence" also has the ability to form amphiphilic alpha helices. As for nuclear proteins, they contain the "nuclear localization signal" consisting of either one (monopartite) or two (bipartite) stretches of basic amino acids (*3*). The sugar residues of proteins destined to lysosomes contain mannose-6-phosphate which bind to the mannose-6-phosphate receptor to direct lysosomal sorting (*4*). Moreover, proteins can also contain "codes" enabling them to be retained in a specific

cellular location. The lysine-aspartic acid- glutamic acid-leucine (KDEL) sequence in mammals (*5*) and histidine-aspartic acid-glutamic acid-leucine (HDEL) sequence in yeast (*6*) serve as endoplasmic reticulum (ER) retention signals while a stretch of 20 hydrophobic residues flanked by basic residues results in a Golgi retention signal (*7*). Finally, proteins that are endocytosed contain tyrosine and di-leucine motifs (*8*). All of the above sequences are sufficient to redirect proteins to the specific organelle defined by the code.

However, a similar canonical "code" has not been established for directing proteins to dense core secretory granules (DCSG). These cytoplasmic organelles, found uniquely in endocrine and neuroendocrine cells, store hormones, proteases and signaling molecules until the cell receives a signal for their release. For example, the inactive proinsulin prohormone is activated within DCSGs by specific proteases and will be released when blood glucose levels are elevated (*9*). As such, DCSGs are the key component in the regulated secretory pathway. Why has the identification of DCSG sorting signals been such an elusive goal?

CELLULAR LOCALIZATION	SIGNAL SEQUENCE
Secretory pathway	7-12 hydrophobic residues at N-terminus
Mitochondria	20-50 residues enriched in positively charged, hydroxylated and hydrophilic sequences
Nucleus	One or two stretches of basic residues
Lysosomes	Mannose-6-phosphate tags
ER retention signal	KDEL (mammals); HDEL (yeast)
Golgi retention signal	20 hydrophobic residues flanked by basic residues
Endocytosis	Tyrosine and di-leucine motifs

Table 1.1: Examples of canonical sorting signals identified for specific cellular compartment

1.2 Dense core secretory granules within the secretory pathway

1.2.1 Early secretory pathway

There has been a lot of debate not only about how DCSG sorting occurs, but also just where in the cell this triage takes place. All cells have the capacity to rapidly secrete proteins after their transit through the constitutive secretory pathway. Thus, proteins destined for the secretory pathway will first transit to the ER. It is here that the N-terminal secretory pathway signal peptide sequence (Table 1.1; secretory pathway) will be recognized by the signal recognition particle located on the signal recognition receptor found on the ER membrane (*10*). In eukaryotes, the Sec 61/Sec Y complex forms a conducting channel ensuring the co-translational translocation of secretory pathway proteins to the ER lumen (*11*). Upon exiting the ER at specific areas lacking ribosomes (*12*), properly folded secretory proteins will be transported to the Golgi in vesicles coated with coat protein complex-II (COP-II) (*13*) with the proper transfer of cargo from vesicles ensured by specific vesicular adaptors termed soluble N-ethylmaleimide sensitive fusion protein attachment receptors (SNARES) (*14*). SNARES are located on the cytosolic face of vesicles (v-SNARE) and will dock a vesicle via an interaction with a target vesicle's t-SNARE. In the Golgi, proteins are glycosylated in the various cisternae and stacks, which range from the *cis,* the *medial* and ending with the *trans* Golgi networks (TGN) in the anterograde trafficking of secretory proteins. During the 1980s, there was considerable debate on whether trafficking through the Golgi occurred using the same vesicle (cisternal maturation) or using cargo transferred via SNARE adaptors to different vesicles (vesicular trafficking model, For Review see (*15*)). In 2006, both Losev *et al.* (*16*) and Matsuura *et al.* (*17*) showed immunofluorescent images of differentially tagged Golgi cisternae. Using

time-lapse video microscopy, both groups demonstrated that the Golgi cisternae would change color over time indicating that the same vesicle was being trafficked along the Golgi. These findings are consistent with a cisternal maturation model for Golgi trafficking and effectively resolved (for the time being) the vigorous debate.

1.2.2 The trans Golgi network as a molecular sorting station

Upon reaching the terminal Golgi stacks in the TGN, proteins are transported in budding vesicles to specific various destinations. In all mammalian cells, proteins can be trafficked to various destinations. Proteins will either bud off into low density vesicles and be secreted via a non-regulated constitutive pathway or be routed towards the intracellular endosomal pathway (*18;19*). Moreover, certain proteins can be re-routed to earlier compartments via recycling mechanisms (*20*). In polarized cells, there is a differential sorting originating from the TGN to either the apical or basolateral surface of the cell ensuring the asymmetrical distribution of certain proteins (*21*). In contrast, a unique regulated pathway of secretion exists in endocrine and neuroendocrine cells where exocytosis of high density DCSGs is tightly regulated (Figure 1.1). A great deal of evidence supports the view that in the appropriate cell type, DCSG sorting signals can re-direct proteins from the constitutive secretory pathway to DCSGs confirming that it is not a default secretory pathway, but rather a pathway that requires specific biophysical properties of the stored protein or the recognition of a “sorting signal” by the cellular machinery. The nature of this DCSG sorting signal has not been clearly defined to date.

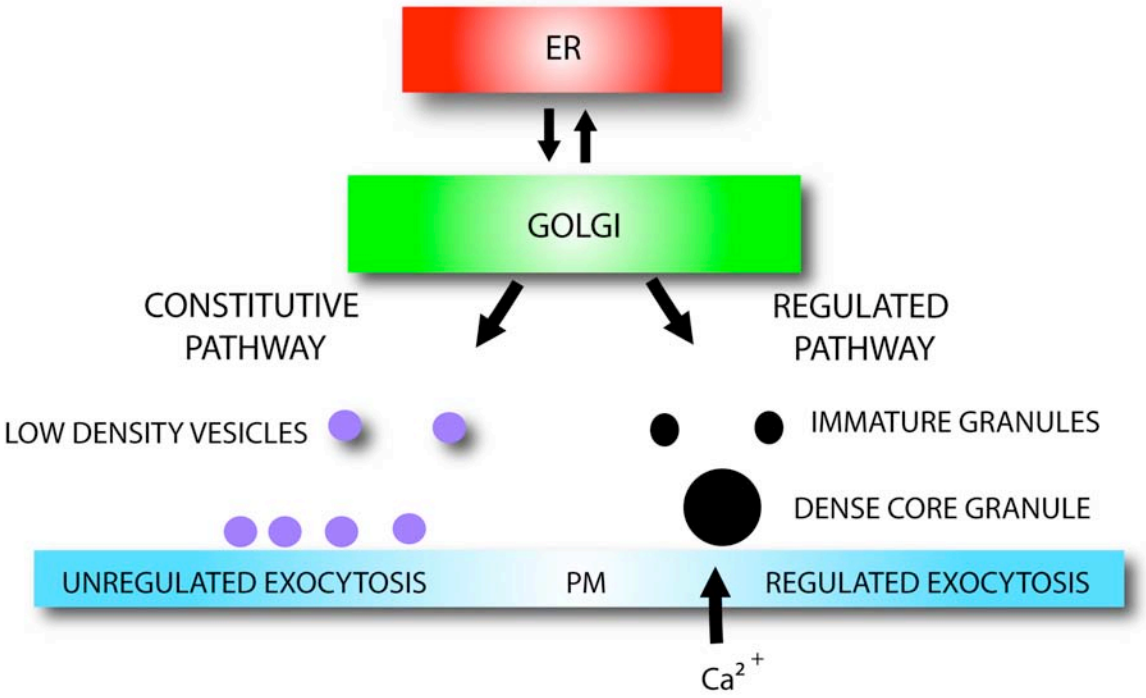


Figure 1.1: Schematic representation of the secretory pathway in endocrine and neuroendocrine cells.

In the anterograde trafficking pathway, proteins pass through the endoplasmic reticulum (ER), then through the cis, medial and trans Golgi networks. The trans Golgi network serves as a sorting station where non-granule proteins are packaged in low-density vesicles and are secreted in an unregulated manner across the plasma membrane (PM). Granule proteins are first packaged in immature secretory granules that fuse and form dense core secretory granules. The dense core secretory granules are secreted when the cell receives an influx of calcium.

Some groups have proposed that DCSG sorting occurs through the action of a sorting "receptor" present in the trans-Golgi network (TGN), which latches onto granule-destined proteins at sites where nascent granules will bud. This model has been referred to as the "sorting by entry" model and implies that only DCSG proteins can be present in this organelle. Studies performed by Chung *et al.* in 1989 provided the first support for the "sorting by entry" hypothesis. They used peptide hormones as affinity ligands to find partner proteins in canine pancreatic tissue. Using this approach, they identified a 25-kDa protein considered as a potential DCSG sorting receptor (*22*). Further experiments revealed that this protein was identical to chymotrypsinogen and was not detectable in every endocrine tissue. Furthermore, chymotrypsinogen bound granule proteins with very low affinity (*23*). One of the limitations of Chung's studies was that it was difficult to distinguish between proteins present in the TGN and DCSG proteins themselves. The inherent difficulty of separating the closely spaced secretory pathway compartments is a complicating factor when identifying DCSG and non-DCSG proteins and this analytical limitation has made the identification of compartment-specific partners difficult. In addition, the presence of the constitutively secreted glycosaminoglycan protein in DCSGs suggests that proteins other than those destined for regulated secretion may enter DCSGs (*24*).

About 10 years later, Cool *et al.* (*25*) postulated that carboxypeptidase E (CPE) could be the *bona fide* DCSG sorting receptor. All of their observations stemmed from an obese mouse line with a spontaneous mutation in CPE: the Cpe $^{fat/fat}$ mouse. Isolation of CPE from the Cpe $^{fat/fat}$ mouse revealed a decreased binding of various peptide hormones to the mutated CPE when compared to native CPE. In addition, the authors reported that the DCSG protein proopiomelanocorticotropin (POMC) was no longer stored in DCSGs in the Cpe $^{fat/fat}$ mouse. However, competitors were quick to point out in independent experiments that hormone secretion was normal in the Cpe $^{fat/fat}$ mouse (*26*). A common argument against CPE as a sorting receptor is that no single receptor can exist to ensure the 1:1 ratio needed to ensure the functionality of the "sorting by entry" theory as the flow of DCSG

proteins traversing the regulated secretory pathway is too large. However, it should be noted that the signal recognition particle receptor is sufficient to handle the large traffic of proteins being co-translationally translocated across the ER membrane.

With all of this conflicting data, “the sorting by entry” view was losing support. Simultaneously, convincing evidence was presented that in cells that generate DCSGs, all of the contents of the TGN are initially encapsulated into nascent granules termed immature secretory granules (ISGs). This finding led to the development of the “sorting by retention” model, which proposes that those proteins destined to be secreted constitutively are progressively extruded in low density vesicles as the granule matures, ultimately leaving only the correct cargo protein in the mature DCSG. A detailed mechanism explaining this extrusion has been clearly identified (Figure 1.2) as well as the mechanism of granule maturation. Thus, the maturation of ISGs involves the progressive acidification of the ISG lumen (*27*) and the homotypic SNARE-dependent fusion between the various ISGs to form the mature DCSG; a process requiring the soluble cytosolic factors N-ethylmaleimide sensitive fusion protein (NSF) and alpha soluble NSF attachment protein (alpha-SNAP) (*28*). A member of the synaptotagmin calcium-sensing family of proteins, synatoptotagmin IV (syt IV), is critical to ensure the ISG homotypic fusion event (*29*). It is adaptor protein-1 (AP-1), a clathrin protein adaptor that assists in the budding of all non-DCSG proteins from the lumen of the ISGs via clathrin coated vesicles. The non-granular proteins in their clathrin coated vesicles are believed to be transported to the endosomal compartment (*30*). The resulting DCSGs produced are then ready to exocytose on the plasma membrane through the interaction of their granule specific vesicle-associated membrane protein-2 (VAMP-2) v-SNARE located on the cytosolic face of the DCSG and the plasma membrane’s t-SNARE (*31*). While the "sorting by retention" model provides clues concerning granule formation and the trafficking of the granule from the TGN to the plasma membrane, the proteins identified to date are all cytosolic and do not explain the selective retention of cargo within the granule core. Perhaps the best such example is provided by a recent proteomics analysis of bovine adrenal medulla granules using a

phosphatidyl inositol (4, 5) bisphosphate pull-down assay (*32*). Phosphatidyl inositol (4,5) bisphosphate is a lipid implicated in the exocytosis of granules and is situated on the cytosolic face of the plasma membrane. The proteomics analysis did not reveal any granule cargo proteins but unsurprisingly identified cytosolic proteins involved exclusively in exocytosis. What about the inside of the granule?

Regardless of which theory is correct, a subset of proteins is somehow correctly stored in DCSGs. These granule resident proteins must contain "codes" allowing efficient targeting to this organelle. Moreover, these proteins may be interacting with partner proteins in order to enter granules while the granular environment itself may also influence the efficient targeting of granule proteins. Can a mechanism be proposed to help explain this critical event?

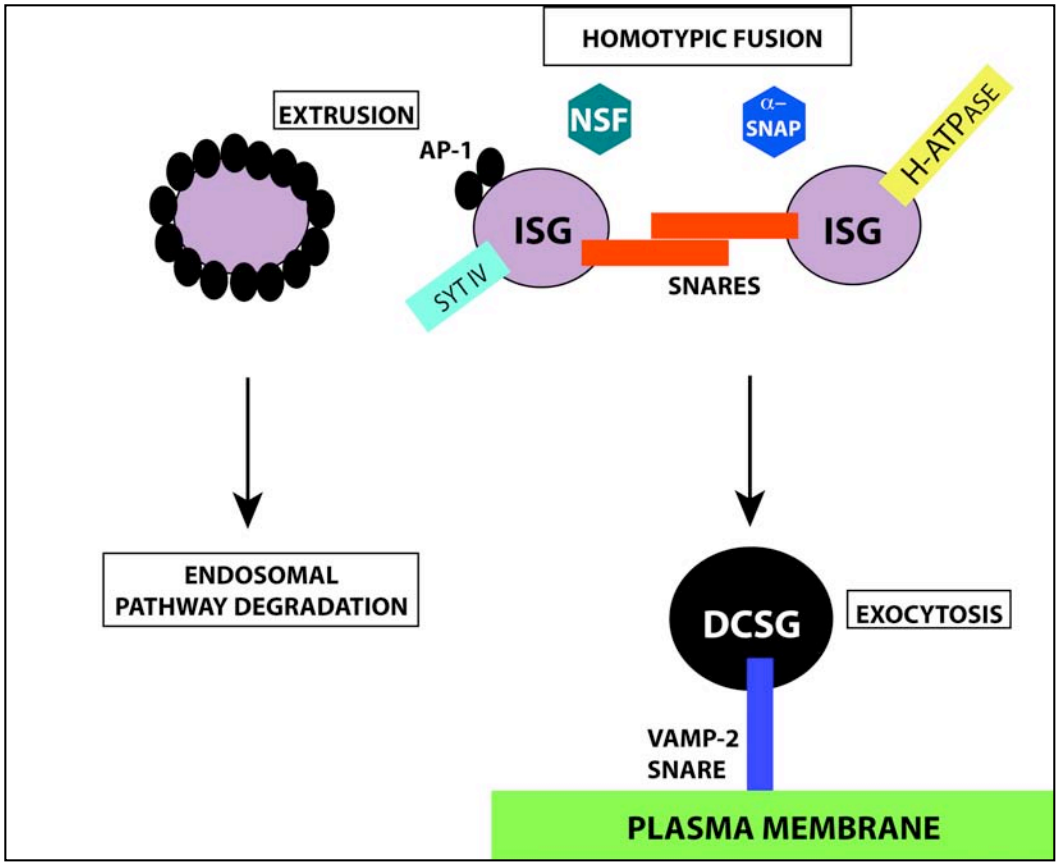


Figure 1.2: Dense core secretory granule maturation.

Equivalent immature secretory granules (ISGs) go through a homotypic fusion event facilitated by SNARE adaptors located on the ISG surface. The homotypic fusion is assisted by the cytosolic factors NSF and alpha-SNAP, the vesicle bound synaptotagmin IV (syt IV) protein and a proton adenosine triphosphate -ase (H-ATPase) assuring the acidification of the ISG lumen. Proteins not destined for DCSGs are extruded via AP-1coated vesicles that attract clathrin and are targeted to the endosomal pathway for degradation. The mature DCSG will fuse with the plasma membrane (PM) and be exocytosed via a specific interaction with the DCSG's VAMP-2 SNARE.

1.3 Three truths and three postulates

The first truth is that regardless of the site at which sorting occurs, a mechanism has to exist that establishes and then maintains the segregation of DCSG cargo proteins from those that are constitutively secreted. Thus, it is a reasonable postulate that some mechanism exists to anchor the appropriate cargo proteins to the DCSG as it forms or matures.

A second truth is that the sorting of proteins to DCSGs is a prerequisite for certain post-translational processing steps in hormone and protease activation. For example, the conversion of proinsulin to active insulin only occurs in the acidic (pH=5.5) DCSG compartment (*9*). Orci *et al.* showed that ionophores disrupting the proton exchange mechanism in the late secretory pathway also blocked the activation of proinsulin (*9*). The cleavage of POMC to its many active peptides including adrenocorticoptropic hormone (ACTH) is equally influenced by the specific ion composition present in the DCSG (*33*). In addition, the proteolytic activation of prorenin to renin can also only occur in DCSGs (*34*).

All of the above examples indicate that the precursors are encapsulated in the nascent secretory granules. This is an efficient mechanism for the organism because it ensures that the secretion of the active hormones or proteases is under appropriate physiological control. However, for granule-restricted activation to occur it is necessary that both the protein precursors and the appropriate processing enzymes end up in the same DCSG. In the case of proinsulin, this means that the proprotein convertases 1/3 and 2 (PC1/3 and PC2) as well as CPE, all of which are required for generation of active insulin, have to be co-targeted with proinsulin in the budding granules. Thus, a second postulate is that a mechanism exists to ensure efficient co-targeting of protein precursors and their processing enzymes in the same organelle.

DCSGs also share by definition the distinguishing trait of a core that appears dark or dense in electron micrographs. However, in spite of this common appearance, there may be important functional and mechanistic differences in DCSGs. For example, the gonadotropes of the pituitary store luteinizing hormone and follicle stimulating hormone in separate DCSGs and their release is independently controlled (Reviewed in (*35*)). Likewise, there are two types of DCSGs in chromaffin cells containing either epinephrine or norepinephrine and these are morphologically distinct (*36*). Norepinephrine granules are larger and electron opaque with a prominent halo between the granule membrane and the dense core. The epinephrine granules are smaller, finely granular structures that fill the enclosing membrane and have no halo. It appears that these differences can depend on the biophysical characteristics of the cargo itself (*37*). Accordingly, Sobota *et al.* demonstrated that proteins that can self-aggregate would be stored in distinct DCSG from proteins that lack tertiary structure (*37*). Thus, cargo itself can dictate the composition of a DCSG independently of the maturation of the granule.

The signals for targeting proteins to DCSGs also show tissue-specific variations: Removal of 90 amino acids from the C-terminus of the granin chromogranin A (CgA) prevents its sorting to DCSGs in pituitary growth hormone 4 cells (GH4), but has no effect on DCSG sorting in sympathoadrenal pheochromocytoma 12 cells (PC12) (*38*). Likewise, POMC is efficiently stored in DCSGs when transfected into cultured pituitary cells, but not in sympathetic neurons (*39*). Thus, a third truth is that not all DCSGs are alike and it is a reasonable postulate that DCSGs can be assembled, even within the same cell, through more than one mechanism.

Could some of these truths explain the difficulties in reaching consensus on the protein signals necessary for DCSG targeting?

1.4 A plethora of signals, a paucity of consensus

There has been no shortage in the variety of DCSG sorting mechanisms proposed in the last 2 decades: these include protein domains that interact with or that traverse membranes and that may or may not interact with additional proteins on the cytoplasmic side of the DCSG, proteins proposed to be a “master switch” for granule formation, universal granule cargo receptors, protein domains that mediate aggregation in the late TGN, certain paired basic protease cleavage sites or alpha helices in secretory proteins, disulfide constrained loops, acidifying proton pumps and other mechanisms. As a result, investigators have become progressively entrenched in defending their favorite mechanisms and commonly use the descriptors “controversial” and “difficult to repeat” to describe the work of others in their publications. Nevertheless, it is possible to accommodate most of these findings into a model that subdivides targeting function into three components (Figure 1.3): membrane associated (or traversing) tethers, tether-associated cargo and aggregation.

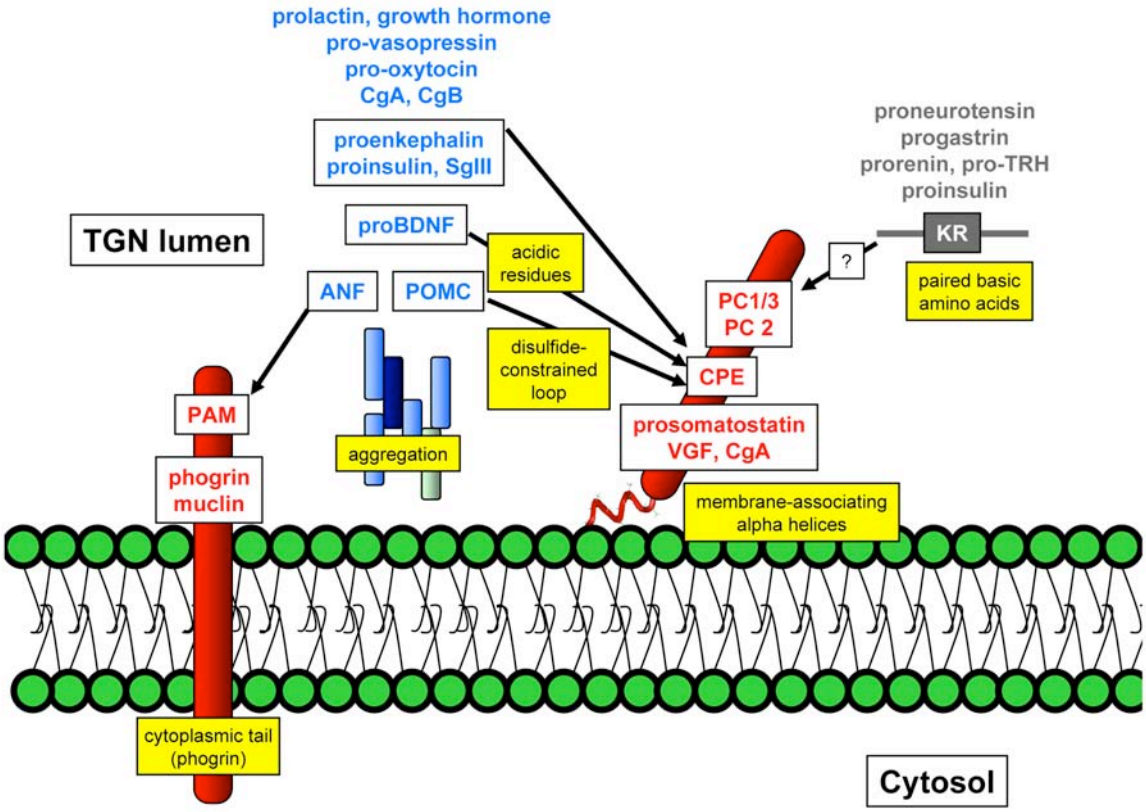


Figure 1.3: Proteins sorted to dense core secretory granules (DCSGs) can be functionally divided into three groups.

Tethers (shown in red) either traverse or associate with membranes. Many DCSG cargo proteins also aggregate to form the dense core (shown in blue) and these aggregates may contain more that one protein. Some DCSG proteins associate with membrane tethers (arrows). The highlighted yellow boxes indicate the various protein domains or mechanisms that have been implicated in DCSG sorting. Note that some proteins (such as insulin) may have multiple DCSG sorting mechanisms. See text for details and abbreviation

1.4.1 Membrane-associated tethers

The fact that many DCSG proteins are either membrane-associated or traverse the membrane is significant. Petidyl-α-amidating monooxygenase (PAM), muclin and phogrin are all type I membrane proteins. PAM is an enzyme catalyzing the alpha-amidation of extended peptides containing glycine residues (*40*). In the pituitary and brain, PAM is in the lumen of the DCSG and anchored via its carboxy terminal transmembrane domain (*40*). Muclin is anchored in the DCSG of pancreatic acinar cells where it binds O-sulfated proteins (*41*). The fact that both PAM and muclin are membrane anchored in granules ensures that both these proteins will not be secreted constitutively. Phogrin is a protein tyrosine phosphatase present in many neuronal and endocrine cell types with a granule sorting domain located in the cytoplasmic tail of the protein (*42;43*). Although the exact nature of the signal is still debated, it appears that this domain can bind the clathrin adaptor proteins AP-1 and adaptor protein-2 (AP-2) *in vitro* (*44*). When one considers that vesicular transport proteins are present on the cytosolic face of vesicles, the interactions described between phogrin and the AP-1 and AP-2 proteins provide a unique means of communication between the granule cargo proteins and the membrane domains or cytoplasmic proteins that will define the budding DCSG.

DCSG proteins can also interact with the membrane without traversing it. The membrane-binding domain of the granule-resident protein CPE is located in the final 22 residues of its C-terminal domain and is proposed to have a shallow membrane interaction (*45*). The CPE DCSG sorting domain adopts an alpha helical secondary structure (*45*). The prohormone convertases PC1/3 (*46*), and PC2 (*47*) are also targeted to DCSGs and as for CPE there is agreement that the granule sorting is mediated by short alpha helical domains. PC1/3 contains a region predicted to form an amphipathic alpha helix in its C-terminal domain between residues 711-753 (*48*). An alpha helical domain has also been

shown to be important for targeting prosomatostatin (*49*), CgA (*50*) and VGF (*51*) to DCSGs. Interestingly, both the alpha helices in the targeting sequences of prosomatostatin and CgA are amphipathic like PC1/3 apart from being located on the N-terminus. Specifically for prosomatostatin, two leucine residues located at positions 7 and 11 of the alpha helix respectively, form a hydrophobic pocket critical in targeting prosomatostatin to DCSGs (*49*) suggesting yet again a possible membrane interaction. In summary, this group of DCSG proteins could therefore be tethered to the membranes of the TGN or the maturing granule. Since many of the DCSGs proteins are membrane tethered could the membrane itself play an important role in the sorting process?

1.4.2 Membrane lipids implicated in vesicular traffic

Since a large number of DCSG sorting domains interact with membranes, the glycerophospholipids, sphingolipids and cholesterol present in eukaryotic cells may play a pivotal role in targeting proteins to DCSGs.

Membrane lipids can be divided in 3 large subclasses. First, glycerophospholipids contain a glycerol backbone and a polar phosphate group esterified to a choline (Figure 1.4; phosphatidylcholine (PtdCho), shown as PC in figure), an ethanolamine (phosphotidylethanolamine, PE; not shown in Figure 1.4), a serine (phosphatidylserine, PS; not shown in Figure 1.4) or an inositol head group (phosphatidylinositol, PI). Two non-polar side chains of varying length are attached to the glycerol backbone. Accordingly, these lipids can be modified resulting in phosphorylated inositol head groups (Fig. 1.4; phosphatidylinositol phosphate, (shown as PIP in figure) and phosphatidyl inositol 2-phosphate, shown as PIP2 in figure). In contrast, the second class of membrane lipids: sphingolipids contain a ceramide (Figure 1.4; ceramide, Cer) backbone instead of the glycerol backbone found in glycerophospholipids (*52*). Structurally, most of the glycerophospholipids adopt a perfect cylindrical geometry due to the diametrical

equivalency of both the head groups and non-polar side chains (Figure 1.4: Space-filling model structure of PtdCho shown as PC in figure). In contrast, the sphingolipids will form very narrow cylinders due to their saturated highly compact tails (Figure 1.4; Structure of sphingomyelin, SM). This results in very tightly packed lipids that give rise to a gel-like state, which does not abide by the classical fluid-like mosaic hypothesis postulated for membranes. As such, a third class of lipids: sterols such as cholesterol (Figure 1.4; Cholesterol) are localized in areas where sphingolipids are present giving a more fluid membrane (*52*). Membrane patches rich in sphingolipids and cholesterol are defined as lipid rafts (*53*).

Most lipids are synthesized at the ER and are then trafficked to various organelles. The ER is composed of PtdCho, PE, PI, PS, phosphatidic acid (PA), Cer, galactoceramide, cholesterol and triacylglycerol while the mitochondrion is composed of PE, phosphatidylglycerol (PG), cardiolipin and PA. Mono- and bi- and triphosphorylated inositol lipids are found on the cytosolic leaflet of the plasma membrane as are sphingolipids (*52*). At the Golgi, some specific lipids are localized on both the cytosolic and lumenal leaflets. In the lumenal leaflet of the TGN, where protein sorting to DCSGs occurs, a large number of sphingolipids are synthesized including sphingomyelin, galactocerebroside and lactosylceramide (*52*). These sphingolipids co-exist with cholesterol at the lumen of the TGN (*53*). As for glycerophospholipids, certain varieties are localized on the cytosolic leaflet of the Golgi such as PtdCho, PE, and PI-4 phosphate. A phosphotransferase capable of removing the phosphate group of PtdCho produces diacylglycerol at the cytosolic leaflet of the TGN (*54*). The diacylglycerol is interconvertable with PA via a diacylglycerol kinase also present on the cytosolic leaflet of the TGN (*55*). PA may also be obtained directly from PtdCho using phospholipase D 1 (*56;57*). The existence of lipid flippases, capable of transferring both diacylglycerol and PA from the cytosolic Golgi leaflet to the lumenal leaflet of the TGN, has been proposed (*58*). Compelling evidence has also been obtained for the implication of glycerophospholipids on sorting to DCSGs. For instance, protein kinase D is capable of

binding the diacylglycerol present at the TGN and regulate vesicular budding (*54*). Unfortunately, this observation has not been extended for the regulated pathway and is limited to constitutively secreted vesicles. Instead, work by Shields and colleagues demonstrated that inhibition of PA synthesis by 1-butanol altered the appearance of the Golgi apparatus and decreased secretion in an endocrine cell type (*59*). Thus, can Golgi lipids be involved in vesicular trafficking and specifically DCSG protein targeting?

CPE preferentially binds sphingolipids and cholesterol-rich membrane fractions (*60*) while both the prohormone convertases PC1/3 (*46*) and PC2 (*47*) bind liposomes enriched in sphingolipids and cholesterol. The lipid raft patches may serve as anchors in the TGN where the alpha helical sequences presented in the C-termini of PC1/3, PC2 and CPE are tethered. Moreover, cholesterol depletion by statin drugs diverted CPE from DCSGs to constitutively secreted vesicles (*60*). Specific inhibition of sphingolipid synthesis with the drug fuminosin resulted in the re-routing of PC2 to the constitutive pathway without affecting another granule protein CgA (*61*). This raises the possibility that different granule proteins may be interacting with different lipids but also that direct membrane interactions may not be sufficient for all proteins to be correctly targeted to DCSGs. Furthermore, cholesterol depletion in endocrine cells completely eliminated the regulated pathway (*62*). Thus, the present tools to study lipids in regulated secretion are limiting especially since membranes are an integral part of the cell. An animal model was used to circumvent the difficulty in blocking lipids by pharmacological agents as is done in cell culture models. The mouse model reiterates the necessity for cholesterol in granule formation (*63*). The Smith-Lemli-Opitiz dehydrocholesterol $7^{-/-}$ (Dhc7 $^{-/-}$) mice are deficient for the 7-Dhc reductase gene, the final step in cholesterol synthesis. The Dhc7 $^{-/-}$ mice contained abnormally sized granules compared to control mice. A biophysical characterization of liposomes containing minimal cholesterol quantities such as in the Dhc7 $^{-/-}$ mice showed a decrease in membrane curvature leading the authors to believe that diminished cholesterol quantities prevent budding of vesicles from the TGN.

In summary, while specific lipid content has been shown to be important for the sorting of proteins to DCSGs, no specific lipid-protein interactions have yet been identified that would explain DCSG sorting.

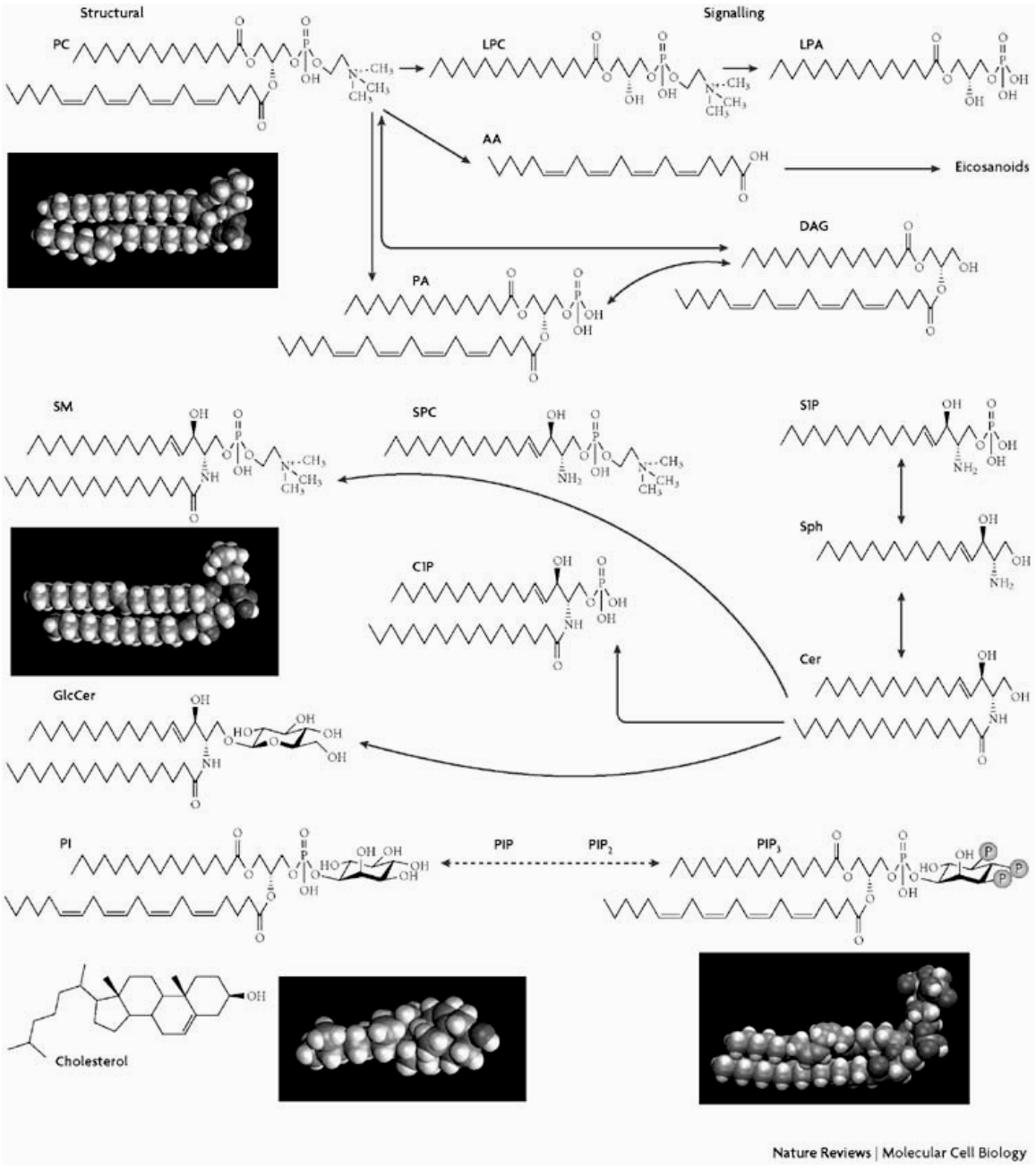


Figure 1.4: Membrane lipids separated as structural or signaling molecules.

The main eukaryotic membrane lipids are the glycerophospholipids such as phosphatidylcholine (PtdCho; PC). Their diacylglycerol (DAG) backbone carries a

phosphate (phosphatidic acid; PA) esterified to a choline (forming PtdCho), ethanolamine (forming phosphatidylethanolamine (PE); not shown), serine (forming phosphatidylserine (PS); not shown), or inositol (forming phosphatidylinositol; PI). The phosphosphingolipid sphingomyelin (SM) and the glycosphingolipid glucosylceramide (GlcCer) have a ceramide (Cer) backbone, consisting of a sphingoid base (such as sphingosine; Sph), which is amide-linked to a fatty acid. Lipids can be interconverted by the actions of kinases and phosphatases.

