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REVIEW PAPER

Chromogranin A as a Crucial Factor in the Sorting of Peptide Hormones to Secretory Granules

Salah Elias · Charlène Delestre · Maite Courel · Youssef Anouar · Maite Montero-Hadjadje

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Abstract Chromogranin A (CgA) is a soluble glycoprotein stored along with hormones and neuropeptides in secretory granules of endocrine cells. In the last four decades, intense efforts have been concentrated to characterize the structure and the biological function of CgA. Besides, CgA has been widely used as a diagnostic marker for tumors of endocrine origin, essential hypertension, various inflammatory diseases, and neurodegenerative disorders such as amyotrophic lateral sclerosis and Alzheimer's disease. CgA displays peculiar structural features, including numerous multibasic cleavage sites for prohormone convertases as well as a high proportion of acidic residues. Thus, it has been proposed that CgA represents a precursor of biologically active peptides, and a "granulogenic protein" that plays an important role as a chaperone for catecholamine storage in adrenal chromaffin cells. The widespread distribution of CgA throughout the neuroendocrine system prompted several groups to investigate the role of CgA in peptide hormone sorting to the regulated secretory pathway. This review summarizes the findings and theoretical concepts around the molecular machinery used by CgA to exert this putative intracellular function. Since CgA terminal regions exhibited strong sequence conservation through evolution, our work focused on the implication of these domains as potential functional determinants of CgA. Characterization of the molecular signals implicating CgA in the intracellular traffic of hormones represents a major biological issue that may contribute to unraveling the mechanisms defining the secretory competence of neuroendocrine cells.

Keywords Granins · Hormones · Targeting · Regulated secretion

Introduction

In pluricellular organisms, cell communication is based on the release of an important arsenal of bioactive molecules by secretory granules fusing to the plasma membrane upon stimulation. The intense study of hormone secretion during the last three decades led to the dissection of the molecular basis of biogenesis, trafficking, and exocytosis of secretory granules in endocrine cells. These organelles mainly contain hormones but also glycoproteins of high molecular weight called granins. Secretory granules originate from the trans-Golgi network (TGN) where hormone sorting takes place through interactions with granins leading to the formation of protein aggregates (Chanat and Huttner 1991). Concomitantly, these complexes interact with the TGN membrane and induce the budding of immature secretory granules (ISG), whereas the other proteins are destined to constitutive secretory vesicles. All these vesicular structures are routed along microtubules toward the F-actin-rich cortex where ISG mature and undergo exocytosis in response to an increase of cytosolic calcium concentration (Rudolf et al. 2001). In contrast, constitutive vesicles immediately release their cargo independently of any stimulation (Kelly 1985). In this review, we will focus on

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S. Elias · C. Delestre · M. Courel · Y. Anouar (✉) · M. Montero-Hadjadje (✉)
Laboratory of Neuronal and Neuroendocrine Differentiation and Communication, INSERM U982, University of Rouen, Mont-St-Aignan Cedex, France
e-mail: youssef.anouar@univ-rouen.fr

M. Montero-Hadjadje
e-mail: maite.montero@univ-rouen.fr

the implication of the well-known granulogenic protein chromogranin A (CgA) in the molecular mechanisms driving peptide hormone sorting through the regulated secretory pathway of endocrine cells. We will also discuss CgA involvement in secretion dysregulation associated to neurodegenerative diseases and endocrine tumors.

The Chromogranin A Features and Hormone Aggregation

Around 40 years ago, the analysis of catecholamine secretion from bovine chromaffin cells revealed the release of an acidic and high molecular weight protein called chromogranin A (Blaschko et al. 1967). This protein exhibits a N-terminal disulfide-bonded loop, a high proportion of acidic residues, and numerous dibasic cleavage sites suggesting the capability to produce peptide hormones via processing by prohormone convertases. CgA belongs to the granin family of soluble, thermostable and Ca^{2+}-binding glycoproteins. After its biosynthesis in the rough endoplasmic reticulum, CgA is trafficked and stored in the TGN lumen which exhibits high Ca^{2+} concentration (10–15 mM) and a low pH (6–6.5) (Kim et al. 2006).

