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Review

Chromogranin A: current status as a precursor for bioactive peptides and a granulogenic/sorting factor in the regulated secretory pathway

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1. Introduction

The chromogranin/secretogranin proteins are acidic polypeptides, of apparent molecular mass 70–120 kDa, found ubiquitously in the soluble matrix of dense-core secretory granules in neurons and endocrine cells [1]. The so-called 'granins' include chromogranins A and B and secretogranin II [2]. Recently, a fourth protein, tentatively called secretogranin III and originally called 1B1075, has been added to this group, based on its net negative charge and pairs of basic amino acids, localization to neurons of the CNS and endocrine cells of the pituitary, and presence in secretory vesicles [3]. Huttner and colleagues have also suggested consideration of both the HISL-19 antigen, and 7B2, acidic proteins found in endocrine cells, as secretogranins IV and V, respectively [4]. 7B2 is found in the pituitary as well as the adrenal, thyroid and brain, and is co-secreted from chromaffin cells of the adrenal medulla along with catecholamines [5,6]. Its aggregatory properties have not been examined, and no physiological functions have yet been proposed for it. Since HISL-19 has not yet been identified as a product secreted from an endocrine cell [7], its designation as a 'secretogranin' may likewise be premature. Nevertheless, considering these proteins as granins, as suggested by Huttner, Gerdes and Rosa [4] may stimulate further research on their physico-chemical properties, uncovering new characteristics common to all granins and leading to a deeper appreciation of their function in neuroendocrine cells. We will concentrate on the most abundant and first-discovered chromogranin, chromogranin A (CGA), with occasional comparisons to chromogranin B (CGB) and secretogranin II (SgII), in order to understand the current status of the granin field, and identify gaps and areas of new investigation. For the purposes of this review, the term 'granins' will refer to these three proteins.

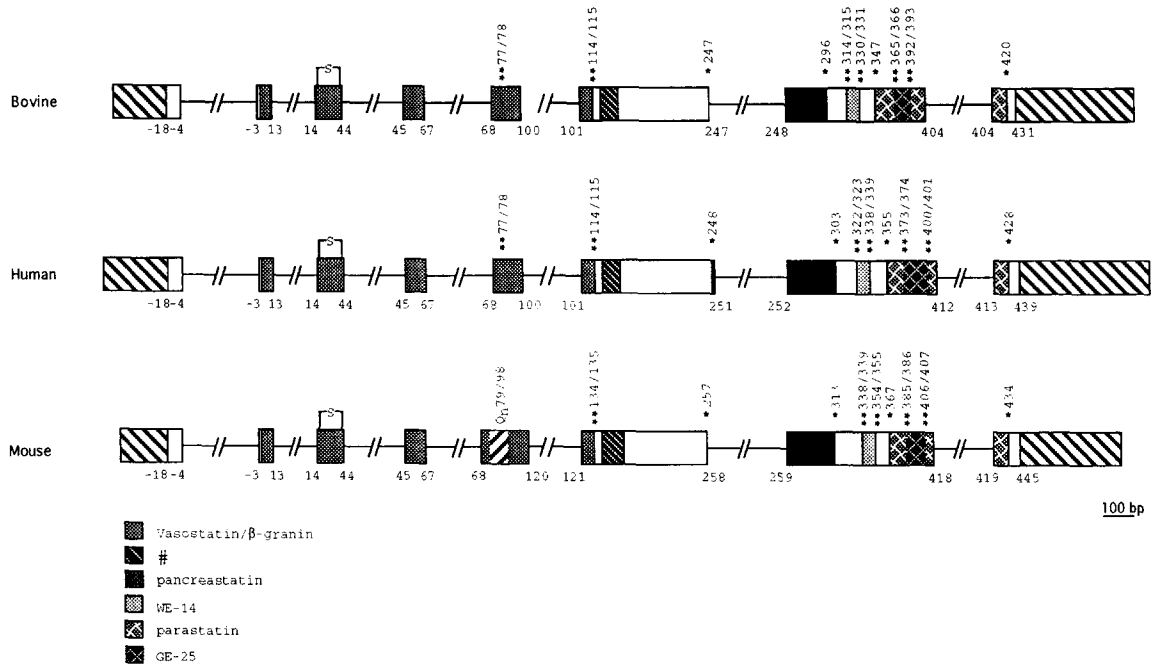

Fig. 1. Exonic organization of the CGA genes of human, cow and mouse. Schematic representation of bovine [25], human [26], and mouse [24] chromogranin A gene organization. Exons are shown as boxes, with numbers above indicating position of putative processing sites, and numbers below indicating the N- and C-terminal amino acid positions of the CGA protein encoded by that exon. Hatched areas represent the 5′- and 3′-untranslated regions. Shaded areas in coding region depict isolated peptides derived from processing of CGA molecule as shown. Asterisks represent single basic amino acid (*) or basic amino acid pair (**). Q_n represents the polyglutamine region of mouse CGA. The shaded area designated as (#) represents the sequence previously designated as 'chromostatin' (see footnote to Fig. 3).

The granins are found, along with various peptides derived from them by proteolytic processing, in secretory granules throughout the diffuse neuroendocrine system [2,8–21]. The granin proteins have been variously considered for a number of intracellular and extracellular roles in neuroendocrine function because of their ubiquity and abundance. The proposed functions of the chromogranins include (i) vesicle aggregation based on calcium binding properties, (ii) chaperone or sorting function based on segregation to secretory vesicles and ability to bind other hormones and (iii) prohormone function, based on the presence within granin proteins of amino acid sequences flanked by pairs of basic amino acids, the sine qua non of the peptide within the prohormone precursor. Recent reviews [22,23] have focused mainly on the prohormone properties of the chromogranins. Our purposes here are to establish general criteria for assigning each of these three endocrine roles to chromogranin A and its peptide fragments, assess current progress in establishing each of these three endocrine roles for chromogranin A and its fragments, and identify likely future avenues for chromogranin research. Significant progress in the cell biology, biochemistry and physiology of the granin family needs to be made to fully understand the biological importance of these highly abundant and ubiquitous neuroendocrine proteins.

2. Structure of the CGA gene and regulation of CGA gene transcription

Chromogranin A is encoded within eight separate exons in the mouse, bovine and human genome (Fig. 1) [24–26]. Exon one encodes the 5′ untranslated region of the CGA mRNA and most of the signal peptide of CGA. Exons two through five encode the sequences for the putative biologically active peptides β-granin and vasostatin. Both cysteines of the highly conserved disulfide loop of CGA are contained within exon three. Exon five contains the poly(Q) domain specific to murine CGA. This sequence appears to represent an insertion into a domain of CGA otherwise fairly well-conserved across mammalian species, rather than an alternative splicing event that includes a polynucleotide sequence contained in a CGA intron of all species. Exon six contains the peptide previously designated as chromostatin (see footnote to Fig. 1), and is the exon containing the most variable peptide sequences across species. Exon seven contains the sequences encoding pancreastatin, WE-14, and GE-25 (see below). The 3′ end of exon seven and the 5′ end of exon eight encode the sequences of the putative paracrine factor parastatin. Exon eight contains the C-terminus of the protein, including the last dibasic amino acid pair, and the 3′ untranslated region of the CGA mRNA. The bovine CGA gene contains an apparent polymorphism in the pancreastatin coding region, resulting in peptide variants containing either an arginine or a histidine residue. Reports of the presence of both peptides in a single bovine tissue suggest this is an allelic variation [27], since but a single mammalian CGA gene appears to exist [25,26,28], localized in the human genome to chromosome 14 [29,30].

The exonic organization of the CGB gene is similar to that of CGA in the regions corresponding to the N- and C-termini of the proteins. In particular, the disulfide loop of CGB is also contained in exon three of the CGB gene and the final exon of CGB contains sequences of the protein highly homologous to CGA [31]. The structures of the CGA and CGB genes suggest that they may be related via a gene duplication event. The structurally dissimilar secretogranin II gene encodes a protein dissimilar to CGA and CGB in overall homology, but with similar physicochemical characteristics [32].