1.4.3 Cargo interactions via membrane tethers

1.4.3.1 Carboxypeptidase E

A second group of granule-sorting domains may act by binding cargo proteins to the granule-tethered proteins described in the previous sections. For example, CPE has been proposed to interact with a number of granule cargo proteins including proenkephalin, proinsulin, POMC (*64*) and secretogranin III (SgIII) (*65*). POMC binds CPE via an amino-terminal disulfide constrained hydrophobic loop (*64*). Brain-derived neurotropic factor (BDNF) is also targeted to the regulated secretory pathway through an interaction with membrane-tethered CPE (*66*). Analysis of superimposed X-ray structural models identified key acidic residues implicated in a complex between CPE and BDNF. Interestingly, some of these cargo-CPE interactions promote retention in secretory granules, even though some of the cargos are not enzymatic substrates of CPE reinforcing the claim for a tether. Furthermore, no common mechanism for interaction of these cargo proteins with CPE has yet emerged.

1.4.3.2 Paired Basic Amino Acids

Paired basic amino acids have also been reported to direct DCSG sorting in some proteins including proneurotensin (*67*), prorenin (*68*), prothyrotropin releasing hormone (*69*) and progastrin (*70*) and to increase the sorting efficiency of proinsulin (*71*). In the analyses carried out to date, it appears that these paired basic amino acids must constitute a cleavage site for one of the granule-resident prohormone convertases (PC1/3 or PC2) to function as a granule sorting domain since changing the cleavage site to one recognized by

furin (another member of the family which cleaves its substrates in the early secretory pathway) causes the proteins to be secreted through the default constitutive pathway (*68*). These results raise the possibility that certain DCSG-targeted proteases can act as sorting chaperones for their substrates in addition to being processing proteases. The interaction of substrates with membrane-tethered proteases is thus another potential granule sorting mechanism.

1.4.3.3 Interaction with DCSG transmembrane proteins

DCSG transmembrane domains are not inert and they too are capable of assisting the correct entry of granule-resident proteins. In fact, muclin has been suggested to act as a granule cargo "receptor" in pancreatic cells through its binding of sulfate groups on O-linked glycosylated proteins (*41*). Atrial natriuretic factor (ANF) has also been shown to be tightly bound to the membranes of atrial myocyte secretory granules through its interaction with PAM (*72*), although it is not a substrate of PAM. Since there are no-known alpha-amidated peptides in the cardiac atrium (*72*), no enzymatic role can be postulated for PAM apart from a DCSG sorting role. Recently, PAM has been found to interact with the new partner proteins: Kalirin/Trio. Overexpression of the Rho guanine nucleotide exchange factors Kalirin/Trio diverted the granule protein POMC to the constitutive secretory pathway and resulted in the secretagogue-independent secretion of unprocessed POMC (*73*). Pharmacological inhibition of the guanine nucleotide exchange activity of Kalirin/Trio restored POMC localization to DCSGs and processing of POMC to its many peptides. Since Kalirin/Trio both interact with the DCSG integral membrane protein PAM, this study indicates how partner proteins can be affected by signaling molecules and subsequently regulate DCSG protein localization thereby ensuring substrate activation. Thus, a variety of interactions with "tethers" may serve to target proteins to secretory granules. Notably, if this mechanism is correct, it would in some cases provide a means to ensure that processing enzymes and their substrates end up in the same DCSGs.

1.5 Aggregation

A third category of granule targeting mechanisms involves formation of high molecular weight protein complexes or aggregates. Indeed, many granule targeted cargo proteins have the ability to multimerize or aggregate, leading in most cases to the formation of electron dense cores. What causes this aggregation and how does it affect the formation of DCSGs?

1.5.1 The granular milieu

The protein concentration in the late secretory pathway is highly elevated. For example, proinsulin has a measured concentration of 42 mM in pancreatic beta-cell granules (*74*) where it can form hexamers (*75*). Granule resident proteins do not exhibit aggregation in the early secretory pathway. Cellular fractionation studies of pituitary cells expressing the granule resident hormones prolactin and growth hormone demonstrate that the latter hormones are soluble in the cis and medial regions of the Golgi (*76*). Prolactin and growth hormone share the unique property of being soluble in Lubrol detergent to test their aggregation (*77*). Thus, Lubrol solubility was only required for TGN and DCSG fractions of growth hormone and prolactin (*77*). Furthermore, Lubrol solubility is reversible after 30 minutes demonstrating the inherent nature of these hormones to aggregate. Strikingly, expression of these hormones in non-endocrine CV-1 in origin (COS) fibroblast cells resulted in no aggregation (*78*) suggesting that there is something unique about the TGN/DCSG environment provided by endocrine cells permitting this “molecular crowding”. What make the TGN so unique in these cell types?

Initial experiments relied on the mildly acidic and divalent-cation rich DCSG environment to study aggregation. Purified secretogranin II (SgII), a granin family granule-

resident protein, aggregated in the presence of 10 mM calcium at pH = 5.2 (*79*). In vivo, packaging of SgII in DCSGs was inhibited by the addition of ammonium chloride which increases the pH (*79*). Furthermore, rough endoplasmic reticulum (RER) specific permeabilization of 1-10 mM calcium buffered at pH = 6.4 aggregated both SgII and a second granin chromogranin B (CgB) in the early secretory pathway where both CgB and SgII are normally non-aggregated (*80*). A direct correlation between the ability to aggregate *in vitro* and to be sorted to secretory granules in transfected cells has also been reported for another granin family member: CgA (*81*). Because granins are acidic proteins that can cluster in the slightly acidic environment present in DCSGs (For review see (*82*)) it has been suggested that aggregation may serve to prevent their extrusion from the maturing granule. Indeed, it has been shown that treatment of PC12 cells with bafilomycin A1, a specific inhibitor of vacuolar H-ATPase, resulted in a decrease in regulated secretion of CgA, with a concomitant decrease in visible DCSGs (*83*) suggesting that regulated secretion of CgA and acid-dependent dense core formation are linked in DCSGs. The pH effect on CgA may be modulated by CgA's response to the calcium present in the late secretory pathway. In fact, CgA is a high capacity but low affinity calcium binding protein (*84*). While CgA is present as a dimer in the early secretory pathway (ER and cis Golgi) it can form higher order multimers when calcium-bound in the DCSG (*85*). Calcium mediated aggregation through direct binding to a protein has also been demonstrated for pro-ANF and this directly correlates with the ability of pro-ANF to be correctly targeted to DCSGs (*86*).

Other divalent ions present in the late secretory pathway of pituitary cells have also correlated with granule protein aggregation. Histochemistry identified a major difference between the TGN from pituitary cells and the TGN from fibroblasts, which do not contain any DCSGs. In fact, pituitary cells contained a higher concentration of the divalent ion zinc (*87*) and zinc is present in high enough concentrations to ensure a 1:1 complex with granule hormones (*88*). A second divalent ion, copper, has also been measured in high concentrations in the late secretory pathway of pituitary cells (*89*). Chelation of both zinc

and copper reduced the aggregation of both prolactin and growth hormone and their subsequent storage to DCSGs (*78*). Moreover, proinsulin can also bind zinc in the late secretory pathway resulting in proinsulin crystal hexamers in the DCSGs of pancreatic alpha cells (*75*)

1.5.2 Aggregation chaperones

Proteins can also act as chaperones to increase the aggregation of partner proteins. However, tags placed on proteins to facilitate their visualization in a cell or their purification can lead to an increase of aggregation for proteins that will be stored in DCSGs or to the misrouting of non-granules proteins to the regulated pathway. Secreted alkaline phosphatase (SEAP) is a constitutively secreted protein that specifically increased the aggregation of CgA in the presence of millimolar calcium concentrations (*81*). However, this was later found to be due to a hexa-histidine tag placed at the C-terminus of SEAP which bound the free calcium present in the secretory pathway resulting in a novel form of aggregation which did not involve hydrophobic interactions (*90*). Similar observations have been made with green fluorescent protein (GFP) which can form disulphide bonds and efficiently target GFP tagged proteins to DCSGs (*91*). Great caution must therefore be taken when analyzing results with hexa-histidine and GFP tagged proteins (*92*). Moreover, 7B2 is a granin family member which assists the granule targeting of PC2 (*93*). Expression of 7B2 also results in the aggregation and enhanced granule-localization of proenkephalin (*92*). Thus, while 7B2 does not cause PC2 to aggregate, it can cause the aggregation of a different partner raising the possibility that aggregation may be sequence specific as has been described for the coiled-coiled domains of CgA (*94*).

Thus, aggregation not only forms a visible core, it appears to play a role in the sorting retention of cargo protein as well.

1.5.3 A role for cargo in directing DCSG biogenesis?

DCSGs are essentially budded off regions of TGN that are densely packed with cargo. In 2002, Kim *et al*. reported that silencing CgA expression in PC12 cells results in impaired regulated secretion of transfected prohormones, a loss of DCSG proteins and the loss of visible DCSGs. The authors concluded that CgA is not only a component of the dense core, but that it is also a "master regulator" of DCSG biogenesis (*95*). Moreover, transfection of CgA in a fibroblast cell type lacking a regulated secretory pathway resulted in the formation of DCSGs rendering CgA a granule "on/off switch" (*95*). Similar experiments were carried out by Courel *et al*. using the DCSG sorting domain of CgA located in the N-terminal region between residues 1-115 (*96*). Transfection of CgA (1-115) in the A35C PC12 sympathoadrenal cell line lacking a secretory pathway biogenerated *de novo* DCSGs and targeted growth hormone to these *de novo* granules as in normal sympathoadrenal cells. A functional secretory pathway was fully restored as the granule-rerouted growth hormone was secreted in a regulated fashion (*96*). Thus, the DCSG sorting domain of CgA also functions as a granulogenic determinant. A mechanism explaining CgA's effect may be provided by the gene repressing RE-1 silencing transcription factor (REST). REST is expressed in A35C cells but not in normal PC12 cells and regulates CgA's promoter region (*97*). CgA and other granule proteins are transcriptionally repressed in the A35C cell line. Thus, the re-introduction of transfected CgA may compensate for the lack of CgA in A35C cells, which will aggregate at the TGN and cause budding to form DCSGs. New evidence suggests that alpha-helical coiled-coiled regions of CgA are responsible for granule core condensation by forming homomultimers (*94*).

CgA has also been reported to induce the expression of protease nexin-1 (PN-1), a serine protease inhibitor that slows the turnover of a number of DCSG cargo proteins (*98*), which could provide an additional mechanism for increasing DCSG aggregate formation. Since CgA binds to another granin partner, SgIII, which in turn can associate with

cholesterol (*99*) as well as CPE (*65*), aggregation may synergize with protein-protein and protein-membrane interactions to improve the retention of cargo proteins in the maturing granule and their regulated secretion. Does all of this data suggest that the appearance of DCSGs can be solely explained by CgA?

Malosio *et al.* (*100*) were unable to confirm the data of Kim *et al* (*95*). They were unable to establish a correlation between DSCG content and CgA expression in isolated clonal lines of PC12 cells which express varying levels of CgA and this suggests that a multitude of proteins could be contributing to DCSG appearance. In fact, expression of a number of other DCSG cargo proteins, including pro-vasopressin, pro-oxytocin, POMC, SgII and CgB, is sufficient to induce aggregate-containing cytoplasmic vesicles even in cells with no regulated secretory pathway (*101*) although these probably do not display all of the functional characteristics of DCSGs (*31*). Indeed, Malosio *et al.* demonstrated that transfection of CgA in non-endocrine cells gives rise to TGN-derived vesicles but that these vesicles co-localize with lysosomal protein markers (*100*). Thus, the complexity of granule formation may not be explained by the aggregation of a single protein.

Regulating the formation of the aggregate may also be physiologically important: Knoch *et al*. reported that a polypyrimidine binding protein (PTB) which is up-regulated under conditions of high insulin demand stabilizes messenger RNAs of many of these same DCSG cargo proteins in insulin-producing cells and leads to increased granule formation (*102*). Knockdown of PTB expression in insulinoma-1 (INS-1) cells causes the specific disappearance of DCSGs without affecting any other cellular organelle (*102*). The authors of these studies did not wish to call PTB “a master gene” since it is ubiquitously expressed. Instead, specific factors present in endocrine cells capable of interacting with PTB are more likely to play a role. In fact, PTB binds to specific untranslated regions of the proinsulin processing enzymes PC1/3 and PC2 increasing the stability of their mRNA (*102*). In summary, aggregation mechanisms are multi-faceted and can either be influenced by specific ionic concentrations of the DCSG, chaperone proteins and specific protein

sequences. These factors along with the newly described transcriptional regulators may ultimately lead to the formation of the granule.

1.6 *In vivo* models of granule biogenesis

In spite of the compelling arguments presented for these various DCSG sorting mechanisms, examining their process in the whole animal has been anything but simple. One example of this difficulty is the proposed role of CgA as a master regulator of granule formation. Although down-regulation of CgA expression was reported to result in the loss of detectable DCSGs in cultured PC12 cells, CgA gene inactivation in mice leads to either a "reduction" (*103*) or no discernable effect (*36*) on DCSG formation in the CgA-rich adrenal chromaffin cells in two independent studies using different mice lines. In spite of the differences in the effects on DCSG morphology, both studies report a similar and dramatic effect on cathecholamine secretion in the CgA-deficient mice while one of the two groups demonstrated that the CgA null mice are hypertensive (*103*) proving that CgA deficiency is not entirely without consequence. How can these apparent differences in the requirement for CgA be explained? One obvious possibility is that other DCSG cargo proteins can complement the function of CgA in the formation of the dense core *in vivo*, but cannot compensate for its absence in catecholamine storage and secretion. In support of this explanation, the group that saw no effect of CgA inactivation on DCSG formation reported an up-regulation of CgB and SgII in the adrenal glands of the mutant CgA null mice (*36*). Thus, while CgA may affect DCSG formation in some cultured cells, this particular function can obviously be replaced *in vivo*. Nevertheless, while experiments to date have not identified a "master" regulator of DCSG formation, the concept may not be entirely wrong in specific cell types: ANF inactivation in mice leads to a complete loss of visible DCSGs in the cardiac atrium (*104*) with an increase in salt-sensitive hypertension. Moreover, inactivation of the renin gene leads to a complete disappearance of DCSGs in

the juxtaglomerular cells of the kidney (*105*). It's important to note, however, that regulated secretion can occur in the absence of a dense core as it does in many neurosecretory vesicles. In the case of the ANF and renin-deficient mice, it will be intriguing to determine if the remaining cargo proteins are still packaged in such vesicles in the absence of the aggregating partner.

A similar conundrum exists with CPE as a "sorting receptor" for a variety of DCSG cargo proteins. Cool *et al.* originally proposed CPE as the regulated secretory pathway sorting receptor because they observed endocrine disorders in the Cpe $^{fat/fat}$ mouse which harbors a mutation in the CPE gene (*25*). Proinsulin and POMC are among the several proteins that were shown to bind to CPE and which were proposed to enter DCSGs by this association (*25*). However, concurrent results demonstrate that both proinsulin and POMC are correctly targeted to DCSGs in CPE $^{fat/fat}$ mice (*26*). Can all of these seemingly disparate results be reconciled?

1.7 Synergy and diversity in granule sorting mechanisms

Although there may be many reasons why it's been hard to derive a consensus for the mechanisms and components of the DCSG sorting machinery, the most intuitive is that we are the victims of our own scientific reductionism. In our search for a simple canonical sorting mechanism we have developed a grossly overly simplified view of the way in which proteins enter DCSGs. Close to 100% of the proinsulin produced in pancreatic beta cells enters DCSGs (*106*) while only about one quarter of the prorenin in the secretory pathway of kidney juxtaglomerular cells is sorted to DCSGs (*107*). Is there an explanation for these differences? Proinsulin contains numerous potential DCSG sorting domains such as a binding domain for CPE (*64*), two paired basic amino acid protease cleavage sites recognized by the membrane tethered proteases PC1/3 and PC2 (*108*) and the ability to hexamerize and subsequently aggregate in the presence of the divalent cation zinc

(*75*) while prorenin only contains a single DCSG sorting domain: a paired basic amino acid protease cleavage site (*68*). In the case of prorenin, changing even a single one of these basic amino acids completely eliminates DCSG targeting in endocrine tissue culture cells (*68*). In contrast, neither the mutation of the protease cleavage sites (*109*) nor the hexamerization domain (*75*) of proinsulin appear to affect its DCSG sorting. Combined with the finding that proinsulin is still efficiently sorted to DCSG in CPE-deficient mice (*26*), it has been tempting to dismiss the function of these putative sorting signals. However, another possible explanation is that with its many DCSG sorting signals, proinsulin might be able to compensate for the loss of any single sorting domain. There is in fact some evidence to support the view that DCSG sorting signals can synergize: duplicating the disulfide constrained loop DCSG sorting signal normally found at the N-terminus of CgB results in a greater sorting efficiency to DCSGs than the native protein (*110*). Furthermore, combining the alpha helical sorting signal of the PC1/3 protease and the paired basic amino acid sorting domains of prorenin on either the same protein or on two proteins capable of dimerizing led to a dramatic increase in DCSG sorting over proteins containing either individual domain (*111*). Thus, diverse sorting signals may be able to functionally complement each other even through protein-protein interactions. Complementarity in cellular sorting machineries may also occur as missing sorting components can easily be replaced by parallel sorting machineries. Hosaka *et al.* also reported that pituitaries of the Cpe $^{fat/fat}$ mouse contain elevated levels of both SgIII and CgA which might compensate for the loss of CPE (*65*). Both SgII and CgA were able to target POMC to the regulated pathway in the absence of its usual sorting receptor CPE. This raises the issue of specificity and how various sorting machineries can not only recognize their own cargo but also increase their expression levels in the absence of parallel sorting mechanisms.

All of these cases are consistent with the existence of multiple sorting mechanisms, each of which can contribute to the overall efficiency of protein sorting or retention in DCSGs. Cell types and the nature and/or the number of the sorting domains contained in

the cargo protein would ultimately determine the extent to which each mechanism is active. Multimerization and aggregation could add synergy between mechanisms used by other DCSG cargo proteins in the aggregate. With such a model, it's also easy to imagine how changing conditions within the cell could alter DCSG sorting efficiency of a protein, a potential control point that has important implications for hormone secretion but that has received little attention to date.

1.8 Granule active Prohormone Convertases as a model to study sorting mechanisms of a DCSG cargo/tether

In 1967, Donald Steiner demonstrated that large peptide hormones were post-translationally cut into smaller hormones by specific proteases and that this occurred at paired basic residues (*112*). During the same year, Michel Chrétien and Choh Hoh Li discovered that beta-melanocyte stimulating hormone, gamma-lipotropin and beta-lipotropin were all derived from the same precursor: POMC (*113*). Almost twenty years later, the PC family of calcium dependent mammalian serine proteases was established with the identification of furin (*114*). The PC family's characterization was established by the structural similarities between furin and both the yeast kexin 2 and the bacterial subtilisin enzymes (*115*). Indeed, the yeast kexin 2 enzyme was capable of cleaving mammalian peptide hormones in endocrine cells (*116*). Thus, PC enzymes will post-translationally activate large inactive precursor hormones by recognizing dibasic residues (usually arginines) within a general motif: (R/K)-(X) $_n$-R↓; where n= 0, 2, 4 or 6, ↓ represents the cleavage site and X is any amino acid except cysteine and rarely proline (*117*). There are currently 7 known members of the PC family of which three: PC1/3, PC2 and PC5/6A are enzymatically active only in DCSGs: Why would these three PC enzymes be different? A close examination of the primary sequences of all PC enzymes reveals that they share

highly homologous domains. In fact, all PC enzymes (Figure 1.5) contain a Pro domain restricting enzymatic activity in the early secretory pathway, a catalytic domain comprising the D, H, S catalytic triad of residues and a P-domain involved in both calcium-binding and stabilization (*117*). The least homologous domain between all PC family members is the C-terminal domain (*117*). Can this explain why the activity of PC1/3, PC2 and PC5/6A is restricted to granules?

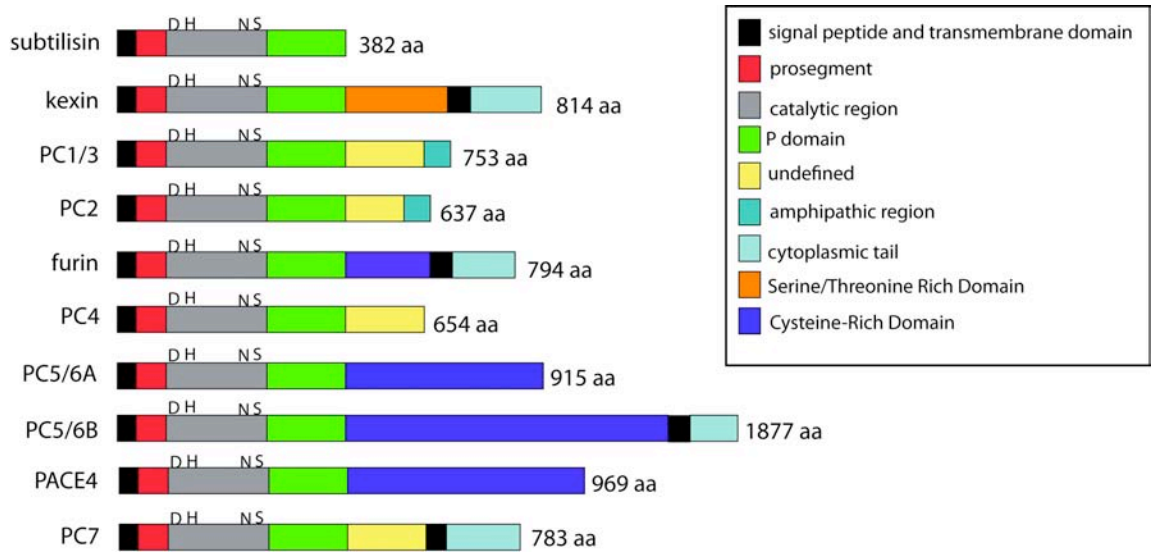


Figure 1.5: The Prohormone Convertases

Schematic diagram comparing the structures of the various members of the basic amino acid specific members of the PC protease family. Note that the C-termini are the least conserved in this family

1.8.1 The cell biology of the granule-localized PC enzymes

1.8.1.1 PC1/3 and PC2

Both PC1/3 and PC2 have a restricted distribution in mammals and are co-expressed in neurons (*118*), the hypophysis (*119*), the cardiovascular system (*120*), the pancreas (*121*), the thyroid gland (*122*), and in the adrenal gland (*123*). Mouse knockout models of both PC1/3 and PC2 exist with the following phenotypes. The PC1/3 knockout mouse has severe growth defects, hyperproinsulinaemia, defective growth hormone releasing hormone (GHRH) and POMC processing (*124*) while the PC2 knockout mouse has defects in hypoglycaemia, proinsulinaemia, glucagon deficiency and defects in opioid processing (*125*). A quantitative neuropeptidomic analysis of both knockout models revealed a degree of redundancy in both enzymes. Indeed, in the PC1/3 knockout mouse, activated levels of PC1/3 substrates remained unchanged leading the authors to believe that PC2 is compensating for the loss of PC1/3 (*126*). Neuropeptidomic analysis of the PC2 knockout mouse displayed a 33% reduction in the processing of prohormones (*127*). Regardless of the PC, both knockout models reduced the number of substrates being cleaved in DCSGs making the correct targeting of PC1/3 and PC2 to this organelle critical for endocrine homeostasis. Perhaps the most striking effect was observed in a patient lacking active PC1/3 (*128*). The patient had severe early obesity (at 6 yrs of age), adrenocortical insufficiency and hyperproinsulinaemia; all consequences of inactive PC1/3 being trapped in the ER (*128*).

Since the PC proteases are themselves synthesized as inactive precursors, the N-terminal Pro domain is cleaved in both PC1/3 and PC2 rendering both enzymes active. It has been reported that the Pro domain of PC1/3 is autocatalytically cleaved in the endoplasmic reticulum while PC2's Pro region is cleaved in a later secretory compartment

(TGN or ISGs) that is more acidic (*129*). Single amino acid substitutions in the Pro region of PC1/3 modify the inhibitory profile of PC1/3 (*130*) making a direct interaction between PC1/3's Pro region and the catalytic site possible. As for PC2's propeptide, it also inhibits the enzyme's activity. In this case, the 7B2 granin chaperone will remove the propeptide within granules making targeting of both PC2 and 7B2 to DCSGs a critical event (*93*). The activation of PC1/3 is also not fully complete until the enzyme reaches the DCSGs. An additional autocatalytic cleavage of the C-terminal domain after a dibasic cleavage site at positions 617-618 renders the enzyme fully active (*131*). Co-transfection of a substrate containing a dibasic cleavage site recognized by PC1/3 with either full length PC1/3 or a PC1/3 deletion mutant devoid of its C-terminal domain demonstrates that full length PC1/3 can cleave substrates uniquely when its C-terminal domain has been removed in DCSGs while the C-terminal truncated mutant is active in a non-granular compartment (*131*). In addition, co-transfection of the substrate with PC1/3 in a non-endocrine cell line resulted in no enzymatic PC1/3 activity while a similar experiment using the C-terminal truncated PC1/3 produced cleaved substrate products (*131*). Thus, removal of the C-terminal of PC1/3 renders the enzyme active and this removal is dependent on the enzyme's localization in DCSGs. Rabah *et al*. demonstrated that the C-terminal domain of PC1/3 inhibits PC1/3 activity in a non-competitive manner in the presence of its own Pro domain (*132*). Thus, the propeptide may bind the catalytic center in the early secretory pathway and the C-terminal domain would bind elsewhere in the molecule. An additional inhibitory mechanism restricting PC1/3 activity may exist. Pro-SAAS (non acronymic) is a peptide present in DCSGs of neuroendocrine cells capable of inhibiting PC1/3 function *in vitro* (*133*). Unfortunately, a pro-SAAS null mouse was made without any significant effect on PC1/3 activity (*134*). In fact, the pro-SAAS null mice were obese as are the PC1/3 knockout mice . It's highly likely that pro-SAAS is a poor substrate for PC1/3 as it contains two non-optimal PC1/3-like recognition sites (*133*). As for the DCSG targeting for PC1/3, it appears to be coordinated by alpha helices located within the C-terminus. Indeed, three separate domains have been identified. A first is located after the C-terminal autocatalytic

cleavage site between residues 617-638 and this domain is predicted to form a transmembrane alpha helix (*135*) though this characteristic remains controversial (*136*). Two separate regions between residues 667-753 are also predicted to form alpha helices and both these sequence are sufficient to sort a heterologous constitutively secreted fusion protein to granules (*48*). A disruption of the second predicted helix located between 738-751 with two proline residues in lieu of two leucine residues at positions 745 and 749 respectively blocked sorting to granules. In addition, a fusion protein expressing the native PC1 sequence between residues 711-753 (thus including the predicted helix between residues 738-753) was consistently located in the membrane fraction in transfected cells while the non-sorting proline mutant was mostly soluble. A recent proteomics analysis of bovine secretory granules revealed that PC1/3 is present in both the membrane and soluble fractions (*137*). These data are consistent with a membrane-anchoring role of alpha helices in sorting of PC1/3 to DCSGs followed by an intra-granular release of PC1/3 devoid of its C-terminal domain where it would be able to cleave substrates. Moreover, PC2 also contains an alpha helical region in its C-terminal domain. Deletion of these residues also blocked PC2 sorting (*47*). Is sorting of PC5/6A, the third granule resident PC also dependent on regions in its C-terminus that can form alpha helices?

1.8.1.2 PC5

PC5 expression is restricted to neuronal cells (*138*), glial cells (*138*), the thyroid gland (*122*), the gut (*139*), the adrenal (*123*) and the cardiovascular system (*123*). There are two PC5 isoforms produced from differentially spliced mRNAS generating PC5/6A, a 915 residue long DCSG protein and PC5/6B, an 1877 residue long Golgi-localized protein with a C-terminal cytosolic tail (*140*). Just like PC1/3 and PC2, the granule sorting sequence of PC5/6A has been localized in the last 38 residues of its C-terminal domain (*140*) without any specific motif having been detected. The C-terminal domain also contains a cysteine-rich region, which is anchored on the cell surface where it can interact

with tissue inhibitors of metalloproteases (*141*). *In vitro* data suggests that the PC5/6A's Pro domain blocks its own activity as with the other two granule resident PCs (*142*). Interestingly, a block in PC5/6A expression results in an increase of PC2 expression (*143*) in a mouse intestinal tumor cell line. Thus, the granule resident PC enzymes are capable of compensating for each other's loss in animal models. The PC5 null mouse resulted in a lethal phenotype demonstrating that the gene regulating PC5's expression is essential (*144*).

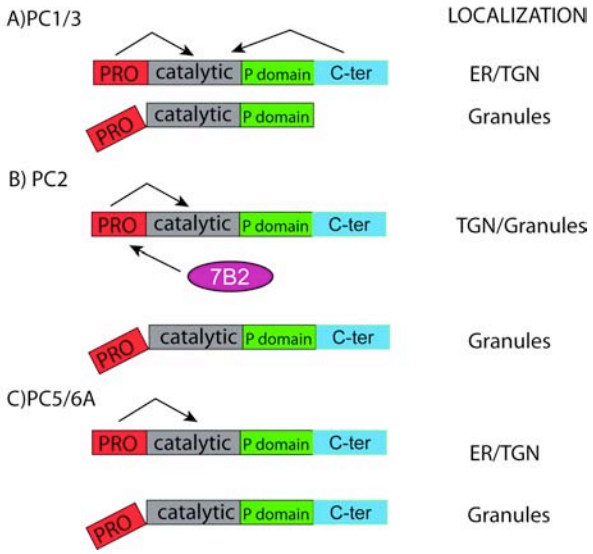


Figure 1.6: The Granule Resident members of the PC family.

(A) PC1/3 activity is inhibited (arrows) by both its PRO and C-terminal domains (C-ter) in the ER/TGN. Once in granules, the PRO domain is cleaved but remains bound to PC1 while the C-terminal domain is autocatalytically cleaved resulting in an active enzyme. (B) Repression of the PRO domain of PC2 is negated (arrow) by the 7B2 chaperone in granules. (C) PC5/6A activity is also repressed (arrow) by its PRO domain in the early secretory pathway

1.9 Objectives of the dissertation

In endocrine and neuroendocrine cells, the secretory pathway contains a regulated component ensuring the activation and subsequent release of hormones within DCSGs.

The correct targeting of prohormones and their proteases to granules is thus critical for cellular function and depends on the diversion of granule-destined proteins at the TGN membrane. The ability of primary sequences capable of forming alpha helices to act as DCSG sorting signals has been observed in granule resident proteins including PC1/3 (*48*) and PC2 (*47*). *Thus, we hypothesize that alpha helices are responsible for redirecting proteins to DCSGs and that this targeting is mediated by protein-lipid interactions.*

In the first objective, we will attempt to determine the biophysical characteristics of sorting helices by testing a series of synthetically engineered alpha helices that vary in residue composition, charge, amphipathicity and hydrophobicity.

In the second objective, we will test whether an alpha helix is necessary for the DCSG targeting of PC5/6A as required for both PC1/3 and PC2. Moreover, we will also assess the efficiency of entry into granules and compare differences between the three granule-resident PC enzymes. A biophysical analysis will be performed to compare differences in the sorting domains of proteins from a same family.

In the final objective, we will solve the three-dimensional solution structure of one of the PC1/3 helical DCSG sorting domains. The function of the individual amino acids making up this DCSG sorting domain will be tested by site-directed mutagenesis.

Taken together, these three objectives will advance our understanding of protein targeting to DCSGs by not only studying the sorting phenomenon but by also observing the structural characteristics of a DCSG sorting domain. Indeed, the functional anatomy of a DCSG sorting domain will be defined by the characteristics of its alpha helix and its ability to interact with the DCSG milieu.

Chapter 2

A Hydrophobic Patch in a Charged Alpha Helix is Sufficient to Target Proteins to Dense Core Secretory Granules

Jimmy D. Dikeakos, Marie–Josée Lacombe, Chantal Mercure, Matei Mireuta and Timothy L. Reudelhuber

Journal of Biological Chemistry 2007; 282 (2) 1136-43

In the following article, Marie–Josée Lacombe and Chantal Mercure provided technical assistance with the cell culture studies while Matei Mireuta assisted in the preparation of the plasmids. The first author under the supervision of Timothy L. Reudelhuber did all other experiments as well as the writing of the manuscript.

ABSTRACT

Many endocrine and neuroendocrine cells contain specialized secretory organelles called dense core secretory granules. These organelles are the repository of proteins and peptides that are secreted in a regulated manner when the cell receives a physiological stimulus. The targeting of proteins to these secretory granules is crucial for the generation of certain peptide hormones including insulin, adrenocorticotropic hormone (ACTH) and others. While previous work has demonstrated that proteins destined to a variety of cellular destinations including secretory granules contain targeting sequences, no single consensus sequence for secretory granule sorting signals has emerged. We have previously shown that alpha helical domains in the carboxy-terminal tail of the prohormone convertase PC1/3 play an important role in the ability of this region of the protein to direct secretory granule targeting. In the current study we show that a variety of alpha helical domains are capable of directing a heterologous secretory protein to granules. By testing a series of synthetic alpha helices we also demonstrate that the presence of charged (either positive or negative) amino acids, spatially segregated from a hydrophobic patch in the alpha helices of secretory proteins likely plays a critical role in the ability of these structures to direct secretory granule sorting.

INRODUCTION

Many enzymes and peptide hormones, such as renin, insulin, adrenocorticotropic hormone (ACTH) and others, are synthesized as precursors that are proteolytically activated before secretion from the cell. In most cases, this proteolytic activation only occurs after the precursors are selectively targeted to specialized organelles found in endocrine and neuroendocrine cells called dense core secretory granules (1;2). This process depends on the efficient co-targeting of the activating protease and its substrate from the trans-Golgi network (TGN) to nascent secretory granules where the protease processes the precursor. Since the resulting polypeptide hormones are stored within secretory granules until the cell receives a signal for their release, this mode of protein export from the cell is called the regulated secretory pathway.

Several protein domains have been implicated in the process of sorting proteins to dense core secretory granules. The function of these domains can be grossly subdivided into three groups: The first group involves formation of high molecular weight protein complexes or aggregates. Indeed, many granule targeted proteins have the ability to multimerize or aggregate, leading in most cases to the formation of electron dense cores (reviewed in (3)). Expression of a variety of granule cargo proteins including chromogranin A (4;5), pro-vasopressin, pro-oxytocin, pro-opiomelanocortin (POMC), secretogranin II and chromogranin B (5) is sufficient to induce aggregate-containing cytoplasmic vesicles in cells that do not normally contain secretory granules, although these vesicles do not have all of the characteristics of secretory granules (5). Proinsulin, POMC and engineered proteins containing the carboxy-terminal tail of prohormone convertase PC1/3 also form multimers; however this property alone is insufficient to direct granule sorting (6;7). Thus, aggregation or multimerization may contribute to secretory granule targeting efficiency although it has not been shown in many cases to be essential. A second group of granule-sorting domains may act by binding cargo proteins to granule-tethered proteins. For

example, carboxypeptidase E (CPE) has been proposed to interact with a number of granule cargo proteins including POMC, proenkephalin, growth hormone and proinsulin and to promote their retention in secretory granules, even though some of these are not enzymatic substrates of CPE (8-10). In addition, paired basic amino acids (that may form semi-stable complexes with granule-resident proteases) have been reported to direct secretory granule sorting in some proteins including prosomatostatin (11), prorenin (12) and progastrin (13). Muclin, a type I membrane protein granule-resident protein, has also been suggested to act as a granule cargo "receptor" in pancreatic cells through its binding of sulfate groups on O-linked glycosylated proteins (14). Thus, a variety of interactions with "tethers" may serve to target proteins to secretory granules. A third class of granule targeting proteins domains involves the direct association of proteins with the secretory granule membrane. Peptidyl-α-amidating monooxygenase (PAM) (15), phogrin (16) and muclin (14) are all type 1 membrane-spanning proteins that are targeted to dense core secretory granules. Proteins may also interact, but not span, granule membranes: For example, the membrane-binding domains of the granule-resident protein carboxypeptidase E (17;18) and the prohormone convertases PC1/3 (7;19), and PC2 (20) are key for their targeting to dense core secretory granules. In each of these cases, as well as in prosommatostatin (21) and the neuronal and endocrine polypeptide VGF (22), the granule sorting domain includes one or more short alpha helical domains. Taken together, these results raise the possibility that the presence of alpha helical domains plays an important role in the targeting of certain proteins to nascent secretory granules. In the current study we have sought to obtain a better understanding of the mechanism by which granule sorting alpha helices function. A systematic analysis of a series of alpha helices varying in either their polar or non-polar residues or their amphipathicity indicates that the hydrophobic face of an alpha helix is likely critical for targeting proteins to secretory granules.