The comparison of CgA primary sequences in vertebrates revealed that terminal domains ($CgA_{1–76}$ and $CgA_{344–377}$) are highly conserved, as well as the N-terminal disulfide-bonded loop (CgA_{17}–CgA_{38}), the global acidity and the multiple processing sites (Fig. 1), suggesting their important role in the biological activity of the protein (Montero-Hadjadje et al. 2008). The disulfide bridge has been shown to contribute to the aggregation ability (Thiele and Huttner 1998). The high content in acidic amino acids of CgA confers the ability to bind Ca^{2+} with low affinity and high capacity, and to aggregate with prohormones at low pH (Chanat and Huttner 1991). In these particular conditions, CgA adopts a coiled-coil structure which promotes the initiation of a core for aggregate nucleation (Mosley et al. 2007). Earlier studies have shown that the C-terminal domain of CgA is involved in Ca^{2+}/pH-dependent homodimerization/homotetramerization (Yoo and Lewis 1993), allowing the aggregation-mediated sorting of CgA to secretory granules (Cowley et al. 2000). These aggregates of high molecular weight sort away prohormones from constitutively secreted proteins (Dannies 2001). Homophilic or heterophilic aggregation is related to the tertiary conformation of the prohormones which direct specific interactions to give rise to various secretory granule populations in a same cell type (Kim et al. 2006).

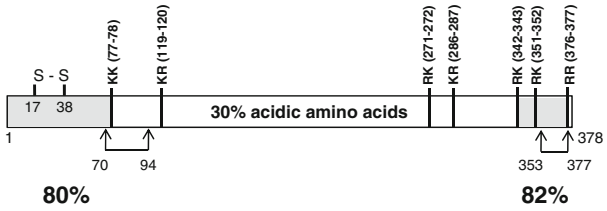

Fig. 1 Schematic representation of the conserved features of CgA. An alignment of mature CgAs from different vertebrate species was performed using the Clustal Multiple Alignment program, and the percentages of homology were calculated. The *gray-colored regions* represent the peptides exhibiting the higher homology percentage (indicated *below*), which can be potentially generated by proteolytic cleavage at conserved putative dibasic processing sites. CgA sequences used are: human (GenBank accession number NP001266), macaca (accession number XP001092629), bovine (accession number NP851348), porcine (accession number P04404), mouse (accession number NP031719), rat (accession number NP067687), chicken (accession number XP421330), frog (accession number AAD38522) and zebrafish (accession number NP001006059). *Arrow-delimited sequences* indicate the conserved predicted α-helix domains between vertebrate CgAs. The secondary structure of mature CgAs was predicted with NNPREDICT and further analyzed with the NPS@ consensus secondary structure prediction program that includes 11 secondary structure prediction algorithms as follows: DPM, DSC, GOR1, GOR3, GOR4, HNNC, PHD, Predator, SIMPA96, SOPM, and SOPMA

Chromogranin A and the Biogenesis of Secretory Granules

A pioneer study suggested that CgA is a crucial factor in the processes leading to the formation of secretory granules since (i) the inhibition of CgA synthesis by antisense RNA induced a dramatic decrease in the number of secretory granules and altered the regulated secretion of hormones from PC12 cells, (ii) CgA expression in AtT20 cells devoid of regulated secretory pathway (6T3 cells) induced a regulated secretion of proopiomelanocortin (POMC), and (iii) CgA expression in fibroblastic CV-1 cells provoked the formation of dense granular structures containing CgA (Kim et al. 2001). More studies performed in non-endocrine cells as well as in PC12 cells devoid of regulated secretory pathway (A35C cells) revealed that CgA expression induced the biogenesis of dense-core granular structures, distinct from lysosomes, containing CgA and co-expressed hormones, and able to release their content upon Ca^{2+} influx (Huh et al. 2003; Beuret et al. 2004; Courel et al. 2006; Montero-Hadjadje et al. 2009; Stettler et al. 2009). In contrast, other studies reported that expression of CgA in non-endocrine cells induced its targeting to the constitutive secretory pathway (Malosio et al. 2004). Nevertheless, we observed that CgA expression in the COS-7 fibroblastic cell line induced the biogenesis of CgA-containing dense-core granules (Montero-Hadjadje et al. 2009) that did not contain GFP-tagged vesicular stomatitis virus glycoprotein, a marker of the constitutive

secretory pathway, and displayed distinct dynamics than those of constitutive carriers (Elias et al., unpublished data). Further investigations performed in CgA null mice and transgenic mice expressing antisense RNA against CgA also revealed a reduction in the number of secretory granules in chromaffin cells that was associated with an impairment of catecholamine storage (Mahapatra et al. 2005; Kim et al. 2005). In CgA knockout mice, the introduction of the gene expressing human CgA restored the regulated secretory phenotype (Mahapatra et al. 2005). In contrast, a different CgA null mice strain exhibited no detectable effect on secretory granule formation that may be explained by a compensatory overexpression of other granins (Hendy et al. 2006). Yet, an elevation of urine catecholamines was measured in these mice. In a recent study performed on chromaffin cells isolated from CgA KO mice, the measurement of catecholamine quanta using the amperometry technique demonstrated an alteration of catecholamine content and secretion, thus confirming that CgA deficiency is associated with hormone storage impairment in secretory granules of neuroendocrine cells (Montesinos et al. 2008).