CGA is expressed in a wide variety of neuroendocrine cell types, each of which co-express a unique and cell-specific polypeptide hormone or neurotransmitter ([1], and references therein). To achieve this co-expression the chromogranin gene must be able to respond to the wide variety of developmental and hormonal signals that drive polypeptide expression in each of these cell types (see Table 1). The very high expression of CGA in pituitary, adrenal medulla, and parathyroid for example have prompted the study of the regulation of CGA biosynthesis by estrogen, glucocorticoids, vitamin D and calcium. CGA mRNA and protein abundance is suppressed by estrogen in the pituitary [33–35], and up-regulated by glucocorticoids in the pituitary and adrenal [25,36–38]. CGA biosynthesis is enhanced by 1,25-dihydroxyvitamin D-3 in the parathyroid gland [39,40], although parathyroid hormone biosynthesis is inhibited by di-

hydroxyvitamin D-3 [39]. Under these conditions, CGA is released at a higher rate, but total synthesis is not significantly affected [40]. CGA biosynthesis is likewise not enhanced upon depolarization-induced secretion from chromaffin cells of the adrenal medulla, unlike the genes encoding the co-stored neuropeptides, which are up-regulated by increased secretory activity of the adrenal medulla [41,42]. Thus, while CGA is co-expressed with characteristic hormones in a wide variety of neuroendocrine cells, 'fine-tuning' of its biosynthesis does not appear to parallel that of the characteristic secreted hormone of the tissue and in some cases may even be oppositely regulated.

Table 1
Hormonal, trans-synaptic and second-messenger regulation of granin mRNA levels in neuroendocrine tissues and cell lines

Treatment/maneuver	CGA	CGB	SGII
Corticosteroid	++ [a,b,d-g]	0 [a], − [j]	0 [c]
Estrogen	−− [h,i]	+ [i,j]	− [h], + [i]
Vitamin D-3	+ [k]		
Insulin shock	0 [l]	0 [l]	0 [m]
K^+ depol.	0 [o,v]	0/+ [q,v]	+ [o]
cAMP/forskolin	0/+ [n-p,s]	+ [q,p,r]	− [r],0 [p],+ [o]
Phorbol ester	− [n,t,u],0 [p,s]	0 [q],+ [p]	0 [p],+ [o,u]

Key: drug treatment or endocrine maneuver causes a marked decrease (− −), decrease (−), no change (0), increase (+), or marked increase (+ +) in granin mRNA levels.
[a] Rat adrenal medulla, [37]; [b] rat anterior pituitary, [38,154] [c] rat adrenal medulla, [50]; [d] AtT20, [45]; [e] bovine chromaffin cells [25]; [f] PC12 cells, [155]; [g] parathyroid cells [47]; [h] rat pituitary and pituitary cells in culture (decrease in CGA and SgII mRNA), [33,34]; [i] rat pituitary (decrease in CGA, slight increase in CGB and SgII mRNA), [35]; [j] rat pituitary, increases with estrogen, decrease with dexamethasone in CGB mRNA, [156]; [k] parathyroid [40]; [l] rat adrenal, [37]; [m] rat adrenal, [50]; [n] bovine chromaffin cells (slight increase in CGA mRNA with forskolin treatment after 48 h, decrease with PMA alone and block of dexamethasone increase by PMA), [25]; [o] bovine chromaffin cells (no change in CGA mRNA after potassium or nicotine depolarization; slight but not significant increase in CGA mRNA after 24 h with forskolin; increased SgII mRNA after 24 h with forskolin and potassium) [50]; [p] PC12 cells, no change in CGA or SgII mRNA with forskolin or PMA and increased CGB mRNA after both forskolin and PMA, [55]; [q] bovine chromaffin cells, CGB mRNA: occasional slight increase with K^+, slight increase forskolin, no change after PMA treatment, C.-M. Hsu, L. Eiden, unpublished observations; [r] PC12 cells, increase in CGB mRNA and decrease in SgII mRNA after treatment with forskolin or 8-Br-cAMP, [157,158]; [s] rat insulinoma (RIN cells), CGA mRNA: no change after forskolin, blockade of dexamethasone induction with PMA but no effect of PMA alone, [25]; [t] SK-N-SH human neuroblastoma cells, decreased CGA mRNA after PMA, S.-H. Hahm, L. Eiden and A. Iacangelo, unpublished observations; [u] SY-5Y human neuroblastoma cells, decreased CGA mRNA and increased SgII mRNA after PMA, [52]; [v] bovine chromaffin cells, no significant change in CGB or CGA mRNA after K^+, [41].

The overall pattern of CGA biosynthesis regulation in the diffuse neuroendocrine system suggests that CGA is normally produced in excess in secretory cells and that episodes of increased secretion are accompanied by decreased degradation of CGA, rather than increased CGA gene transcription [39,43], although examples of up-regulation of CGA transcription in response to secretagogue stimulation may well be uncovered in future. The 'constitutive' regulation of CGA biosynthesis, compared to strong coupling of secretion and biosynthesis for other vesicle constituents [37] seems consistent with a role for CGA in granulogenesis (see below).

The bovine CGA gene was initially reported to contain a cyclic AMP response element (CGACGTCA; present in mouse as CGACGTAA) and an estrogen response element half-site [24,25]. Further investigations of the human and mouse gene by the laboratories of Hendy and O'Connor respectively [26,44], have demonstrated that a region of about 100 bases, including 55–70 bases of gene immediately 5′ to the start of transcription and the first 32–45 bases of the 5′ untranslated region of exon 1 (Fig. 2), are sufficient to impart endocrine-cell specific expression of reporter gene constructs of the CGA gene. This region includes the CRE conserved in CGA genes from all species examined to date (Fig. 2 and references therein). More recently, O'Connor and co-workers have localized a glucocorticoid-response element approximately 600 bases upstream of the start of transcription of the rat gene imparting glucocorticoid responsiveness in endocrine cells [45]. A Pit-1 (TATNCAT) site, which on the growth hormone and prolactin genes imparts somatotroph- and lactotroph-specific expression, is also present in the CGA gene, potentially imparting increased expression in pituitary [44,46]. A hierarchy of neuroendocrine sublineage-specific cis-active elements may be 'nested' in the CGA promoter allowing its expression in a wide variety of cells. Calcium and dexamethasone interaction in regulation of CGA gene transcription in parathyroid cells may be an example of such hierarchical regulation [47]. Interac-

tion between the protein kinase C and glucocorticoid signalling pathways in regulating CGA gene transcription in chromaffin and insulinoma cells may be another [25].

Unlike neuropeptide genes which respond to secretagogue stimulation with increased gene transcription, or 'stimulus-secretion-synthesis coupling' [48], the CGA gene, as mentioned above, appears to be constitutively expressed upon secretagogue stimulation or depolarization of endocrine cells. Phorbol esters, at least in some cells, down-regulate CGA gene expression [25]. Although CGA mRNA is not up-regulated by increased secretory activity, or by protein kinase C stimulation, incorporation of radiolabeled amino acids into CGA is increased by depolarization and stimulation with phorbol esters [49]. The explanation for this may lie in decreased degradation of constitutively expressed CGA in the face of increased demand for incorporation of CGA into newly formed secretory vesicles, although this is as yet unproved.

Although the CGA gene contains a consensus CRE, stimulation of the protein kinase A pathway elicits only modest increases in CGA mRNA levels in chromaffin cells and neuroblastoma cell lines [25,50] (Hahm et al., unpublished observations). The CGA CRE may function as a non-protein kinase A-dependent enhancer for cell-specific expression, akin to the regulation of the somatostatin CRE by the Isl-1 protein in endocrine cells in an apparently non-protein kinase A-dependent fashion [51]. The reciprocal regulation of CGA and SgII abundance in human neuroblastoma cell lines by PMA [52] (Hahm et al., unpublished observations) suggests that elements of each gene are cis-active for determining the level of expression of the chromogranins in neuroendocrine sub-lineages. The neurotrophins or hormones that may represent the first messengers activating