EXPERIMENTAL PROCEDURES

Recombinant plasmid construction

Naturally occurring peptide fragments to be analyzed for secretory granule sorting were derived from the mouse PC1/3 (NM_013628) and the human prorenin cDNAs (NM_000537). The numbering used to identify the protein domains is relative to initiator methionine of both proteins. Protein fragments were tested for their ability to sort heterologous proteins to secretory granules by attachment to a fragment of mouse immunoglobulin IgG2b (referred to as Fc) as previously described ((7) and illustrated in Figure 2.1C). Fusion proteins were constructed by selective amplification of corresponding fragments using polymerase chain reaction (PCR). Attachment of the K/L+W synthetic amphipathic alpha helices KLLKLLLKLWLKLLKLLL (23) to the Fc fusion protein containing PC1/3 711-740 (Figures 2.1, 2.2) or the Fc protein alone (Figure 2.3) was accomplished by insertion of double stranded oligonucleotides with the following forward sequences: **K/L+W:** AAG CTG TTG AAA CTA TTG CTG AAA CTG TGG CTA AAG CTG TTG AAG CTA CTG CTA TGA. Remaining synthetic helices (Figure 2.3 and Table 2.2) were similarly constructed by using the following forward nucleotides: **K/V+W:** AAG GTG GTT AAA GTC GTG GTT AAG GTC TGG GTG AAG GTT GTC AAA GTG GTT GTC TGA; **Non-amphi.:** AAG AGA CTG AAA TTG CTA CTG AAG TTG TGG CTA AAA CTG TTG AAG CTA AAA AAG TGA; **E/L+W:** GAG CTG TTG GAA CTA TTG CTG GAG CTG TGG CTA GAA CTG TTG GAG CTA CTG CTA TGA; **D/L+W:** GAC CTG TTG GAT CTA TTG CTG GAC CTG TGG CTA GAT CTG TTG GAC CTA CTG CTA TGA; **K/A+W:** AAG GCT GCA AAA GCC GCA GCG AAG GCA TGG GCG AAG GCT GCG AAA GCT GCC GCA TGA; **K/A:** AAG GCT GCA AAA GCC GCA GCG AAG GCA GCT GCG AAG GCT GCG AAA GCT GCC GCA TGA; **G/L+W:** GGC CTG TTG GGT CTA TTG CTG GGG CTG TGG CTA GGG CTG TTG GGT CTA CTG

CTA TGA. All of the resulting coding sequences were verified in their entirety by DNA sequencing and were inserted into the pRSV globin mammalian expression vector (7).

Mammalian cell culture, transfection and secretion analysis

Mouse corticotrophic AtT-20 cells were grown in Dulbecco's minimal essential medium (DMEM; Invitrogen, Burlington, Ontario, CANADA) containing 10% fetal bovine serum (FBS) in a humidified incubator at 37 ºC in 10% CO_2. Expression vectors were stably transfected into AtT-20 cells by co-transfection with pSV-Neo (Invitrogen, Burlington, Ontario, CANADA) and selection of stable pools was carried out in Geneticin (G418, Invitrogen, Burlington, Ontario, CANADA). G418-resistant pools of cells were used for all subsequent studies.

For metabolic labeling, 4.5 X 10^5 stably transfected cells were plated in each of two 35mm dishes. Twenty-four hours later, the medium was replaced with 0.5 ml of pre-warmed methionine-free DMEM containing 10% dialyzed FBS for 1 hrs. Labeling was achieved by addition of 300 μCi of ^{35}S-methionine/cysteine (Trans-^{35}S Label; MP Biomedicals, Irvine, CA) for 2 hours. Medium was then replaced with pre-warmed complete medium for 16 hrs. (chase). To test for regulated secretion the cells were rinsed in complete medium and in one of the wells, the cells were incubated for an additional 3 hours in complete medium to measure constitutive secretion while in the other well the cells were incubated in complete medium supplemented with 10μM forskolin (Sigma-Aldrich, St. Louis), a secretagogue which stimulates secretory granule release. The corresponding culture supernatants were then immunoprecipitated with Protein-A Sepharose (Sigma-Aldrich, St. Louis) and the immunoprecipitated proteins were separated by SDS-PAGE. The gels were incubated with 3 changes of 10% 2,5-Diphenyloxazole (PPO; Sigma-Aldrich, St. Louis) in dimethyl sulfoxide, rinsed in water, dried and subjected to fluorography. Dried gels were subsequently exposed to storage phosphor screens and emissions were quantified using a Storm Phosphorimager (GE Healthcare, Mississauga, Ontario). Results were compared by

one-way ANOVA using Dunnet's post-test and are expressed in Table 2.I as mean ± SEM. The forskolin-stimulated secretion of the endogenous granule cargo peptide beta-endorphin (2.00 ± 0.14, n=15) was determined by radioimmunoassay in each experiment and was used to ensure that the stimulation of AtT-20 cell granule release was comparable in all experiments.

Protein secondary structure predictions.

Secondary structure predictions (Table 2.2) were carried out with the NNPREDICT (24), GOR III (25) and PROF (26) algorithms. Helical wheel projections and isoelectric point calculation (pI) were carried out with the EMBOSS (The European Molecular Biology Open Software Suite) software package (27). Hydrophobic cluster analysis was carried out by the method of Gaboriaud et al. (28).

Immunocytochemistry and confocal microscopy

AtT-20 cells stably transfected with the appropriate expression vector were seeded onto Lab Tek Glass Chambers (Nalgene Nunc, Napierville, Il) at a density of 10,000 cells/chamber. Twenty four hours later, the cells were fixed with 4% paraformaldehyde in Tris-buffered saline (TBS) for 10 minutes at room temperature, rinsed in TBS and permeabilized with absolute methanol at –20 °C for 10 min. Non-specific binding was blocked for 30 minutes with 5% non-fat milk in TBS at 4°C for 1 hr. Slides were incubated with a polyclonal rabbit anti-ACTH antibody (1:300, obtained from Nabil G. Seidah, IRCM) and an anti-mouse IgG antibody (Invitrogen, Burlington, Ontario, CANADA) conjugated to ALEXA 488 (1:200) for 1 hr. at room temperature in blocking buffer. The slides were subsequently washed with TBS and incubated with anti-Rabbit IgG antibody conjugated to rhodamine (1:100; Chemicon, Temecula, Ca) for 1 hr. at room temperature.

Slides were mounted using SlowFade Light Antifade Kit, (Invitrogen, Burlington, Ontario, CANADA) and visualized using a Zeiss LSM 510 Confocal Microscope.

RESULTS

Exposed alpha helices direct secretory proteins to granules

To compare the ability of various alpha helical domains to redirect a constitutively secreted protein into secretory granules, we analyzed the secretory properties of a series of pulse-labeled recombinant fusion proteins in mouse pituitary AtT-20 cells either in the absence or presence of forskolin, a secretagogue that increases intracellular cAMP resulting in the release of secretory granules. AtT-20 cells contain dense core secretory granules in which endogenous proopiomelanocortin (POMC) is processed into adrenocorticotropic hormone (ACTH) by a series of proteolytic cleavages involving PC1/3 (reviewed in (29)). As we have previously shown (7), a recombinant protein expressing a single chain fragment of the mouse immunoglobulin heavy chain constant region is secreted constitutively (i.e., not retained in granules) when expressed in these cells as evidenced by equivalent levels of secretion in absence (-F; constitutive secretion) and presence (+F; regulated secretion) of forskolin (Figure 2.1C, Fc). By contrast, attaching the region of the PC1/3 C-terminal tail comprising amino acids 711-753 onto the C-terminus of the Fc fragment (Fc:PC1/3 711-753) leads to its increased secretion in the presence of forskolin, demonstrating that the fusion protein had been diverted to the regulated secretory pathway. This portion of the PC1/3 C-terminal tail contains a region predicted to form an alpha helix (Figure 2.1A) which is crucial for its ability to direct secretory granule sorting (7). The importance of the alpha helix in directing sorting is confirmed by the loss of forskolin-stimulated release of the fusion protein in which the helix domain is deleted (Figure 2.1; Fc: PC1 711-740). To determine if there is some unique characteristic of alpha helices that are capable of sorting proteins to secretory granules, we replaced the natural helix predicted to reside in the last 13 amino acids of PC1/3 C with an entirely synthetic amphipathic alpha helix (23). This alpha helix (Figure 2.1B K/L+W; also referred to as Hel 13-5 in references 30-33) has been

shown to bind with high affinity to both model membranes and biomembranes (30;31) and to reorganize Golgi-specific phospholipid micelles into nanotubular structures (32). Surprisingly, substitution of the native helical peptide with the synthetic alpha helix led to regulated secretion of the fusion protein in AtT-20 cells (Figure 2.1C; Fc: PC1/3 711-740 K/L+W). To further test whether the simple exposure of alpha helical domains on secretory proteins could be sufficient to target proteins to secretory granules, we attached the first 31 amino acids of the human prorenin prosegment to the C-terminus of the Fc fragment (Figure 2.1C; Fc:hProren 1-31). While this region of human prorenin contains two predicted alpha helical domains (33) (Figure 2.1B), these play no role in secretory granule sorting in the native protein context (12). However, when placed C-terminal to the Fc fragment, the prorenin prosegment has the ability to confer regulated secretion to the fusion protein (Figure 2.1C; Fc:hProren 1-31). Quantification of granule sorting efficiency and comparison to the forskolin-stimulated secretion of endogenous beta-endorphin (a peptide product of POMC cleavage in secretory granules) confirms that all of the helix-containing domains divert the normally constitutively secreted Fc fragment into the regulated secretory pathway (Table 2.1).

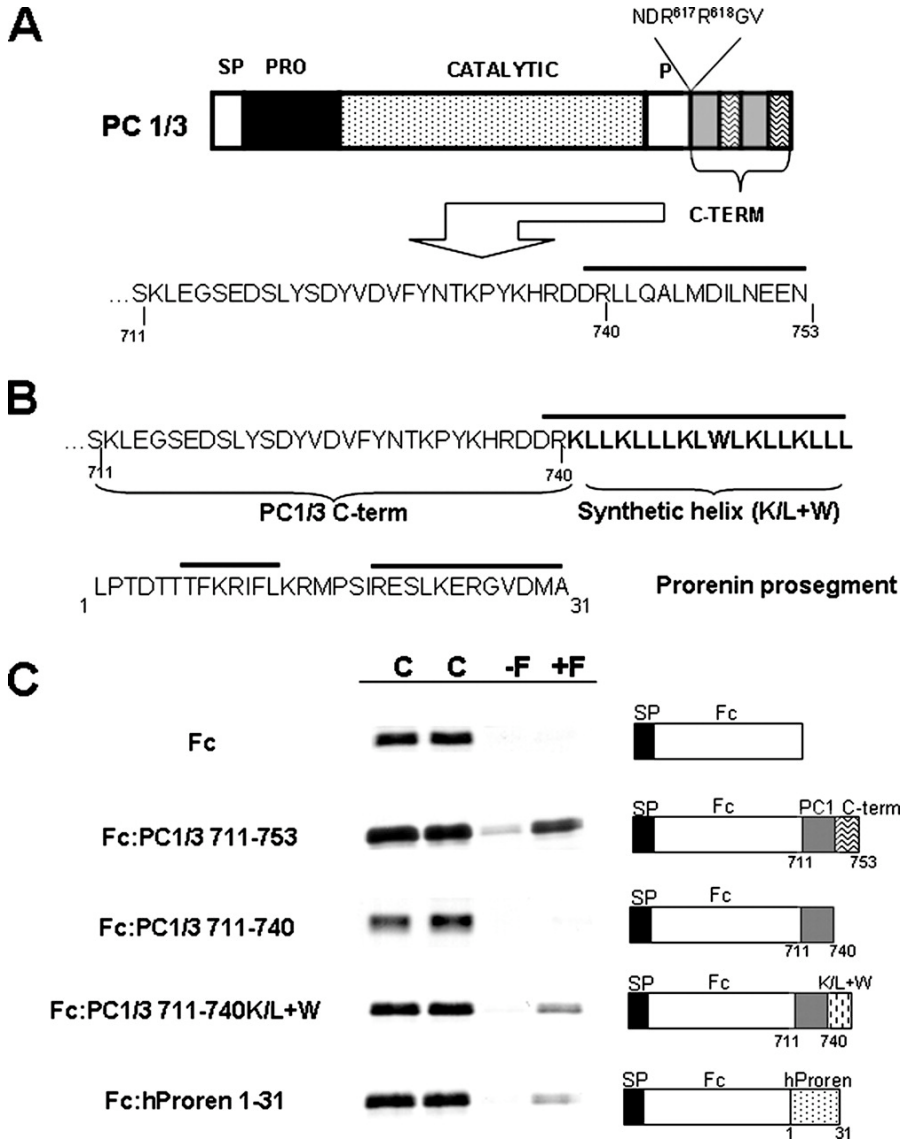


Figure 2.1: A variety of alpha helices are able to target proteins to secretory granules.

(A) The C-terminal tail of PC1/3 from amino acids 711-753 is sufficient to target heterologous proteins to the regulated secretory pathway (7). **(B)** Heterologous alpha helical domains tested for granule targeting. (Top) A fusion between a portion of the PC1 C-terminal tail in which the native granule-sorting alpha helical region has been deleted and replaced with a synthetic helix (bold type). The novel predicted helical region is overlined. (Bottom) A portion of the pro region of prorenin between amino acids 1 and 31 contains

two predicted alpha helical regions (overlined) neither of which are involved in secretory granule targeting in the native protein (12). **(C)** Transfection assay to test for secretory granule targeting. The fusion proteins (depicted in schematic form on the right) consisted of a signal peptide (sp) and a fragment of the mouse immunoglobulin heavy chain (Fc) in addition to the putative sorting domains described above. Parallel wells of stably transfected AtT-20 cell pools expressing the various fusion proteins were pulse-labeled for 2 hrs and chased with unlabeled medium for an additional 16 hrs. After the chase period, the supernatants were collected from the parallel wells (two lanes labeled C) and the cells were subsequently incubated for an additional 3 hrs either in the absence (-F) or presence (+F) of the secretagogue Forskolin. Fc containing proteins in the culture supernatants were immunoprecipitated with Protein A Sepharose, separated by SDS-PAGE and detected by fluorography.

FUSION PROTEIN	**n=**	**SORTING INDEX (+F/-F)**
Fc	8	0.94 ± 0.10
Fc:PC1/3 711-753	5	4.82 ± 0.55**
Fc:PC1/3 711-740	3	1.25 ± 0.39
Fc:PC1/3 711-740Synth	4	3.24 ± 0.12**
Fc:hProren 1-31	8	3.25 ± 0.16**

Table 2.1: Secretory granule sorting efficiency of fusion proteins in transfected AtT-20 cells.

Immunoprecipitated fusion proteins from autoradiograms similar to those shown in Figure 2.1 were quantified by exposure to a storage phosphor screen. Values shown are the mean ± SEM for multiple independent experiments.**P< 0.01 as compared to Fc.

As an independent confirmation of the ability of these alpha helices to direct secretory granule targeting, we compared the sub-cellular distribution of the various Fc fusion proteins to that of ACTH, an endogenous granule-resident protein in AtT20 cells (Figure 2.2). Labeling of the transfected AtT-20 cells with an anti-ACTH antibody (Figure 2.2, middle column) results in staining of POMC, the precursor of ACTH in the Golgi apparatus (asterisks) as well as speckled staining of ACTH in dense core secretory granules which are concentrated in cytoplasmic extensions ((34); closed arrowheads). When either the Fc protein alone or the Fc protein attached to the 711-740 fragment of PC1/3 are expressed, staining is localized primarily in the Golgi and perinuclear area (left column, asterisks), indicating inefficient sorting to granules as predicted by our biochemical studies, above. In contrast, attachment of either the 711-753 region of PC1/3 or the human prorenin prosegment to the Fc fragment or the synthetic K/L+W alpha helix to Fc:PC1/3 711-740 leads to a dramatic increase of fusion protein detection in cytoplasmic extensions (Figure 2.2). This staining corresponds to secretory granules as evidenced by its co-localization with ACTH (right column). Thus, the sub-cellular distribution of the fusion proteins as determined by confocal microscopy confirms the results obtained by biochemical assays and strongly suggests that any exposed alpha helical domain in a secretory protein can direct the protein to dense core secretory granules.

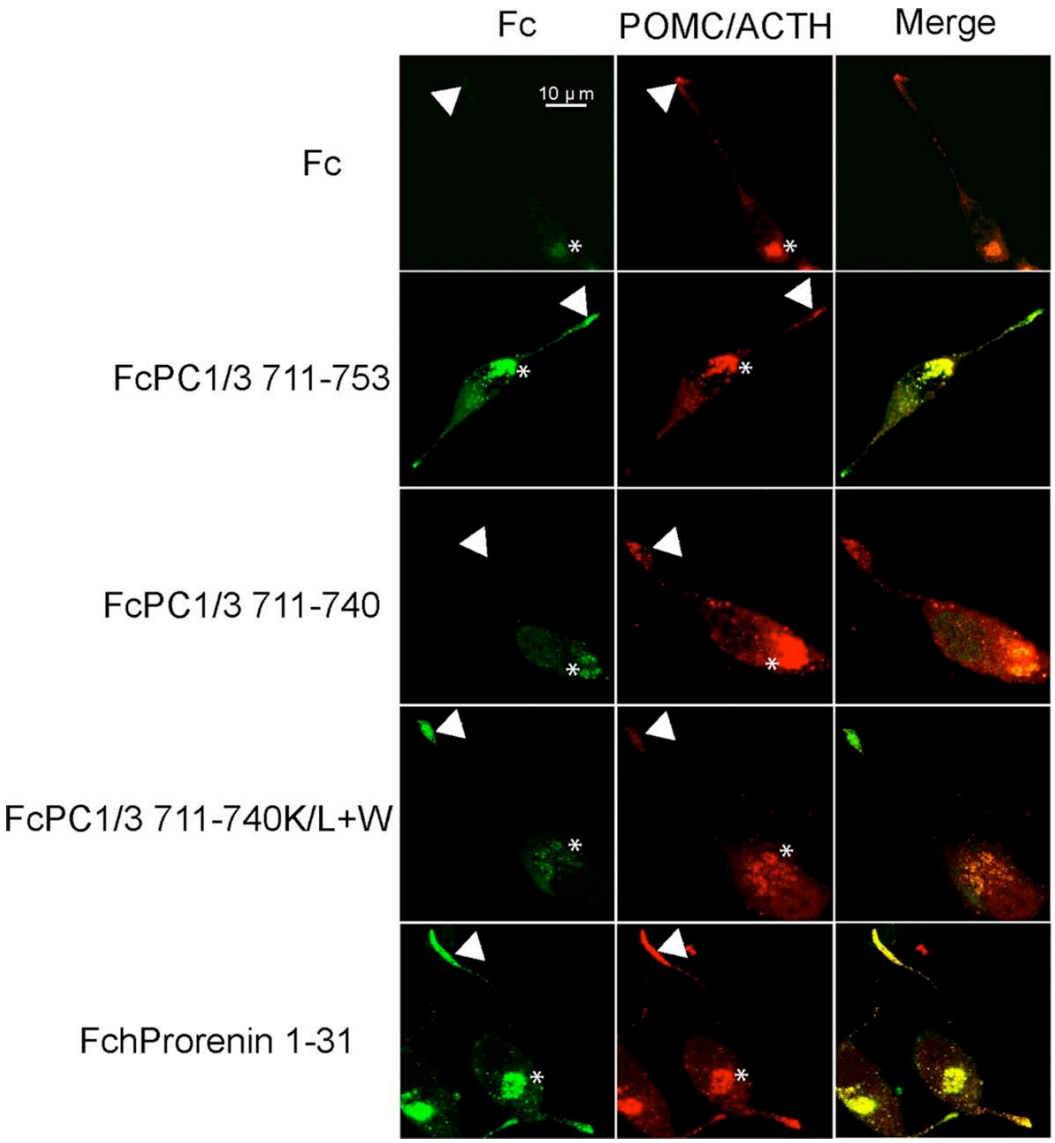


Figure 2.2: A variety of alpha helix-containing domains target fusion proteins to secretory granules.

Stably transfected mouse pituitary AtT-20 cells were simultaneously immunolabeled with antibody to the various fusion proteins (Fc, left panel) and endogenous ACTH (middle panel). The asterisks denote the Golgi apparatus that stains for all of the fusion proteins as well as the ACTH precursor POMC. The closed arrowheads denote the cytoplasmic

extensions where ACTH-containing secretory granules are concentrated. Note that the relative staining intensity of the fusion proteins (green, left panels) in the granule-containing cytoplasmic extensions increases dramatically when either of the three helix-containing domains is attached to the fusion protein. Original magnification 63X. Reference size bar is in the upper left panel.

Biochemical characteristics of granule-targeting alpha helices

Although the data presented above demonstrate that the presence of an alpha helix is necessary for the granule sorting activity of the protein fragments tested, they do not test whether an alpha helix is sufficient to constitute a granule-sorting signal. To test this possibility, we have attached the K/L+W helix directly to the C-terminus of the Fc protein and examined its sorting to secretory granules in stably transfected AtT-20 cells. Because the fusion proteins attached to various synthetic alpha helices were secreted with variable kinetics from the transfected cells (data not shown), we could not directly compare their sorting by pulse-chase analysis. For this reason and because the cellular distribution of the fusion proteins by microscopy confirms the pulse chase analysis (Figure 2.2), we chose to test for the steady-state distribution of the helix-containing fusion proteins in transfected AtT-20 cells (Figure 2.3). Analysis of the distribution of the Fc protein fused to the K/L+W helix showed an identical pattern of distribution of Fc and ACTH staining, both in the Golgi apparatus (asterisks) as well as the cytoplasmic extensions (closed arrowheads). Thus, it appears that the K/L+W helix is sufficient to re-direct the constitutively secreted Fc protein to secretory granules. To derive an estimate of the efficiency of granule sorting with this fusion protein, the fraction of Fc-expressed cells that contained the fusion protein in the granule-containing cytoplasmic extensions was determined (Table 2.3). The K/L + W helix was present in 70.40 ± 3.06 % of the cells in the cytoplasmic extension, suggesting a high efficiency of targeting to secretory granules.

The finding that synthetic helices are sufficient to direct secretory granule targeting in fusion proteins permits the analysis of the biochemical requirements for the sorting activity by introducing systematic amino acid substitutions. As shown in the helical wheel projection in Table 2.2, the K/L+W helix is amphipathic (23) with a predicted isoelectric point (pI) of 11.4. To test for the contribution of the positive charge of the helix, we substituted the lysines with negatively charged glutamic acid residues. Thus, the E/L+W helix is also predicted to be amphipathic but has a pI of 3.3 (Table 2.2). Nevertheless,

confocal microscopy shows that the negatively charged E/L+W helix also redirected the fusion protein to secretory granules as evidenced by its co-localization with ACTH in the cytoplasmic extensions in 63.27 ± 3.37 % of the cells (Figure 2.3, closed arrowheads). Interestingly, replacing the glutamic acid residues with aspartic acid (D/L+W), while not significantly changing the pI of the helix (Table 2.2) led to reduced co-localization of the fusion protein with ACTH in cytoplasmic extensions and a more diffuse staining throughout the cell body with only 36.17 ±1.57 % of the fusion protein being present in cytoplasmic extensions (Figure 2.3, D/L+W). Moreover, a comparison of the distribution of the Fc fusion proteins linked to K/L+W, E/L+W and D/L+W reveals that only the helix containing the glutamic acids (E/L+W) co-localizes with POMC in the perinuclear Golgi apparatus.

To test the importance of the hydrophobic face of the helices in sorting, we progressively decreased the hydrophobicity of the amino acids on that side of the helix. Accordingly, the K/V+W, K/A+W helices gradually reduce the size and hydrophobicity of the side chains on the hydrophobic face, whereas the K/A helix also removes the bulky tryptophan hydrophobic group. While the K/V+W helix led to a degree of co-localization with ACTH comparable to that seen with the K/L+W helix (66.86 ± 1.45 of K/V +W in cytoplasmic extensions), the K/A+W helix shows more staining of the fusion protein in the Golgi apparatus than in the granule containing extensions with only 3.20 ±1.60 % of the fusion protein present in cytoplasmic extensions (K/A+W, closed arrowheads). Strikingly, removal of the bulky hydrophobic tryptophan residue from the uncharged face of the helix leads to detection of the fusion protein only in the Golgi apparatus (K/A, <0.9% of the fusion protein is present in cytoplasmic extensions). Thus, the ability of the helix to direct proteins to secretory granules appears to correlate with its hydrophobicity.

To test whether amphipathicity is necessary for the sorting activity of the helix, we replaced three leucine residues on the hydrophobic face of the amphipathic helix with three positively charged amino acids (Table 2.2, Non-amphi., Helical wheel projection). Confocal microscopy of transfected AtT-20 cells revealed that this non-amphipathic helix

efficiently redirects the fusion protein to secretory granule-containing cytoplasmic extensions where it co-localizes with ACTH in 72.05 ± 1.95 % of the cells (Figure 2.3, Non-amphi., closes arrowheads). In order to determine if a charged face on the helix is important for its activity in directing secretory granule sorting, a helix was designed with non-polar glycine residues replacing the charged residues while maintaining the hydrophobic face (Table 2.2, G/L+W). This G/L+W helix-containing fusion protein shows no co-localization with POMC or ACTH in either the Golgi apparatus or the cytoplasmic extensions of the transfected cells, but rather shows a weak and diffuse cytoplasmic staining pattern in permeabilized cells (Figure 2.3, G/L+W). Immunocytochemistry of the G/L + W helix under non-permeabilizing conditions resulted in staining of the plasma membrane suggesting that the fusion protein containing the G/L + W helix is restricted to the membrane surface. Surprisingly, when the cells were prepared under non-permeabilizing conditions, a strong generalized staining pattern for the G/L+W helix-containing fusion protein was detected, consistent with the distribution of the fusion protein on the cell surface (Figure 2.4, lower right panel). This staining pattern was not seen in cells expressing the fusion protein containing the very similar helix in which the glycines were replaced with lysines (Figure 2.4, upper right panel). Indeed, we have been unable to detect secretion of the G/L+W helix-containing fusion protein in culture supernatants after metabolic labeling (data not shown). Thus, although amphipathicity per se does not seem to play a critical role in the ability of the helix to direct sorting of the fusion protein to secretory granules, maintenance of a charged surface on the helix is essential in allowing a protein to dissociate from the plasma membrane and be efficiently secreted.

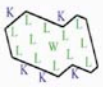
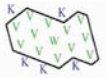
Peptide name	Primary sequence	pI	Predicted structure	Helical wheel projection	Hydrophobic cluster projection
K/L+W	KLLKLLLKLWLKLLKLLL	11.4	helix[A-D]		
E/L+W	ELLELLLELWLELLELLL	3.3	helix[A-C]		
D/L+W	DLLDLLLDLWLDLLDLLL	3.1	helix[A-C]		
K/V+W	KVVKVVVKVWVKVVKVVV	11.4	helix[A,B] strand[C]		
K/A+W	KAAKAAAKAWAKAAKAAA	11.4	helix[A-C]		
K/A	KAAKAAAKAAAKAAKAAA	11.4	helix[A-C]		
Non-amphipathic	KRLKLLLKLWLKLLKLKK	12.5	helix[A-C]		
G/L+W	GLLGLLLGLWLGLLGLLL	6.1	helix[A-C]		

Table 2.2: Biochemical characteristics of the synthetic helices tested for granule sorting activity:

Isoelectric point (pI) and helical wheel projections were determined using the EMBOSS software package (27). Secondary structure was either predicted with the NNPREDICT[A], GOR III[B] and PROF [C] algorithms(24) (25) (26) or determined by circular dichroism[D] (23). Hydrophobic cluster analysis was carried out by the method of Gaboriaud et al. (28). The filled diamond shapes represent the positions of the non-polar glycine residues. Circled sequences represent hydrophobic patches.

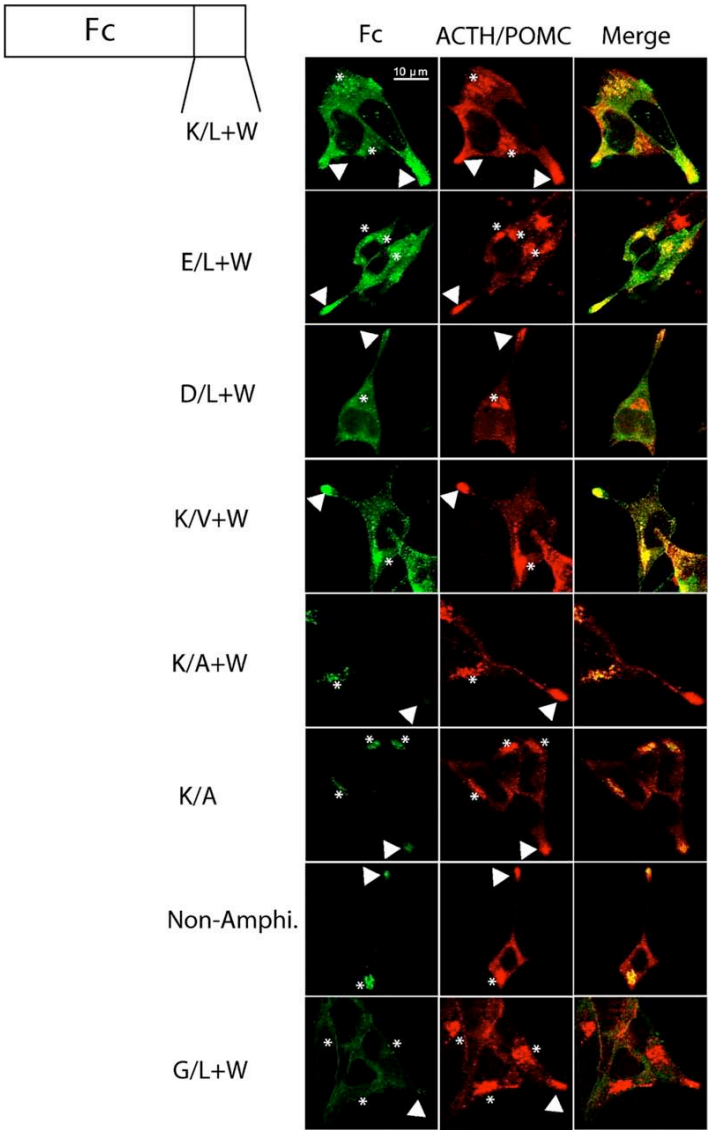


Figure 2.3: Granule-targeting efficiency of alpha helices with differing biochemical properties.

Fc fusion proteins attached to various non-natural alpha helices were stably transfected in mouse pituitary AtT-20 cells and simultaneously immunolabeled with antibody to detect the fusion proteins (Fc, left panel) and endogenous ACTH (middle panel). See Table 2.2 for the primary sequence and structural characteristics of the helical domains tested. The asterisks denote the Golgi apparatus that stains for all of the fusion proteins as well as the

ACTH precursor POMC. The closed arrowheads denote the cytoplasmic extensions where ACTH-containing secretory granules are concentrated. Yellow staining in the right panel denotes co-localization of staining for the Fc fusion proteins and POMC/ACTH. Original magnification was 63X. Reference size bar is in the upper left panel.

Fc Fusion protein	**Percent expressing cells showing localization in cytoplasmic extensions (%)**
Fc	2.15 ± 1.57
K/L + W	70.40 ± 3.06 ***
E/L + W	63.27 ± 3.37 ***
D/L + W	36.17 ± 1.57 ***
K/V + W	66.86 ± 1.45 ***
K/A + W	3.20 ±1.60
K/A	0.98 ±0.98
Non-amphipathic	72.05 ±1.95 ***
G/L + W	1.54 ±1.50

Table 2.3: Secretory granule sorting efficiency of Fc fusion proteins containing various alpha helical peptides with differing biochemical properties.

Stably transfected AtT-20 cells were immunostained as in Figure 2.3 and the percent of Fc-expressing cells with staining in granule-containing cytoplasmic extensions was assessed in 100 separate cells each in three independent experiments. Values shown are the mean ± SEM. ***P< 0.001 as compared to Fc.

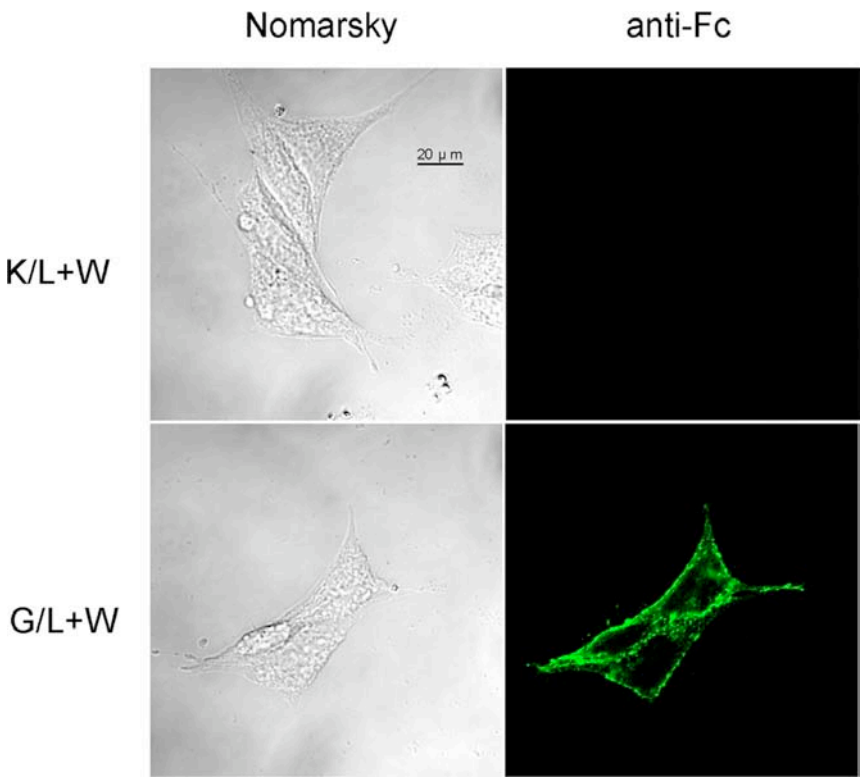


Figure 2.4: **The G/L+W fusion protein is localized to the cell surface**.

Transfected AtT-20 cells stably expressing Fc fusion proteins with either the K/L+W or G/L+W peptides at their C-terminus were stained with fluorescent antibody to the Fc protein as described in Figure 2.2 and 3, but without permeabilization. Note that while the G/L+W containing Fc fusion protein showed little intracellular staining (Figure 2.3), abundant staining is detected in the absence of permeabilization, suggesting localization to the cell surface (G/L+W, right lower panel). In contrast, the K/L+W peptide does not direct the Fc fusion protein to the cell surface (right, upper panel). Original magnification was 63X. Reference size bar is in the upper left panel

DISCUSSION

In this study, we have provided evidence that the simple exposure of an alpha helical domain is sufficient to direct a linked secretory protein to dense core secretory granules. Although it is clear that alpha helices are present in numerous secretory proteins that do not enter secretory granules, placement of these helices in the context of our fusion proteins may expose them, making them able to interact with membranes or other granule sorting components in the secretory pathway. This would explain the ability of the prorenin alpha helices to direct granule sorting in the fusion protein while they have no such ability in the native protein. The alpha helices in the C-terminal tail of PC1/3 may, themselves, be masked until the enzyme undergoes a conformational change in the TGN where the tail has been proposed to unfold and render the enzyme fully active (29;35). In this model, the autocatalysis of the C-terminal tail would have to take place after formation of the immature secretory granule since the tail would provide the anchor for retention of PC1/3 in the nascent granule buds.

The most surprising finding of the current study is that a completely synthetic alpha helix is sufficient to redirect a constitutively secreted protein into the regulated secretory pathway, thereby confirming that it is the helix itself, and not adjacent sequences, that directs the secretory granule sorting of the fusion protein. This finding made it possible to systematically characterize the biochemical requirements for this sorting activity. The initial peptide that we tested (K/L+W) was previously shown to form an amphipathic helix and to rearrange Golgi-enriched liposomes into nanotubular structures in vitro (23). For direct comparison of the activity of this helix in in vitro liposome deformation and in vivo secretory granule sorting, we included the bulky hydrophobic tryptophan residue placed originally in this helix to measure tryptophan quenching in vitro (23). Our results demonstrated that the presence of the hydrophobic leucine and tryptophan residues in the helix played a critical role in conferring sorting activity (Figure 2.3 and Table 2.3). In

addition, although there was no strict requirement for amphipathicity (non-amphipathic helix) (Figure 2.3 and Table 2.3), there did seem to be a correlation between the ability of the helix to direct sorting and clustering of hydrophobic residues (Table 2.2) and the degree of hydrophobicity of the residues in the cluster (Leu > Val > Ala). In addition to the requirement for hydrophobic residues, sorting helices also required segregated charged residues since removal of the charges (e.g., G/L+W) completely eliminated the sub-cellular sorting of the fusion protein to the secretory granule and caused the fusion protein to be accumulated at the cell surface. The finding that both negatively and positively charged amino acids could be substituted in the helix supports the role of the charge beyond a primary recognition event in the sorting (e.g. with another protein or with a charged lipid). The seemingly more efficient sorting of the helix containing glutamic acids (E/L+W) as compared to the helix containing aspartic acids (D/L+W) remains difficult to understand. Notably, the aspartic acid side chain is the smallest among the charged amino acids we tested, raising the possibility that larger charged side chains are more efficient in directing granule sorting. Interestingly, the natural sorting helices of PC1/3 (Figure 2.1), PC2 (20) and CPE (18) are rich in glutamic acids. One possible explanation for the role of the charged amino acids is that they maintain a shallow interaction of the helix with the inner leaflet of the TGN membrane. This model is supported by the behavior of the G/L+W peptide, in which elimination of the charged residues caused the helix to behave as if it were anchored in the membrane and unable to be secreted.