Functional Determinants of Chromogranin A in Hormone Sorting

In the TGN, aggregates of CgA with prohormones were believed for a long time to enter the budding granule in a passive manner, as CgA was considered to be wholly soluble (Chanat and Huttner 1991; Jain et al. 2002). Alternatively, it has been shown that a fraction of CgA is tightly associated with the membrane of secretory granules via its N-terminal region (Kang and Yoo 1997), suggesting that CgA/hormone aggregates might interact with TGN membrane at the level of lipid-rich microdomains, where peripheral or integral proteins are concentrated and act as sorting receptors to secretory granules. In vitro and in vivo studies revealed that depletion of cholesterol altered ISG biogenesis (Dhanvantari and Loh 2000; Gondre-Lewis et al. 2006). Cholesterol has been shown to interact specifically with integral proteins involved in ISG biogenesis such as vacuolar proton pumps (V-ATPases) (Thiele et al. 2000), carboxypeptidase E (CPE) and secretogranin III (SgIII) (Hosaka et al. 2005), as well as the prohormone convertases PC1/3 and PC2 (Arnaoutova et al. 2003; Assadi et al. 2004). CPE, which binds various soluble protein precursors such as SgIII (Cool and Loh 1998; Hosaka et al. 2005), interacts with lipid rafts of the TGN membrane leading to the budding of ISG (Dhanvantari et al. 2002). In PC12, AtT20, and pancreatic β cells, CgA has been shown to interact with SgIII, which therefore targets CgA multimeric complexes to the regulated secretory pathway (Hosaka et al. 2002, 2004, 2005; Han et al. 2008). Interestingly, these authors demonstrated that although SgIII is essential for the sorting of CgA, the agglomeration rate of CgA could also influence transport of CgA and prohormones to the secretory granules. Besides, the CgA domain of interaction with SgIII ($CgA_{41–109}$) is not fully conserved through evolution (Fig. 1), and SgIII is not expressed in all cell types nor in non-endocrine cells, indicating that hormone sorting is probably a cell-dependent process and various mechanisms of CgA-mediated hormone sorting exist and remain to be elucidated.

CgA exhibits multiple dibasic sites (Fig. 1) and is the substrate for various processing enzymes such as PC1/3 and PC2 (Udupi et al. 1999; Doblinger et al. 2003). The substitution of the cleavage sites for PC1/3 and PC2 by furin motifs led to the leakage of hormone precursors to the constitutive secretory pathway, suggesting that dibasic sites act also as sorting domains (Brechler et al. 1996). Interestingly, PC1/3 gene knockout in AtT20 cells impaired CgA processing and its regulated secretion (Eskeland et al. 1996), while overexpression of PC1/3 in patients with type 2 diabetes is associated with an increase in the processing and the regulated release of CgA and insulin (Lankat-Buttgereit et al. 2008). The implication of CgA dibasic sites in hormone sorting could be explained by the participation of these residues in the proper conformation of the granin, and/or to the generation of CgA-derived peptides involved in hormone secretion. Altogether, these observations suggest that CgA enter the nascent granule by inducing negative curvatures of the TGN membrane directly via its possible association with lipid rafts and/or indirectly via interactions with SgIII and PCs.

Recent studies revealed that PC1/3, PC2, and PC5/6A are targeted to secretory granules through α helices-rich hydrophobic domain(s) identified in their C-terminal parts (Jutras et al. 2000; Dikeakos et al. 2007; 2009) and that CPE also interacts with lipid rafts through a α helix-rich hydrophobic domain (Dhanvantari et al. 2002). The targeting function of such structural feature was also proposed for CgA and secretogranin II (Taupenot et al. 2002; Courel et al. 2006, 2008). Concerning CgA, such an amphipathic α helix was predicted in the N-terminal region of the protein (Taupenot et al. 2002). Interestingly, secondary structure prediction algorithms revealed the existence of a second α helix in the C-terminal part of vertebrate CgA sequences (Fig. 1), raising the possibility that this region may also act as a targeting motif.