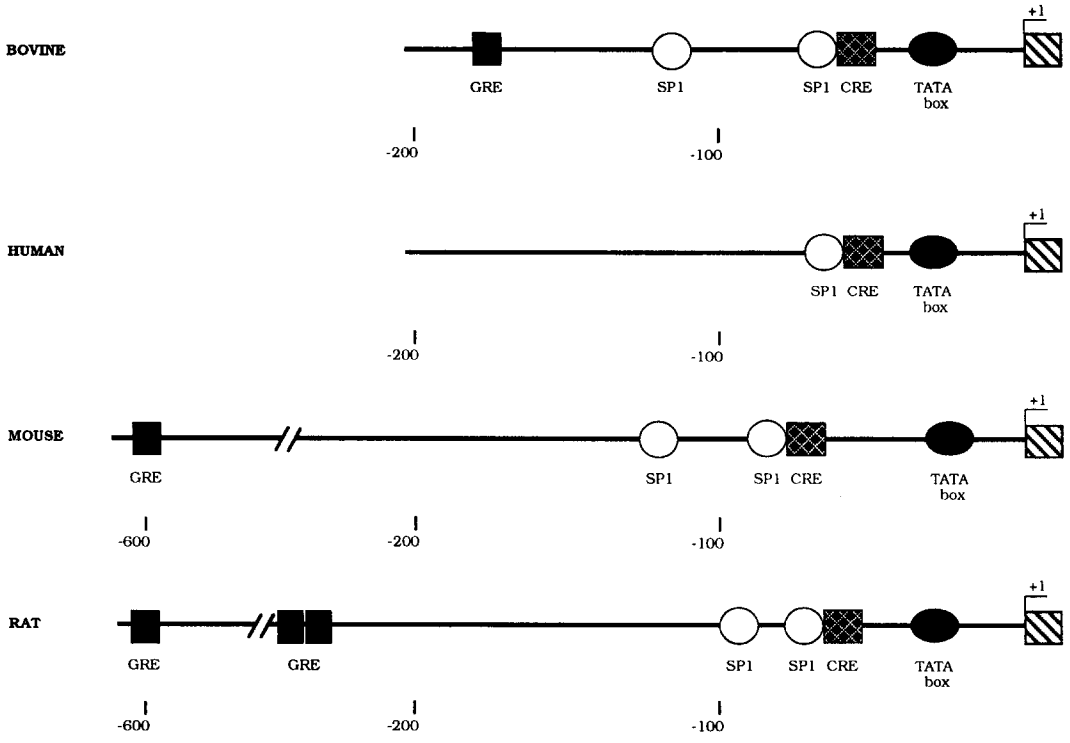

Fig. 2. Potential regulatory elements in the 5′ flanking region of the CGA promoter. Sequences shown are 5′ flanking regions of the bovine [25], human [26], mouse [24] and rat [45] genes.

such lineage programs in neuroendocrine development through the protein kinase C pathway have yet to be identified. CGB has significant homology to CGA and similar expression in adrenal medulla and pituitary. Its regulation by the protein kinase A and C signalling pathways is significantly different from that of CGA (Table 1), although both genes contain consensus CREs in the proximal promoter [31]. The structural basis for CGB gene expression awaits detailed analysis.

The regulation of SgII, also an acidic glycoprotein expressed throughout the diffuse neuroendocrine system, appears to be more akin to that of other neuropeptides than to the other granins. Thus in contrast to CGA and CGB, SgII mRNA is up-regulated by depolarization of bovine chromaffin cells [50]. However, unlike mRNA encoding the neuropeptides enkephalin, NPY, galanin and CGRP [37,53,54] (Anouar and Eiden, in press, 1995), SgII mRNA is not up-regulated by insulin-induced trans-synaptic stimulation of the adrenal medulla in vivo [50], nor is it up-regulated by nicotinic stimulation as are chromaffin cell neuropeptides and their mRNAs [48,50]. Thus, SgII may represent an 'intermediate' between neuropeptide prohormone precursors, which are robustly up-regulated by secretory cell activity at the transcriptional level, and the constitutively expressed chromogranins A and B. In addition, some variation in granin gene regulation by calcium as well as protein kinase A and C signalling pathways could arise from differences in basal levels of expression in the various cells and cell lines studied (Table 1).

Also in contrast to CGA and CGB, SgII is up-regulated by stimulation of the protein kinase C pathway in bovine chromaffin and human neuroblastoma cells [50,52]. Regulation by protein kinase C may be either cell- or species-specific, since Laslop and co-workers [55] reported that PMA stimulation of PC12 cells did not result in the up-regulation of SgII mRNA (Table 1). Like both the CGA and CGB genes, the SgII promoter contains a consensus CRE. An additional AP-1 element in the SgII promoter may be responsible for its regulation by phorbol esters in some systems [32]. It will be of considerable interest to compare the cis-acting elements on the CGA and SgII genes that confer PMA-responsive negative and positive regulation, respectively, on these two granin proteins.

3. Chromogranin A: aggregatory properties and their relevance to endocrine cell function

A property shared by all of the granins including secretogranin II, but studied most extensively for CGA, is the ability to aggregate in the presence of high levels of calcium (reviewed by [56]). This and other properties of CGA are consistent with intracellular functions for it, including (i) formation of the secretory vesicle itself via budding of the immature vesicle from the trans-Golgi network (granulogenesis), (ii) chaperoning of other secreted proteins into the secretory granule (sorting), and (iii) formation of storage complexes with nucleotides, cations, and neurotransmitters within the granule, serving to render the secretory matrix osmotically inert.

Because of its original detection in chromaffin granules, which contain high concentrations of ATP, catecholamines and calcium, the potential role of CGA in maintaining these small molecules in an osmotically inert form in storage vesicles received attention soon after the discovery of CGA and its characterization in these organelles (reviewed in [57]). A variety of techniques was used to demonstrate interactions between CGA or chromogranin protein mixtures and calcium adenosine triphosphate, and catecholamines (e.g., [58–60]). Subsequently, Gratzl and co-workers used highly purified CGA preparations to demonstrate aggregation in the presence of calcium, and defined a moderate-affinity binding site for calcium with a K_d of about 55 μM and a capacity of about 22 mol of calcium per mol of CGA [61]. Both CGB and SgII exhibit moderate-affinity calcium binding in vitro as well [62,63], although the calcium-induced aggregation of SgII appears to occur from much more dilute solution (as little as 35 μg/ml of SgII) than the calcium-induced aggregation of CGA or CGB [62]. In extensive studies employing purified CGA, Yoo and co-workers demonstrated nucleotide binding to CGA [64], and the presence of low affinity, high capacity ($\sim$ 50 mol Ca^{2+}/mol CGA; K_d 4 mM) and pH-dependent binding sites for calcium on CGA conferring aggregation at and above pH 5.5 [65,66]. Similar results were reported by Videen et al., who also demonstrated catecholamine binding to purified CGA, using equilibrium dialysis rather than analytical ultracentrifugation methods [67], and Westermann et al.,

who employed direct binding of catecholamines and calcium to antibody-immobilized CGA [68]. The last two reports mentioned included a demonstration of calcium-inhibited catecholamine binding to CGA, and calcium-dependent catecholamine binding to CGA, respectively. This discrepancy remains to be resolved.

The characteristics of CGA self-aggregation and interaction with calcium and other granule components relevant to a role for CGA in granulogenesis have been studied by several laboratories. Calcium-CGA binding appears not to be dramatically altered upon pH shift from 5.5 (the pH of the granule interior) to neutral pH (7.4, the pH of the extracellular milieu to which the granule matrix would be exposed upon exocytosis of the granule contents) [67]. Yoo and Albanesi reported that maximal calcium binding to CGA at pH 7.5 was about half that of calcium binding at pH 5.5, but the affinity of calcium for CGA at pH 7.5 was 2.7 mM, compared to 4 mM at pH 5.5 [65]. The effects of these relatively modest pH-dependent effects measured in vitro on granule dissolution upon exocytosis at physiological pH and calcium concentration in vivo cannot easily be predicted. However, Cohn and coworkers showed that calcium-dependent aggregation of CGA is accompanied by increased binding of CGA to secretory cell membranes [69], and Yoo has demonstrated that enhanced affinity of CGA for secretory cell membranes at acid pH is reversed at neutral pH [70]. Similar membrane-binding properties of CGB have been observed using similar techniques [71]. Yoo speculates that tetramerization of CGA at pH 5.5 may contribute to both aggregation and membrane association of CGA [66]. Reversal of CGA tetramerization at pH 7.4 may equally contribute to granule dissolution and dissociation from the secretory granule membrane following exocytosis. Consistent with this is the finding of Pimplikar and Huttner that CGB exists (10% or more) as a tightly secretory membrane-bound form as well as a 'soluble' granular form, and that the former may play a role in sorting of the latter [72].