The importance of the hydrophobic amino acids and the relative unimportance of the charge of the polar residues have been reported in other membrane-deforming helices. Both positively and negatively charged synthetic alpha helices are equally capable of deforming liposomes into tubular structures in vitro (32). Mutation of the hydrophobic residues in a non-membrane spanning alpha helical region of the membrane-interacting protein epsin abolishes its ability to curve membranes (36). Similarly, mutation of hydrophobic residues in Sar1p (a component of the COPII complex) abolished its ability to deform lipid vesicles in vitro whereas mutation of the charged residues within the helix had

no effect (37). The current results suggest that alpha helices involved in sorting proteins to secretory granules might also act by interacting directly with membranes. Indeed, PC1/3 (7;19), PC2 (20) and CPE (18) have been reported to bind membranes through their helical sorting domains. These hydrophobic-plasma membrane interactions may function to assist in the pinching off of the TGN membrane to form the secretory granules. This argues for a shallow interaction between alpha helices and membrane components permitting targeting to secretory granules and subsequent secretion in the extracellular milieu.

In summary, we have shown that simple exposure of an alpha helix at the C-terminal end of a constitutively secreted protein redirects this protein to the regulated secretory pathway. The major characteristic of an alpha helix important for targeting proteins to secretory granules would be the presence of both a hydrophobic cluster and segregated charged amino acids.

ACKNOWLEDGMENTS

The authors wish to acknowledge Drs. Christian Deschepper, Daniel Catanzaro, James Omichinski and Gaétan Thibault for their helpful comments and Dr. Nabil Seidah for his generous gift of anti-ACTH antibody. This work was supported by Operating Grant MOP-53177 (TR) from the Canadian Institutes of Health Research.

REFERENCES

1. Orci, L., Ravazzola, M., Amherdt, M., Madsen, O., Perrelet, A., Vassalli, J. D., and Anderson, R. G. (1986) *J Cell Biol.* **103,** 2273-2281

2. Tanaka, S., Yora, T., Nakayama, K., Inoue, K., and Kurosumi, K. (1997) *J Histochem. Cytochem.* **45,** 425-436

3. Dannies, P. S. (2001) *Mol. Cell Endocrinol.* **177,** 87-93

4. Kim, T., Tao-Cheng, J. H., Eiden, L. E., and Loh, Y. P. (2001) *Cell* **106,** 499-509

5. Beuret, N., Stettler, H., Renold, A., Rutishauser, J., and Spiess, M. (2004) *J Biol. Chem.* **279,** 20242-20249

6. Quinn, D., Orci, L., Ravazzola, M., and Moore, H. P. (1991) *J Cell Biol.* **113,** 987-996

7. Jutras, I., Seidah, N. G., and Reudelhuber, T. L. (2000) *J. Biol. Chem.* **275,** 40337-40343

8. Cool, D. R., Normant, E., Shen, F., Chen, H. C., Pannell, L., Zhang, Y., and Loh, Y. P. (1997) *Cell* **88,** 73-83

9. Cool, D. R. and Loh, Y. P. (1998) *Mol. Cell Endocrinol.* **139,** 7-13

10. Shen, F. S. and Loh, Y. P. (1997) *Proc. Natl. Acad. Sci. U. S. A* **94,** 5314-5319

11. Brakch, N., Cohen, P., and Boileau, G. (1994) *Biochem. Biophys. Res. Commun.* **205,** 221-229

12. Brechler, V., Chu, W. N., Baxter, J. D., Thibault, G., and Reudelhuber, T. L. (1996) *J. Biol. Chem.* **271,** 20636-20640

13. Bundgaard, J. R., Birkedal, H., and Rehfeld, J. F. (2004) *J Biol. Chem.* **279,** 5488-5493

14. Boulatnikov, I. and De Lisle, R. C. (2004) *J Biol. Chem.*

15. O'Donnell, P. J., Driscoll, W. J., Back, N., Muth, E., and Mueller, G. P. (2003) *J Mol. Cell Cardiol.* **35,** 915-922

16. Wasmeier, C., Bright, N. A., and Hutton, J. C. (2002) *Traffic* **3,** 654-665

17. Mitra, A., Song, L., and Fricker, L. D. (1994) *J Biol. Chem.* **269,** 19876-19881

18. Dhanvantari, S., Arnaoutova, I., Snell, C. R., Steinbach, P. J., Hammond, K., Caputo, G. A., London, E., and Loh, Y. P. (2002) *Biochemistry* **41,** 52-60

19. Arnaoutova, I., Smith, A. M., Coates, L. C., Sharpe, J. C., Dhanvantari, S., Snell, C. R., Birch, N. P., and Loh, Y. P. (2003) *Biochemistry* **42,** 10445-10455

20. Assadi, M., Sharpe, J. C., Snell, C., and Loh, Y. P. (2004) *Biochemistry* **43,** 7798-7807

21. Mouchantaf, R., Kumar, U., Sulea, T., and Patel, Y. C. (2001) *J Biol. Chem.* **276,** 26308-26316

22. Garcia, A. L., Han, S. K., Janssen, W. G., Khaing, Z. Z., Ito, T., Glucksman, M. J., Benson, D. L., and Salton, S. R. (2005) *J. Biol. Chem.* **280,** 41595-41608

23. Lee, S., Furuya, T., Kiyota, T., Takami, N., Murata, K., Niidome, Y., Bredesen, D. E., Ellerby, H. M., and Sugihara, G. (2001) *J. Biol. Chem.* **276,** 41224-41228

24. Kneller, D. G., Cohen, F. E., and Langridge, R. (1990) *J. Mol. Biol.* **214,** 171-182

25. Gibrat, J. F., Garnier, J., and Robson, B. (1987) *J. Mol. Biol.* **198,** 425-443

26. Rost, B., Yachdav, G., and Liu, J. (2004) *Nucleic Acids Res.* **32,** W321-W326

27. Rice, P., Longden, I., and Bleasby, A. (2000) *Trends Genet.* **16,** 276-277

28. Gaboriaud, C., Bissery, V., Benchetrit, T., and Mornon, J. P. (1987) *FEBS Lett.* **224,** 149-155

29. Seidah, N. G. and Prat, A. (2002) *Essays Biochem.* **38,** 79-94

30. Kitamura, A., Kiyota, T., Tomohiro, M., Umeda, A., Lee, S., Inoue, T., and Sugihara, G. (1999) *Biophys. J.* **76,** 1457-1468

31. Kiyota, T., Lee, S., and Sugihara, G. (1996) *Biochemistry* **35,** 13196-13204

32. Furuya, T., Kiyota, T., Lee, S., Inoue, T., Sugihara, G., Logvinova, A., Goldsmith, P., and Ellerby, H. M. (2003) *Biophys. J.* **84,** 1950-1959

33. Heinrikson, R. L., Hui, J., Zurcher-Neely, H., and Poorman, R. A. (1989) *Am. J. Hypertens.* **2,** 367-380

34. De, B., I, Marcinkiewicz, M., Malide, D., Lazure, C., Nakayama, K., Bendayan, M., and Seidah, N. G. (1996) *J Cell Biol.* **135,** 1261-1275

35. Ueda, K., Lipkind, G. M., Zhou, A., Zhu, X., Kuznetsov, A., Philipson, L., Gardner, P., Zhang, C., and Steiner, D. F. (2003) *Proc. Natl. Acad. Sci. U. S. A* **100,** 5622-5627

36. Ford, M. G., Mills, I. G., Peter, B. J., Vallis, Y., Praefcke, G. J., Evans, P. R., and McMahon, H. T. (2002) *Nature.* **419,** 361-366

37. Lee, M. C., Orci, L., Hamamoto, S., Futai, E., Ravazzola, M., and Schekman, R. (2005) *Cell.* **122,** 605-617

Chapter 3

PC1/3, PC2 and PC5/6A are Targeted to Dense Core Secretory Granules by a Common Mechanism

Jimmy D. Dikeakos, Chantal Mercure, Marie-Josée Lacombe, Nabil G. Seidah and Timothy L. Reudelhuber

FEBS Journal 2007; 274 (16) 4094-102

In the following article, Marie–Josée Lacombe and Chantal Mercure provided technical assistance with the cell culture studies. Nabil G. Seidah was a collaborator on this project. The first author under the supervision of Timothy L. Reudelhuber did all other experiments as well as the writing of the manuscript.

SUMMARY

There are seven members of the Prohormone Convertase (PC) family of secreted serine proteases that cleave their substrates at basic amino acids thereby activating a variety of hormones, growth factors and viruses. PC1/3, PC2 and PC5/6A are the only members of the PC family that are targeted to dense core secretory granules where they carry out the processing of proteins that are secreted from the cell in a regulated manner. Previous studies have identified alpha helices in the C-termini of the PC1/3 and PC2 proteases that are required for this sub-cellular targeting. In the current study, we demonstrate that a predicted alpha helix in the C-terminus of PC5/6A is also critical for the ability of this domain to target a heterologous protein to the regulated secretory pathway of mouse endocrine AtT-20 cells. Analysis of the subcellular distribution of fusion proteins containing the C-terminal domains of PC1/3, PC2 and PC5/6A confirmed that all three domains have the capacity to redirect a constitutively secreted protein to the granule-containing cytoplasmic extensions. Analysis of the predicted structures formed by these three granule sorting helices shows a correlation between their granule sorting efficiency and the clustering of hydrophobic amino acids in their granule targeting helices.

INTRODUCTION

The Proprotein Convertases (PC) constitute a distinct family of serine proteases related to bacterial subtilisin and the yeast kexin proteases. The PC enzymes cleave their substrates after paired basic amino acids and they are known to participate in the proteolytic activation of a variety of hormones, growth factors, enzymes, receptors and viruses either in the secretory pathway or after secretion from the cell (for review see [1]). Upon entry into the Trans-Golgi Network (TGN), the majority of the PC enzymes, including furin, PC4, PACE4 and PC7, enter low-density secretory vesicles and are secreted from cells in a constitutive manner. Only three of the seven known basic amino acid-specific PC enzymes, PC1/3, PC2 and PC5/6A, are selectively targeted to dense core secretory granules of endocrine and neuroendocrine cells where they activate their substrates. Targeting of proteins to dense core secretory granules requires the recognition of one or more sorting signals in the TGN and granule-resident proteins are either selectively included or retained in nascent secretory granules (reviewed in [2]). The resulting secretory granules subsequently undergo a series of maturation steps that include processing of hormone precursors, condensation to form a dense core and docking at the plasma membrane. Because dense core secretory granules are released from the cell in response to a physiological stimulus, this mechanism of secretion is referred to as the regulated secretory pathway. While the transit time through the regulated secretory pathway is in the order of hours, transit through the constitutive secretory pathway can be completed within minutes.

The various PC enzymes share a common general structure that includes an amino-terminal prosegment which is autocatalytically cleaved, a central catalytic domain comprising the catalytic triad amino acids aspartic acid, histidine and serine and a stabilizing P- domain involved in the binding of calcium [1]. The C-terminal domains of the PC enzymes exhibit the least amount of homology between the family members. Several lines of evidence suggest that the granule sorting signals for PC1/3, PC2 and

PC5/6A reside in the C-terminal domain of these enzymes. PC1/3 devoid of its C-terminal domain is efficiently expressed and enzymatically active, but no longer enters the regulated secretory pathway [3;4]. A predicted amphipathic alpha helix in the last 43 amino acids at the C-terminus of PC1/3 is necessary for this domain to target a heterologous fusion protein to secretory granules and mediates the interaction of this domain with the membrane fraction of expressing cells [3]. Likewise, a protein domain in the C-terminal tail of PC2 is capable of redirecting heterologous proteins to secretory granules [5;6]. This sorting activity is contained in the last 25 amino acids of PC2 and has been reported to form an amphipathic alpha helix capable of interacting with raft resident lipids [6].

The granule-targeting domain of the PC5/6A protease has been less well defined. Alternative splicing produces two forms of PC5/6A that differ in their C-termini [1]: The longer form (PC5/6B) contains a C-terminal transmembrane domain that retains the enzyme in the Golgi apparatus. The shorter form, PC5/6A, is secreted by both the constitutive and regulated secretory pathways. Similar to PC1/3, C-terminal tail of PC5/6A is removed by a proteolytic cleavage once it enters secretory granules [7]. Engineered deletion of the last 38 residues within this C-terminal tail of PC5/6A leads to its exclusive secretion from the constitutive secretory pathway [8], consistent with the existence of a secretory granule sorting signal in this domain. In the current study, we sought to define the secretory sorting signals in the PC5/6A C-terminus and to compare these to the granule sorting domains in the other granule-targeted PC family enzymes. Our results suggest that PC1/3, PC2 and PC5/6A share a common sorting mechanism defined by an alpha helix whose efficiency correlates with the clustering of hydrophobic residues on a face of the helix.

MATERIALS AND METHODS

Recombinant plasmid construction

Naturally occurring peptide fragments to be analyzed for secretory granule sorting were derived from the mouse PC1/3 (NM013628), the mouse PC2 (NM 008792) and the mouse PC5/6A (BC12619). The numbering used to identify the protein domains used is relative to initiator methionine. Protein fragments were tested for their ability to sort heterologous proteins to secretory granules by attachment to a fragment of mouse immunoglobulin IgG2b (referred to as Fc) as previously described [3;21]. Fusion proteins were constructed by selective amplification of corresponding fragments using polymerase chain reaction (PCR). All of the resulting coding sequences were verified in their entirety by DNA sequencing and were inserted into the pCDNA3 mammalian expression vector (BF052232).

Mammalian cell culture, transfection and secretion analysis

Mouse corticotrophic AtT-20 cells were grown in Dulbecco's minimal essential medium (DMEM; Invitrogen, Burlington, Ontario, CANADA) containing 10% fetal bovine serum (FBS) in a humidified incubator at 37 ºC in 10% CO_2. Stable transfection of expression vectors into AtT-20 cells was carried out by electroporation as previously described [3]. Selection of stable pools was carried out in Geneticin (G418, Invitrogen, Burlington, Ontario, CANADA). G418-resistant pools of cells were used for all subsequent studies.

For secretion analysis, 4.5 X 10^5 stably transfected cells were plated in each of two 35mm dishes. Twenty-four hours later, the medium was replaced with 0.5 ml of pre-warmed methionine-free DMEM containing 10% dialyzed FBS for 1 hrs. Labeling was achieved by addition of 300 µCi of ^{35}S-methionine/cysteine (Trans-^{35}S Label; MP Biomedicals, Irvine, CA) for 2 hours. Medium was then replaced with pre-warmed complete medium for 16 hrs. (chase). To test for regulated secretion the cells were rinsed in complete medium and in one

of the wells, the cells were incubated for an additional 3 hours in complete medium to measure constitutive secretion while in the other well the cells were incubated in complete medium supplemented with 10μM forskolin (Sigma-Aldrich, St. Louis), a secretagogue which stimulates secretory granule release. The corresponding culture supernatants were then immunoprecipitated with Protein-A Sepharose (Sigma-Aldrich, St. Louis) and the immunoprecipitated proteins were separated by SDS-PAGE. The gels were incubated with 3 changes of 10% 2,5-Diphenyloxazole (PPO; Sigma-Aldrich, St. Louis) in dimethyl sulfoxide, rinsed in water, dried and subjected to fluorography. Dried gels were subsequently exposed to storage phosphor screens and emissions were quantified using a Storm Phosphorimager (GE Healthcare, Mississauga, Ontario). The forskolin-stimulated secretion of the endogenous granule cargo peptide beta-endorphin was determined by radioimmunoassay in 15 parallel cultures in order to ensure that the stimulation of AtT-20 cell granule release was efficient and comparable in all experiments.

For comparison of fusion protein expression levels in stably transfected AtT20 clones (Figures 3.3B, and Figure 3.4) G418-resistant cell clones were picked, seeded in 24 well plates and tested for fusion protein expression using an ELISA assay for the mouse Ig2b fragment (Assay Designs, Ann Arbor, MI). Clonal cultures were subsequently verified for uniform expression of the fusion proteins by fluorescence microscopy using an anti-mouse IgG antibody conjugated to ALEXA 488 (Molecular Probes; Eugene, OR)(see below). To verify expression levels in clones, 5 X 10^5 cells were plated into 25 mm wells. The next day, the cells were labeled with 300 μCi of ^{35}S-methionine/cysteine for 1 hour. Labeling medium was then replaced with pre-warmed complete medium for 3 hours. (Chase). The culture supernatants corresponding to this chase period were immunoprecipitated with equal mixtures of Protein-A sepharose and Protein-A sepharose which had previously been pre-coupled to an antibody recognizing the N-terminus of PC1/3 (antibody 7690-06, ref. [22]). The resulting fluorogram (Figure 3.3B) allows a comparison of fusion protein expression levels relative to the endogenous PC1/3.

Immunocytochemistry and confocal microscopy

Mouse corticotropic AtT-20 cells stably transfected with the appropriate expression vector were seeded onto Lab Tek Glass Chambers (Nalgene Nunc, Napierville, Il) at a density of 20,000 cells/chamber. Twenty-four hours later, the cells were fixed with 4% paraformaldehyde, washed in TBS and permeabilized with –20 °C absolute methanol for 10 min. Slides were immunostained with a polyclonal rabbit anti-ACTH antibody (1:300) and an anti-mouse IgG antibody conjugated to ALEXA 488 (Molecular Probes, Eugene, OR) (1:200) for 1 hour at room temperature. Slides were subsequently stained with anti-Rabbit IgG antibody conjugated to rhodamine (Chemicon, Temecula, Ca) (1:100) for 1 hr. at room temperature. Slides were mounted using SlowFade Light Antifade Kit, (Molecular Probes) and visualized using an Zeiss LSM 510 Confocal Microscope.

Protein secondary structure predictions.

Predictions of helical wheel and helical net structures were carried out with the EMBOSS (The European Molecular Biology Open Software Suite) software package [23]. Additional helical structure predictions were carried out with the Jnet [24] or the PredictProtein (PROF) [11] algorithms.

Statistical analysis

Results (Figures 3.1, 3.2) are expressed as the mean +/- SEM and were compared by one-way ANOVA using Dunnet's Multiple Comparisons post-test.

RESULTS

The secretory granule sorting domain of PC5/6A is contained in the last 38 amino acids of the C-terminus

Previous results had shown that PC5/6A with a 38 amino acid truncation at the C-terminal failed to enter secretory granules [8]. In order to further define the PC5/6A secretory granule sorting signal, both the entire PC5/6A C-terminal tail (688-915) and the last 38 amino acids were tested for the ability to re-direct a constitutively secreted protein into the secretory granules of mouse corticotropic AtT20 cells (Figure 3.1) either in the absence or presence of forskolin, a secretagogue that increases intracellular cAMP resulting in the release of secretory granules [9]. AtT-20 cells contain dense core secretory granules in which endogenous proopiomelanocortin (POMC) is processed into adrenocorticotropic hormone (ACTH) by a series of proteolytic cleavages involving PC1/3 (reviewed in [1]). As we have previously shown [3], a recombinant protein containing a single chain fragment of the mouse immunoglobulin heavy chain constant region is secreted constitutively (i.e., not retained in granules) when expressed in these cells as evidenced by its continued secretion into the supernatant after a 16 hr chase period (Figure 3.1B, Fc). After the chase period, there is a roughly 1.5-fold stimulation of secretion of the small amount of the Fc protein remaining in the cells as determined by comparing levels secreted in the absence (-F; constitutive secretion) and presence (+F; regulated secretion) of forskolin (Figure 3.1C, Fc). By comparison, the secretion of endogenous granule-resident beta-endorphin is stimulated roughly 2.1-fold by the same treatment (2.1-fold +/- 0.12 (SEM), n=15). The low abundance of intracellular protein retention and forskolin-stimulated secretion of the Fc protein thereby constitute the baseline for analyzing potential granule sorting domains. Attachment of the entire 228 amino acid C-terminal tail of PC5/6A to the Fc fusion protein causes a significant increase in its retention in the cell and its regulated secretion (Figure

3.1 B and C; 688-915) confirming that this region of the protein contains a granule sorting signal. Notably, attachment of the last 38 amino acids of the C-terminus to the fusion protein results in an equivalent redirection of the fusion protein to the regulated secretory pathway (Figure 3.1 B and C; 878-915) suggesting that the PC5/6A secretory granule sorting signal is entirely contained within the C-terminal 38 amino acids of PC5/6A.

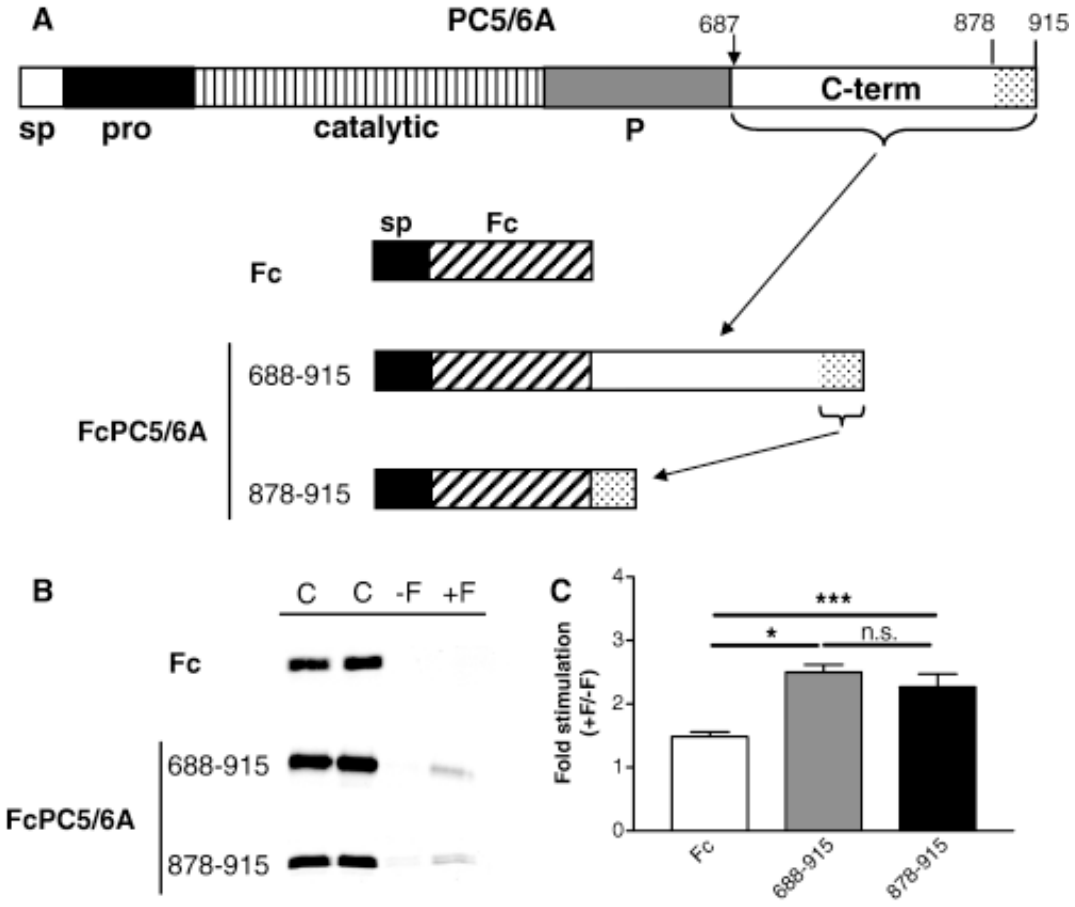


Figure 3.1 The PC5/6A granule-sorting domain is contained in the last 38 amino acids of its C-terminus.

(A) Upper: Schematic representation of PC5/6A showing the signal peptide (sp) prosegment (pro), catalytic, P domain (P) and the autocatalytically cleaved C-terminal domain (C-term). The speckled area represents the region deleted by de Bie et al. [8] that resulted in loss of secretory granule sorting. Lower: Schematic representation of the fusion proteins used to test for secretory granule targeting. sp, signal peptide. Fc, portion of the mouse IgG2b immunoglobulin. PC5/6A, various portions of the PC5/6A C-terminus as indicated (numbering is relative to the initiator methionine). **(B)** Representative pulse-chase assay for regulated secretion of the fusion proteins in AtT-20 cells. Parallel wells of stably transfected AtT-20 cell pools expressing the various fusion proteins were pulse-labeled for 2 hrs and chased with unlabeled medium for an additional 16 hrs. After the chase period,

the supernatants were collected from the parallel wells (two lanes labeled C) and the cells were subsequently incubated for an additional 3 hrs either in the absence (-F) or presence (+F) of the secretagogue Forskolin. Fc containing proteins in the culture supernatants were immunoprecipitated with Protein A Sepharose, separated by SDS-PAGE and detected by fluorography. **(C)** Autoradiograms similar to those shown in panel B were exposed to storage phosphor screen and quantified. Shown are the ratios (mean +/- SEM) of fusion protein content in the regulated (+F) versus constitutive (-F) secretion incubations. n=4-12 independent transfections.***P<0.001, *P<0.05, versus Fc by one-way ANOVA with Dunnet's post test. n.s., not significant.

The PC5/6A secretory granule sorting domain is predicted to form an alpha helix

The secretory granule sorting domains of PC1/3 and PC2 correspond to regions predicted to form alpha helices [3;6]. In order to determine if the same is true for the granule-sorting domain of PC5/6A, we analyzed this domain using two different protein structure prediction algorithms. Both Jnet [10] and PROF [11] predict the formation of a helix in the C-terminal half of this domain, roughly centered over residues 897-910, as well as a short region in the N-terminal portion of the fragment (Figure 3.2, 880-884, overlines). To test whether the C-terminal helix corresponds to the secretory granule sorting activity, serial deletions that remove either part or all of the predicted helix were made. Secretion analysis demonstrates that both of the fusion proteins containing C-terminal deletions show reduced sorting efficiency as compared to protein containing the intact 38 amino acid domain (Figure 3.2 B and C, compare 878-906 and 878-891 to 878-915). Moreover, this reduction in sorting efficiency correlates with the disruption of the predicted alpha helix in this region (see overlines on Figure 3.2 A, 878-906 and 878-891). To further confirm that the observed effects were due to deletion of functional sorting elements, a 5 amino acid deletion was made in the N-terminal end of the PC5/6A peptide, resulting in a disruption of the short helix predicted in that portion of the molecule (see Figure 3.2 A, 883-915, missing overlines). Secretion analysis in transfected AtT-20 cells reveals that this fusion protein sorts to secretory granules with the same efficiency as the fusion protein containing the entire 38 amino acid sorting domain (Figure 3.2 B and C, compare 878-915 to 883-915). In conclusion, the PC5/6A C-terminal tail contains a secretory granule-sorting signal whose function, like those of PC1/3 and PC2, correlates with the predicted formation of an alpha helix.

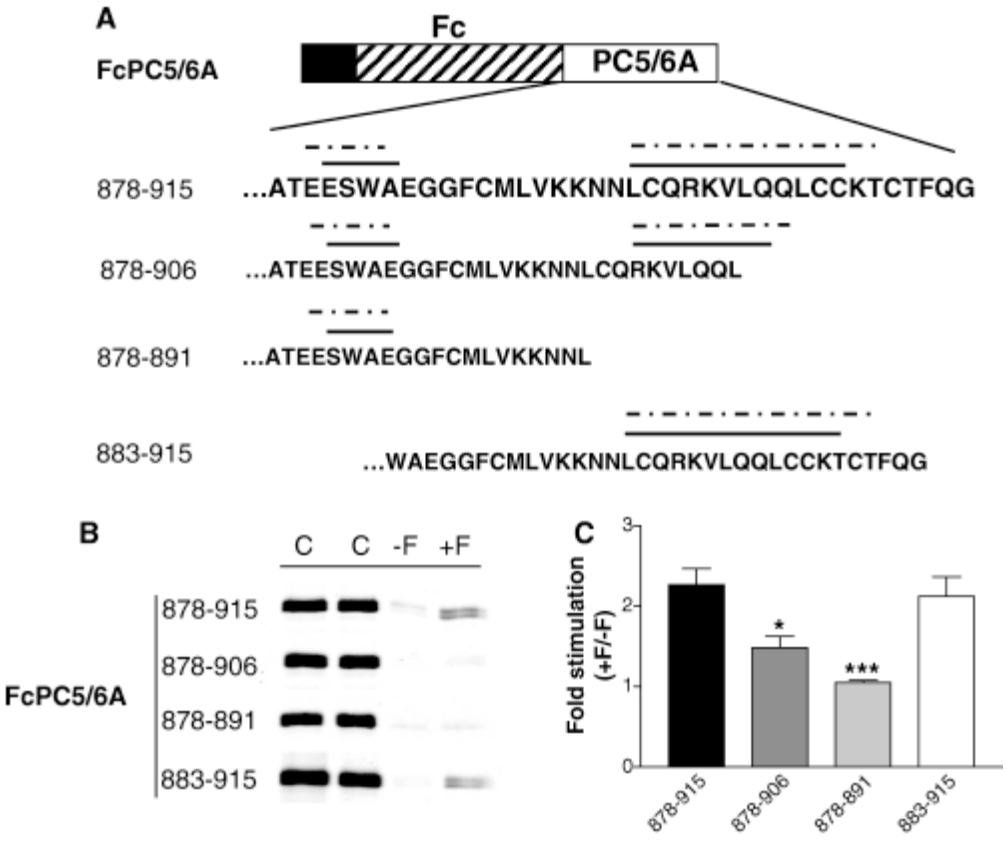


Figure 3.2: The PC5/6A C-terminus contains a granule-sorting domain predicted to form an alpha helix.

(A) Schematic representation of the PC5/6A C-terminal domains tested for regulated secretion. Overlined regions were predicted to form alpha helices by either the JPred (solid line) or PROF (hatched/dotted line) algorithms. **(B)** Representative fluorogram of supernatants from transfected AtT-20 cells. **(C)** Quantitative analysis of fusion protein sorting to the regulated secretory pathway. n=4-12 independent transfections

The minimal granule sorting domains of PC1/3, PC2 and PC5/6A selectively re-direct a constitutive protein to granules

To compare the sorting properties of the PC5/6A C-terminal tail to those previously identified in PC1/3 and PC2, we tested their ability to re-direct the Fc protein to the granule-containing regions in AtT-20 cells. In order to reduce experimental artifact that might result from varying levels of fusion protein expression, we isolated clonal lines of stably transfected AtT-20 cells that were selected for comparable levels of expression of the various fusion proteins (Figure 3.3B). In addition, to ensure that the levels of expression of the fusion proteins did not saturate the endogenous sorting machinery, we verified that endogenous PC1/3 secretion and its conversion from the 87Kd form to the 66Kd C-terminal truncated form (a secretory granule phenomenon) were not affected (Figure 3.3B, endogenous PC1/3). In agreement with our previous results [3], staining of transfected cells with an antibody to the mouse immunoglobulin (Fc) domain of the fusion protein revealed its presence predominantly in the TGN (Figure 3.4, open arrows) and in a diffuse pattern throughout the cytoplasm of expressing cells. This is the pattern expected for a constitutively secreted protein which transits from the TGN to low-density secretory vesicles. In contrast, inclusion of the C-terminal domains of either FcPC1/3, PC2 or PC5/6A results in the detection of the fusion protein not only in the TGN (open arrows) but also in cytoplasmic extensions (closed arrow), with the most intense staining being in the extensions. Concomitant staining with an antibody that detects both POMC and ACTH shows an identical spatial distribution of staining, with roughly equivalent localization in the TGN (POMC) and granule-containing cytoplasmic extensions (ACTH). Thus, the C-terminal domains of PC1/3, PC2 and PC5/6A are all equally capable of re-directing a constitutively secreted protein to granule-containing cytoplasmic extensions in AtT-20 cells.

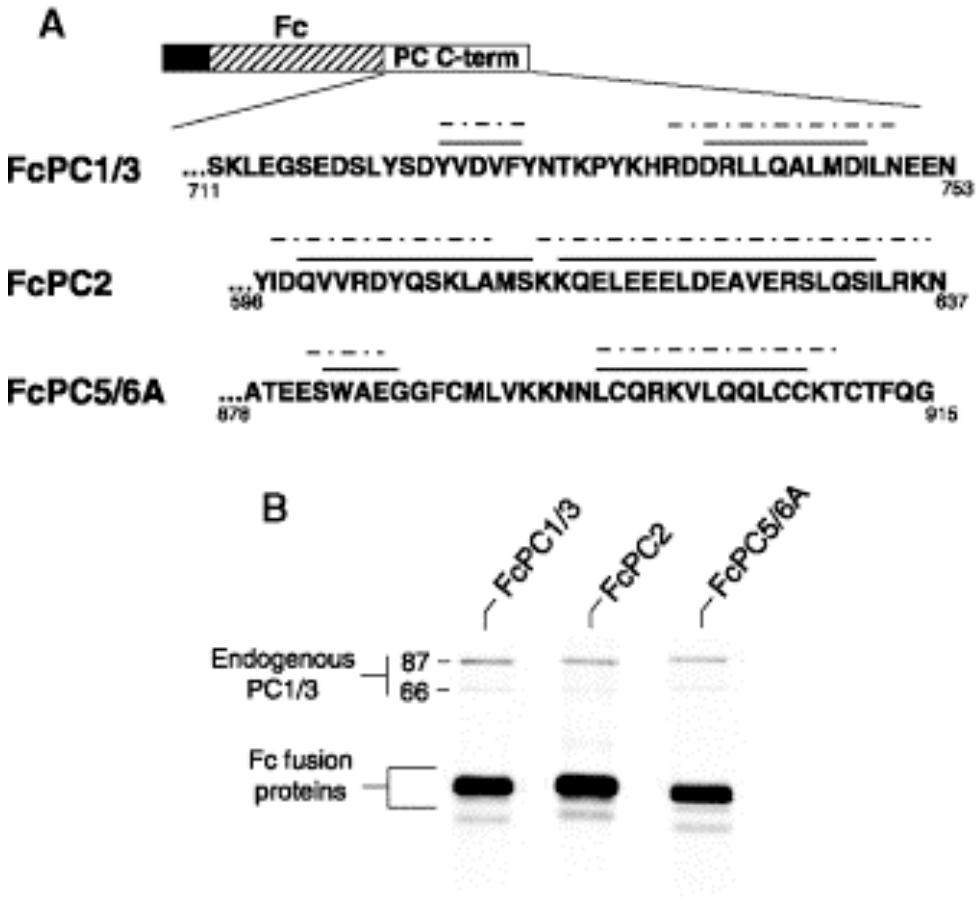


Figure 3.3 (A) and (B): Comparison of the sorting capacity of fusion proteins containing various PC family C-termini:

(A) Schematic representation of the C-terminal domains tested for regulated secretion. Overlined regions were predicted to form alpha helices by either the JPred (solid line) or PROF (hatched/dotted line) algorithms. **(B)** Clonal cell lines were selected from AtT-20 cell pools, labeled with ^{35}S-methionine for 1 hour and chased for 2 hours in complete medium. The chase supernatant was simultaneously immunoprecipitated for the fusion protein and endogenous PC1/3 and the precipitated proteins were subjected to SDS-PAGE and fluorography. Note that the level of secretion of each of the various fusion proteins was comparable between the different cell lines. In addition, expression of the fusion proteins did not interfere with secretion of the endogenous PC1/3 (87 kDa and 66 kDa forms).