The N-terminal disulfide-bonded loop was the primary candidate in the quest for sorting motifs within granins. Surprisingly, mutant CgA lacking this structure was correctly sorted to the regulated secretory pathway in GH4C1 cells (Cowley et al. 2000). Nevertheless, further studies

have revealed that the deletion of the N- or C- terminal domains of CgA altered its sorting in other endocrine cells (Cowley et al. 2000; Hosaka et al. 2002; Taupenot et al. 2002; Courel et al. 2006), suggesting that the process is cell-type dependent. Although the sequence identity between frog CgA (fCgA) and its mammalian orthologs is limited to the terminal peptides (Fig. 1), its expression in COS-7 cells induces the biogenesis of CgA-containing granules which release coexpressed neuropeptide Y (NPY) and growth hormone upon stimulation (Fig. 2) (Montero-Hadjadje et al. 2009). In AtT20 cells, overexpression of CgA increased POMC levels in the secretory granules, and POMC release after high potassium stimulation, suggesting that CgA may function as a helper protein for sorting of POMC to the regulated secretory pathway (Fig. 3) (Montero-Hadjadje et al. 2009). Besides, expression of CgA lacking N- and/or C-terminal conserved regions led to the sorting of truncated CgA and NPY to the constitutive secretory pathway in COS-7 cells (Fig. 2) and the retention of POMC in the perinuclear area in AtT20 cells (Fig. 3), indicating that these domains play a key role in prohormone sorting to secretory granules (Montero-Hadjadje et al. 2009). These CgA-conserved domains could mediate molecular interactions between CgA, lipid rafts, and other secretory helper proteins to ensure such a function.

Clinical Aspects

Since CgA and CgA-derived peptides are secreted, their concentration has the potential to account for the presence of various pathologies such as endocrine tumors, neurodegenerative diseases or metabolic disorders, making CgA and CgA-derived peptides useful biological markers in these diseases. Colombo et al. (2002) have shown that CgA expression in neoplastic cells affects tumor growth and morphogenesis in mouse models, suggesting that abnormal secretion of CgA by neuroendocrine neoplastic cells could affect tumorigenic processes. Serum CgA has been well documented as a marker for sympathoadrenal activity underlying normal cardiovascular regulation and essential hypertension (O'Connor and Bernstein 1984; Takiyyudin et al. 1994). It has been shown that CgA genetic variants may cause profound changes in human autonomic activity, and may associate with the risk of developing hypertension (Rao et al. 2007). Similarly, elevation of the CgA-derived peptide pancreastatin has been observed in type II diabetes,