Calcium-induced aggregation of chromogranins may play a role in sorting of other secreted proteins to the regulated pathway. Two models of recruitment to this secretory pathway in neuroendocrine cells have been proposed. One model is that a 'sorting signal' exists on regulated secretory proteins allowing them to bind to the membrane of nascent immature secretory granules and be included in them [73]. Another model is that selective co-aggregation of regulated proteins allows their inclusion in nascent immature secretory vesicles based on membrane envelopment of aggregates of characteristic size, and independent of specific interactions between secreted proteins and secretory granule membranes [74]. CGA may mediate sorting to the regulated pathway by both mechanisms. Selective inclusion of other secretory proteins occurs in CGA as well as CGB aggregates in vitro [63]. C-terminal self-association of CGA as well as CGB leading to enhanced membrane binding, and bridging to calcium-granin aggregates, may trigger interactions with Golgi membranes favorable for budding into immature secretory granules [71,75]. Thus, if granin aggregation and attachment to specific membranes and other secreted proteins is a concerted process leading to the formation of dense-core granules, both 'sorting-signal' and 'aggregation' mechanisms for formation of secretory granules may operate.

The formation of secretory granules in endocrine cells appears to proceed from development of a dense-cored aggregate in the trans-Golgi network to its processing into a membrane-bounded aggregate and budding into immature secretory vesicles [76,77]. All of the properties of CGA described above suggest its potential role in each stage of this process. At the trans-Golgi network (TGN), a Ca-ATPase [78] allows increased calcium influx from the cytosol [79–82] and acidification due to the action of a vacuolar ATPase [83]. Under these conditions, aggregation of CGA would be predicted to occur based on its physical properties as determined in vitro. In fact, when the TGN of secretory cells has been isolated and permeabilized with preservation of these acidic conditions, CGB and SgII have been reported to be trapped in aggregated form in this compartment [84]. This aggregatory process is predicted to be exclusionary of non-secretory granule proteins also found in the RER and TGN. If in addition to aggregation of the dense core, tetramerization of a portion of the CGA in the TGN has already occurred, perhaps at intermediate calcium and pH levels, segregation of a membrane-bound population of CGA could bridge the core and the secretory granule membrane

with its characteristic proteins [70], allowing budding of immature secretory vesicles with the correct components to occur from the TGN. Additional acidification of the granule, and transport of catecholamines and nucleotides would complete maturation with CGA binding to these components contributing to maintenance of a low osmotic pressure within the granule [85]. Upon exocytosis, pH would rise, and the process of disaggregation and matrix dissolution would begin. If intragranular pH begins to rise with the formation of a fusion pore in exocytosis, matrix dissolution could contribute increased osmotic pressure to the fusion process. Complete intragranular communication with the extracellular space would then allow complete dissolution of the granule protein aggregate/matrix into the circulation. Each of these steps remains speculative in the absence of their demonstration in reconstituted cell systems or in vivo. If CGA is a relatively ancient protein [86], other proteins of the secretory granule may have evolved containing domains for interacting with it. In this regard, Yoo and co-workers have identified a pH-and Ca-dependent interaction between an IP3 receptor located on the chromaffin granule membrane and CGA [70]. Identification of additional granin-interaction domains on other granule membrane proteins, as well as secreted proteins of the granule matrix such as the neuropeptide prohormone precursors, would strengthen this hypothesis.

Dissection of the domains of granins imparting both hetero-and homomultimerization properties important in sorting and granulogenesis will require further work with both chimeric proteins and synthetic peptide fragments. Parmer et al. have made a significant beginning to this line of investigation with chimeric CGA proteins in which the N-terminus of CGA is capable of sorting a heterologous polypeptide (chloramphenicol acetyltransferase) into the regulated pathway as a fusion protein with CGA [87]. Yoo has demonstrated that synthetic C-terminal CGA peptides are capable of dimerization and may represent the region responsible for self-aggregation of CGA [75].

Winkler and Fischer-Colbrie have expressed the opinion that since "many neurosecretory granules contain only small amounts of chromogranins...a general hypothesis which concentrates on these minor components apparently neglects the role of the major granule constituent, i.e., the respective hormones, for the granule formation process." [1]. There are two difficulties in the assumption that CGA, CGB, or SgII need be abundant in all regulated secretory granules in all endocrine cells in order to play a role in granulogenesis in any of them. First, concentrations of the granins in mature secretory granules may not reflect their concentrations in the immature granule immediately following granulogenesis. The concentration of unprocessed granin in mature granules appears to be inversely proportional to the amount of co-stored processed peptide(s), which are directly proportional to the concentration of the prohormone converting (PC) enzymes PC1 and PC2 within the granule [88,89]. Thus, the granins could well be present in relatively high levels in the trans-Golgi network, where they contribute to granulogenesis, and in relatively low concentrations in the mature secretory granules following processing [17,88,90,91]. The second is that it is unlikely that CGA, CGB and SgII are the only regulated granule constituents that contribute to granulogenesis. It has been pointed out that 7B2, HISL-19 and 1B1075 are all endocrine-expressed, acidic amino acid-rich, vesicle-associated proteins that could function like the granins [4]. If so, and if various combinations of granins can co-aggregate to form granules, then one would predict granulogenesis whenever the total granin concentration in the trans-Golgi network reached a critical level. This critical concentration would depend in turn on the critical calcium concentration required for aggregation for each granin protein. Differences in critical concentration for self-aggregation, and calcium binding affinity may dictate the differential distribution and potential functions of CGA and CGB, as well as other granins, in the nervous system [92–94] and endocrine cells [20].

The domains of granins as well as hormones capable of metal-dependent aggregation, such as prolactin and insulin, responsible for aggregation and generation of granules of characteristic morphology is an area of granin research that merits further intense investigation. We have recently observed that expression of bovine CGA at high levels in non-secretory granule-containing monkey fibroblastoid cells results in the formation of dense-cored granule profiles by electron microscopical examination, suggesting that CGA may contain structural information

for granulogenesis apart from additional helper factors present in neuroendocrine cells (A.L.I., J.-H. Tao-Cheng, and L.E.E., unpublished observations).

It must be kept firmly in mind that most of the experiments implicating the granins in secretory granule morphogenesis, and secretory protein sorting, have been circumstantial, relying on inferences about the behavior of the granins in intact cells based on their properties in quite specialized in vitro systems. Further detailed reconstitution experiments in otherwise non-secretory granule-containing cells, or genetic lesioning experiments in secretory granule-producing cells or whole organisms will be required to move from the discussion of 'properties of granins consistent with involvement in granulogenesis' to the 'role of granins in granulogenesis'.

4. Processing of CGA and tissue distribution of processed forms of CGA

Chromogranin A was identified as the major soluble protein of the chromaffin cell secretory granule core more than 25 years ago [1]. Its co-release with catecholamines from the adrenal medulla upon electrical stimulation was an important part of the bio-