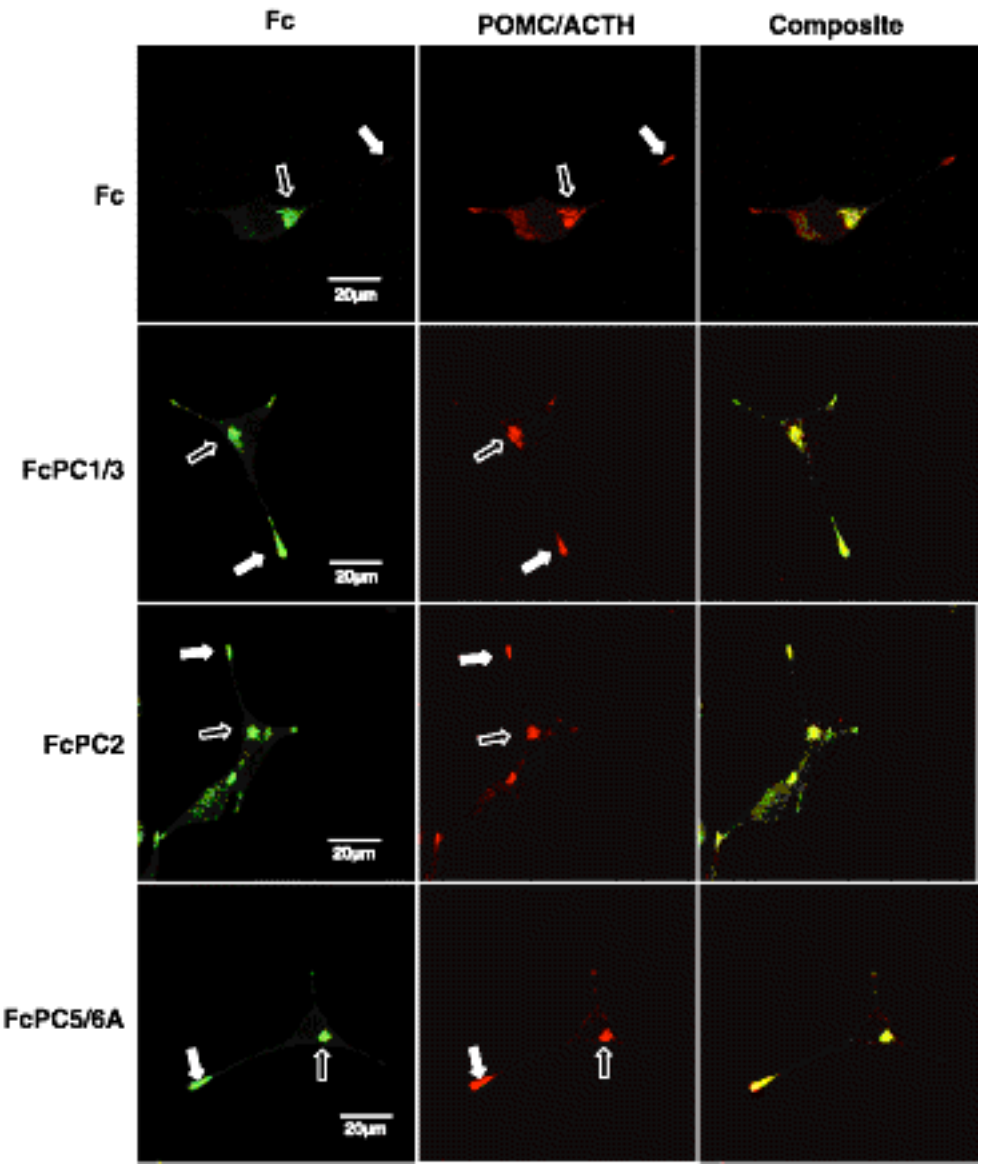


Figure 3.4: Comparison of the sorting capacity of fusion proteins containing various PC family C-termini:

Sub-cellular distribution of fusion proteins in transfected AtT-20 cells immunolabeled with antibody to the various fusion proteins (Fc; left panel) or endogenous POMC/ACTH (middle panel). The red staining (middle panels) shows the distribution of endogenous ACTH (present primarily in dense core secretory granules) and its precursor

proopiomelanocortin (primarily present in the endoplasmic reticulum and Golgi apparatus). Note the relative staining distribution of the fusion proteins between the TGN (open arrows), the cytoplasmic region and the granule-containing cytoplasmic extensions (closed arrows). The micrographs shown are typical of the staining pattern seen in >50 cells examined in 4 independent experiments.

Structural correlates of sorting efficiency

In an effort to better understand the granule sorting properties of the C-terminal alpha helices in PC1/3, PC2 and PC5/6A, we compared their predicted biophysical characteristics. The efficiency of sorting of the predicted helices did not correlate with their length; while the sorting helices of PC1/3 and PC5/6A are predicted to cover 14 amino acids, the PC2 helix extends over 28 amino acids (Figure 3.3 A, overlines). In addition, the sorting efficiency did not correlate with predicted isoelectric points since the PC1/3 and PC2 helices are predicted to be acidic while the PC5/6A sorting helix is very basic (Figure 3.5, pI). Helical wheel projections revealed that while both the PC1/3 and PC2 helices were amphipathic (i.e., had a segregation of hydrophobic and polar faces on the helix), the PC5/6A helix had a relatively uniform distribution of hydrophobic residues (boxed) around the helix (Figure 3.5, left). Interestingly, helical net projections, which represent a side view of the helix as if it had been sliced open and flattened, reveals that the more hydrophobic residues (L, I and V) are present in clusters on the surface of all three helices, but in the PC 1/3 helix these residues are more abundant and are predicted to be more tightly clustered on the helix surface (Figure 3.5, right).

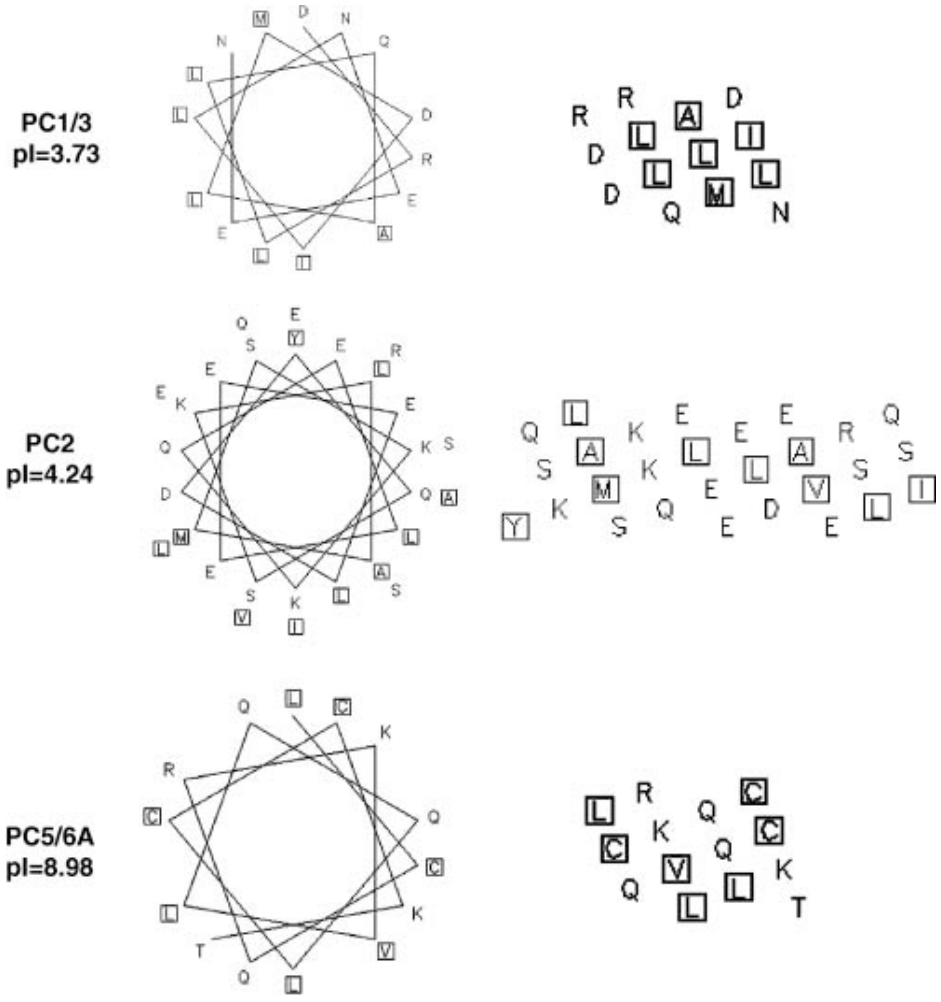


Figure 3.5: Predicted biophysical properties of C-terminal granule sorting helices in PC enzymes.

Shown are the predicted isoelectric points (pI), helical wheel projections (left) and helical net projections (center) for the regions predicted to form alpha helices in the C-termini of PC1/3, PC2 and PC5/6A. Hydrophobic amino acids are boxed. See text for details.

DISCUSSION

Many peptide hormones (such as insulin, ACTH and others) are only bioactive after selective cleavage of their precursor proteins in secretory granules by PC enzymes such as PC1/3, PC2 or PC5/6A. Understanding how these enzymes and their substrates are targeted to secretory granules is thus critical in understanding this key cellular process in endocrinology. The current results suggest that secretory granule-targeting domain of PC5/6A is comprised of a C-terminal region predicted to form an alpha helix as had been previously reported for PC1/3 and PC2 and suggests that the three members of the PC family that are targeted to dense core secretory granules share a common sorting mechanism. Although these studies were carried out using engineered fusion proteins, several studies have now shown that removal of the C-terminal tails in the otherwise intact PC1/3, PC2 and PC5/6A enzymes prevents their sorting to dense core secretory granules [3-6;8], confirming the importance of these domains in the context of the native proteins.

Alpha helical sequences involved in sorting proteins to secretory granules have also been observed in other proteins: prosomatostatin contains an alpha helix in its N-terminal region sufficient for targeting to secretory granules [12]. Carboxypeptidase E also contains an alpha helix in its C-terminus that is critical for sorting the protein to secretory granules which has been reported to traverse the granule membrane [13]. Recently, a protease cleavage site located within an alpha helix was found to mediate sorting of VGF to secretory granules [14]. Thus, alpha helices may represent a family of sorting signals used by a number of secretory granule cargo proteins. The exact mechanism by which these helices mediate secretory granule targeting has not yet been determined. However, by studying the secretory granule sorting activity of a variety of synthetic helices, Dikeakos et al. [15] demonstrated that the most efficient helices were characterized by a hydrophobic patch in a charged helix and their efficiency was unaffected by the nature of the charge (i.e., they could be either acidic or basic), properties that also characterize the natural

granule sorting helices of PC1/3, PC2 and PC5/6A. Previous reports have suggested that PC1/3 and PC2 associate with detergent-resistant membrane microdomains within the secretory pathway [3;6;16-18] and that this association could be disrupted by either deletion or mutation of the alpha helical region of the sorting domain [3;6]. This raises the possibility that the secretory granule targeting of the PC enzymes is mediated by interaction of the helices in their C-termini with specific membrane domains in the secretory pathway. Such a mechanism could be important for anchoring certain secretory granule cargo proteins (such as the PC enzymes) to the vesicle membrane so that they can be retained for storage. In a recent proteomic analysis of endocrine cell secretory granules derived from bovine chromaffin cells, Wegrzyn et al. [19] reported that PC1/3 was indeed a component of both the soluble and membrane fractions of the granule preparation, lending support to this model. Whether or not the granule-targeting helices of the PC enzymes also play a role in triggering granule budding as has been suggested for other membrane-binding helices [20] will be an interesting topic for further study.

ACKNOWLEDGMENTS

The authors wish to thank Dr. James Omichinski for helpful discussions. This work was supported by Operating Grants MOP-53177 (to TLR) and MOP 44363 (to NGS) from the Canadian Institutes of Health Research.

REFERENCES

[1] Seidah NG & Prat A (2002) Precursor convertases in the secretory pathway, cytosol and extracellular milieu. *Essays Biochem.*, **38,** 79-94.

[2] Dikeakos JD & Reudelhuber TL (2007) Sending proteins to dense core secretory granules: still a lot to sort out. *J. Cell Biol.*, **177,** 191-196.

[3] Jutras I, Seidah NG, & Reudelhuber TL (2000) A predicted alpha -helix mediates targeting of the proprotein convertase PC1 to the regulated secretory pathway. *J. Biol. Chem.*, **275,** 40337-40343.

[4] Zhou A, Paquet L, & Mains RE (1995) Structural elements that direct specific processing of different mammalian subtilisin-like prohormone convertases. *J Biol. Chem.*, **270,** 21509-21516.

[5] Creemers JW, Usac EF, Bright NA, Van de Loo JW, Jansen E, Van d, V, & Hutton JC (1996) Identification of a transferable sorting domain for the regulated pathway in the prohormone convertase PC2. *J. Biol. Chem.*, **271,** 25284-25291.

[6] Assadi M, Sharpe JC, Snell C, & Loh YP (2004) The C-terminus of prohormone convertase 2 is sufficient and necessary for Raft association and sorting to the regulated secretory pathway. *Biochemistry*, **43,** 7798-7807.

[7] Barbero P, Rovere C, De Bie, I, Seidah N, Beaudet A, & Kitabgi P (1998) PC5-A-mediated processing of pro-neurotensin in early compartments of the regulated secretory pathway of PC5-transfected PC12 cells. *J. Biol. Chem.*, **273,** 25339-25346.

[8] De Bie I, Marcinkiewicz M, Malide D, Lazure C, Nakayama K, Bendayan M, & Seidah NG (1996) The isoforms of proprotein convertase PC5 are sorted to different subcellular compartments. *J Cell Biol.*, **135,** 1261-1275.

[9] Schmidt WK & Moore HP (1995) Ionic milieu controls the compartment-specific activation of pro-opiomelanocortin processing in AtT-20 cells. *Mol. Biol. Cell*, **6,** 1271-1285.

[10] Cuff JA, Clamp ME, Siddiqui AS, Finlay M, & Barton GJ (1998) JPred: a consensus secondary structure prediction server. *Bioinformatics.*, **14,** 892-893.

[11] Rost B, Yachdav G, & Liu J (2004) The PredictProtein server. *Nucleic Acids Res.*, **32,** W321-W326.

[12] Mouchantaf R, Kumar U, Sulea T, & Patel YC (2001) A conserved alpha-helix at the amino terminus of prosomatostatin serves as a sorting signal for the regulated secretory pathway. *J Biol. Chem.*, **276,** 26308-26316.

[13] Dhanvantari S, Arnaoutova I, Snell CR, Steinbach PJ, Hammond K, Caputo GA, London E, & Loh YP (2002) Carboxypeptidase E, a prohormone sorting receptor, is anchored to secretory granules via a C-terminal transmembrane insertion. *Biochemistry*, **41,** 52-60.

[14] Garcia AL, Han SK, Janssen WG, Khaing ZZ, Ito T, Glucksman MJ, Benson DL, & Salton SR (2005) A prohormone convertase cleavage site within a predicted alpha-helix mediates sorting of the neuronal and endocrine polypeptide VGF into the regulated secretory pathway. *J. Biol. Chem.*, **280,** 41595-41608.

[15] Dikeakos JD, Lacombe MJ, Mercure C, Mireuta M, & Reudelhuber TL (2007) A hydrophobic patch in a charged alpha-helix is sufficient to target proteins to dense core secretory granules. *J. Biol. Chem.*, **282,** 1136-1143.

[16] Blazquez M, Docherty K, & Shennan KI (2001) Association of prohormone convertase 3 with membrane lipid rafts. *J. Mol. Endocrinol.,* **27,** 107-116.

[17] Blazquez M, Thiele C, Huttner WB, Docherty K, & Shennan KI (2000) Involvement of the membrane lipid bilayer in sorting prohormone convertase 2 into the regulated secretory pathway. *Biochem. J*, **349 Pt 3,** 843-852.

[18] Arnaoutova I, Smith AM, Coates LC, Sharpe JC, Dhanvantari S, Snell CR, Birch NP, & Loh YP (2003) The prohormone processing enzyme PC3 is a lipid raft-associated transmembrane protein. *Biochemistry*, **42,** 10445-10455.

[19] Wegrzyn J, Lee J, Neveu JM, Lane WS, & Hook V (2007) Proteomics of Neuroendocrine Secretory Vesicles Reveal Distinct Functional Systems for Biosynthesis and Exocytosis of Peptide Hormones and Neurotransmitters. *J. Proteome. Res.*,Apr 5; [Epub ahead of print] **..**

[20] Lee MC, Orci L, Hamamoto S, Futai E, Ravazzola M, & Schekman R (2005) Sar1p N-terminal helix initiates membrane curvature and completes the fission of a COPII vesicle. *Cell.,* **122,** 605-617.

[21] Brechler V, Chu WN, Baxter JD, Thibault G, & Reudelhuber TL (1996) A protease processing site is essential for prorenin sorting to the regulated secretory pathway. *J. Biol. Chem.,* **271,** 20636-20640.

[22] Benjannet S, Reudelhuber T, Mercure C, Rondeau N, Chretien M, & Seidah NG (1992) Proprotein conversion is determined by a multiplicity of factors including convertase processing, substrate specificity, and intracellular environment. Cell type-specific processing of human prorenin by the convertase PC1. *J. Biol. Chem.,* **267,** 11417-11423.

[23] Rice P, Longden I, & Bleasby A (2000) EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet.*, **16,** 276-277.

[24] Cuff JA & Barton GJ (2000) Application of multiple sequence alignment profiles to improve protein secondary structure prediction. *Proteins.*, **40,** 502-511.

Chapter 4

Functional Anatomy of a Secretory Granule Sorting Domain: Solution Structure of the C-terminal Helical Domain of the Protease PC1/3

Jimmy D. Dikeakos, Paola Di Lello, Marie-Josée Lacombe, Pascale Legault, Timothy L. Reudelhuber and James. G. Omichinski

To be submitted to the Journal of Cell Biology

In the following article, Paola Di Lello collected the NMR data while Marie–Josée Lacombe provided technical assistance by performing the cell culture studies. Pascale Legault implemented the NMR sequences. The first author under the supervision of Timothy L. Reudelhuber and James G. Omichinski performed all other experiments as well as the writing of the manuscript.

ABSTRACT

Peptide hormones such as insulin and glucagon are synthesized as inactive precursors. It is only upon the proper co-targeting of prohormones and their processing enzymes, the prohormone convertases (PCs), to dense core secretory granules (DCSGs) that the precursors are activated to exert their physiological functions. PC1/3 is targeted to DCSGs via its carboxy terminal granule-sorting domain (PC1/3 $_{711-753}$). In the current study, we have determined the three-dimensional solution structure of PC1/3 $_{711-753}$. The structure reveals that PC1/3 $_{711-753}$ contains two amphipathic alpha helices located between residues 722-728 and 738-750, respectively. A functional targeting study demonstrates that the second helix (PC1/3 $_{738-750}$) is necessary and sufficient to target a constitutively secreted protein to granules and that leucine 745 (L745) within the second helix is critical in mediating this function. Moreover, NMR chemical shift mapping studies identified a calcium-binding region located in the carboxy terminal tail between residues 750-753. These results provide key information regarding the mechanism alpha helical regions use to target proteins to DCSGs.

INTRODUCTION

Dense core secretory granules (DCSGs) are a repository in endocrine and neuroendocrine cells. It is only upon entering this organelle that inactive prohormones are activated by processing enzymes and subsequently released when the cell receives the correct physiological stimulus [For Review see (*1*)]. This process is termed the regulated secretory pathway and diverges from the constitutive secretory pathway where proteins are packaged in low-density vesicles and released in an unregulated manner. Thus, the correct targeting of hormone substrates and their concomitant processing enzymes ensures the availability of critical hormones such as insulin, glucagon and renin at the proper rate and time.

Various mechanisms have been postulated to explain protein sorting to DCSGs [For Review see (*2*)] and they can be divided in two models. The "sorting by entry'' model postulates that proteins are triaged at the Trans Golgi Network (TGN) via a protein-receptor interaction. Proteins interacting with this receptor would be diverted into the regulated secretory pathway. Carboxypeptidase E (CPE) may play the receptor role (*3*) although this classification is not clear-cut (*4*). A second model is 'sorting by retention' where all proteins, regardless of their final destination, enter an immature secretory granule which matures through acidification (*5*) and prohormone processing (*6*) steps. Non-DCSG proteins would be extruded and only the remaining proteins will form the mature DCSG. Molecular players have now been identified explaining this maturation step. N-ethylmaleimide-sensitive fusion protein (NSF) (*7*), alpha soluble NSF attachment protein (α-SNAP) (*7*) and synaptotagmin IV (*8*) play distinct roles in the homotypic fusion of immature secretory granules whereas cytosolic adaptor protein 1 (AP-1) plays a role in the retrieval of non-DCSG proteins from the immature secretory granules (*9*). While these two models are compelling they both fail to define how cargo is targeted to the DCSG.

There is no known canonical sorting signal sequence but there are multiple mechanisms by which sorting to DCSGs can occur (*2*). For example, dibasic processing sites (most notably KR sequences) in unprocessed precursor hormones can direct proteins to DCSGs. Prorenin (*10*), progastrin (*11*) and proneurotensin (*12*) are examples of an intact basic processing site being required to ensure DCSG targeting. Alternatively, the inherent ability of proteins to aggregate in the environment of the late secretory pathway, which is high in calcium, (*13*) is another example of a DCSG sorting signal. Proteins such as atrial natriuretic factor (ANF) (*14*) contain a calcium binding site mediating its aggregation. Also, the ability of proteins to tether to a membrane by either associating with or traversing the membrane can also result in their targeting to the DCSG. Phogrin is an example of a transmembrane protein capable of interacting with cytosolic proteins which assist in its trafficking to DCSGs (*15*). Alpha helices have been described as targeting signals in DCSG proteins such as CPE (*16*), VGF (*17*), chromogranin A (CgA) (*18*), prosomatostatin (*19*) and secretogranin II (SgII) (*20*). We have demonstrated that the three DCSG members, PC1/3, PC2 and PC5A/6, of the prohormone convertase family are targeted to DCSG by a common alpha helical targeting region (*21*).

The prohormone convertases are a family of serine proteases [For Review see (*22*)] containing an amino-terminal Pro domain that functions to repress enzymatic activity in the early secretory pathway, a catalytic domain and a P-domain implicated in protein stability. The pro, catalytic and P domains have a high degree of homology between all seven known PC family members. It is the carboxy-terminal domain of the PC enzymes that displays the least amount of sequence homology. Interestingly, DCSG- targeting signals for PC1/3, PC2 and PC5A/6 are all found in the non-conserved carboxy-terminal domain. Specifically, PC1/3 requires the presence of residues 617-753 to enter DCSGs as this domain contains three regions predicted to form alpha helices that are capable to function as DCSG-sorting signals. First, a helical sequence between residues 617-638 can redirect a constitutively secreted protein to DCSGs (*23*). Alternatively, we demonstrated that DCSG-sorting signals are also found between residues 665-682 and residues 711-753 respectively (*24*). All three

of the proposed DCSG-sorting signals are predicted to form alpha helices. Moreover, we demonstrated that the region between residues 711-753 was necessary and sufficient to target a heterologous fusion protein to the DCSGs (*24*). The signal targeting PC2 to DCSG is in a region forming an alpha helix localized in the carboxy terminal domain of PC2 (*25*). Moreover, we showed that PC5A/6 is targeted to DCSGs via a predicted alpha helix localized in the carboxy-terminus of PC5A/6 between residues 891-915 (*21*). While all of the above data explaining the potential role of alpha helices in sorting proteins to DCSGs has been predicted using secondary structure prediction algorithms, no experimental data has verified the actual structure of a DCSG-sorting domain. In the current manuscript, we have determined the nuclear magnetic resonance (NMR)-solution structure of the PC1/3 DCSG-sorting domain localized between residues 711-753 and use this information to systematically identify the mechanism PC1/3 utilizes to enter DCSG. The structure represents the first high-resolution structure of a DCSG-sorting domain. The structure demonstrates that the domain contains two amphipathic alpha helices separated by a short eight-residue linker. The structure in combination with *in vivo* functional experiments helps us in defining how PC1/3 interacts with membranes and binds calcium.

MATERIALS AND METHODS

^{15}N and $^{15}N/^{13}C$-labeled protein expression and purification

The mouse PC1/3 cDNA encoding for the amino acids 711-753 was inserted into the pGEX-4T (GE Healthcare) vector, which contains an amino-terminal glutathione-S-transferase (GST) tag followed by a thrombin-cleavage site. A single colony was picked and grown in 2 L of minimal media containing 1 g/L of $^{15}NH_4Cl$ and 2.5 g/L ^{13}C-glucose. Log phase cultures were induced with 0.66 mM IPTG for 3 hours at 37˚ C. Bacterial pellets were resuspended in 100 mL of Buffer A (20 mM Tris, pH = 7.4, 1 M NaCl, 1 mM EDTA and 1 mM DTT) and lysed. Bacterial lysates were centrifuged and the supernatant was incubated with 10 mL of Glutathione-Sepharose resin (GE Healthcare). Bound resin was washed three times with Buffer A and twice times with phosphate-buffered saline (PBS). Subsequent overnight incubation of the resin with 100 units of thrombin protease (GE Healthcare) allowed the release of PC1 $_{711-753}$ from the resin into the supernatant. The resulting supernatant was dialyzed overnight against 5% acetic acid and was further purified by reverse-phase high-pressure liquid chromatography (HPLC) (C_4 Vydac column) and eluted with a 25-45 % acetonitrile gradient over 40 minutes. The acetonitrile was removed via roto-evaporation and the pure peptide was lyophilized.

NMR sample preparation and data collection

For NMR studies, ^{15}N- or $^{13}C/^{15}N$-labeled PC1/3 $_{711-753}$ peptide was resuspended at a concentration of 1 mM in 20 mM d-11 Tris (Cambridge Isotopes) (pH = 6.85), 20 mM 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Fisher) and either

90% H_20/10% D_20 or 99.9% H_20. NMR spectra were collected at 26.6˚C on Varian Unity Inova 500, and 600 and 800 MHz NMR spectrometers. The backbone and aliphatic side chain resonances were assigned using a combination of experiments, including two-dimensional (2D) ^{1}H-^{15}N Heteronuclear Single Quantum Coherence (HSQC) (*26*), 2D ^{1}H-^{13}C constant time HSQC (CT-HSQC), 2D HBCBCGCDHD (*27*), 2D HBCBCGCDCEHE (*27*), three-dimensional (3D) HNCACB (*28*), 3D (HB)CBCA(CO)NNH (*29*), 3D HNCO (*30*), 3D H(CCO)NNH, 3D (H)C(CO)NNH (*29;30*), 3D HNHA (*31*) and 3D HCCH-correlation spectroscopy (COSY). Backbone dynamics were assessed by measuring ^{15}N-^{1}H heteronuclear Nuclear Overhauser Effects (NOEs) (*32*). Intermolecular NOEs between the CHAPS micelle and $^{15}N/^{13}C$-labeled $PC1/3_{711\text{-}753}$ were obtained from a 3D $^{15}N/^{13}C$ (F1)-filtered, (F3)-edited NOESY (*33*)}. The NMR data were processed with NMRPipe/NMRDraw (*34*) and analyzed with NMRView (*35*).

Structure calculations

The interproton distances were estimated from the intensities of the cross peaks observed in the 3D ^{15}N-edited Nuclear Overhauser Effect Spectroscopy (NOESY)-HSQC (*36;37*) and ^{13}C-edited heteronuclear multiple quantum coherence-total correlation (HMQC)-NOESY experiments (*38*). The backbone dihedral angles were obtained from the analysis of ^{1}H, ^{15}N, $^{13}C'$, $^{13}C^{\alpha}$, and $^{13}C^{\beta}$ chemical shifts using the program TALOS (*39*). Structure calculations were performed with the program CNS (*40*) using a combination of torsion angle dynamics (TAD) and Cartesian dynamics. Starting from an extended structure with standard geometry, 50 structures were calculated using NOE-derived interproton distances and backbone dihedral angles. These 50 structures did not have any NOE violation greater than 0.2 Å and no backbone dihedral angle restraint violation greater than 2˚. Structural statistics were performed using Procheck (*41*). Illustrations of the structure were prepared with the programs PYMOL (www.pymol.org), Chimera (*42*) and MOLMOL (*43*).

Recombinant plasmid construction

Peptide fragments to be analyzed for secretory-granule sorting were derived from the mouse PC1/3 (NM013628). The numbering used to identify the protein domains is relative to the initiator methionine of both proteins. Protein fragments were tested for their ability to sort heterologous proteins to secretory granules by attachment to a fragment of mouse immunoglobulin IgG2b (referred to as Fc) as previously described (*21*). The PC1/3 fusion protein was constructed by selective amplification of corresponding fragments. Mutations were made using PCR overlap extension (*44*). All of the resulting coding sequences were verified in their entirety by DNA sequencing and were inserted into the pRSV globin mammalian expression vector (*24*).

Mammalian cell culture, transfection and secretion analysis

Mouse corticotrophic AtT-20 cells were grown in Dulbecco's minimal essential medium (DMEM; Invitrogen, Burlington, Ontario, CANADA) containing 5% fetal bovine serum (FBS), 0.01% gentamicin (Invitrogen) and 0.1% SerXtend (Irvine Scientific, Santa Anna, CA) in a humidified incubator at 37 ºC in 5% CO_2. Expression vectors were stably transfected into AtT-20 cells by cotransfection with pSV-Neo (Invitrogen, Burlington, Ontario, CANADA) and selection in Geneticin (G418, Invitrogen, Burlington, Ontario, CANADA). G418-resistant pools of cells were used for all subsequent studies. For metabolic labeling, 4.5 X 10^5 stably transfected cells were plated in each of two 35mm dishes. After twenty-four hours, the medium was replaced with 0.5 mL of prewarmed methionine-free DMEM containing 10% dialyzed FBS and 300 µCi of ^{35}S-methionine/cysteine (Trans-^{35}S Label; MP Biomedicals, Irvine, CA) for 2 hours and incubated at 37 ºC. Labeling medium was then replaced with prewarmed complete medium

for 16 hours (chase). To test for regulated secretion, the cells were rinsed in complete medium and in one of the wells, the cells were incubated for an additional 3 hours in complete medium to measure constitutive secretion while in the other well the cells were incubated in complete medium supplemented with 10 μM Forskolin, a secretagogue which stimulates granule release. The corresponding culture supernatants were then immunoprecipitated with Protein-A Sepharose (Sigma, St. Louis) and the immunoprecipitated proteins were separated by SDS-PAGE. The gels were incubated with 3 changes of 10% PPO in dimethyl sulfoxide, rinsed in water, dried and subjected to fluorography. Dried gels were subsequently exposed to storage phosphor screens (GE Healthcare) and emissions were quantified using a Molecular Dynamics Storm Phosphorimager. Results were compared by one-way ANOVA using Bonferonni's post-test and expressed as mean values with a standard error on the mean (SEM)

RESULTS

DCSG-sorting domain of PC1/3 in a micellar environment.

We sought to characterize the structure of the DCSG sorting domain of PC1/3 located within residues 711-753 to understand how this domain is able to redirect the PC1/3 processing enzyme to DCSG. NMR and crystallization studies of DCSG sorting domains have been limited by the fact that these domains are associated with membranes and have limited solubility in physiological buffers (*2*). Indeed, a two-dimensional ^{1}H-^{15}N HSQC spectrum of ^{15}N-labeled PC1/3 $_{711-753}$ contains signals that appear to be heterogeneous with respect to each other in typical physiological buffer conditions (Figure 4.1, A). The heterogeneous signal intensities suggest that the protein is partially aggregated (*45*). In contrast, ^{15}N-labeled PC1/3 $_{711-753}$ in the presence of 20 mM CHAPS gave a homogenous set of signals suggesting that the protein was no longer aggregated (Figure 4.1, B). Identical results were obtained using a dodecyl phosphocholine (DPC) micelle solution (data not shown). CHAPS is a zwitterionic detergent that has a critical micellar concentration between 4-6 mM depending on the buffer used (*46*). CHAPS micelles have been used to characterize the structure of several proteins by NMR including calcineurin-B (*45*) and eukaryotic translation initiation factor 4E (eIF4e) (*47*). The addition of 20 mm CHAPS to PC1/3 $_{711-753}$ allowed for the complete ^{1}H, ^{15}N and ^{13}C chemical shift assignment of PC1/3 $_{711-753}$. As a result, we pursued the structure elucidation of PC1/3$_{711-753}$ in the presence of 20 mM CHAPS.

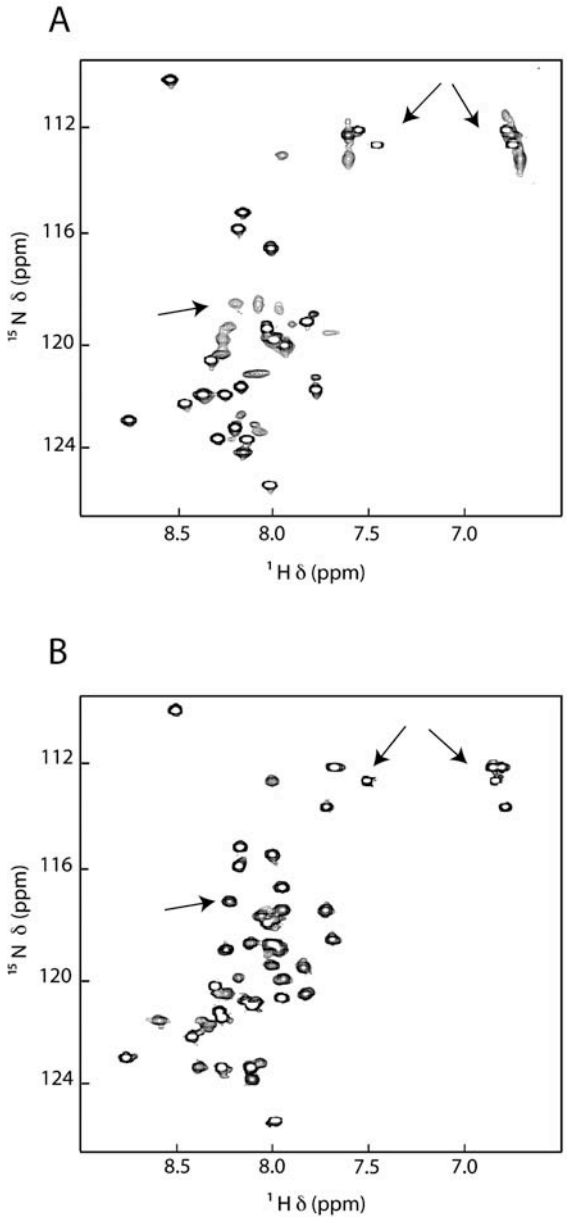


Figure 4.1: PC1/3 $_{711\text{-}753}$ requires a micelle for structure determination

Two-dimensional ^{1}H-^{15}N-HSQC spectra for ^{15}N-labeled PC1/3 $_{711\text{-}753}$ in the absence (**A**) and in the presence (**B**) of 20 mM CHAPS. All spectra were recorded in 20 mM d-11 Tris (pH= 6.5) at 26.6˚C with or without CHAPS. Arrows denote regions of the spectra displaying peak heterogeneity without CHAPS (**A**) and subsequent peak homogeneity with CHAPS (**B**)

The NMR solution structure of the PC1/3 DCSG-sorting domain reveals two alpha helical regions.

The NMR solution structure of the DCSG-sorting domain of PC1/3 from residues 711-753 indicates the presence of two amphipathic alpha helices. The first α-helix is between residues 722-728 whereas the second α-helix is between residues 738-750 (Figure 4.2 and Figure 4.3). The precise angle between the two helices in the sorting domain of PC1/3 is difficult to define by NMR due to the absence of long range NOEs between the two helices. A total of 50 structures was calculated, all of them satisfying the experimental constraints with no NOE violation larger then 0.2 Å and no backbone dihedral angle violation greater then 2°. The 20 structures with the lowest energies were selected for statistical analysis (Table 4.1).

Interestingly, the second helix overlaps with the previously identified minimal domain of PC1/3 required for sorting (*24*). However, a phylogenetic analysis of the carboxy terminal region of PC1/3 using the ClustalW program (*48*) (Figure 4.4) demonstrates that residues 717-749 are highly conserved from fish to man. This sequence conservation includes both helices as well as the loop between the two helices.

Restraints used for the structure calculations	
Total NOE distance restraints	275
Short-range (intraresidue)	135
Medium-range ($\|i - j\| \leq 4$)	140
Dihedral angle restraints, ϕ, ψ	38
Structural statistics	
RMSDs from idealized geometry	
Bonds (Å)	0.00070 ± 0.00005
Angles (deg)	0.3226± 0.0009
Impropers (deg)	0.092± 0.002
Dihedral restraints (deg)	0.05± 0.02
Distance restraints (Å)	0.007±0.001
Ramachandran statistics (%)	
Residues in most favorable regions	69.2
Residues in additional allowed regions	25.1
Residues in generously allowed regions	3.3
Residues in disallowed regions	2.3
Coordinate Precision (Å)	
Atomic pairwise rmsd for backbone atoms (C', C^{α}, N) $_{(722\text{-}728)}$	0.3 ± 0.2
Atomic pairwise rmsd for backbone atoms (C', C^{α}, N) $_{(738\text{-}750)}$	0.4 ± 0.3
Atomic pairwise rmsd for all heavy atoms $_{(722\text{-}728)}$	1.0 ± 0.3
Atomic pairwise rmsd for all heavy atoms $_{(738\text{-}750)}$	1.6 ± 0.3

Table 4.1 Structural statistics for of PC1/3 $_{711\text{-}753}$

Figure 4.2: Overlay of the 20 lowest-energy structures of PC1/3 $_{711\text{-}753}$.

The structures were superimposed using the backbone atoms C′, C^{α}, and N of the first helix between residue S^{722} and residue F^{728} (**A**) and the second helix between residue D^{738} and residue N^{750} (**B**).