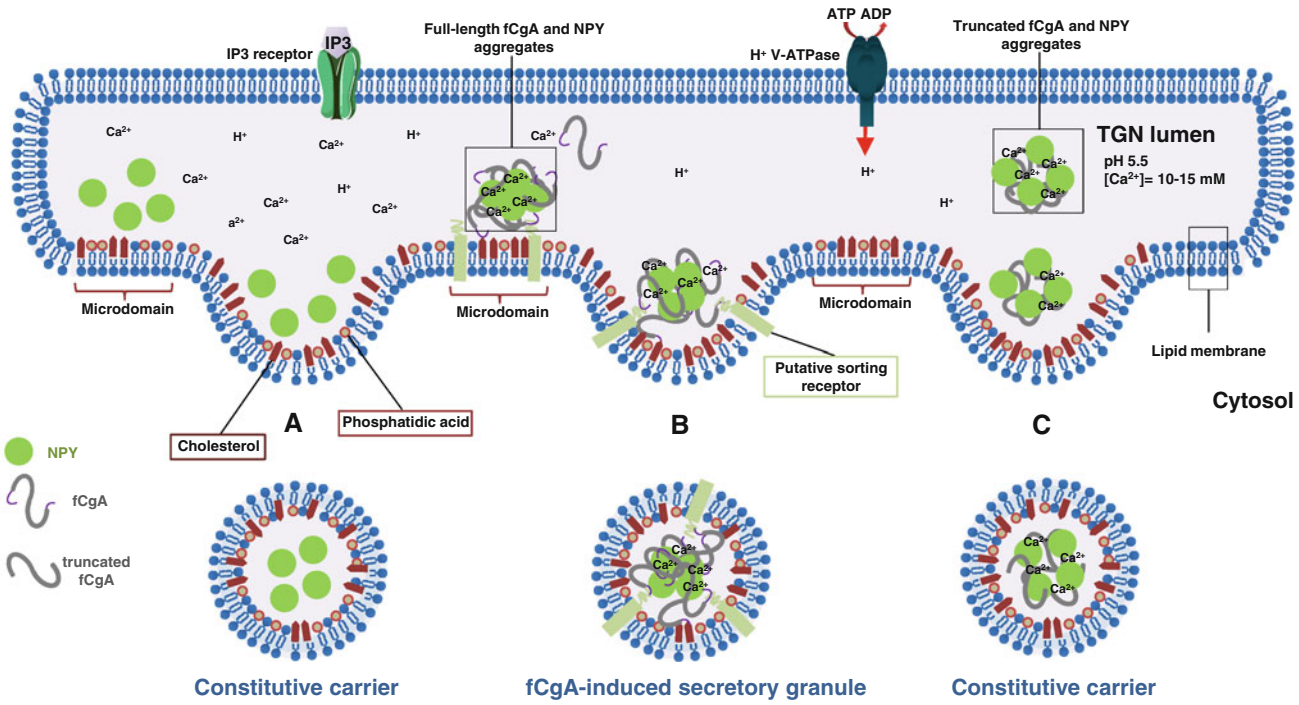

Fig. 2 Sorting model involving CgA in COS-7 cells. *A* NPY is sorted to constitutive carriers in the absence of frog CgA (fCgA). *B* Expression of fCgA induces its aggregation with NPY. Aggregates associate via the conserved terminal regions of fCgA, which potentially interact with the lipid rafts and/or putative membrane-associated sorting receptors to trigger the biogenesis of secretory granules. *C* Deletion of the conserved domains of fCgA alters its granulogenic function, and provokes its missorting along with NPY to the constitutive secretory pathway