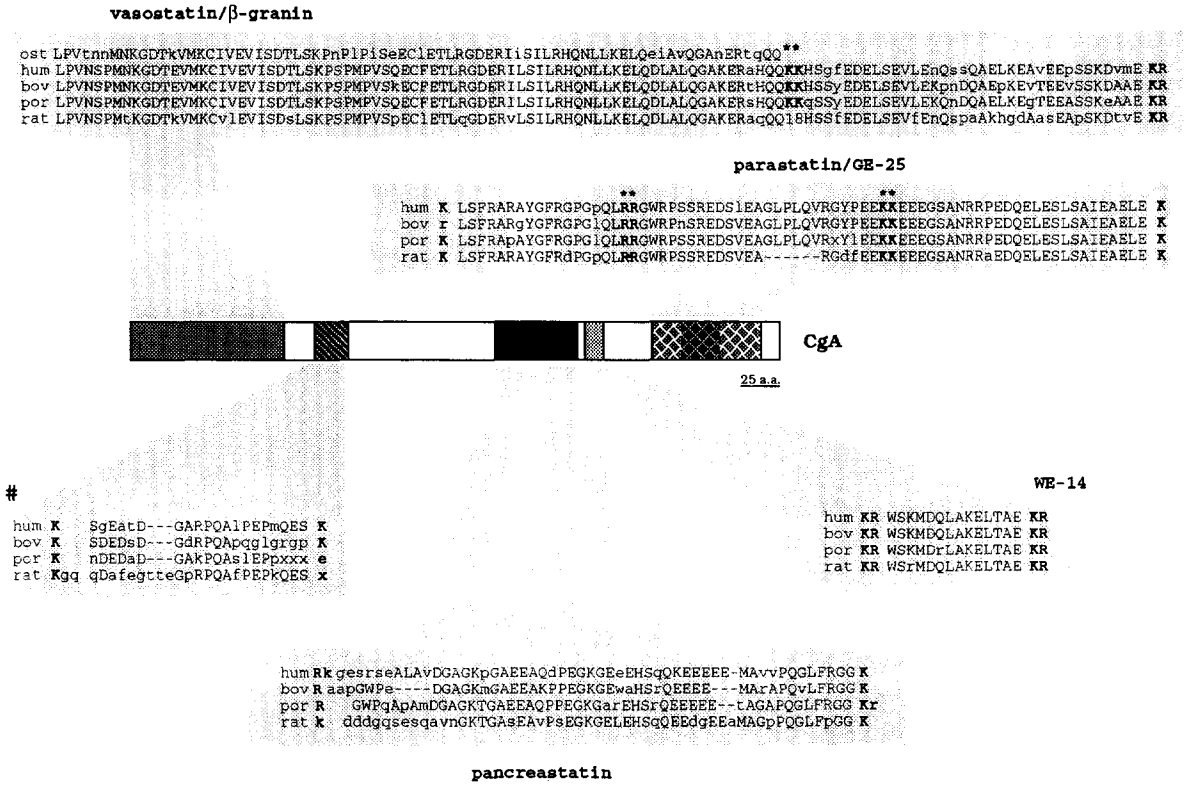

Fig. 3. Species conservation of the structure and flanking processing signals of CGA-derived peptides. Amino acid sequence comparison of the CGA-derived peptides from human, bovine, porcine, and rat are shown above and below the bar indicating the CGA protein. Uppercase letters indicate consensus, with lowercase indicating differences. Letters in bold indicate putative processing signals. Asterisks indicate putative proteolytic signals that generate vasostatin and GE-25. Amino acids sequences are those predicted from cDNA clones for human [150], bovine [97,98], porcine [102] and rat [151]. Abbreviations: ost, ostrich; hum, human; bov, bovine; por, porcine. #Indicates a sequence formerly called chromostatin. A tryptic digest of chromaffin granule soluble extract yielded a biological activity that inhibited secretagogue-stimulated catecholamine release from the adrenal medulla [130]. Upon purification of the material, a fraction containing the sequence shown here for bovine chromostatin was obtained, sequenced, and shown to have activity as a synthetic peptide [152]. However, this material was shown to be contaminated by low molecular-weight, non-peptide material which was responsible for its biological activity [153].

chemical evidence establishing exocytosis as the mechanism of hormone/neurotransmitter release from the adrenal medulla and sympathetic nerves [10,11]. In 1982, Cohn and Winkler and their collaborators established that CGA was immunologically and biochemically similar, if not identical to secretory protein I (SP-I), co-stored and secreted with parathromone from the parathyroid gland [13]. Using specific antibodies against purified CGA O'Connor and co-workers established that chromogranin A immunoreactivity was present in most endocrine organs examined, neuronal tissue, and the brain [14,15,95]. Similar work was carried out with a monoclonal antibody to human CGA in human tissues by Wilson and Lloyd [18]. Several laboratories soon established that CGA was ubiquitous to neuroendocrine tissues in which hormones were stored in secretory granules, and absent from endocrine tissues such as the adrenal cortex in which hormone secretion occurs immediately upon biosynthesis without intervening storage in and exocytosis from, secretory granules [9,16,17]. Chromogranin B and secretogranin II were subsequently shown to have a similar, but somewhat more restricted distribution compared to that of CGA [19,20]. The ubiquity and abundance of high-molecular weight (unprocessed) chromogranin in the diffuse neuroendocrine system, particularly in chromaffin granules in which a high concentration of low-molecular weight components are co-stored, was responsible for the early emphasis on the role of the granins in granulogenesis, and in complexation leading to reduction of osmotic pressure of the granule contents (see above). However, several factors then led to a consideration of the role of chromogranins as potential precursors for biologically active peptides. First, high-resolution two-dimensional gel electrophoresis of CGA and CGB immunoreactivity revealed that multiple forms of chromogranin existed in various endocrine tissues [20]. Secondly, biosynthesis of neuropeptides from high-molecular weight precursors via specific proteolytic cleavage at paired and single basic amino acid residues within the secretory granule was established as a general neuroendocrine principle ([96] and references therein). Third, the cDNA cloning of CGA and the generation of anti-peptide antibodies firmly established that the lower molecular-weight chromogranins, shown to be antigenically related to CGA, were in fact contained within the CGA sequence, and the pattern of processing could be predicted based on the pattern of placement of dibasic amino acid pairs within the CGA molecule [97–99]. Finally, the observation that a portion of bovine CGA was homologous to the biologically active porcine peptide pancreastatin [100,101], and subsequently that CGA was in fact the precursor for pancreastatin [102], established a precedent for granins as prohormones.

Precursors for enkephalin, β-endorphin, cholecystokinin and virtually all other neuropeptides were discovered as a deliberate search for the precursors for identified biologically active peptides. In contrast, the granins had been characterized for some time before it was suspected that they could function as prohormones. The full picture of tissue-specific processing of the granins has yet to emerge, and the rigorous identification of biologically active and endocrinologically relevant CGA-derived peptides will depend on it. Several studies indicate that in tissues in which CGA processing is most complete, four major peptide derivatives of CGA exist (Fig. 3). These are (i) the vasostatin/β-granin peptides, comprising the N-terminal 113 amino acids (β-granin/vasostatin II) of CGA and in some tissues (except in rat) a shorter N-terminal 76-amino acid polypeptide (vasostatin I) [91,103,104], (ii) pancreastatin [21,105], (iii) WE-14 [86,106,107] and (iv) the parastatin/GE-25 peptides [88,108,109]. Each of the peptides above have been demonstrated to exist in endocrine tissues by extraction, isolation, purification and peptide sequencing. The single exception is parastatin, the existence of which is inferred from the presence of a 14 kDa C-terminal CGA fragment generated in rat insulinoma cells, and the biological activity of the parastatin fragment generated by endoproteinase Lys-C digestion of purified CGA [88,108]

The processing of CGA seems to occur preferentially at some but not all pairs of basic amino acids, and at some single basic amino acids, at the C- and N-termini of the molecule [27,103,110,111]. The extent of chromogranin A processing is highly tissue-dependent: processing appears to be least complete in the adrenal medulla and progressively greater in intestine, stomach, peripheral nerves, and pancreas [27,103,110–112]. The endocrine pancreas appears to exhibit the highest level of processing based on

the ratio of intact CGA to fully processed β-granin as well as pancreastatin [21,27,88]. CGA intragranular proteolytic processing in the endocrine pancreas is apparently completely calcium-dependent, and CGA is a much better substrate for PC2 than for PC1/3 kex2-like prohormone converting enzymes [88]. According to Arden and co-workers, the higher concentrations of PC2 in pancreas compared to adrenal medulla may explain why the former tissue contains CGA polypeptides in more processed form than the latter [88]. More complete processing of SgII in bovine splenic nerve secretory granule than in chromaffin granules may also be due to the presence of higher concentrations of prohormone converting activity in the former compared to the latter secretory granules [89].

A critical question that remains unanswered is whether CGA processing actually occurs in qualitatively different patterns, yielding tissue-specific CGA products, or whether CGA processing is more or less complete, but occurring through the same pattern of proteolysis in various endocrine tissues. Based on the distribution of CGA processing to pancreastatin it would appear that the extent rather than the pattern of CGA processing is tissue-specific, but this remains to be determined systematically for additional CGA-derived peptides [21,90]. The expression of CGA-processing enzymes must be critical to the distribution of CGA-derived peptides in various endocrine tissues. Besides PC2 and possibly PC1/3 [113,114], carboxypeptidase H would be required for intracellular generation of β-granin/vasostatin, pancreastatin, parastatin, GE-25 and WE-14 [115,116], and glycine-dependent amidating (PAM) activity required for C-terminal amidation of pancreastatin [117,118]. Thus, the levels of these enzymes in various endocrine tissues could contribute to tissue-specific generation of CGA-derived biologically active peptides. Post-translational modification of CGA could conceivably also affect processing in a tissue-specific manner: both sulfation and glycosylation patterns of CGA may differ between parathyroid and adrenal glands [119,120].