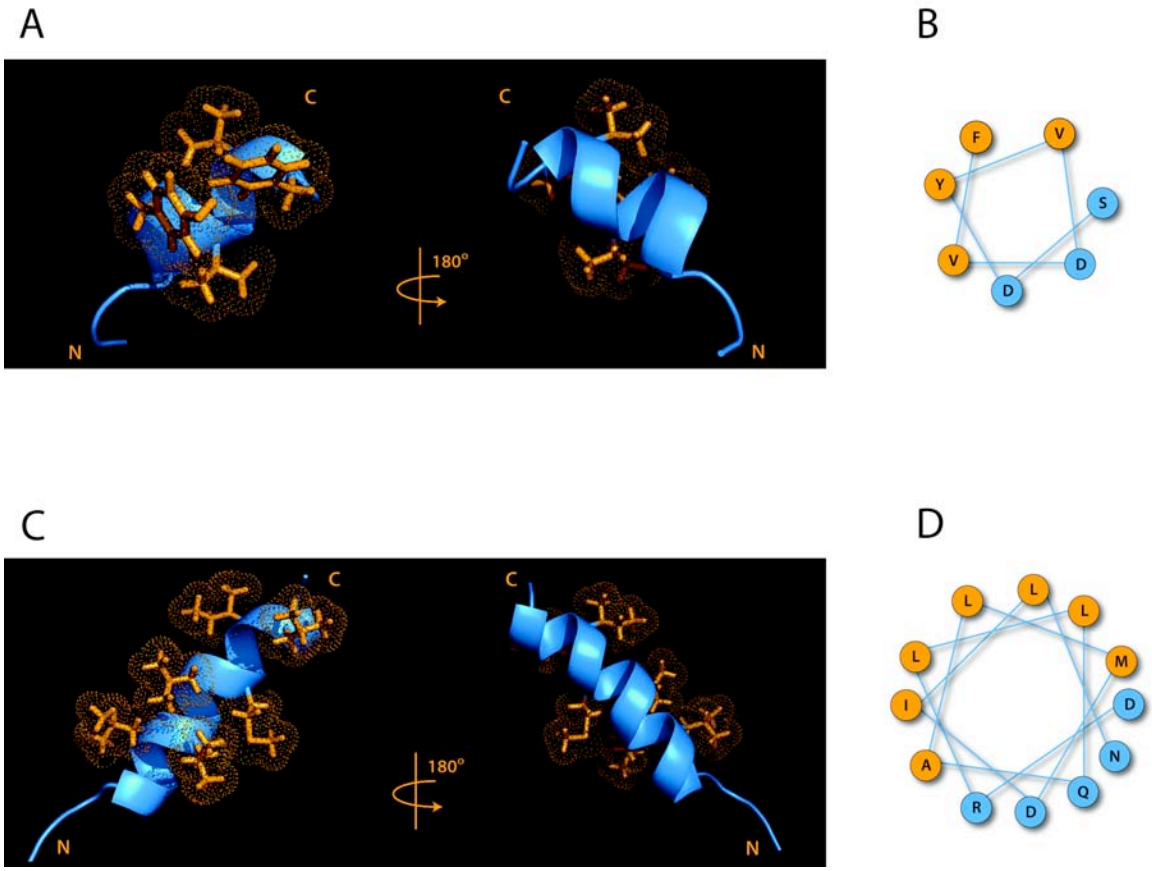


Figure 4.3: PC1/3 $_{711\text{-}753}$ contains two amphipathic helices.

Ribbon representation of the first alpha helix of PC1/3 $_{711\text{-}753}$ (**A**) and the second alpha helix of PC1/3 $_{711\text{-}753}$ (**B**) with the hydrophobic sides chains highlighted in orange. **(B)** Clusters of hydrophobic (orange) and hydrophilic (blue) residues of the first alpha helix of PC1/3 $_{711\text{-}753}$ between residue S^{722} and residue F^{728} (Panel **C**) and the second alpha helix of PC1/3 $_{711\text{-}753}$ between residue D^{738} and residue N^{750}.

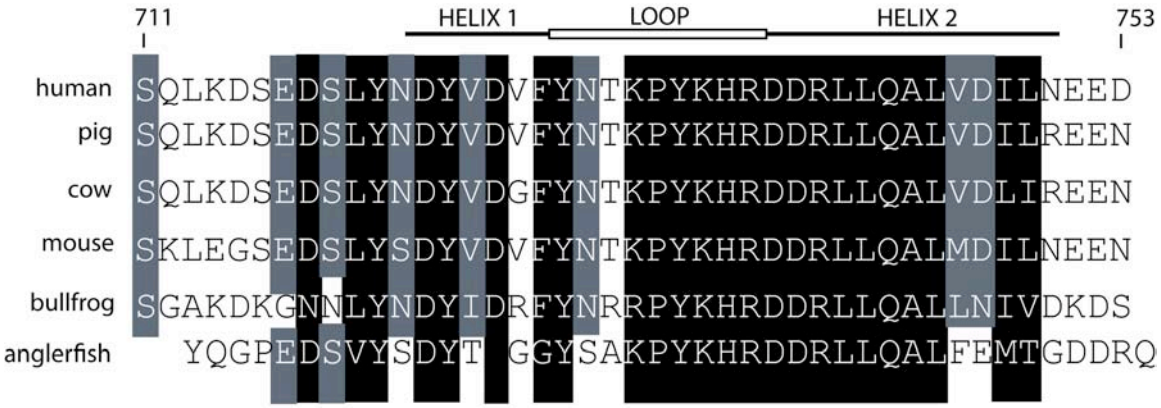


Figure 4.4: PC1/3 $_{711\text{-}753}$ phylogenetic analysis

The amino acid sequence alignment of PC1/3 $_{711\text{-}753}$ from several species was generated using the CLUSTALW algorithm (*48*). The sequences are highly conserved between residues 717 and 749. The secondary structure elements derived from the structure of PC1/3 $_{711\text{-}753}$ (Helix 1, Loop and Helix 2) are indicated above the sequences.

Correlation between the structure of PC1/3 $_{711\text{-}753}$ and its ability to act as a DCSG sorting domain

We next sought to determine if both alpha helices of PC1/3$_{711\text{-}753}$ are capable of independently targeting a constitutively secreted protein to DCSGs in AtT20 neuroendocrine cells. Vectors either expressing a fragment of the non-DCSG targeted immunoglobulin (Fc) protein (*49*) or fusion proteins consisting of FcPC1/3 $_{711\text{-}753}$, FcPC1/3 $_{711\text{-}738}$ and FcPC1/3 $_{738\text{-}753}$ were transfected in AtT20 cells (Figure 4.5). The ability of the various Fc-fusion proteins to be secreted in a regulated manner was tested in the presence of the forskolin secretagogue, which increases cytoplasmic calcium levels and promotes regulated exocytosis. In this assay, the FcPC1/3 $_{711\text{-}753}$ fusion protein is secreted in a regulated manner with high efficiency, $p < 0.005$ (sorting ratio: 0.29 ± 0.07) compared to the Fc protein alone (sorting ratio: 0.05 ± 0.02). Statistical analysis of the sorting ratios proved that the DCSG-sorting information for PC1/3 is localized in the second helix as the sorting ratios of Fc PC1/3 $_{711\text{-}738}$ and Fc PC1/3 $_{738\text{-}753}$ fusion proteins were equivalent ($p < 0.005$). The first helix (Fc PC1/3 $_{722\text{-}728}$) did not function as a sorting domain on its own and displayed an equivalent sorting ratio to Fc alone, $p < 0.005$ (sorting ratio: 0.07 ± 0.07). These results are consistent with previous results that PC1/3 $_{738\text{-}751}$ is necessary and sufficient for DCSG sorting (*24*).

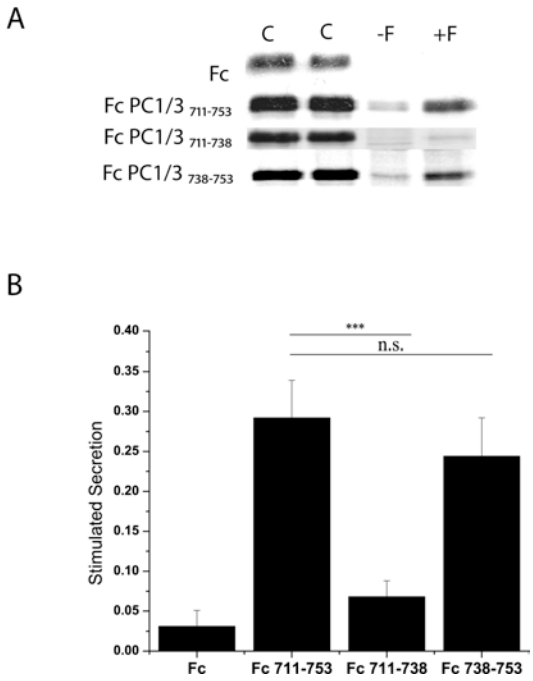


Figure 4.5: Functional assay of the two helices in PC1/3 $_{711-753}$

(A) Secretion assay testing the two individual helices of PC1/3 $_{711-753}$ fused with the portion of the mouse IgG2b immunoglobulin Fc. Proteins are composed of either Fc alone (Fc), both helices (FcPC1/3 $_{711-753}$), the first helix (FcPC1 $_{711-738}$) or the second helix (FcPC1/3 $_{738-753}$). Representative pulse-chase assay for regulated secretion of fusion proteins in AtT-20 endocrine cells are shown. Parallel wells of stably transfected AtT-20 cell pools expressing the various proteins were pulse-labeled for 2 hours and chased with unlabeled medium for an additional 16 hours. After the chase period, the supernatants were collected from the parallel wells (lanes C) and the cells were subsequently incubated for an additional 3 hours either in the absence (-F) or in the presence (+F) of the secretagogue Forskolin. Fc containing proteins in the culture medium were imunoprecipitated with Protein A Sepharose, separated by SDS-PAGE and detected by fluorography. **(B)** Autoradiograms similar to those shown in panel A were exposed to storage phosphor screen and quantified. Shown are the ratios (mean +/- SEM) of fusion protein (for simplicity the term PC1/3 was removed in the figure legend whenever residue numbers

appear) content in the regulated (+F) versus constitutive (-F) secretion incubations. N = 4-6 independent transfections. ***P< 0.005, FcPC1/3 $_{738-753}$ and FcPC1/3 $_{711-753}$ versus Fc by one-way ANOVA with a Tukey post test. No significant (n.s.) difference was observed between FcPC1/3 $_{711-738}$ and Fc alone.

Mutational analysis of the second helix of PC1/3 $_{711\text{-}753}$

In order to define specific residues involved in sorting of PC1/3$_{711\text{-}753}$ to DCSG within the second alpha helix, we tested a series of alanine point mutants (Figure 4.6). As described above, a DCSG targeting assay was performed in AtT20 endocrine cells stably expressing the Fc PC1/3 $_{711\text{-}753}$ mutants. Quantification of the amount of fusion protein released upon secretagogue stimulation revealed that most mutants did not significantly affect DCSG targeting efficiency apart from the mutant FcPC1/3 $_{711\text{-}753}$ (L745A), $p < 0.005$ (sorting ratio: 0.11 ± 0.03). Interestingly, the L745A mutation within PC1/3 $_{711\text{-}753}$ reduced the sorting efficiency of PC1/3 to a level equivalent to the presence of Fc alone or a fusion protein containing only the first helix (FcPC1/3 $_{711\text{-}738}$), $p < 0.005$.

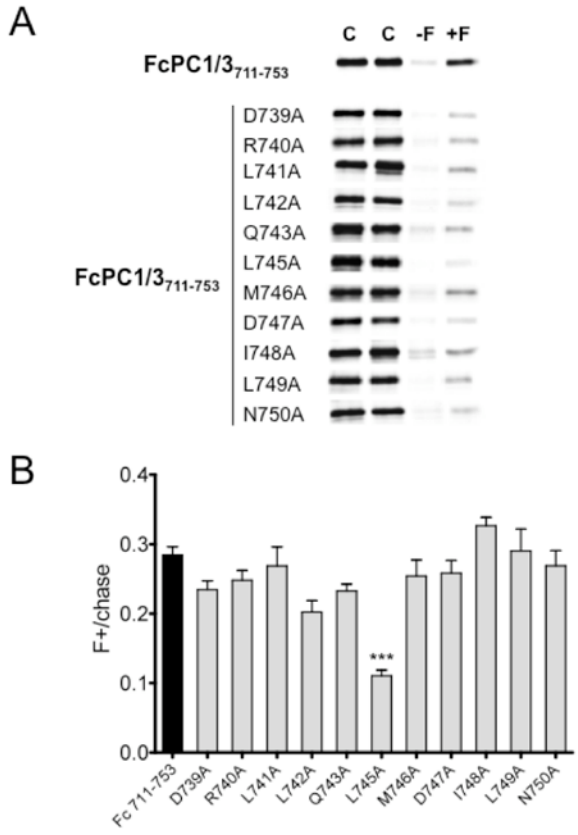


Figure 4.6: Site-directed mutagenesis of the second helix of PC1/3$_{711\text{-}753}$

(A) PC1/3$_{711\text{-}753}$ point mutant proteins used to test regulated secretion in AtT20 endocrine cells. Proteins containing the Fc portion of the mouse IgG2b immunoglobulin and fragments of the carboxy-terminal domain of PC1/3 comprising either Fc alone (Fc), both helices (FcPC1/3 $_{711\text{-}753}$) or alanine point mutants of FcPC1/3 $_{711\text{-}753}$ were stably transfected in endocrine AtT20 cells. The same type of assay described in the legend of Figure 4.5 was performed and Fc containing proteins in the culture medium were imunoprecipitated with Protein A Sepharose, separated by SDS- PAGE and detected by fluorography. **(B)** Autoradiograms similar to those shown in panel A (for simplicity the term PC1/3 was

removed in the figure legend whenever residue numbers appear) were exposed to storage phosphor screen and quantified. Shown are the ratios (mean +/- SEM) of fusion protein content in the regulated (+F) versus constitutive (-F) secretion incubations. n = 4-8, independent transfections. ***P< 0.005, Fc PC1/3$_{711\text{-}753}$ (L745A) versus Fc PC1/3$_{711\text{-}753}$ by one-way ANOVA with a Tukey post-test.

Association of the PC1/3 DCSG sorting domain with micelles

We had previously demonstrated that PC1/3 $_{711\text{-}753}$ was present in the membrane fraction when transfected in endocrine cells (*24*). Based on our structural and mutagenesis data, we attempted to determine by NMR spectroscopy if PC1/3$_{711\text{-}753}$ interacts with membrane-mimicking micelle components. CHAPS is an analog of cholesterol and cholesterol is an integral part of the TGN membrane where the sorting of DCSG proteins occurs (*2*). We titrated ^{15}N-labelled PC1/3 $_{711\text{-}753}$ with incremental quantities of CHAPS (0-20 mM) to specifically determine which residues of the protein are involved in interactions with the CHAPS micelles (Figure 4.7, A). Quantification of chemical shift variations between ^{15}N-labelled PC1/3 $_{711\text{-}753}$ in the absence of CHAPS and ^{15}N-labeled PC1/3 $_{711\text{-}753}$ in 20 mM CHAPS allowed us to define two regions of PC1/3 $_{711\text{-}753}$ displaying significant chemical shift variations, and they correspond to residues 724-729 and 741-750 (Figure 4.7, B). These regions virtually coincide with the two alpha helical regions of PC1/3 $_{711\text{-}753}$ that are located between residues 722-728 and 738-750, respectively. The amphipathic nature of the helices is further supported by the fact that intermolecular NOEs are observed between the CHAPS and only hydrophobic residues in both the first (V^{725}) and second (L^{741}, L^{742}, L^{745}, M^{746}, and I^{748}) helix (data not shown). Thus both alpha helical regions of PC1/3 $_{711\text{-}753}$ are capable of interacting with membrane-like micelle components.

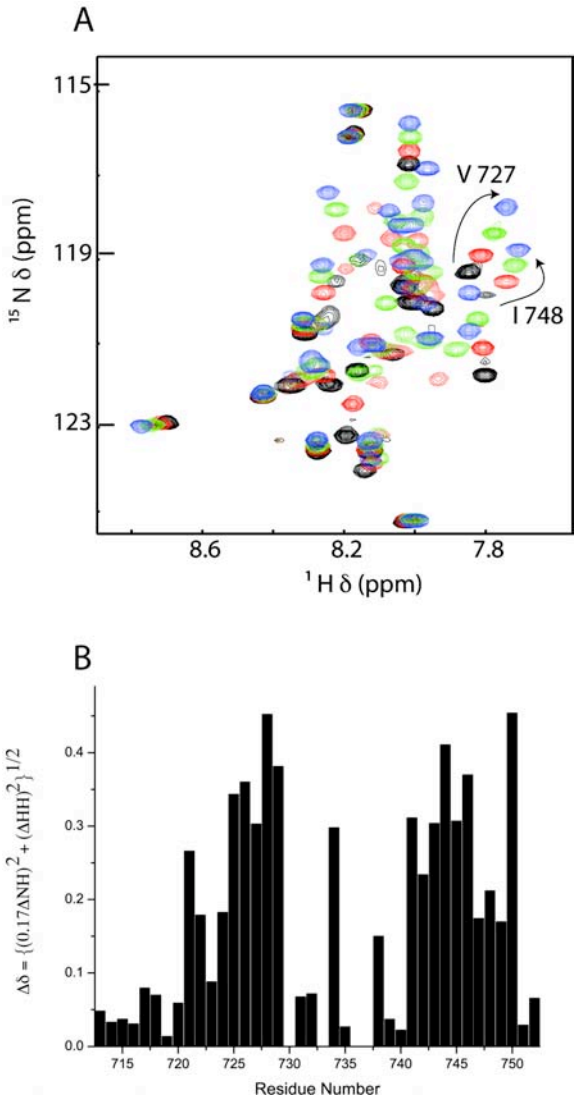


Figure 4.7: PC1/3 $_{711\text{-}753}$ interactions with a CHAPS micelle

(A) Overlay of a selected region from the two dimensional ^{1}H-^{15}N HSQC spectra for ^{15}N-labeled PC1/3 $_{711\text{-}753}$ in the free form (black), in the presence of 4 mM CHAPS (green), 8 mM CHAPS (blue) and 20 mM CHAPS (red). The chemical shift variations observed for residues V^{727} and I^{748} are highlighted with arrows. **(B)** Histogram of the variations in chemical shifts [$\Delta\delta_{(ppm)}$ (*50*)] of ^{15}N-labeled PC1/3 $_{711\text{-}753}$ between the free form and in the presence of 20 mM CHAPS.

Identification of a calcium-binding site in PC1/3

It has been proposed that calcium plays a role in sorting based on the high concentration of calcium in the DCSGs (10 mM) (*13*). However, the exact role of calcium in the sorting of PC1/3 to DCSGs remains unknown. In order to determine if the DCSG-sorting domain of PC1/3 bound calcium, we performed chemical shift mapping studies in the presence of varying concentrations of $CaCl_2$ (1-10 mM). Addition of 10 mM $CaCl_2$ to ^{15}N-labeled PC1/3 $_{711-753}$, resulted in significant changes in the chemical shifts of several residues of PC1/3 $_{711-753}$, as determined by two-dimensional ^{1}H-^{15}N HSQC experiments (Figure 4.8, A). The most significant chemical shift changes were observed for residues I^{748} and E^{751}. Analysis of the variations indicated that the largest chemical shift changes occurred at residue E^{751} (Figure 4.8, B). An electrostatic potential map of PC1/3 $_{711-753}$ reveals that E^{751} is located in a highly negatively charged region containing residues 750-753 (Figure 4.9). In the absence of calcium, the region between residues 751-753 showed a high degree of backbone motion on the picosecond-to-nanosecond time scale, as derived from ^{1}H-^{15}N heteronuclear NOE experiments (Figure 4.10). Residues displaying low ^{1}H-^{15}N heteronuclear NOE values have an intrinsic flexibility. The ^{1}H-^{15}N heteronuclear NOE experiment indicates the presence of a flexible and disordered region localized between residues 751-753, which is most likely accessible for calcium binding.

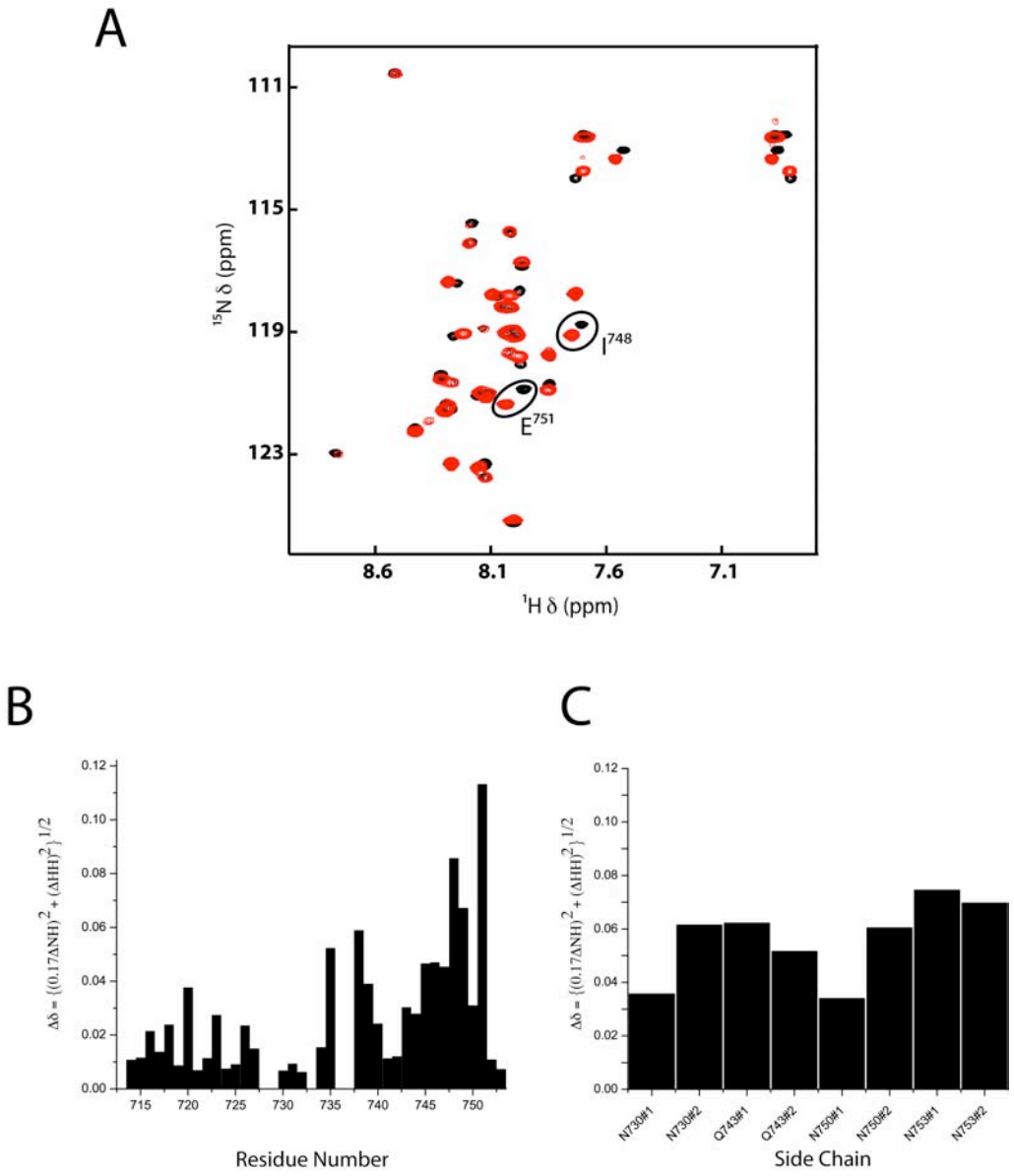


Figure 4.8: PC1/3 $_{711\text{-}753}$ interacts with calcium

(A) Overlay of the two-dimensional ^{1}H-^{15}N spectra for ^{15}N-labeled PC1/3 $_{711\text{-}753}$ in the free form (black) and in the presence of 10 mM $CaCl_2$ (red). Examples of shifted signals are circled. Histogram of the variations in either backbone **(B)** or side chain **(C)** chemical shifts [$\Delta\delta_{(ppm)}$ (*50*)] of ^{15}N-labeled PC1/3 $_{711\text{-}753}$ between the free form and in the presence of 10 mM $CaCl_2$.

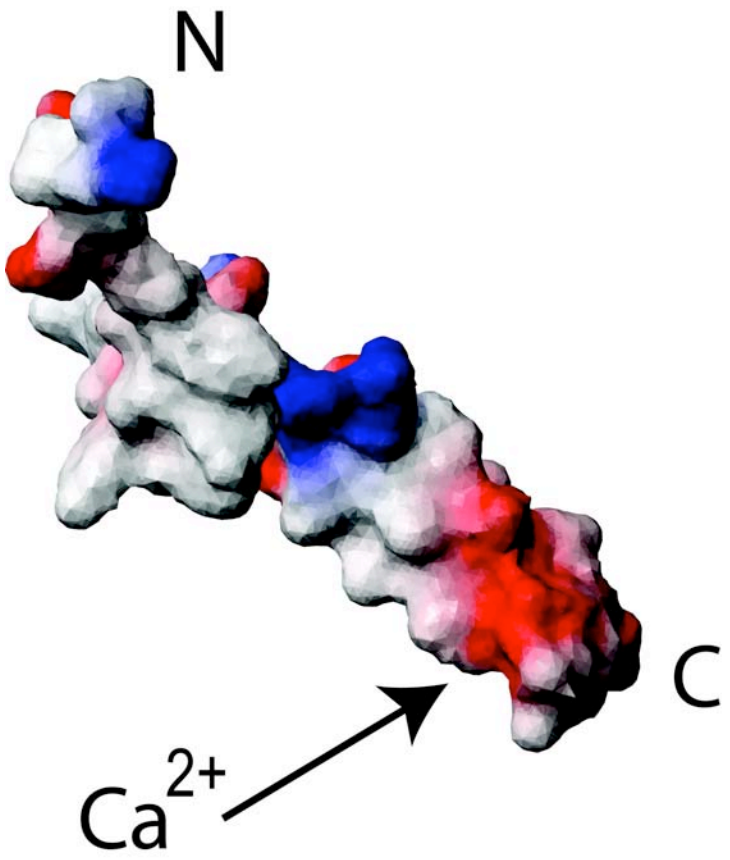


Figure 4.9: Calcium-binding site in PC1/3 $_{711\text{-}753}$

Electrostatic potential for PC1/3 $_{711\text{-}753}$ mapped on the molecular surface between –0.9 (red) and +0.9 (blue) kT using the program Pymol (www.pymol.org). Arrow denotes negatively charged region at the carboxy-terminus corresponding to the proposed calcium-binding site which comprises residues 751-753 of PC1/3.

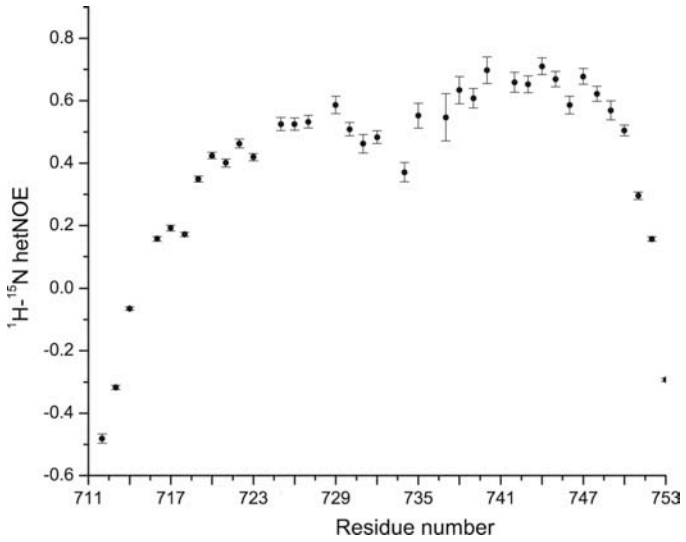


Figure 4.10: PC1/3 $_{711-753}$ backbone dynamics

Plot of the heteronuclear ^{15}N-^{1}H NOEs for PC1/3 $_{711-753}$

Mutational analysis of the calcium-binding site of PC1/3 $_{711-753}$

In order to determine if calcium binding to PC1/3 $_{711-753}$ plays a role in sorting, we tested the ability of point mutants [FcPC1/3$_{711-753}$ (E751A), FcPC1/3$_{711-753}$ (E752A) and FcPC1/3$_{711-753}$ (N753A)] and a deletion mutant (Fc PC1/3 $_{711-750}$) (Figure 4.11) to redirect the constitutively secreted Fc protein to the regulated secretory pathway. Quantification of the amount of fusion protein released upon secretagogue stimulation revealed that only the FcPC1/3$_{711-753}$ (E752A) mutant had an effect on the sorting of PC1/3 to DCSGs effectively decreasing regulated secretion when compared to Fc PC1/3 $_{711-753}$ (Figure 4.10; $p < 0.05$). Surprisingly, the FcPC1/3 $_{711-750}$ deletion mutant still sorted as well as FcPC1/3 $_{711-753}$ despite the fact that it no longer contains the calcium-binding region.

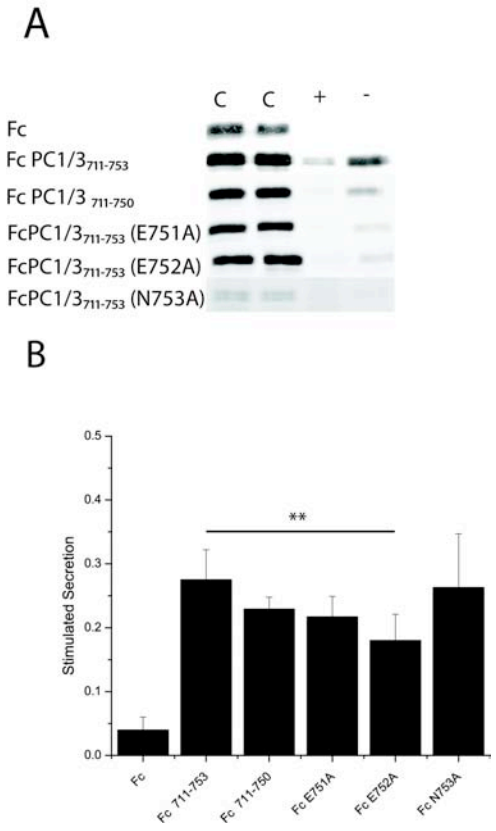


Figure 4.11: Mutational analysis of the calcium binding site on the sorting of PC1/3$_{711-753}$ to secretory granules.

(A) A similar stimulated secretion assay as described in Figures 4.5 and 4.6 was performed on either point mutants of the calcium-binding regions of PC1/3 [FcPC1/3$_{711-753}$ (E751A), FcPC1/3$_{711-753}$ (E752A) or FcPC1/3$_{711-753}$ (N753A)] or a deletion mutant that eliminates the calcium-binding region (FcPC1/3 $_{711-750}$). **(B)** Autoradiograms similar to those shown in panel A (for simplicity the term PC1/3 was removed in the figure legend whenever residue numbers appear) were exposed to storage phosphor screen and quantified. Shown are the ratios (mean +/- SEM) of fusion protein content in the regulated (+F) versus constitutive (-F) secretion incubations. n = 4-8; independent transfections.

**P< 0.05, Fc E752A versus Fc PC1 $_{711-753}$ by one-way ANOVA with a Tukey post-test.

DISCUSSION

In the current manuscript, we have determined the first high-resolution structure of a DCSG-sorting domain from the carboxy terminal of PC1/3 between residues 711 and 753. The solubility of DCSG proteins has been a major hurdle in solving structures in an aqueous environment since they are often membrane-associated and the use of micelle-forming conditions such as CHAPS and DPC was crucial to solving the structure of PC1/3 $_{711\text{-}753}$. The sorting domain of PC1/3 contains two alpha helices separated by a short eight-residue linker. The sequence of the domain is highly conserved in both helices as well as in the linker. Both helices are amphipathic and they interact with the CHAPS micelle through the hydrophobic face of the helix. Since both helices are interacting with the micelle and one does not observe long range NOEs between the two helices, this suggests that the sorting domain of PC1/3 is effectively laying on the micelle surface and interacts with the micelle in a carpet-like mechanism [For Review See (*51*)]. The second helix (PC1/3 $_{738\text{-}750}$) is necessary and sufficient to target a constitutively secreted protein to DCSGs and L^{745} is critical in mediating this targeting step. L^{745} is localized on the hydrophobic face of a non-dynamic region. Moreover, a calcium-binding region was found in a negatively charged and flexible region localized at the extreme carboxy terminus.

The interaction of PC1/3 $_{711\text{-}753}$ with the CHAPS and DPC micelle is consistent with the DCSG-sorting domain being anchored on the membrane surface at the level of the TGN and thereby not being imported to the default constitutive secretory pathway. Functionally, the presence of two helices in PC1/3's sorting domain and the necessity of only the second helix located between residues 738-750 requires further investigation. Interestingly, two similar DCSG sorting motifs have been observed in secretogranin II (*20*). Secretogranin II contains both an amino-terminal and a carboxy-terminal sorting domain and both of these domains have been predicted to form a similar helix-loop-helix motif. Moreover, in both of

these motifs the second helix was necessary and sufficient to direct secretogranin II to granules. The loop region between both helices for these sorting domains may ensure that the proteins are not forming transmembrane domains hindering their subsequent extracellular release. Furthermore, the length of a helix may be critical for its granule targeting capacity. It has been shown that engineered alpha helices were capable of deforming vesicles when they contained 18 residues while 12 residue helices did not to deform these same vesicles (*52*). Thus, length may be critical in not just the membrane-tethering step at the TGN but in the subsequent budding and DCSG formation stages.

In PC1/3 $_{711\text{-}753}$, L^{745} was critical in mediating the sorting of PC1/3 to granules. A surface representation of PC1/3 $_{711\text{-}753}$ (Figure 4.12, A) demonstrates that PC1/3 contains a hydrophobic patch in the region surrounding L^{745}. This clustering of hydrophobic residues appears to be critical in tethering PC1/3$_{711\text{-}753}$ to the TGN membrane allowing sorting to DCSGs. Recently, our group studied a series of alpha helices and determined that the clustering of hydrophobic residues within a charged alpha helix was necessary and sufficient to target a constitutively secreted protein to DCSGs (*49*). Disruption of the hydrophobic cluster resulted in the constitutive secretion of the tested proteins. Indeed, a surface representation of PC1/3 $_{711\text{-}753}$ (Figure 4.12, B) indicates that the alanine at position 745 (arrow) disrupts the hydrophobic cluster present in the second helix of PC1/3 $_{711\text{-}753}$. Moreover, we had previously shown that the hydrophobic cluster localized in the second helix ensured a more efficient targeting of PC1/3 $_{711\text{-}753}$ to DCSGs than either of the alpha-helical sorting domains of the two other PC granule resident proteins: PC2 and PC5/6A (*21*). A biophysical comparison of the helical-sorting domains of PC1/3, PC2 and PC5A/6 demonstrated that the helix containing the more hydrophobic cluster (PC1/3) was capable of redirecting a constitutively secreted fusion protein more efficiently to DCSGs (*21*). In terms of interacting with membranes, the presence of hydrophobic residues may supersede the actual presence of the alpha helix itself. Sar1P contains an amino-terminal helix critical in targeting the protein to COP1 coated vesicles (*53*). Mutation of the hydrophobic residues of Sar1p and not the alpha-helical structure itself abrogated targeting of Sar1p to

COP1 vesicles. Recently, it has been demonstrated that luteinizing hormone is targeted to granules by a non-alpha helical leucine-rich sequence situated at its carboxy-terminus (*54*). While the authors did not investigate the membrane-binding capabilities of luteinizing hormone, they did observe that the protein formed a dimer through the leucine-rich granule-targeting region. Thus, hydrophobic regions may not be exclusively involved in membrane binding but also in forming high molecular weight complexes that will be stored in DCSGs and not be secreted in low-density constitutively secreted vesicles.

The DCSG sorting domain of PC1/3 was also found to interact with calcium. Since the FcPC1/3$_{711\text{-}753}$ (E752A) point mutant slightly decreased the sorting of PC1/3 to DCSGs, we tested whether calcium binding is important for sorting. The role of calcium in the targeting of PC1/3 to DCSGs has been stipulated in the activation step of the enzyme as 10 mM is required to activate PC1/3 processing of POMC (*13*) and activation of PC1/3 occurs in the DCSG (*55*). The autocatalytic cleavage of the carboxy-terminal domain (PC1/3 $_{617\text{-}753}$) within DCSGs results in a fully active PC1/3 enzyme. Calcium may assist in the autocatalytic cleavage of PC1/3 $_{617\text{-}753}$ thereby activating PC1/3. In our current study, we identified a negatively charged region in PC1/3 between residues 750-753, which displayed significant chemical shift variations upon the addition of 10 mM $CaCl_2$. Thus, $CaCl_2$ is binding a disordered and highly flexible region situated at the extreme carboxy-terminal end of PC1/3. While the observed chemical shift variations do not suggest a conformational change, they may result in a change in the electrostatics of PC1/3 ensuring the proper anchoring of PC1/3 on the membrane. Indeed, the FcPC1/3 $_{711\text{-}750}$ deletion mutant, which eliminates the calcium-binding region, did not affect sorting of PC1/3 to DCSGs suggesting that calcium binding serves to mask the negative charges present between residues 751-753 thereby ensuring that the hydrophobic face of the helix approaches the TGN membrane. This mechanism would be an efficient way to ensure the membrane tethering of PC1/3 in an intracellular compartment where calcium concentration rises. A similar mode of membrane binding has been described for the exocytic protein synaptotagmin I, which exhibits calcium dependent phospholipid binding (*56*) and does not undergo a

conformational change in the presence of calcium but rather an electrostatic modification. Alternatively, it has been shown that calcium can also modulate the aggregation state of certain granule proteins such as the highly acidic chromogranin A (CgA) protein (*57*). CgA binding to calcium results in high-molecular weight complexes, which are stored to DCSGs. We have shown that a fusion protein containing PC1/3 $_{711\text{-}753}$ has the ability to form dimers (*24*). Calcium may assist in the dimerization of PC1/3 at the TGN membrane thereby ensuring a more efficient targeting to DCSGs.