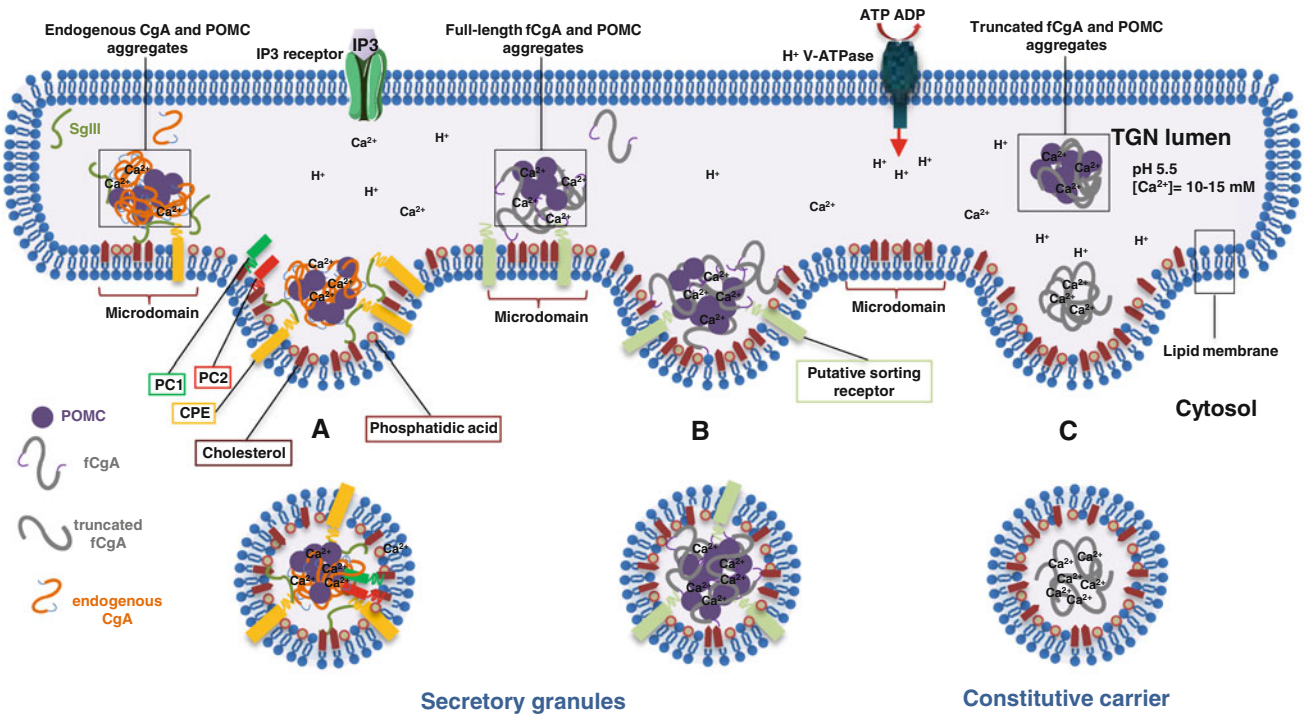

Fig. 3 Sorting model involving CgA in AtT20 cells. *A* In non-transfected cells, aggregates formed with endogenous CgA and POMC interact with SgIII, which in turn tethers to lipid-rich microdomains on the TGN membrane. SgIII interacts specifically with CPE to potentiate the efficiency of prohormone sorting to secretory granules. The lipid-rich microdomains are enriched in phosphatidic acid that triggers, along with cholesterol, membrane negative curvatures for secretory granule biogenesis. Processing enzymes, in particular PC1/3 and PC2 are recruited to the lipid-rich platform to participate in the sorting mechanisms. In secretory granules, prohormones are processed to intermediate peptides. *B* In fCgA-transfected cells, the full-length granin aggregates with POMC to mediate its sorting to secretory granules through the conserved terminal regions of fCgA, which potentially interact with the lipid rafts and/or putative membrane-associated sorting receptors. *C* Deletion of the conserved domains of fCgA obliterates the sorting of POMC and results in missorting of fCgA to the constitutive secretory pathway

and its sequence variation is linked to alteration of insulin secretion (O'Connor et al. 2005). In neurodegenerative diseases such as Alzheimer's disease, CgA expression is enhanced, accumulating in the senile and preamyloid plaques and in dystrophic neurites (Munoz 1991; Yasuhara et al. 1994). This phenomenon is associated with impaired neurotransmission and altered dense-core vesicle function (Lechner et al. 2004). In amyotrophic lateral sclerosis (ALS), CgA has been reported to interact with mutant forms of superoxide dismutase (SOD1) acting as a chaperone protein to promote their secretion that triggers microgliosis and neural death (Urushitani et al. 2006). Recently, a CgB genetic modification has been reported to cause a defective sorting of CgB into secretory granules, associated with an earlier age of ALS onset by almost a decade in both sporadic and familial ALS cases (Gros-Louis et al. 2009). Collectively, these data suggest that CgA and probably other granin family members are involved in the pathogenic mechanisms of metabolic and neurological diseases. It remains to determine to what extent these disorders are related to the role of CgA and granins in general in the regulated secretory pathway.

Concluding Remarks and Perspectives

While aggregation ability of CgA allows the segregation of secretory granule cargo proteins from those that are constitutively secreted in all studied cell models, the anchor of the CgA-bound cargo proteins to the secretory granule as it forms or matures seems to result from a less universal mechanism. The nature and/or the number of sorting domains within the cargo protein would ultimately determine the extent to which each mechanism is active (Dikeakos and Reudelhuber, 2007). The study of CgA-expressing cells, which possess multiple types of secretory granules and thus the ability to selectively secrete different cocktails of biologically active components (Dannies 2002), may be informative on the variety of existing CgA-mediated sorting mechanisms. The identification of the membrane patches into the TGN recognized by the protein cargo, and their communication with the cytoplasmic proteins would help to describe the transport, docking at the membrane, and exocytosis of secretory granules. What is already known is that CgA and its conserved N- and C-terminal peptides are not only important

for promoting the biogenesis of bona fide mobile secretory granules, but also for the targeting and release of hormones through the regulated secretory pathway (Montero-Hadjadje et al. 2009). These observations raise the interesting possibility that CgA through its conserved domains could be a relevant factor in the mobilization of secretory granules, probably by promoting the interactions between secretory granule proteins and the cytoskeleton elements. Because several studies performed on neuroendocrine cells have provided rather indirect clues regarding the involvement of CgA in dense-core granule trafficking, a non-endocrine cell model or an endocrine cell model devoid of regulated secretory pathway expressing CgA could constitute a useful and appropriate tool to further characterize the mechanisms by which CgA, through its terminal regions, establishes a regulated secretory pathway. The characterization of the proteome of CgA-containing granules in such model may help to further dissect the protein machineries involved along with CgA in biogenesis, trafficking, and regulated exocytosis of secretory granules.

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