CGA processing in neurons, especially in the central nervous system, is not yet fully characterized. The hippocampus, like the adrenal during development contains a high-molecular weight proteoglycan with CGA as its core [121,122]. Although principal ganglion cells of the parasympathetic nervous system express relatively little CGA mRNA, it is abundantly expressed in cholinergic motoneurons [94,123]. Characterization of the form of CGA expressed in mammalian motoneurons may be of interest in comparison to the acidic proteoglycan vesiculin abundantly expressed in cholinergic vesicles of the *Torpedo* electric organ [124].

Since a full inventory of CGA-derived peptides in various endocrine tissues does not yet exist, identification of CGA mRNA-expressing cells, in particular those in which little or no immunoreactivity for CGA is found, offers the most systematic approach for detecting cells in which CGA is fully processed to low-molecular weight forms which may not be recognized by commonly employed CGA antibodies. It remains to be seen whether the presence of peptides processed at monobasic sites (parastatin; pancreastatin) compared to those processed at dibasic sites (vasostatin-II/β-granin; WE-14) depends primarily on differential expression of processing enzymes specific for dibasic and monobasic processing sites. Although parastatin, vasostatin and WE-14 have been identified in various endocrine tumors including pheochromocytoma, the ratios of monobasic- and dibasic-cleavage-derived products has not been studied.

An interesting 'sidebar' to the question of CGA processing is the possible role of CGA as a competitive inhibitor of the prohormone convertase PC2, and other processing enzymes in situ, thereby affecting processing rates of other co-stored hormones [125]. CGA levels are often up-regulated by hormone treatments (Vitamin D, glucocorticoids) that do not affect, or even down-regulate, tissue specific hormones co-stored with CGA (see Table 1). Thus, increased CGA expression could comprise a second line of regulation modulating the rate of post-translational processing and therefore the availability of the tissue-unique hormones such as enkephalin, NPY, adrenomedullin, and atrial natiuretic peptide in adrenal and heart, and PTH in parathyroid.

Chromostatin is a fragment of chromogranin A (CGA-(124–143) in the cow) which represents a special case of potential extracellular processing of CGA. Simon and co-workers described an inhibitory effect of purified chromogranin A on catecholamine release from the adrenal medulla. They reported that

this effect was seen only after incubation with conditioned medium, and could be mimicked with trypsin treatment. A peptide fragment generated by tryptic digest of CGA appeared to have this activity, and it was dubbed chromostatin. The biological activity of chromostatin helped drive the hypothesis that CGA could be processed into biologically active peptide(s) extracellularly. Recently, preparations of purified chromostatin were found to be contaminated with a non-peptide material that inhibited catecholamine secretion, and removal of this material from chromostatin preparations rendered them biologically inactive (see Fig. 3). Thus, the question of generation of a biologically active peptide fragment after trypsin treatment of chromogranin A remains open. Chromostatin itself has never been demonstrated to exist in serum, an apparent requirement for assignment of potential biological function. It should be pointed out, however, that the full range of chromogranin-derived peptides present in serum, both under normal and pathophysiological or stressed conditions, has not been catalogued. Determination of whether any of these are generated following release, a la the bradykinin and angiotensin systems, rather than by intracellular processing, awaits a complete investigation of the chromogranin-related peptides present in serum. Leduc and co-workers investigated the peptides released from purified bovine CGA by human plasma kallikrein, and were unable to detect fragments corresponding to any known biologically active fragments of CGA, i.e., β-granin/vasostatin, pancreastatin or parastatin [126]. One fragment obtained by digestion of bovine CGA corresponded to a C-terminally truncated and non-amidated congener of pancreastatin, but this fragment was not bioassayed in any cell system in which pancreastatin had been demonstrated to be active. Since no unique biologically active CGA fragments generated by putative extracellular processing pathways have been identified in serum, there is no need at this time to invoke extracellular processing as a necessary step in generating biologically active material from the CGA precursor. Furthermore, all of the CGA-derived peptides shown to have biological activity (with the exception of chromostatin, see legend to Fig. 3) have also been identified in extracts of cells, and therefore can be presumed to be generated by intracellular rather than extracellular processing. One of the goals of future research on CGA processing will be to identify the enzyme(s) in addition to PC2 required to generate vasostatin/β-granin, pancreastatin, and parastatin peptides in secretory granules.

5. Biological activities of chromogranin peptides

A number of biological activities for several CGA-derived peptides have been measured, in a variety of endocrine secretory systems both in vitro and in vivo (Table 2). In addition to biological activities associated with discrete CGA-derived peptides, Fasciotto and co-workers have demonstrated that material secreted by parathyroid cells inhibits secretion of CGA and PTH from parathyroid cells, and that this inhibition is abrogated by introduction of anti-CGA antiserum into the culture medium [127]. Similarly, Wand et al. have reported that material secreted by the rat AtT20 corticotroph cell line blocks POMC peptide secretion, and ascribed this activity to CGA or CGA-derived peptides ([128], but see [129]). So far, all evidence points to a negative modulatory, probably autocrine or paracrine role of the major CGA-derived peptides vasostatin, pancreastatin, and parastatin, in regulating secretory function throughout the neuroendocrine system. However, major issues relating to the biological activity of CGA and its processing products, and the sites at which CGA processing occurs, remain to be resolved before significant further progress in this aspect of CGA function can be expected.

In many cases it is not established whether the activity of a given CGA-derived peptide is shared by unprocessed CGA itself, but in several cases proteolysis of intact CGA appears to be required before biological activity could be demonstrated [127,130]. Simon and co-workers demonstrated that trypsin digestion of intact CGA resulted in a peptide cocktail that inhibited nicotine-stimulated secretion from chromaffin cells, and that conditioned medium from chromaffin cells developed inhibitory activity after several hours [130]. These data imply that unprocessed CGA has no biological activity and that extracellular processing is critical for the activity of CGA-derived. However, a candidate processed peptide for this biological activity, chromostatin, has been withdrawn from consideration for this role, and

Table 2
Biological activities of CGA-derived peptides

Peptide	Species	Tissue of origin	Target tissue	Action
Vasostatin/β-granin				
CGA 1–76	bovine	adrenal medulla	veins^{hum}	inhibits blood vessel segment contraction [a,b]
CGA 1–76	bovine	synthetic	parathyroid^{bov}	inhibits PTH secretion [c]
CGA 1–76	ostrich	pituitary	unknown	unknown [d]
CGA 1–128	rat	pancreatic β-cells	unknown	unknown [e]
CGA 1–113	bovine	parathyroid	parathyroid^{bov}	inhibits PTH and CGA secretion [f,g]
Pancreastatin				
CGA 240–288	porcine	pancreas, synthetic	pancreas^{rodent}	inhibits insulin and glucagon secretion [h]
		synthetic	pancreas$^{g\text{-}pig}$	inhibits CCK-8 stimulated amylase secretion [i]
		synthetic	parietal cell^{rab}	inhibition of acid secretion [j]
		synthetic	parathyroid^{pig}	inhibits PTH and CGA secretion and transcription [k]
CGA 247–296	bovine	pancreas, pituitary	pancreas^{rat}	inhibits CCK-8 stimulated amylase secretion [l]
CGA 248–303	human	synthetic	pancreas^{rat}	inhibits insulin secretion [m]
	human	glucagonoma, carcinoid tumor		unknown [n,o]
CGA 289–314	rat	synthetic	pancreas^{rat}	inhibits insulin secretion [p]
		synthetic	adr medulla^{rat}	decreased plasma epinephrine [q]
Parastatin *				
CGA 347–419	porcine	parathyroid	parathyroid^{pig}	inhibits PTH and CGA secretion [r]
WE-14				
CGA 324–337	human	pheo. and ileal carcinoid	unknown	unknown [s,t]
GE-25				
CGA 375–399	human	frontal cortex	unknown	unknown [u]
CGA 367–391	bovine	frontal cortex	unknown	unknown [u]
Proteolytic products				
CGA trypsin digest	bovine	adrenal medulla	chromaffin cells^{bov}	inhibition of catecholamine secretion [v]