In summary, by elucidating the structure of PC1/3 $_{711\text{-}753}$ we have functionally characterized the key residue implicated in DCSG targeting. We have also documented a calcium-binding site present in PC1/3 $_{711\text{-}753}$ potentially masking a negatively charged region on PC1/3 thereby ensuring efficient granule targeting.

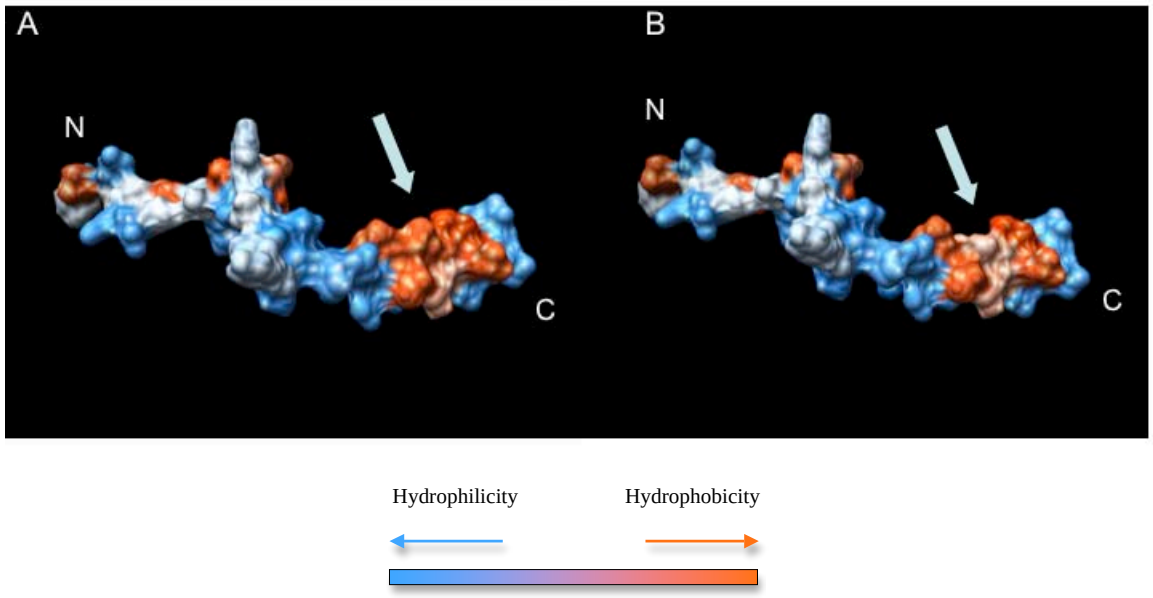


Figure 4.12: Hydrophobic molecular surface representation of PC1/3 $_{711\text{-}753}$

(**A**) Surface representation of PC1/3$_{711\text{-}753}$ with the more hydrophobic residues colored in red and the more hydrophilic residues colored in blue generated with Chimera (*42*). The arrow indicates a highly hydrophobic cluster of residues in the vicinity of L^{745}. (**B**) Model hydrophobic surface representation of PC1/3$_{711\text{-}753}$ (L745A) mutant. Arrow indicates the area with a decrease in hydrophobicity.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Canadian Institute of Health Research to JGO and TLR. JDD is a recipient of a studentship from the Fonds de Recherche en Santé du Québec whereas PDL is a recipient of a Fellowship from the Canadian Institute of Health Research. The NMR instruments were supported by grants from the Canada Foundation for Innovation (800 MHz, 600 MHz and 500 MHz), the Québec Ministère de la recherche en science et technologie (800 MHz, 600 MHz and 500 MHz), and McGill University (800 MHz).

REFERENCES

1. Meldolesi, J., Chieregatti, E., and Luisa, M. M. (2004) Requirements for the identification of dense-core granules, *Trends Cell Biol. 14*, 13-19.

2. Dikeakos, J. D. and Reudelhuber, T. L. (2007) Sending proteins to dense core secretory granules: still a lot to sort out, *J. Cell Biol. 177*, 191-196.

3. Cool, D. R., Normant, E., Shen, F., Chen, H. C., Pannell, L., Zhang, Y., and Loh, Y. P. (1997) Carboxypeptidase E is a regulated secretory pathway sorting receptor: genetic obliteration leads to endocrine disorders in Cpe(fat) mice, *Cell 88*, 73-83.

4. Irminger, J. C., Verchere, C. B., Meyer, K., and Halban, P. A. (1997) Proinsulin targeting to the regulated pathway is not impaired in carboxypeptidase E-deficient Cpefat/Cpefat mice, *J. Biol. Chem. 272*, 27532-27534.

5. Taupenot, L., Harper, K. L., and O'Connor, D. T. (2005) Role of H+-ATPase-mediated acidification in sorting and release of the regulated secretory protein chromogranin A: evidence for a vesiculogenic function, *J. Biol. Chem. 280*, 3885-3897.

6. Orci, L., Ravazzola, M., Amherdt, M., Madsen, O., Perrelet, A., Vassalli, J. D., and Anderson, R. G. (1986) Conversion of proinsulin to insulin occurs coordinately with acidification of maturing secretory vesicles, *J. Cell Biol. 103*, 2273-2281.

7. Urbe, S., Page, L. J., and Tooze, S. A. (1998) Homotypic fusion of immature secretory granules during maturation in a cell-free assay, *J. Cell Biol. 143*, 1831-1844.

8. Ahras, M., Otto, G. P., and Tooze, S. A. (2006) Synaptotagmin IV is necessary for the maturation of secretory granules in PC12 cells, *J. Cell Biol. 173*, 241-251.

9. Austin, C., Hinners, I., and Tooze, S. A. (2000) Direct and GTP-dependent interaction of ADP-ribosylation factor 1 with clathrin adaptor protein AP-1 on immature secretory granules, *J. Biol. Chem. 275*, 21862-21869.

10. Brechler, V., Chu, W. N., Baxter, J. D., Thibault, G., and Reudelhuber, T. L. (1996) A protease processing site is essential for prorenin sorting to the regulated secretory pathway, *J. Biol. Chem. 271*, 20636-20640.

11. Bundgaard, J. R., Birkedal, H., and Rehfeld, J. F. (2004) Progastrin is directed to the regulated secretory pathway by synergistically acting basic and acidic motifs, *J. Biol. Chem. 279*, 5488-5493.

12. Feliciangeli, S., Kitabgi, P., and Bidard, J. N. (2001) The role of dibasic residues in prohormone sorting to the regulated secretory pathway. A study with proneurotensin, *J. Biol. Chem. 276*, 6140-6150.

13. Schmidt, W. K. and Moore, H. P. (1995) Ionic milieu controls the compartment-specific activation of pro-opiomelanocortin processing in AtT-20 cells, *Mol. Biol. Cell 6*, 1271-1285.

14. Canaff, L., Brechler, V., Reudelhuber, T. L., and Thibault, G. (1996) Secretory granule targeting of atrial natriuretic peptide correlates with its calcium-mediated aggregation, *Proc. Natl. Acad. Sci. U. S. A 93*, 9483-9487.

15. Wasmeier, C., Burgos, P. V., Trudeau, T., Davidson, H. W., and Hutton, J. C. (2005) An extended tyrosine-targeting motif for endocytosis and recycling of the dense-core vesicle membrane protein phogrin, *Traffic. 6*, 474-487.

16. Dhanvantari, S., Arnaoutova, I., Snell, C. R., Steinbach, P. J., Hammond, K., Caputo, G. A., London, E., and Loh, Y. P. (2002) Carboxypeptidase E, a prohormone sorting receptor, is anchored to secretory granules via a C-terminal transmembrane insertion, *Biochemistry 41*, 52-60.

17. Garcia, A. L., Han, S. K., Janssen, W. G., Khaing, Z. Z., Ito, T., Glucksman, M. J., Benson, D. L., and Salton, S. R. (2005) A prohormone convertase cleavage site within a predicted alpha-helix mediates sorting of the neuronal and endocrine polypeptide VGF into the regulated secretory pathway, *J. Biol. Chem. 280*, 41595-41608.

18. Taupenot, L., Harper, K. L., Mahapatra, N. R., Parmer, R. J., Mahata, S. K., and O'Connor, D. T. (2002) Identification of a novel sorting determinant for the regulated pathway in the secretory protein chromogranin A, *J. Cell Sci. 115*, 4827-4841.

19. Mouchantaf, R., Kumar, U., Sulea, T., and Patel, Y. C. (2001) A conserved alpha-helix at the amino terminus of prosomatostatin serves as a sorting signal for the regulated secretory pathway, *J. Biol. Chem. 276*, 26308-26316.

20. Courel, M., Vasquez, M. S., Hook, V. Y., Mahata, S. K., and Taupenot, L. (2008) Sorting of the Neuroendocrine Secretory Protein Secretogranin II into the Regulated Secretory Pathway: ROLE, *J. Biol. Chem. 283*, 11807-11822.

21. Dikeakos, J. D., Mercure, C., Lacombe, M. J., Seidah, N. G., and Reudelhuber, T. L. (2007) PC1/3, PC2 and PC5/6A are targeted to dense core secretory granules by a common mechanism, *FEBS J. 274*, 4094-4102.

22. Seidah, N. G. and Chretien, M. (1999) Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides, *Brain Res. 848*, 45-62.

23. Lou, H., Smith, A. M., Coates, L. C., Cawley, N. X., Loh, Y. P., and Birch, N. P. (2007) The transmembrane domain of the prohormone convertase PC3: a key motif for targeting to the regulated secretory pathway, *Mol. Cell Endocrinol. 267*, 17-25.

24. Jutras, I., Seidah, N. G., and Reudelhuber, T. L. (2000) A predicted alpha -helix mediates targeting of the proprotein convertase PC1 to the regulated secretory pathway, *J. Biol. Chem. 275*, 40337-40343.

25. Assadi, M., Sharpe, J. C., Snell, C., and Loh, Y. P. (2004) The C-terminus of prohormone convertase 2 is sufficient and necessary for Raft association and sorting to the regulated secretory pathway, *Biochemistry 43*, 7798-7807.

26. Kay, L. E., Keifer, P., and Saarinen, T. (1992) Pure Absorption Gradient Enhanced Heteronuclear Single Quantum Correlation Spectroscopy with Improved Sensitivity, *Journal of the American Chemical Society 114*, 10663-10665.

27. Yamazaki, T., Formankay, J. D., and Kay, L. E. (1993) 2-Dimensional Nmr Experiments for Correlating C-13-Beta and H-1-Delta/Epsilon Chemical-Shifts of Aromatic Residues in C-13-Labeled Proteins Via Scalar Couplings, *Journal of the American Chemical Society 115*, 11054-11055.

28. Wittekind, M. and Mueller, L. (1993) Hncacb, A High-Sensitivity 3D Nmr Experiment to Correlate Amide-Proton and Nitrogen Resonances with the Alpha-Carbon and Beta-Carbon Resonances in Proteins, *Journal of Magnetic Resonance Series B 101*, 201-205.

29. Muhandiram, D. R. and Kay, L. E. (1994) Gradient-Enhanced Triple-Resonance 3-Dimensional Nmr Experiments with Improved Sensitivity, *Journal of Magnetic Resonance Series B 103*, 203-216.

30. Grzesiek, S., Ikura, M., Clore, G. M., Gronenborn, A. M., and Bax, A. (1992) A 3D Triple-Resonance Nmr Technique for Qualitative Measurement of Carbonyl-H-Beta J Couplings in Isotopically Enriched Proteins, *Journal of Magnetic Resonance 96*, 215-221.

31. Vuister, G. W., Wang, A. C., and Bax, A. (1993) Measurement of 3-Bond Nitrogen Carbon-J Couplings in Proteins Uniformly Enriched in N-15 and C-13, *Journal of the American Chemical Society 115*, 5334-5335.

32. Kay, L. E., Torchia, D. A., and Bax, A. (1989) Backbone Dynamics of Proteins As Studied by N-15 Inverse Detected Heteronuclear Nmr-Spectroscopy - Application to Staphylococcal Nuclease, *Biochemistry 28*, 8972-8979.

33. Zwahlen, C., Legault, P., Vincent, S. J. F., Greenblatt, J., Konrat, R., and Kay, L. E. (1997) Methods for measurement of intermolecular NOEs by multinuclear NMR spectroscopy: Application to a bacteriophage lambda N-peptide/boxB RNA complex, *Journal of the American Chemical Society 119*, 6711-6721.

34. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) Nmrpipe - A Multidimensional Spectral Processing System Based on Unix Pipes, *Journal of Biomolecular Nmr 6*, 277-293.

35. Johnson, B. A. and Blevins, R. A. (1994) Nmr View - A Computer-Program for the Visualization and Analysis of Nmr Data, *Journal of Biomolecular Nmr 4*, 603-614.

36. Marion, D., Kay, L. E., Sparks, S. W., Torchia, D. A., and Bax, A. (1989) 3-Dimensional Heteronuclear Nmr of N-15-Labeled Proteins, *Journal of the American Chemical Society 111*, 1515-1517.

37. Marion, D., Driscoll, P. C., Kay, L. E., Wingfield, P. T., Bax, A., Gronenborn, A. M., and Clore, G. M. (1989) Overcoming the Overlap Problem in the Assignment of H-1-

Nmr Spectra of Larger Proteins by Use of 3-Dimensional Heteronuclear H-1-N-15 Hartmann-Hahn Multiple Quantum Coherence and Nuclear Overhauser Multiple Quantum Coherence Spectroscopy - Application to Interleukin-1-Beta, *Biochemistry 28*, 6150-6156.

38. Zuiderweg, E. R. P., Mcintosh, L. P., Dahlquist, F. W., and Fesik, S. W. (1990) 3-Dimensional C-13-Resolved Proton Noe Spectroscopy of Uniformly C-13-Labeled Proteins for the Nmr Assignment and Structure Determination of Larger Molecules, *Journal of Magnetic Resonance 86*, 210-216.

39. Cornilescu, G., Delaglio, F., and Bax, A. (1999) Protein backbone angle restraints from searching a database for chemical shift and sequence homology, *Journal of Biomolecular Nmr 13*, 289-302.

40. Brunger, A. T., Adams, P. D., Clore, G. M., Delano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination, *Acta Crystallographica Section D-Biological Crystallography 54*, 905-921.

41. Laskowski, R. A., Rullmann, J. A. C., MacArthur, M. W., Kaptein, R., and Thornton, J. M. (1996) AQUA and PROCHECK-NMR: Programs for checking the quality of protein structures solved by NMR, *Journal of Biomolecular Nmr 8*, 477-486.

42. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF chimera - A visualization system for exploratory research and analysis, *Journal of Computational Chemistry 25*, 1605-1612.

43. Koradi, R., Billeter, M., and Wuthrich, K. (1996) MOLMOL: A program for display and analysis of macromolecular structures, *Journal of Molecular Graphics 14*, 51-&.

44. Higuchi, R., Krummel, B., and Saiki, R. K. (1988) A General-Method of Invitro Preparation and Specific Mutagenesis of Dna Fragments - Study of Protein and Dna Interactions, *Nucleic Acids Research 16*, 7351-7367.

45. Anglister, J., Grzesiek, S., Wang, A. C., Ren, H., Klee, C. B., and Bax, A. (1994) H-1, C-13, N-15 Nuclear-Magnetic-Resonance Backbone Assignments and Secondary Structure of Human Calcineurin-B, *Biochemistry 33*, 3540-3547.

46. Chattopadhyay, A. and Harikumar, K. G. (1996) Dependence of critical micelle concentration of a zwitterionic detergent on ionic strength: Implications in receptor solubilization, *Febs Letters 391*, 199-202.

47. Matsuo, H., Li, H., McGuire, A. M., Fletcher, C. M., Gingras, A. C., Sonenberg, N., and Wagner, G. (1997) Structure of translation factor eIF4E bound to m7GDP and interaction with 4E-binding protein, *Nat. Struct. Biol. 4*, 717-724.

48. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Clustal-W - Improving the Sensitivity of Progressive Multiple Sequence Alignment Through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice, *Nucleic Acids Research 22*, 4673-4680.

49. Dikeakos, J. D., Lacombe, M. J., Mercure, C., Mireuta, M., and Reudelhuber, T. L. (2007) A hydrophobic patch in a charged alpha-helix is sufficient to target proteins to dense core secretory granules, *J. Biol. Chem. 282*, 1136-1143.

50. Farmer, B. T., Constantine, K. L., Goldfarb, V., Friedrichs, M. S., Wittekind, M., Yanchunas, J., Jr., Robertson, J. G., and Mueller, L. (1996) Localizing the NADP+ binding site on the MurB enzyme by NMR, *Nat. Struct. Biol. 3*, 995-997.

51. Shai, Y. and Oren, Z. (2001) From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides, *Peptides 22*, 1629-1641.

52. Lee, S., Furuya, T., Kiyota, T., Takami, N., Murata, K., Niidome, Y., Bredesen, D. E., Ellerby, H. M., and Sugihara, G. (2001) De Novo-designed Peptide Transforms Golgi-specific Lipids into Golgi-like Nanotubules, *J. Biol. Chem. 276*, 41224-41228.

53. Lee, M. C., Orci, L., Hamamoto, S., Futai, E., Ravazzola, M., and Schekman, R. (2005) Sar1p N-terminal helix initiates membrane curvature and completes the fission of a COPII vesicle, *Cell 122*, 605-617.

54. Jablonka-Shariff, A., Pearl, C. A., Comstock, A., and Boime, I. (2008) A Carboxyl-terminal Sequence in the Lutropin {beta} Subunit Contributes to the Sorting of Lutropin to the Regulated Pathway, *J. Biol. Chem. 283*, 11485-11492.

55. Jutras, I., Seidah, N. G., Reudelhuber, T. L., and Brechler, V. (1997) Two activation states of the prohormone convertase PC1 in the secretory pathway, *J. Biol. Chem. 272*, 15184-15188.

56. Shao, X., Fernandez, I., Sudhof, T. C., and Rizo, J. (1998) Solution structures of the Ca2+-free and Ca2+-bound C2A domain of synaptotagmin I: does Ca2+ induce a conformational change?, *Biochemistry 37*, 16106-16115.

57. Yoo, S. H. and Albanesi, J. P. (1990) Ca2(+)-induced conformational change and aggregation of chromogranin A, *J. Biol. Chem. 265*, 14414-14421.

Chapter 5

Conclusion

5.1 The Carboxy-terminal domain of PC1/3 has a dual function

Studies of the biosynthesis and of the DCSG-sorting signal of PC1/3 have led to many insights regarding its activity as a secretory granule convertase. In addition to the autocatalytic removal of its inhibitory amino-terminal prosegment in the early secretory pathway, PC1/3 also catalyzes the proteolytic removal of its own carboxy-terminal tail only in cells containing dense core secretory granules (*131*). Removal of the PC1/3 carboxy-terminal tail leads to full activation of the enzyme and provides an explanation for the restraint of the enzymatic activity in the early, pre-granule secretory pathway. In addition, the carboxy-terminal tail is required for correct sorting of PC1/3, as recombinant PC1/3 enzymes in which the carboxy-terminal tail has been removed no longer enter secretory granules (*131*). The PC1/3 carboxy-terminal domain is sufficient to direct secretory granule targeting of linked proteins that would normally be secreted by the default or constitutive secretory pathway (*131*). Thus, the carboxy-terminal domain of PC1/3 is a bi-functional domain that represses PC1/3's enzymatic activity in the pre-granular secretory pathway in addition to correctly targeting PC1/3 to DCSGs.

PC1/3's granular fate is determined at the TGN membrane or through an extrusion mechanism in an immature secretory granule. At these locations, proteins enter either the constitutive or the regulated secretory pathways. A membrane-associated amphipathic alpha-helix located between residues 738-750 serves as a DCSG sorting signal for PC1/3. Leucine 745 (L745), located in the amphipathic alpha helix between residues 738-750, is critical for the proper DCSG localization of PC1/3. There is nothing unique about the ability of the PC1/3 alpha helix to act as a DCSG sorting domain. In fact, artificial sequences that form alpha helices functionally replaced the native helix of PC1/3 and redirected a constitutively secreted fusion protein to DCSGs (Chapter 2). In order to function as DCSG-sorting domains, alpha-helices need to contain a hydrophobic cluster extending to the degree of hydrophobicity of the residues in the cluster (L>V>A) in

addition to charged (positively or negatively) residues. Thus, L745, located in PC1/3's alpha helix, may serve to stabilize a hydrophobic cluster ensuring efficient DCSG targeting.

Moreover, further investigation of the three PC family members localized in DCSGs, PC1/3, PC2 and PC5/6A, again revealed a common alpha helical targeting mechanism (Chapter 3). The carboxy-terminal helices of the three granule-resident PC enzymes are able to target a fusion protein to granules with varying efficiencies. The biophysical comparison of these C-terminal helices demonstrated that PC1/3 contained an amphipathic helix with the highest hydrophobic moment and was more efficiently targeted to DCSGs than both PC5/6A and PC2. Why would hydrophobic clusters be more efficient in sorting proteins to DCSGs?

Lipid domains have been proposed to be critical for sorting proteins to DCSGs. Hydrophobic residues may provide key contact points with the TGN membrane ensuring the tethering of granule-resident proteins. The proposed hydrophobic residue-plasma membrane interactions may function to anchor granule-targeted cargo proteins to the secretory granule membrane thereby segregating granule-resident proteins from the default constitutive pathway.

5.2 Models on how the late secretory pathway environment contributes to DCSG sorting

Calcium concentrations increase and the pH decreases in the late secretory pathway and both of these changes may contribute to DCSG sorting of select cargo proteins (*33*). The calcium-rich environment within the TGN may also dictate the tethered state of PC1/3 at the TGN membrane or simply promote the aggregation of PC1/3. The solution structure of the granule-targeting region of PC1/3 $_{711\text{-}753}$ revealed a calcium-binding site located in the negatively charged flexible carboxy-terminal residues 750-753 (Chapter 3). However, deletion of the calcium-binding site did not have any effect on sorting of PC1/3

to DCSGs. The calcium-binding site may serve to mask negative charges to allow better anchoring of PC1/3 in the membrane. The proposed masking of the charged residues in PC1/3 $_{750\text{-}753}$ may also expose hydrophobic clusters that cause PC1/3 to aggregate and thereby increase tethering at the TGN membrane. Indeed, divalent cation binding to proteins has been demonstrated to expose a hydrophobic surface in various proteins including GroEL (*145*) resulting in their aggregation. In addition, the late secretory pathway's acidic environment may enhance the exposure of a hydrophobic surface. In fact, at the intra-granular pH (pH = 5.5), CgA's carboxy-terminal region undergoes a conformational change exposing its carboxy-terminal domain (*146*). Interestingly, the isolelectric point (pI) of CgA $_{407\text{-}431}$ is 4.34 almost identical to the pI value of PC1/3 $_{711\text{-}753}$ (pI = 4.1), making both these proteins negatively charged and capable of binding calcium in the pH ranges (pH = 5.5-7.0) present in the late secretory pathway. While a specific calcium-binding site has not been identified for CgA, the alpha helical region in CgA $_{407\text{-}431}$ may serve this function by creating an exposed hydrophobic surface. How would the results presented in this dissertation fit in the context of the whole PC1/3 carboxy-terminal domain (PC1/3 $_{619\text{-}753}$)?

Once PC1/3 has entered the granule, the pH decreases further (pH=5.5). Our structural studies demonstrate that PC1/3 $_{711\text{-}753}$ is insoluble at this pH (data not shown), presumably due to the proximity of the pH to the protein's pI (PC1/3 $_{711\text{-}753}$, pI = 4.1). At this juncture, the anchored DCSG targeting domain may be insoluble and disrupt PC1/3's fold. In this state, the carboxy-terminal domain would no longer repress PC1/3's enzymatic activity allowing PC1/3 to be an active enzyme within the DSCG.

In Chapter 2, we demonstrated that charge reversed alpha helices could be efficiently targeted to DCSGs. Since a positively charged helix cannot bind calcium and will not become insoluble in the DCSGs, how would this fit with the model? The activity of PC1/3 in the context of the entire PC1/3 molecule was not tested in the charge-reversed helices. The positively charged helices studied in Chapter 2 contained hydrophobic clusters that may have been sufficient to act as DCSG-sorting domains. In future studies, we need

to determine if positively charged helices are able to activate PC1/3 by causing the release of the carboxy-terminal domain and subsequently unfold the inhibitory portion of the carboxy-terminal domain from the rest of the PC1/3 molecule and enhance the auto-catalytic cleavage of the inhibitory carboxy-terminal domain. If the proposed model is correct (Figure 5.1), positively charged sorting domains would not be affected by the drop in pH within the DCSG and this would result in constitutively inactive PC1/3.

Moreover, two additional alpha helices present in the C-terminal domain of PC1/3 have been demonstrated to act independently as DCSG-sorting signals. First, a sequence between residues 617-638 can redirect a constitutively secreted protein to DCSGs (135). A hydrophobic cluster analysis (*147*) reveals that this sequence contains two hydrophobic clustered regions (Figure 5.2). A similar hydrophobic cluster analysis of a second DCSG sorting alpha helical region localized between residues 665-682 (*48*) also reveals two hydrophobic clusters. These hydrophobic clusters may contribute to anchoring PC1/3 to the TGN membrane and PC1/3 would be targeted to the DCSG. The difference between these alpha helices (PC1/3 $_{617-638}$ and PC1 $_{665-682}$) and the alpha helix studied in this dissertation (PC1/3 $_{711-753}$) is the charge. Both PC1/3 $_{617-638}$ and PC1/3 $_{665-686}$ have basic pIs (pI around 11) as opposed to PC1/3 $_{711-753}$'s acidic pI (pI = 4.1). As noted above, while the basic alpha helices (PC1/3 $_{617-638}$ and PC1/3 $_{665-682}$) can target a constitutively secreted protein to DCSGs, their effect on the activation of full-length PC1/3 needs be tested.

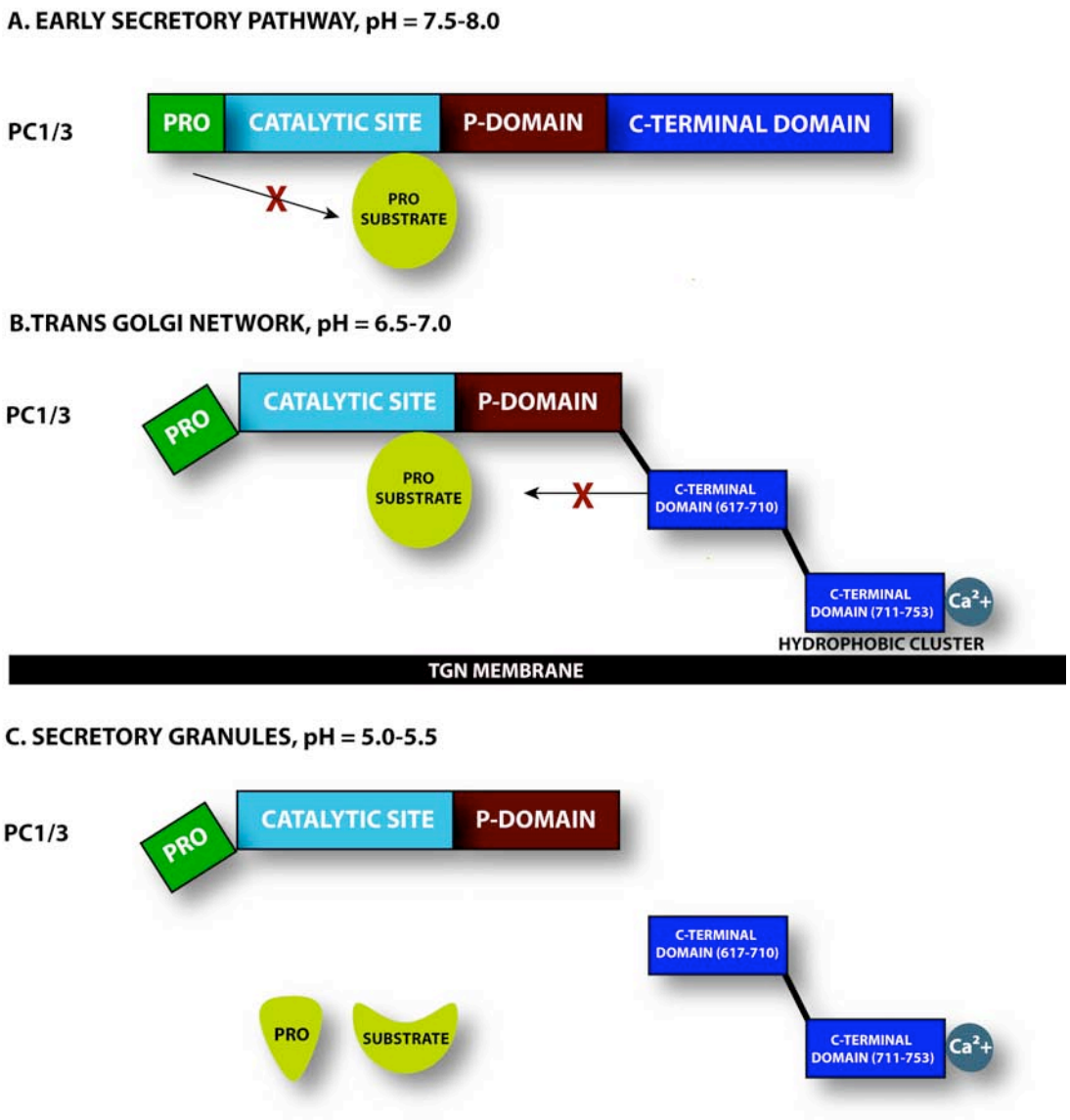


Figure 5.1: Model for the sorting of PC1/3 to DCSGs

(A) In the early secretory pathway, the PRO domain functions to repress the catalytic activity of PC1/3. Bound substrates are not converted to active hormones (prosubstrates). **(B)** At the Trans Golgi Network, the PRO domain of PC1/3 undergoes an autocatalytic cleavage event. The carboxy-terminal (C-terminal) domain represses the enzymatic activity of PC1/3. We propose that this inhibition event occurs via residues 619-710 since residues 711-753 contains a hydrophobic cluster proposed to interact with the TGN membrane. The binding of calcium to a negatively charged region comprising residues 750-

753 may enhance this interaction. **(C)** Once in the secretory granule (pH = 5.5), the carboxy-terminal domain would become uncharged and aggregate. We propose that this action enhances the autocatalytic cleavage of the carboxy-terminal domain of PC1/3. This would eliminate the inhibition on the catalytic site of PC1/3 by the carboxy-terminal domain and result in the activation of prohormone substrates.

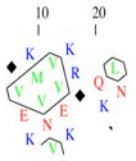
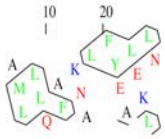
PC1/3 SORTING DOMAINS	ISOELECTRIC POINT	HYDROPHOBIC CLUSTER ANALYSIS
PC1/3 617-638	11.00	
PC1/3 665-682	11.26	
PC1/3 711-753	4.01	

Figure 5.2: Comparison of the sorting domains of PC1/3

Using the method of Gaboriaud et al. (*147*), the three sorting domains located in the carboxy-terminal domain (residues 617-753) were analyzed. The filled diamond shapes represent the positions of the non-polar glycine residues. Isoelectric point (pI) were determined using the EMBOSS software package (*148*).

5.3 Concluding remarks

In summary, we have examined the mechanism whereby alpha helices target proteins to DCSGs. Alpha helices through highly hydrophobic faces are anchored to the membrane ensuring entry in DCSGs. The results presented in this dissertation are in accordance with the characteristics of granule targeting sequences presented in Chapter 1. Indeed, we can effectively reconcile numerous sorting mechanisms using PC1/3 as a model. Thus, PC1/3 is an example of a membrane-tethered protein. The tethering occurs through an alpha-helical DCSG-targeting sequence. Moreover, the interaction between PC1/3 and the membrane may be dependent on the ionic conditions present in the late secretory pathway as calcium is proposed to assist in the membrane-tethering event. The acidic conditions in the late secretory pathway may be functionally linked to the masking of negatively charged residues by calcium as these acidic pH conditions result in non-protonated PC1/3 protein. Furthermore, the residues located on the hydrophobic face of PC1/3 are essential for the sorting event. In closing, the alpha helical mediated DCSG targeting of PC1/3 depends on interactions between PC1/3 and the late secretory pathway environment.

5.4 Long-term Perspectives

Many key experiments can further explain the sorting of proteins to DCSGs.

1 The proper localization of proteins in granules can be simplified with a more quantifiable secretion assay. Recent advances in fluorescence microscopy, notably deconvolution microscopy, allow three dimension reconstructions of cells improving their visualization (96). Moreover, the attachment of reporter proteins on sorting domains has improved the quantification of the amount of protein secreted

from a granule (96).

2 Sorting to granules takes place on the lumenal side of the TGN membrane. Researchers have failed to identify lumenal partner proteins capable of assisting this sorting event. With the advent of proteomics, a greater effort must be undertaken to identify partner proteins that interact with granule-resident proteins. In contrast, *in vitro* systems can be developed to determine whether or not granule-resident proteins require any partner proteins to be targeted to DCSGs. For example, the KL helix used in Chapter 2 has the capability to deform Golgi-like liposomes into nanotubules (*149*). A similar effect can be tested for the native helices of the PC-enzymes to prove that the helix itself can provide a mechanical force to induce budding of the TGN.

3 The elucidation of the solution structures of the PC2 and PC5/6A sorting domains will prove whether these domains also require a micellar environment in order to not be aggregated. This will most likely be the case for PC2, which adopts an alpha-helical structure only in the presence of liposomes (47). On the other hand, PC5/6A contains a cysteine rich domain in its carboxy-terminal domain adjacent to the region predicted to form an alpha helix representing a potential novel fold. These structures will also help in understanding why these PC enzymes (PC2 and PC5/6A) are sorted less efficiently to DCSGs when compared to PC1/3 (Chapter 3).

4 Novel approaches must be undertaken to study the effect of calcium on sorting. A live cell imaging method can be developped to monitor the subcellular localization of fluorescently-tagged granule proteins upon specific chelatation of calcium present in DCSGs. Care must be taken to maintain normal levels of the extracellular calcium as decreased levels of calcium will inhibit regulated exocytosis.

5 The long-term objective for understanding the mechanism of sorting proteins to granules is a better understanding of hormone peptide production. An animal model can be created to study sorting domains *in vivo*. For example, granule production

can be tested in mice overexpressing the granule sorting region of PC1/3. Possible therapeutic effects can be verified in diabetic mice models that do not produce enough of the PC1/3 cleavage product insulin.

5.5 Where do we go from here?

The past two decades have been marked by many interesting discoveries in DCSG protein targeting. Work to date has largely supported the three postulates outlined in Chapter 1: proteins exist that could explain the anchoring of DCSG cargo to the granule membrane, protein complexes between processing enzymes and their substrates have been proposed that could explain how these proteins end up in the same granules and the diversity of sorting mechanisms helps to understand how there could be distinct DCSGs even within single cells and how there could be such a lack of agreement on the mechanism of DCSG sorting. Unfortunately, this lack of a simple consensus mechanism has limited progress primarily to attempts to determine the role of single-targeting motifs in given proteins in various cell culture models that may or may not be entirely appropriate. While this approach has certainly not been without merit, a better understanding of the complexity of this important cellular event may help to design experiments that will help to significantly advance this field of research. Several important questions remain: How does the DCSG protein cargo identify the membrane patches that will make up the mature granule? Does this occur in the lumen of the TGN? Are specific lipids involved? How do these complexes communicate with the cytoplasmic accessory proteins that are necessary for the formation of the budding DCSG? In fact, while it has been difficult to explain the entry of proteins into DCSG, we can expect that describing the assembly of proteins on the surface of the DCSG which are necessary for their cytoplasmic transport, docking at the membrane and exocytosis will be an equally daunting challenge: A recent report on the components of the functionally related synaptic vesicle identified over 400 associated proteins (*150*).

What is abundantly clear is that in the characterization of this unique organelle, there is still a lot to sort out.

Bibliography (Introduction and Discussion)

1. Halic, M. and Beckmann, R. (2005) The signal recognition particle and its interactions during protein targeting, *Curr. Opin. Struct. Biol. 15*, 116-125.

2. Pfanner, N. (2000) Protein sorting: recognizing mitochondrial presequences, *Curr. Biol. 10*, R412-R415.

3. Lange, A., Mills, R. E., Lange, C. J., Stewart, M., Devine, S. E., and Corbett, A. H. (2007) Classical nuclear localization signals: definition, function, and interaction with importin alpha, *J. Biol. Chem. 282*, 5101-5105.

4. Pfeffer, S. R. (1991) Mannose-6-phosphate receptors and their role in protein sorting along the pathway to lysosomes, *Cell Biophys. 19*, 131-140.