Abbreviations: hum, human; bov, bovine; rab, rabbit; g-pig, guinea pig; pheo, pheochromocytoma.
[a] Vasostatin preparation comprised five peptides – three 20–23 kDa fragments, a 7 kDa fragment (vasostatin I) and a 16 kDa fragment [159]; [b] [160]; [c] 30 and 50% inhibition obtained with 10 nM and 100 nM CGA 1–76 (synthetic) [144]; [d] [161]; [e] [103,162]; [f] [91]; [g] inhibition obtained with 0.5 μg/ml CGA 1–113 [163]; [h] inhibition of insulin secretion observed in vitro with 10 nM pancreastatin [105]; see also [164], inhibition of insulin and glucagon secretion in conscious rat [165], inhibition of insulin secretion in mouse in vivo, [166]; [i] 1–10 nM pancreastatin (synthetic) inhibited release [167]; [j] 0.1 nM to 1 μM and 1–1000 nM pancreastatin 33–49 inhibited carbachol and histamine-stimulated acid secretion, respectively, in a dose-dependent fashion [168,169]; [k] pancreastatin from 0.01–1 nM inhibited secretion of CGA in a dose-dependent manner [170], pancreastatin inhibits PTH and CGA secretion in parathyroid, [132]; pancreastatin causes decrease in parathyroid PTH and CGA mRNA levels [171]; [l] inhibition at 200 pmol/kg/h of pancreastatin [172]; [m] synthetic pancreastatin 1–52 and the C-terminal fragment 24–52 inhibited secretion at 100 nM [165]; [n] [173]; [o] [174]; [p] synthetic C-terminal fragment of rat pancreastatin (26 residues) [165]; [q] [175]; [r] inhibited secretion in a dose-dependent manner (0.1–0.6 μM) [108]; [s] [107]; [t] [106]; [u] GE-25, [109]; [v] a preparation of purified CGA containing the native protein and two degradation products (60 and 43 kDa) inhibits catecholamine secretion at 1 μM [130].

the actual CGA-derived peptide responsible for the activity originally described by Simon et al. remains to be identified. Fasciotto et al. demonstrated that parathyroid cell secretion of CGA levels off after about three hours, and that this apparent auto-inhibition of secretion can be relieved by precipitating out of the culture medium CGA-immunoreactive materials with antibody [127]. The application of conditioned parathyroid cell medium to fresh cells results in prompt rather than delayed inhibition of CGA secretion from the fresh cells. The extracellular processing of CGA released from parathyroid cells was further demonstrated to be inhibited by a serum protease inhibitor (alpha-2-macroglobulin). Demonstration that alpha-2 macroglobulin inhibition of extracellular processing of CGA in fact abrogated autocrine inhibition of parathyroid cell secretion, was not reported by Fasciotto et al. [127]. In the absence of this information, generation of CGA-derived autocrine factors intracellularly, with several hours required for these factors to reach critical concentration in culture medium, remains an equally likely possibility. Thus, the case for extracellular processing of CGA to yield autocrine factors from either chromaffin or parathyroid cells remains to be made. In fact, it is difficult to envisage an autocrine factor with a requirement for extracellular processing over a several hours, since local concentrations of the factor would be diluted by blood during processing outside the cell. Vasculodynamics predict that factors derived from any and all endocrine cells, and activated by extracellular processing in the circulation would act on any and all endocrine cells in contact with the blood compartment. In the final analysis, detailed consideration of the actual species of CGA-derived peptides present in the blood of various mammalian species including man, pig, cow and rat, is an absolute requirement for distinguishing between extracellular and intracellular generation of CGA-derived peptides. While a potential role of extracellular CGA processing in generating bioactive peptides remains a possibility, the overwhelming evidence that CGA is highly processed in secretory granules in many endocrine tissues to yield putative biologically active peptides will be the focus of what follows in this section.

Characterizing the intracellular processing patterns of CGA is a necessary first step in the identification of CGA-derived regulatory peptides. Several criteria must be met, however, before a granin peptide can reasonably be considered a regulatory peptide by modern endocrinological/cell biological standards (Table 3). No CGA-derived peptides identified to date fully meet all of the criteria listed in Table 3. The evidence favoring a regulatory peptide role for each of these three peptides will be reviewed in the context of these criteria below.

5.1. Processing in appropriate tissues

Trypsin- and carboxypeptidase B-like enzyme activities present in secretory granules of endocrine cells can liberate biologically active peptides from many neuropeptide precursors that contain pairs of

Table 3
Criteria for establishing CGA fragments as regulatory peptides

1.	Peptide must be processed from CGA, either intracellularly or extracellularly, by processing enzymes present in the tissue or its surrounds
2.	Peptide must be present in the secretory exudate from cells in which a paracrine/autocrine role is proposed.
3.	Action of peptide must be demonstrated using an in vivo or in vitro bioassay in which criteria 4–6 can be tested. Cross-species effects are pharmacological: biological activities and/or amino acid sequence should be conserved across species.
4.	Action of peptide should be abolished by addition of sequence-specific antisera to the medium of the cell test system.
5.	Action of peptide should be mimicked by addition of exogenous synthetic peptide. The putative activity of the added peptide should be abolished when the putative peptide is destroyed by peptide-specific treatments such as sequence-specific proteolytic digestion, non-specific peptidase digestion before addition to the bioassay system.
6.	Specific receptors for the putative regulatory peptide must be present on target cells.

basic amino acids, but the pattern of dibasic amino acid residues in prohormone precursors does not fully predict the processing pattern of each prohormone polypeptide. In addition, biologically active peptides can be liberated from prohormone precursors by cleavage at single basic amino acids, although predicting which single lysine (K) or arginine (R) residues in a given polypeptide will be processing sites is even more difficult [131]. Thus, the potential for generation of a candidate biologically active peptide from a prohormone precursor in a given cell must be accompanied by empirical evidence that the peptide of interest is actually produced in, and released from neuroendocrine cells in a regulated fashion (see section 4 above). The putative CGA-derived paracrine/autocrine factors listed in Table 2 are divided into those which have been actually isolated from endocrine tissues, under conditions in which artefactual peptide generation during extraction is minimized or avoided, and those which have been generated by processing of CGA by proteolytic enzymes 'ex vivo', without a parallel demonstration that such processing occurs in endocrine cells. Rat, cow, and pig pancreastatin, and rat and cow vasostatin I (CGA-(1–76) and β-granin/vasostatin II have fulfilled these criteria (Fig. 3 and references therein). It is noteworthy that pancreastatin is generated at both single basic amino acid cleavages and dibasic sites in a variety of species.

5.2. *Presence in secretory exudate of autocrine / paracrine / hormonal cell*

If a particular CGA-derived peptide is hypothesized to be active following intracellular processing, it must be possible to demonstrate the presence of the specific processed material in the secretory cell exudate. This confirms its existence by specific processing compared to cell extracts, in which extraction artifacts can confound the identification of bona fide processing products. Pancreastatin and β-granin/vasostatin II, for example, are produced in and secreted from cells of the endocrine pancreas and bovine adrenal medulla (Table 2), but according to Drees and Hamilton, not the parathyroid [132]. Neither vastostatin I nor II have yet been identified in neuronal elements innervating human vascular endothelium. These data imply that vasostatin/β-granin and pancreastatin may function as autocrine or paracrine factors in the endocrine pancreas, but as hormones at the parathyroid and vascular endothelium.

5.3. *Hormonal, paracrine and autocrine biological activity and its conservation across species (pharmacological vs. physiological effects)*

Conservation of the sequences of the putative CGA-derived bioactive peptides across several mammalian species is summarized in Fig. 3. All of the major putative bioactive CGA-derived peptides exhibit rather good conservation of sequence as well as processing signals across species, albeit pancreastatin processing appears to depend on monobasic or dibasic cleavage in a species-specific way. In cases where biologically active CGA-derived peptide sequences differ across species, biological activity should be demonstrated using isospecies natural or synthetic peptides to demonstrate a physiological effect on neuroendocrine cells. Many of the reported biological activities of β-granin/vasostatin, pancreastatin, and parastatin are 'pharmacological' rather than physiological by this criterion (see Table 2).

A difficulty inherent in demonstrating autocrine/paracrine actions of CGA peptides by exogenous application is that CGA peptides are already abundantly released from the cells on which they act and in which the activity of the exogenous peptide is being assayed. Thus, the activity of exogenously added peptide only augments activity already present, and this small increment may be difficult to measure. A parallel approach is to attempt to modulate secretion from intact cells, already secreting a cocktail of CGA-derived peptides as well as characteristic hormones, with specific anti-CGA antibodies. The work of Cohn et al. in parathryoid is an example of this approach to showing an autocrine role for CGA-derived peptides [127].

In the absence of additional physiological or pharmacological data, sequence conservation across species can be a powerful (albeit circumstantial) argument for a putative hormonal role for CGA-derived peptides. The same is true for conservation of processing sites among species in which characteristic prohormone converting activities also appear to

be conserved. Regions of CGA, such as WE-14, are quite well conserved evolutionarily [86]. It remains to be determined how closely CGA is conserved through non-vertebrate metazoan species such as paramecia [133]. This remains an issue of pressing importance in the granin field.

Especially if hormonal, as opposed to autocrine or paracrine, roles are proposed for CGA-derived peptides, it is critical to know what kind and how much of CGA-derived peptides are present in circulation, both normally and in pathophysiological states [109,134–136].

Examination of CGA-like proteins in organisms without neuroendocrine systems per se may also provide useful information. Is there a CGA analog, for example, in yeast suggesting a 'preendocrine' secretory role akin to the roles played by proteins of the 'sec' family in extracellular secretion in yeast compared to endocrine and neuronal secretion in mammals [137].

5.4. Abolition of activity by maneuvers that destroy peptides or alter peptide structure

Surprisingly this criterion is the most easily and least frequently met. It is especially important in studying the biological activities of CGA-derived peptides. Many of the proposed actions of CGA peptides involve modulation of secretory processes mediated by L-type calcium channel operation [138–140]. Potent non-peptide inhibitors of L-type calcium-dependent voltage channels have been identified as low-molecular weight contaminants extractable from plasticware frequently used in peptide purification [141]. Thus, even in purified or synthetic preparations of peptides, no biological activity can reasonably be attributed to the peptide present in the mixture unless biological activity is preserved upon acid treatment or boiling, preserved upon treatment with presumptive processing enzymes, and most importantly, abolished by Pronase treatment (e.g., [142]).

A related control is the use of 'scrambled' or altered-sequence peptides that mimick the overall physical characteristics of the active peptide (e.g., lipophilicity, amphipathicity, charge distribution) but contain amino acid mutations or conserved amino acid composition with altered primary sequence (see for example [143]).

5.5. Antibodies against the peptide should have appropriate effects on secretion of target cell secretory products

Antibodies can be useful in identification of biologically active peptides for which no receptor has been identified, and for which there are no specific receptor antagonists. In cases where chromogranin-derived peptides are in fact negative paracrine or autocrine regulators of endocrine secretion, then specific antibodies to CGA should block the action of exogenously added peptides, distinguishing bona fide peptide effects from the effects of co-purified non-peptide contaminants. Further, anti-CGA and anti-CGA peptide antibodies would be predicted to enhance secretion in the absence of exogenously added peptide. Several laboratories have demonstrated that anti-chromogranin antibodies actually augment secretion of PTH and CGA itself in parathyroid, indicating that the actions of endogenous, co-released CGA-derived peptides need to be taken into account in biological assays [127,144]. It can be anticipated that acquisition of new CGA antibodies, especially those specific for processed forms of CGA such as amidation-specific pancreastatin antibodies, will be useful not only in implicating CGA, but specific CGA-derived peptides, in autocrine and paracrine modulation of secretion, and in distinguishing a dependence on extracellular from a dependence on intracellular processing for biological activities attributed to CGA.

5.6. Receptor identification on target tissues / cells

The work of identifying specific receptors for β-granin/vasostatin, pancreastatin, parastatin, WE-14, or GE-25 is clearly in its infancy. Aardel and co-workers have demonstrated specific binding of vasostatin to endothelial tissue on which it exerts a modulatory action, but full characterization of a receptor for vasostatin is lacking. Initial cross-linking experiments done with vascular smooth muscle cells responsive to vasostatin indicate that this receptor has a molecular weight of about 78,000 [145]. Establishing a quantitative receptor-binding assay for the

density and presence of receptors in various tissues will be required, along with physiological and biochemical characterization of CGA-derived peptides, to make a convincing case for their status as CGA-derived biologically active peptides (neuropeptides).

WE-14 and GE-25 represent peptides that fulfill several criteria for CGA-derived regulatory peptides, including identification of fully processed peptide in a variety of endocrine tissues and brain [106,109]. However, the most important criterion is establishing biological activity. At this time no biological function for WE-14 or GE-25 has been identified. It may be hoped that with the availability of purified WE-14 and GE-25 from several species, the biological activity of these peptides, if they have one, will be uncovered. Such as been the case for secretoneurin, a 33-residue peptide processed from SgII at dibasic amino acid processing sites [146].

In addition to autocrine/paracrine roles for CGA, it has been suggested that CGA may function as a neurotrophic factor, although recent experiments employing purified CGA and CGA-derived fragments have failed to support a neurotrophic function for CGA [147,148].

6. Future directions for chromogranin research

Additional experimental information is required to definitively establish a function for CGA as an aggregation/granulogenetic factor, a sorting factor for other co-stored hormones, or a secreted bioactive peptide precursor.

Definitive demonstration that granins function in granulogenesis and sorting of other hormones to the regulated secretory granule pathway will necessitate reconstitution of granulogenesis in cells lacking granules upon expression of CGA or other granins. Such gain-of-function assays may not be possible, if cell lines in which granins are absent, but additional membrane or soluble factors required for granulogenesis are present, cannot be found. In this case, loss-of-function assays, including abrogation of granin expression with antisense oligonucleotides, or in whole organisms by gene deletion, will be required. Assessment of a protein's role in secretion by examining the phenotype of 'gene knockout' animals can be complicated by the expression of addition, potentially redundant proteins, as in the case of the granins. However several tissues have been identified in which only one of the known members of the granin family are expressed, and in which hormonal phenotype can be examined in knockout animals both in vivo and in vitro.

In summary, a CGA-derived 'regulatory peptide' should meet the following criteria: a bioactive peptide should be present in the granin precursor flanked by appropriate cleavage signals, it should be found in endocrine cells and secreted in a regulated fashion, and it should have a measurable biological action that can be blocked by antibodies and mimicked by exogenous peptide. In future it will be necessary to establish the presence and concentration of candidate peptides in paracrine/autocrine target cells, and characterize their receptors. The lack of a single receptor-binding assay for a CGA-derived peptide is a major impediment to further progress in the field at this time, although preliminary characterization of high-affinity vasostatin binding sites in vascular tissue has been reported [145]. Peptides such as WE-14 and GE-25 (Table 2) are likely candidates for bioactive peptides and will require screening for biological activity in diverse bioassays. The concentrations of all putative granin-derived regulatory peptides in serum need to be determined, as well as their approximate extracellular concentrations at putative autocrine receptors (see, for example, [149]). Does tissue-specific processing destroy as well as create CGA-derived autocrine/paracrine factors in a tissue-specific manner? Since CGA and CGA fragments run anomalously on polyacrylamide gel electrophoresis with or without post-translational processing [98], this will require peptide purification and sequencing of candidate biologically active peptides from all endocrine tissues that contain CGA and CGA fragments, and from plasma. At the same time, detailed structure-activity studies must be done in parietal, parathyroid, chromaffin, gastric acid-secreting and exocrine cells in which secretion can be modulated by CGA-derived peptides. Whether or not intact CGA, present at nanomolar concentrations in plasma, is itself active in any bioassay system, remains an open question. Likewise, the requirement for amidation, and the presence of the ultimate and penultimate C- and N-terminal residues of vasostatin, pancreastatin and parastatin for biological activity

will help to address the issue of whether extracellularly derived CGA-derived peptides (such as those generated by plasma kallakrein digestion) as well as intracellularly derived peptides, can function as regulatory peptides.

Firm evidence has been accumulating for a prohormone function of chromogranin A and secretogranin II, and for a role of all three granin proteins in the intracellular processes of granulogenesis and sorting within the regulated secretory pathway. The granin protein family may represent a primordial or ancestral prohormone/granulogenic factor. There is an evolutionary logic to the combination of these two roles in a single protein. Evolution of any 'packaging' or granulogenic protein would have to have occurred on the basis of a selective advantage imparted by the presence of a regulated secretory pathway. This would be most easily accomplished if a single protein possessed both hormonal and granulogenic activities. Clues for the support of such an hypothesis must come from the analysis of the conservation of the structure and function of the granin family in lower animals, and from a closer examination of the ontogeny of granin expression and regulated secretion in mammals.

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