5. Pelham, H. R. (1990) The retention signal for soluble proteins of the endoplasmic reticulum, *Trends Biochem. Sci. 15*, 483-486.

6. Pelham, H. R., Hardwick, K. G., and Lewis, M. J. (1988) Sorting of soluble ER proteins in yeast, *EMBO J. 7*, 1757-1762.

7. Nilsson, T. and Warren, G. (1994) Retention and retrieval in the endoplasmic reticulum and the Golgi apparatus, *Curr. Opin. Cell Biol. 6*, 517-521.

8. Letourneur, F. and Klausner, R. D. (1992) A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains, *Cell 69*, 1143-1157.

9. Orci, L., Ravazzola, M., Amherdt, M., Madsen, O., Perrelet, A., Vassalli, J. D., and Anderson, R. G. (1986) Conversion of proinsulin to insulin occurs coordinately with acidification of maturing secretory vesicles, *J. Cell Biol. 103*, 2273-2281.

10. Emr, S. D., Hanley-Way, S., and Silhavy, T. J. (1981) Suppressor mutations that restore export of a protein with a defective signal sequence, *Cell 23*, 79-88.

11. Osborne, A. R., Rapoport, T. A., and Van Den Berg. B. (2005) Protein translocation by the Sec61/SecY channel, *Annu. Rev. Cell Dev. Biol. 21*, 529-550.

12. Lavoie, C., Chevet, E., Roy, L., Tonks, N. K., Fazel, A., Posner, B. I., Paiement, J., and Bergeron, J. J. (2000) Tyrosine phosphorylation of p97 regulates transitional endoplasmic reticulum assembly in vitro, *Proc. Natl. Acad. Sci. U. S. A 97*, 13637-13642.

13. Zeuschner, D., Geerts, W. J., van, D. E., Humbel, B. M., Slot, J. W., Koster, A. J., and Klumperman, J. (2006) Immuno-electron tomography of ER exit sites reveals the existence of free COPII-coated transport carriers, *Nat. Cell Biol. 8*, 377-383.

14. Pfeffer, S. R. (2007) Unsolved mysteries in membrane traffic, *Annu. Rev. Biochem. 76*, 629-645.

15. Malhotra, V. and Mayor, S. (2006) Cell biology: the Golgi grows up, *Nature 441*, 939-940.

16. Losev, E., Reinke, C. A., Jellen, J., Strongin, D. E., Bevis, B. J., and Glick, B. S. (2006) Golgi maturation visualized in living yeast, *Nature 441*, 1002-1006.

17. Matsuura-Tokita, K., Takeuchi, M., Ichihara, A., Mikuriya, K., and Nakano, A. (2006) Live imaging of yeast Golgi cisternal maturation, *Nature 441*, 1007-1010.

18. Honing, S., Griffith, J., Geuze, H. J., and Hunziker, W. (1996) The tyrosine-based lysosomal targeting signal in lamp-1 mediates sorting into Golgi-derived clathrin-coated vesicles, *EMBO J. 15*, 5230-5239.

19. Kuliawat, R., Klumperman, J., Ludwig, T., and Arvan, P. (1997) Differential sorting of lysosomal enzymes out of the regulated secretory pathway in pancreatic beta-cells, *J. Cell Biol. 137*, 595-608.

20. Sannerud, R., Saraste, J., and Goud, B. (2003) Retrograde traffic in the biosynthetic-secretory route: pathways and machinery, *Curr. Opin. Cell Biol. 15*, 438-445.

21. Yoshimori, T., Keller, P., Roth, M. G., and Simons, K. (1996) Different biosynthetic transport routes to the plasma membrane in BHK and CHO cells, *J. Cell Biol. 133*, 247-256.

22. Chung, K. N., Walter, P., Aponte, G. W., and Moore, H. P. (1989) Molecular sorting in the secretory pathway, *Science 243*, 192-197.

23. Gorr, S. U., Hamilton, J. W., and Cohn, D. V. (1992) Regulated, but not constitutive, secretory proteins bind porcine chymotrypsinogen, *J. Biol. Chem. 267*, 21595-21600.

24. Brion, C., Miller, S. G., and Moore, H. P. (1992) Regulated and constitutive secretion. Differential effects of protein synthesis arrest on transport of glycosaminoglycan chains to the two secretory pathways, *J. Biol. Chem. 267*, 1477-1483.

25. Cool, D. R., Normant, E., Shen, F., Chen, H. C., Pannell, L., Zhang, Y., and Loh, Y. P. (1997) Carboxypeptidase E is a regulated secretory pathway sorting receptor: genetic obliteration leads to endocrine disorders in Cpe(fat) mice, *Cell 88*, 73-83.

26. Irminger, J. C., Verchere, C. B., Meyer, K., and Halban, P. A. (1997) Proinsulin targeting to the regulated pathway is not impaired in carboxypeptidase E-deficient Cpefat/Cpefat mice, *J. Biol. Chem. 272*, 27532-27534.

27. Urbe, S., Dittie, A. S., and Tooze, S. A. (1997) pH-dependent processing of secretogranin II by the endopeptidase PC2 in isolated immature secretory granules, *Biochem. J. 321 (Pt 1)*, 65-74.

28. Urbe, S., Page, L. J., and Tooze, S. A. (1998) Homotypic fusion of immature secretory granules during maturation in a cell-free assay, *J. Cell Biol. 143*, 1831-1844.

29. Ahras, M., Otto, G. P., and Tooze, S. A. (2006) Synaptotagmin IV is necessary for the maturation of secretory granules in PC12 cells, *J. Cell Biol. 173*, 241-251.

30. Dittie, A. S., Thomas, L., Thomas, G., and Tooze, S. A. (1997) Interaction of furin in immature secretory granules from neuroendocrine cells with the AP-1 adaptor complex is modulated by casein kinase II phosphorylation, *EMBO J. 16*, 4859-4870.

31. Meldolesi, J., Chieregatti, E., and Luisa, M. M. (2004) Requirements for the identification of dense-core granules, *Trends Cell Biol. 14*, 13-19.

32. Osborne, S. L., Wallis, T. P., Jimenez, J. L., Gorman, J. J., and Meunier, F. A. (2007) Identification of secretory granule phosphatidylinositol 4,5-bisphosphate-interacting proteins using an affinity pulldown strategy, *Mol. Cell Proteomics. 6*, 1158-1169.

33. Schmidt, W. K. and Moore, H. P. (1995) Ionic milieu controls the compartment-specific activation of pro-opiomelanocortin processing in AtT-20 cells, *Mol. Biol. Cell 6*, 1271-1285.

34. Taugner, R., Kim, S. J., Murakami, K., and Waldherr, R. (1987) The fate of prorenin during granulopoiesis in epithelioid cells. Immunocytochemical experiments with antisera against renin and different portions of the renin prosegment, *Histochemistry 86*, 249-253.

35. Dannies, P. S. (2002) Mechanisms for storage of prolactin and growth hormone in secretory granules, *Mol. Genet. Metab 76*, 6-13.

36. Hendy, G. N., Li, T., Girard, M., Feldstein, R. C., Mulay, S., Desjardins, R., Day, R., Karaplis, A. C., Tremblay, M. L., and Canaff, L. (2006) Targeted ablation of the chromogranin a (Chga) gene: normal neuroendocrine dense-core secretory granules and increased expression of other granins, *Mol. Endocrinol. 20*, 1935-1947.

37. Sobota, J. A., Ferraro, F., Back, N., Eipper, B. A., and Mains, R. E. (2006) Not all secretory granules are created equal: Partitioning of soluble content proteins, *Mol. Biol. Cell 17*, 5038-5052.

38. Cowley, D. J., Moore, Y. R., Darling, D. S., Joyce, P. B., and Gorr, S. U. (2000) N- and C-terminal domains direct cell type-specific sorting of chromogranin A to secretory granules, *J. Biol. Chem. 275*, 7743-7748.

39. Marx, R., El, M. R., Johns, D. C., and Mains, R. E. (1999) Differences in the ways sympathetic neurons and endocrine cells process, store, and secrete exogenous neuropeptides and peptide-processing enzymes, *J. Neurosci. 19*, 8300-8311.

40. Bell-Parikh, L. C., Eipper, B. A., and Mains, R. E. (2001) Response of an integral granule membrane protein to changes in pH, *J. Biol. Chem. 276*, 29854-29863.

41. Boulatnikov, I. and De Lisle, R. C. (2004) Binding of the Golgi sorting receptor muclin to pancreatic zymogens through sulfated O-linked oligosaccharides, *J. Biol. Chem. 279*, 40918-40926.

42. Wasmeier, C., Bright, N. A., and Hutton, J. C. (2002) The lumenal domain of the integral membrane protein phogrin mediates targeting to secretory granules, *Traffic. 3*, 654-665.

43. Torii, S., Saito, N., Kawano, A., Zhao, S., Izumi, T., and Takeuchi, T. (2005) Cytoplasmic transport signal is involved in phogrin targeting and localization to secretory granules, *Traffic. 6*, 1213-1224.

44. Wasmeier, C., Burgos, P. V., Trudeau, T., Davidson, H. W., and Hutton, J. C. (2005) An extended tyrosine-targeting motif for endocytosis and recycling of the dense-core vesicle membrane protein phogrin, *Traffic. 6*, 474-487.

45. Dhanvantari, S., Arnaoutova, I., Snell, C. R., Steinbach, P. J., Hammond, K., Caputo, G. A., London, E., and Loh, Y. P. (2002) Carboxypeptidase E, a prohormone sorting receptor, is anchored to secretory granules via a C-terminal transmembrane insertion, *Biochemistry 41*, 52-60.

46. Arnaoutova, I., Smith, A. M., Coates, L. C., Sharpe, J. C., Dhanvantari, S., Snell, C. R., Birch, N. P., and Loh, Y. P. (2003) The prohormone processing enzyme PC3 is a lipid raft-associated transmembrane protein, *Biochemistry 42*, 10445-10455.

47. Assadi, M., Sharpe, J. C., Snell, C., and Loh, Y. P. (2004) The C-terminus of prohormone convertase 2 is sufficient and necessary for Raft association and sorting to the regulated secretory pathway, *Biochemistry 43*, 7798-7807.

48. Jutras, I., Seidah, N. G., and Reudelhuber, T. L. (2000) A predicted alpha -helix mediates targeting of the proprotein convertase PC1 to the regulated secretory pathway, *J. Biol. Chem. 275*, 40337-40343.

49. Mouchantaf, R., Kumar, U., Sulea, T., and Patel, Y. C. (2001) A conserved alpha-helix at the amino terminus of prosomatostatin serves as a sorting signal for the regulated secretory pathway, *J. Biol. Chem. 276*, 26308-26316.

50. Taupenot, L., Harper, K. L., Mahapatra, N. R., Parmer, R. J., Mahata, S. K., and O'Connor, D. T. (2002) Identification of a novel sorting determinant for the regulated pathway in the secretory protein chromogranin A, *J. Cell Sci. 115*, 4827-4841.

51. Garcia, A. L., Han, S. K., Janssen, W. G., Khaing, Z. Z., Ito, T., Glucksman, M. J., Benson, D. L., and Salton, S. R. (2005) A prohormone convertase cleavage site within a predicted alpha-helix mediates sorting of the neuronal and endocrine polypeptide VGF into the regulated secretory pathway, *J. Biol. Chem. 280*, 41595-41608.

52. Van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) Membrane lipids: where they are and how they behave, *Nat. Rev. Mol. Cell Biol. 9*, 112-124.

53. Rajendran, L. and Simons, K. (2005) Lipid rafts and membrane dynamics, *J. Cell Sci. 118*, 1099-1102.

54. Yeaman, C., Ayala, M. I., Wright, J. R., Bard, F., Bossard, C., Ang, A., Maeda, Y., Seufferlein, T., Mellman, I., Nelson, W. J., and Malhotra, V. (2004) Protein kinase D regulates basolateral membrane protein exit from trans-Golgi network, *Nat. Cell Biol. 6*, 106-112.

55. Bard, F. and Malhotra, V. (2006) The formation of TGN-to-plasma-membrane transport carriers, *Annu. Rev. Cell Dev. Biol. 22*, 439-455.

56. Ktistakis, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C., and Roth, M. G. (1996) Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi coated vesicles, *J. Cell Biol. 134*, 295-306.

57. Chen, Y. G., Siddhanta, A., Austin, C. D., Hammond, S. M., Sung, T. C., Frohman, M. A., Morris, A. J., and Shields, D. (1997) Phospholipase D stimulates release of nascent secretory vesicles from the trans-Golgi network, *J. Cell Biol. 138*, 495-504.

58. Kooijman, E. E., Chupin, V., de, K. B., and Burger, K. N. (2003) Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid, *Traffic. 4*, 162-174.

59. Siddhanta, A. and Shields, D. (1998) Secretory vesicle budding from the trans-Golgi network is mediated by phosphatidic acid levels, *J. Biol. Chem. 273*, 17995-17998.

60. Dhanvantari, S. and Loh, Y. P. (2000) Lipid raft association of carboxypeptidase E is necessary for its function as a regulated secretory pathway sorting receptor, *J. Biol. Chem. 275*, 29887-29893.

61. Blazquez, M., Thiele, C., Huttner, W. B., Docherty, K., and Shennan, K. I. (2000) Involvement of the membrane lipid bilayer in sorting prohormone convertase 2 into the regulated secretory pathway, *Biochem. J. 349 Pt 3*, 843-852.

62. Wang, Y., Thiele, C., and Huttner, W. B. (2000) Cholesterol is required for the formation of regulated and constitutive secretory vesicles from the trans-Golgi network, *Traffic. 1*, 952-962.

63. Gondre-Lewis, M. C., Petrache, H. I., Wassif, C. A., Harries, D., Parsegian, A., Porter, F. D., and Loh, Y. P. (2006) Abnormal sterols in cholesterol-deficiency diseases cause secretory granule malformation and decreased membrane curvature, *J. Cell Sci. 119*, 1876-1885.

64. Cool, D. R. and Loh, Y. P. (1998) Carboxypeptidase E is a sorting receptor for prohormones: binding and kinetic studies, *Mol. Cell Endocrinol. 139*, 7-13.

65. Hosaka, M., Watanabe, T., Sakai, Y., Kato, T., and Takeuchi, T. (2005) Interaction between secretogranin III and carboxypeptidase E facilitates prohormone sorting within secretory granules, *J. Cell Sci. 118*, 4785-4795.

66. Lou, H., Kim, S. K., Zaitsev, E., Snell, C. R., Lu, B., and Loh, Y. P. (2005) Sorting and activity-dependent secretion of BDNF require interaction of a specific motif with the sorting receptor carboxypeptidase E, *Neuron 45*, 245-255.

67. Feliciangeli, S., Kitabgi, P., and Bidard, J. N. (2001) The role of dibasic residues in prohormone sorting to the regulated secretory pathway. A study with proneurotensin, *J. Biol. Chem. 276*, 6140-6150.

68. Brechler, V., Chu, W. N., Baxter, J. D., Thibault, G., and Reudelhuber, T. L. (1996) A protease processing site is essential for prorenin sorting to the regulated secretory pathway, *J. Biol. Chem. 271*, 20636-20640.

69. Mulcahy, L. R., Vaslet, C. A., and Nillni, E. A. (2005) Prohormone-convertase 1 processing enhances post-Golgi sorting of prothyrotropin-releasing hormone-derived peptides, *J. Biol. Chem. 280*, 39818-39826.

70. Bundgaard, J. R., Birkedal, H., and Rehfeld, J. F. (2004) Progastrin is directed to the regulated secretory pathway by synergistically acting basic and acidic motifs, *J. Biol. Chem. 279*, 5488-5493.

71. Kuliawat, R., Prabakaran, D., and Arvan, P. (2000) Proinsulin endoproteolysis confers enhanced targeting of processed insulin to the regulated secretory pathway, *Mol. Biol. Cell 11*, 1959-1972.

72. O'Donnell, P. J., Driscoll, W. J., Back, N., Muth, E., and Mueller, G. P. (2003) Peptidylglycine-alpha-amidating monooxygenase and pro-atrial natriuretic peptide constitute the major membrane-associated proteins of rat atrial secretory granules, *J. Mol. Cell Cardiol. 35*, 915-922.

73. Ferraro, F., Ma, X. M., Sobota, J. A., Eipper, B. A., and Mains, R. E. (2007) Kalirin/Trio Rho guanine nucleotide exchange factors regulate a novel step in secretory granule maturation, *Mol. Biol. Cell 18*, 4813-4825.

74. Hutton, J. C., Penn, E. J., and Peshavaria, M. (1983) Low-molecular-weight constituents of isolated insulin-secretory granules. Bivalent cations, adenine nucleotides and inorganic phosphate, *Biochem. J. 210*, 297-305.

75. Quinn, D., Orci, L., Ravazzola, M., and Moore, H. P. (1991) Intracellular transport and sorting of mutant human proinsulins that fail to form hexamers, *J. Cell Biol. 113*, 987-996.

76. Rambourg, A., Clermont, Y., Chretien, M., and Olivier, L. (1992) Formation of secretory granules in the Golgi apparatus of prolactin cells in the rat pituitary gland: a stereoscopic study, *Anat. Rec. 232*, 169-179.

77. Lee, M. S., Zhu, Y. L., Chang, J. E., and Dannies, P. S. (2001) Acquisition of Lubrol insolubility, a common step for growth hormone and prolactin in the secretory pathway of neuroendocrine cells, *J. Biol. Chem. 276*, 715-721.

78. Sankoorikal, B. J., Zhu, Y. L., Hodsdon, M. E., Lolis, E., and Dannies, P. S. (2002) Aggregation of human wild-type and H27A-prolactin in cells and in solution: roles of Zn(2+), Cu(2+), and pH, *Endocrinology 143*, 1302-1309.

79. Gerdes, H. H., Rosa, P., Phillips, E., Baeuerle, P. A., Frank, R., Argos, P., and Huttner, W. B. (1989) The primary structure of human secretogranin II, a widespread tyrosine-sulfated secretory granule protein that exhibits low pH- and calcium-induced aggregation, *J. Biol. Chem. 264*, 12009-12015.

80. Chanat, E. and Huttner, W. B. (1991) Milieu-induced, selective aggregation of regulated secretory proteins in the trans-Golgi network, *J. Cell Biol. 115*, 1505-1519.

81. Jain, R. K., Chang, W. T., Geetha, C., Joyce, P. B., and Gorr, S. U. (2002) In vitro aggregation of the regulated secretory protein chromogranin A, *Biochem. J. 368*, 605-610.

82. Taupenot, L., Harper, K. L., and O'Connor, D. T. (2003) The chromogranin-secretogranin family, *N. Engl. J. Med. 348*, 1134-1149.

83. Taupenot, L., Harper, K. L., and O'Connor, D. T. (2005) Role of H+-ATPase-mediated acidification in sorting and release of the regulated secretory protein chromogranin A: evidence for a vesiculogenic function, *J. Biol. Chem. 280*, 3885-3897.

84. Yoo, S. H. and Albanesi, J. P. (1991) High capacity, low affinity Ca2+ binding of chromogranin A. Relationship between the pH-induced conformational change and Ca2+ binding property, *J. Biol. Chem. 266*, 7740-7745.

85. Yoo, S. H. and Albanesi, J. P. (1990) Ca2(+)-induced conformational change and aggregation of chromogranin A, *J. Biol. Chem. 265*, 14414-14421.

86. Canaff, L., Brechler, V., Reudelhuber, T. L., and Thibault, G. (1996) Secretory granule targeting of atrial natriuretic peptide correlates with its calcium-mediated aggregation, *Proc. Natl. Acad. Sci. U. S. A 93*, 9483-9487.

87. Thorlacius-Ussing, O. (1987) Zinc in the anterior pituitary of rat: a histochemical and analytical work, *Neuroendocrinology 45*, 233-242.

88. Cunningham, B. C., Mulkerrin, M. G., and Wells, J. A. (1991) Dimerization of human growth hormone by zinc, *Science 253*, 545-548.

89. Glembotski, C. C., Eipper, B. A., and Mains, R. E. (1984) Characterization of a peptide alpha-amidation activity from rat anterior pituitary, *J. Biol. Chem. 259*, 6385-6392.

90. Jain, R. K., Chang, W. T., Geetha, C., Joyce, P. B., and Gorr, S. U. (2002) In vitro aggregation of the regulated secretory protein chromogranin A, *Biochem. J. 368*, 605-610.

91. Jain, R. K., Joyce, P. B., Molinete, M., Halban, P. A., and Gorr, S. U. (2001) Oligomerization of green fluorescent protein in the secretory pathway of endocrine cells, *Biochem. J. 360*, 645-649.

92. Jain, R. K., Joyce, P. B., and Gorr, S. U. (2000) Aggregation chaperones enhance aggregation and storage of secretory proteins in endocrine cells, *J. Biol. Chem. 275*, 27032-27036.

93. Muller, L., Zhu, P., Juliano, M. A., Juliano, L., and Lindberg, I. (1999) A 36-residue peptide contains all of the information required for 7B2-mediated activation of prohormone convertase 2, *J. Biol. Chem. 274*, 21471-21477.

94. Mosley, C. A., Taupenot, L., Biswas, N., Taulane, J. P., Olson, N. H., Vaingankar, S. M., Wen, G., Schork, N. J., Ziegler, M. G., Mahata, S. K., and O'Connor, D. T. (2007) Biogenesis of the secretory granule: chromogranin A coiled-coil structure results in unusual physical properties and suggests a mechanism for granule core condensation, *Biochemistry 46*, 10999-11012.

95. Kim, T., Tao-Cheng, J. H., Eiden, L. E., and Loh, Y. P. (2001) Chromogranin A, an "on/off" switch controlling dense-core secretory granule biogenesis, *Cell 106*, 499-509.

96. Courel, M., Rodemer, C., Nguyen, S. T., Pance, A., Jackson, A. P., O'Connor, D. T., and Taupenot, L. (2006) Secretory granule biogenesis in sympathoadrenal cells: identification of a granulogenic determinant in the secretory prohormone chromogranin A, *J. Biol. Chem. 281*, 38038-38051.

97. Pance, A., Livesey, F. J., and Jackson, A. P. (2006) A role for the transcriptional repressor REST in maintaining the phenotype of neurosecretory-deficient PC12 cells, *J. Neurochem. 99*, 1435-1444.

98. Kim, T. and Loh, Y. P. (2006) Protease nexin-1 promotes secretory granule biogenesis by preventing granule protein degradation, *Mol. Biol. Cell 17*, 789-798.

99. Hosaka, M., Suda, M., Sakai, Y., Izumi, T., Watanabe, T., and Takeuchi, T. (2004) Secretogranin III binds to cholesterol in the secretory granule membrane as an adapter for chromogranin A, *J. Biol. Chem. 279*, 3627-3634.

100. Malosio, M. L., Giordano, T., Laslop, A., and Meldolesi, J. (2004) Dense-core granules: a specific hallmark of the neuronal/neurosecretory cell phenotype, *J. Cell Sci. 117*, 743-749.

101. Beuret, N., Stettler, H., Renold, A., Rutishauser, J., and Spiess, M. (2004) Expression of regulated secretory proteins is sufficient to generate granule-like structures in constitutively secreting cells, *J. Biol. Chem. 279*, 20242-20249.

102. Knoch, K. P., Bergert, H., Borgonovo, B., Saeger, H. D., Altkruger, A., Verkade, P., and Solimena, M. (2004) Polypyrimidine tract-binding protein promotes insulin secretory granule biogenesis, *Nat. Cell Biol. 6*, 207-214.

103. Mahapatra, N. R., O'Connor, D. T., Vaingankar, S. M., Hikim, A. P., Mahata, M., Ray, S., Staite, E., Wu, H., Gu, Y., Dalton, N., Kennedy, B. P., Ziegler, M. G., Ross, J., and Mahata, S. K. (2005) Hypertension from targeted ablation of chromogranin A can be rescued by the human ortholog, *J. Clin. Invest 115*, 1942-1952.

104. John, S. W., Krege, J. H., Oliver, P. M., Hagaman, J. R., Hodgin, J. B., Pang, S. C., Flynn, T. G., and Smithies, O. (1995) Genetic decreases in atrial natriuretic peptide and salt-sensitive hypertension, *Science 267*, 679-681.

105. Clark, A. F., Sharp, M. G., Morley, S. D., Fleming, S., Peters, J., and Mullins, J. J. (1997) Renin-1 is essential for normal renal juxtaglomerular cell granulation and macula densa morphology, *J. Biol. Chem. 272*, 18185-18190.

106. Rhodes, C. J. and Halban, P. A. (1987) Newly synthesized proinsulin/insulin and stored insulin are released from pancreatic B cells predominantly via a regulated, rather than a constitutive, pathway, *J. Cell Biol. 105*, 145-153.

107. Pratt, R. E., Carleton, J. E., Richie, J. P., Heusser, C., and Dzau, V. J. (1987) Human renin biosynthesis and secretion in normal and ischemic kidneys, *Proc. Natl. Acad. Sci. U. S. A 84*, 7837-7840.

108. Steiner, D. F., Rouille, Y., Gong, Q., Martin, S., Carroll, R., and Chan, S. J. (1996) The role of prohormone convertases in insulin biosynthesis: evidence for inherited defects in their action in man and experimental animals, *Diabetes Metab 22*, 94-104.

109. Halban, P. A. and Irminger, J. C. (2003) Mutant proinsulin that cannot be converted is secreted efficiently from primary rat beta-cells via the regulated pathway, *Mol. Biol. Cell 14*, 1195-1203.

110. Glombik, M. M., Kromer, A., Salm, T., Huttner, W. B., and Gerdes, H. H. (1999) The disulfide-bonded loop of chromogranin B mediates membrane binding and directs sorting from the trans-Golgi network to secretory granules, *EMBO J. 18*, 1059-1070.

111. Lacombe, M. J., Mercure, C., Dikeakos, J. D., and Reudelhuber, T. L. (2005) Modulation of secretory granule-targeting efficiency by cis and trans compounding of sorting signals, *J. Biol. Chem. 280*, 4803-4807.

112. Steiner, D. F. (1967) Evidence for a precursor in the biosynthesis of insulin, *Trans. N. Y. Acad. Sci. 30*, 60-68.

113. Chretien, M. and Li, C. H. (1967) Isolation, purification, and characterization of gamma-lipotropic hormone from sheep pituitary glands, *Can. J. Biochem. 45*, 1163-1174.

114. Roebroek, A. J., Schalken, J. A., Bussemakers, M. J., van, H. H., Onnekink, C., Debruyne, F. M., Bloemers, H. P., and Van, d., V (1986) Characterization of human c-fes/fps reveals a new transcription unit (fur) in the immediately upstream region of the proto-oncogene, *Mol. Biol. Rep. 11*, 117-125.

115. Fuller, R. S., Brake, A. J., and Thorner, J. (1989) Intracellular targeting and structural conservation of a prohormone-processing endoprotease, *Science 246*, 482-486.

116. Thomas, G., Thorne, B. A., Thomas, L., Allen, R. G., Hruby, D. E., Fuller, R., and Thorner, J. (1988) Yeast KEX2 endopeptidase correctly cleaves a neuroendocrine prohormone in mammalian cells, *Science 241*, 226-230.

117. Seidah, N. G. and Chretien, M. (1999) Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides, *Brain Res. 848*, 45-62.

118. Day, R., Schafer, M. K., Cullinan, W. E., Watson, S. J., Chretien, M., and Seidah, N. G. (1993) Region specific expression of furin mRNA in the rat brain, *Neurosci. Lett. 149*, 27-30.

119. Seidah, N. G., Gaspar, L., Mion, P., Marcinkiewicz, M., Mbikay, M., and Chretien, M. (1990) cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases, *DNA Cell Biol. 9*, 789.

120. Beaubien, G., Schafer, M. K., Weihe, E., Dong, W., Chretien, M., Seidah, N. G., and Day, R. (1995) The distinct gene expression of the pro-hormone convertases in the rat heart suggests potential substrates, *Cell Tissue Res. 279*, 539-549.

121. Marcinkiewicz, M., Ramla, D., Seidah, N. G., and Chretien, M. (1994) Developmental expression of the prohormone convertases PC1 and PC2 in mouse pancreatic islets, *Endocrinology 135*, 1651-1660.

122. Hendy, G. N., Bennett, H. P., Gibbs, B. F., Lazure, C., Day, R., and Seidah, N. G. (1995) Proparathyroid hormone is preferentially cleaved to parathyroid hormone by the prohormone convertase furin. A mass spectrometric study, *J. Biol. Chem. 270*, 9517-9525.

123. Zheng, M., Streck, R. D., Scott, R. E., Seidah, N. G., and Pintar, J. E. (1994) The developmental expression in rat of proteases furin, PC1, PC2, and carboxypeptidase E: implications for early maturation of proteolytic processing capacity, *J. Neurosci. 14*, 4656-4673.

124. Zhu, X., Zhou, A., Dey, A., Norrbom, C., Carroll, R., Zhang, C., Laurent, V., Lindberg, I., Ugleholdt, R., Holst, J. J., and Steiner, D. F. (2002) Disruption of PC1/3 expression in mice causes dwarfism and multiple neuroendocrine peptide processing defects, *Proc. Natl. Acad. Sci. U. S. A 99*, 10293-10298.

125. Furuta, M., Zhou, A., Webb, G., Carroll, R., Ravazzola, M., Orci, L., and Steiner, D. F. (2001) Severe defect in proglucagon processing in islet A-cells of prohormone convertase 2 null mice, *J. Biol. Chem. 276*, 27197-27202.

126. Pan, H., Nanno, D., Che, F. Y., Zhu, X., Salton, S. R., Steiner, D. F., Fricker, L. D., and Devi, L. A. (2005) Neuropeptide processing profile in mice lacking prohormone convertase-1, *Biochemistry 44*, 4939-4948.

127. Pan, H., Che, F. Y., Peng, B., Steiner, D. F., Pintar, J. E., and Fricker, L. D. (2006) The role of prohormone convertase-2 in hypothalamic neuropeptide processing: a quantitative neuropeptidomic study, *J. Neurochem. 98*, 1763-1777.

128. Jackson, R. S., Creemers, J. W., Ohagi, S., Raffin-Sanson, M. L., Sanders, L., Montague, C. T., Hutton, J. C., and O'Rahilly, S. (1997) Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene, *Nat. Genet. 16*, 303-306.

129. Muller, L., Cameron, A., Fortenberry, Y., Apletalina, E. V., and Lindberg, I. (2000) Processing and sorting of the prohormone convertase 2 propeptide, *J. Biol. Chem. 275*, 39213-39222.

130. Rabah, N., Gauthier, D., Wilkes, B. C., Gauthier, D. J., and Lazure, C. (2006) Single amino acid substitution in the PC1/3 propeptide can induce significant modifications of its inhibitory profile toward its cognate enzyme, *J. Biol. Chem. 281*, 7556-7567.

131. Jutras, I., Seidah, N. G., Reudelhuber, T. L., and Brechler, V. (1997) Two activation states of the prohormone convertase PC1 in the secretory pathway, *J. Biol. Chem. 272*, 15184-15188.

132. Rabah, N., Gauthier, D., Dikeakos, J. D., Reudelhuber, T. L., and Lazure, C. (2007) The C-terminal region of the proprotein convertase 1/3 (PC1/3) exerts a bimodal regulation of the enzyme activity in vitro, *FEBS J. 274*, 3482-3491.

133. Basak, A., Koch, P., Dupelle, M., Fricker, L. D., Devi, L. A., Chretien, M., and Seidah, N. G. (2001) Inhibitory specificity and potency of proSAAS-derived peptides toward proprotein convertase 1, *J. Biol. Chem. 276*, 32720-32728.

134. Wei, S., Feng, Y., Che, F. Y., Pan, H., Mzhavia, N., Devi, L. A., McKinzie, A. A., Levin, N., Richards, W. G., and Fricker, L. D. (2004) Obesity and diabetes in transgenic mice expressing proSAAS, *J. Endocrinol. 180*, 357-368.

135. Lou, H., Smith, A. M., Coates, L. C., Cawley, N. X., Loh, Y. P., and Birch, N. P. (2007) The transmembrane domain of the prohormone convertase PC3: a key motif for targeting to the regulated secretory pathway, *Mol. Cell Endocrinol. 267*, 17-25.

136. Stettler, H., Suri, G., and Spiess, M. (2005) Proprotein convertase PC3 is not a transmembrane protein, *Biochemistry 44*, 5339-5345.

137. Chen, X., Walker, A. K., Strahler, J. R., Simon, E. S., Tomanicek-Volk, S. L., Nelson, B. B., Hurley, M. C., Ernst, S. A., Williams, J. A., and Andrews, P. C. (2006) Organellar proteomics: analysis of pancreatic zymogen granule membranes, *Mol. Cell Proteomics. 5*, 306-312.

138. Dong, W., Marcinkiewicz, M., Vieau, D., Chretien, M., Seidah, N. G., and Day, R. (1995) Distinct mRNA expression of the highly homologous convertases PC5 and PACE4 in the rat brain and pituitary, *J. Neurosci. 15*, 1778-1796.

139. Seidah, N. G., Day, R., and Chretien, M. (1993) The family of pro-hormone and pro-protein convertases, *Biochem. Soc. Trans. 21 (Pt 3)*, 685-691.

140. De, B., I, Marcinkiewicz, M., Malide, D., Lazure, C., Nakayama, K., Bendayan, M., and Seidah, N. G. (1996) The isoforms of proprotein convertase PC5 are sorted to different subcellular compartments, *J. Cell Biol. 135*, 1261-1275.

141. Nour, N., Mayer, G., Mort, J. S., Salvas, A., Mbikay, M., Morrison, C. J., Overall, C. M., and Seidah, N. G. (2005) The cysteine-rich domain of the secreted proprotein convertases PC5A and PACE4 functions as a cell surface anchor and interacts with tissue inhibitors of metalloproteinases, *Mol. Biol. Cell 16*, 5215-5226.

142. Nour, N., Basak, A., Chretien, M., and Seidah, N. G. (2003) Structure-function analysis of the prosegment of the proprotein convertase PC5A, *J. Biol. Chem. 278*, 2886-2895.

143. Reynolds, N. A., Blum, A., Kitagawa, K., and Beinfeld, M. C. (2006) Inhibition of PC5 expression decreases CCK secretion and increases PC2 expression, *Peptides 27*, 901-904.

144. Essalmani, R., Hamelin, J., Marcinkiewicz, J., Chamberland, A., Mbikay, M., Chretien, M., Seidah, N. G., and Prat, A. (2006) Deletion of the gene encoding proprotein convertase 5/6 causes early embryonic lethality in the mouse, *Mol. Cell Biol. 26*, 354-361.

145. Brazil, B. T., Ybarra, J., and Horowitz, P. M. (1998) Divalent cations can induce the exposure of GroEL hydrophobic surfaces and strengthen GroEL hydrophobic binding interactions. Novel effects of Zn2+ GroEL interactions, *J. Biol. Chem. 273*, 3257-3263.

146. Yoo, S. H. and Ferretti, J. A. (1993) Nature of the pH-induced conformational changes and exposure of the C-terminal region of chromogranin A, *Febs Letters 334*, 373-377.

147. Gaboriaud, C., Bissery, V., Benchetrit, T., and Mornon, J. P. (1987) Hydrophobic cluster analysis: An efficient new way to compare and analyse amino acid sequences, *Febs Letters 224*, 149-155.

148. Rice, P., Longden, I., and Bleasby, A. (2000) EMBOSS: The European Molecular Biology Open Software Suite, *Trends in Genetics 16*, 276-277.

149. Lee, S., Furuya, T., Kiyota, T., Takami, N., Murata, K., Niidome, Y., Bredesen, D. E., Ellerby, H. M., and Sugihara, G. (2001) De Novo-designed Peptide Transforms Golgi-specific Lipids into Golgi-like Nanotubules, *J. Biol. Chem. 276*, 41224-41228.

150. Takamori, S., Holt, M., Stenius, K., Lemke, E. A., Gronborg, M., Riedel, D., Urlaub, H., Schenck, S., Brugger, B., Ringler, P., Muller, S. A., Rammner, B., Grater, F., Hub, J. S., De Groot, B. L., Mieskes, G., Moriyama, Y., Klingauf, J., Grubmuller, H., Heuser, J., Wieland, F., and Jahn, R. (2006) Molecular anatomy of a trafficking organelle, *Cell 127*, 831-846.

Appendix

Figure License

This is a License Agreement between Dimitrios Dikeakos ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

License Number 1896670093344

License date Feb 26, 2008

Licensed content publisher Nature Publishing Group

Licensed content publication Nature Reviews Molecular Cell Biology

Licensed content title Membrane lipids: where they are and how they behave

Licensed content author Gerrit van Meer, Dennis R. Voelker and Gerald W. Feigenson

Volume number: 9

Issue number: 2

Year of publication: 2008

Portion used: Figures / tables

Requestor type: Student

Type of Use: Thesis / Dissertation

High-res requested: No

Total 0.00 USD

Terms and Conditions for Permissions
Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

5. The credit line should read: Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication) For AOP papers, the credit line should read:
Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows: Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows: Translated by permission from Macmillan Publishers Ltd: [JOU reference

citation), copyright (year of publication).

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.