Chromogranin B (secretogranin I), a neuroendocrine-regulated secretory protein, is sorted to exocrine secretory granules in transgenic mice

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Chromogranin B (CgB, secretogranin I) is a secretory granule matrix protein expressed in a wide variety of endocrine cells and neurons. Here we generated transgenic mice expressing CgB under the control of the human cytomegalovirus promoter. Northern and immunoblot analyses, *in situ* hybridization and immunocytochemistry revealed that the exocrine pancreas was the tissue with the highest level of ectopic CgB expression. Upon subcellular fractionation of the exocrine pancreas, the distribution of CgB in the various fractions was indistinguishable from that of amylase, an endogenous constituent of zymogen granules. Immunogold electron microscopy of pancreatic acinar cells showed co-localization of CgB with zymogens in Golgi cisternae, condensing vacuoles/immature granules and mature zymogen granules; the ratio of immunoreactivity of CgB to zymogens being highest in condensing vacuoles/immature granules. CgB isolated from zymogen granules of the pancreas of the transgenic mice aggregated in a mildly acidic (pH 5.5) milieu *in vitro*, suggesting that low pH-induced aggregation contributed to the observed concentration of CgB in condensing vacuoles. Our results show that a neuroendocrine-regulated secretory protein can be sorted to exocrine secretory granules *in vivo*, and imply that a key feature of CgB sorting in the *trans*-Golgi network of neuroendocrine cells, i.e. its aggregation-mediated concentration in the course of immature secretory granule formation, also occurs in exocrine cells although secretory protein sorting in these cells is thought to occur largely in the course of secretory granule maturation.

Keywords: exocrine pancreas/granins/protein sorting/secretory granules/transgenic mice

Introduction

Sorting of secretory proteins to the regulated pathway has been studied extensively (Burgess and Kelly, 1987; Arvan and Castle, 1992; Tooze *et al.*, 1993; Halban and Irminger, 1994; Thiele *et al.*, 1997). Two sorting compartments can be distinguished, the *trans*-Golgi network (TGN) and the immature secretory granule, a vesicular intermediate in secretory granule biogenesis. In the TGN, secretory proteins destined to secretory granules (regulated secretory proteins, RSPs) and other luminal cargo proteins are segregated from each other into immature secretory granules and constitutive secretory vesicles, respectively. The RSPs and other luminal cargo proteins are segregated further from each other in the course of the fission of the immature secretory granule into the mature secretory granule and an AP1 adaptor/clathrin-coated vesicle (Dittié *et al.*, 1996), respectively, with the latter mediating constitutive-like secretion presumably via early endosomes (von Zastrow and Castle, 1987; Arvan and Castle, 1992; Kuliawat and Arvan, 1992; Huttner *et al.*, 1995; Kuliawat *et al.*, 1997). In both sorting compartments, the segregation of the RSPs from other luminal cargo proteins involves their progressive self-interaction, i.e. their aggregation during the formation of the immature secretory granule from the TGN and their further condensation during the conversion of the immature to the mature secretory granule (Gerdes *et al.*, 1989; Tooze *et al.*, 1989, 1993; Arvan and Castle, 1992; Thiele *et al.*, 1997). Though the mechanism of segregation of the RSPs from other luminal cargo proteins utilizes a common principle in both compartments, the extent to which sorting occurs at the level of the TGN versus the immature secretory granule may vary between cell types, depending on the ratio of volume flow from the TGN into immature secretory granules versus constitutive secretory vesicles, and from immature secretory granules into mature secretory granules versus the AP1 adaptor/clathrin-coated vesicles (Thiele *et al.*, 1997).

Cells containing the regulated pathway of protein secretion include exocrine and neuroendocrine cells. However, our understanding as to the extent of conservation of RSP sorting between exocrine and neuroendocrine cells is incomplete. On the one hand, certain RSPs normally produced by exocrine cells such as trypsinogen (Burgess *et al.*, 1985), proline-rich protein (Castle *et al.*, 1992) and amylase (Castle *et al.*, 1997) have been found qualitatively to be sorted to secretory granules upon expression in neuroendocrine cells, although quantitative differences in the sorting efficiency compared with the endogenous neuroendocrine RSPs have been noted (Castle *et al.*, 1997). On the other hand, an anchorless, secretory version of GP2, a luminal protein of pancreatic zymogen granules normally bound to the membrane via a glycerophosphatidylinositol (GPI) anchor, is sorted to secretory

Fig. 1. Transgenic mice expressing CgB under the control of the human CMV promoter. (A) The pCMV-CgB expression vector containing the mouse CgB gene under the control of the human CMV promoter (open box). Filled boxes E1–E5, exons 1–5; hatched box, 1.5 kb *SacI*–*XbaI* fragment used as a probe in Southern blot analysis (B); dotted box, 1.6 kb *EcoRI* fragment used as probe in Northern blot analysis (Figure 2A) and as template for the cRNA probes for *in situ* hybridization (Figure 3). The asterisk indicates the *HindIII* site generated 5' to the *SacI* site to distinguish, in Southern blot analysis of *HindIII*–*EcoRI*-digested genomic DNA, the CgB transgene (T, 3.6 kb fragment) from the endogenous CgB gene (E, 6.3 kb fragment). (B) Southern blot analysis of *HindIII*–*EcoRI*-digested genomic DNA from control (C) and CgB-transgenic (T) mice showing the endogenous CgB gene (arrow) and the CgB transgene (arrowhead).

granules in AR42J cells (Colomer *et al.*, 1994), a pancreatic cell line exhibiting some exocrine features (Rosewicz *et al.*, 1992), but not in the neuroendocrine cell line AtT-20 (Colomer *et al.*, 1994). These observations indicate that an exocrine RSP may, but will not necessarily be sorted to neuroendocrine secretory granules.

Whether or not the converse, the sorting of neuroendocrine RSPs to exocrine secretory granules, may also occur, is unknown. Two neuroendocrine RSPs, pancreatic polypeptide and gastrin, whose biogenesis in neuroendocrine cells involves proteolytic processing of larger precursors and C-terminal amidation in secretory granules, have been recovered in fully processed form from cell extracts upon expression in AR42J cells (Dickinson *et al.*, 1993). However, the significance of these observations with regard to a possible sorting of the two prohormones to exocrine secretory granules is unclear because AR42J cells exhibit not only exocrine, but also neuroendocrine, features (Rosewicz *et al.*, 1992) and a possible storage of the two hormones in exocrine secretory granules was not investigated (Dickinson *et al.*, 1993).

An additional problem with both approaches, the expression of exocrine RSPs in neuroendocrine cells and of neuroendocrine RSPs in exocrine cells, is that cultured cell lines rather than cells in tissues were used as hosts for the foreign RSPs. Hence, the significance of the observed, at least partial conservation of exocrine

Fig. 2. Tissue distribution of CgB mRNA and protein in control and CgB-transgenic mice. (A) Northern blot of total RNA (10 µg) from salivary gland (Sa), brain (Br), muscle (Mu), kidney (Ki), testis (Te), lung (Lu), heart (H), thymus (Thy), liver (Li), pancreas (Pa) and spleen (Spl) of CgB-transgenic (upper panel) and control (lower panel) mice. To document expression of the CgB transgene in the various tissues, an autoradiogram with an overexposed signal of the CgB mRNA in the transgenic pancreas is shown; this accentuates the CgB mRNA fragments resulting from degradation by pancreatic RNase. (B) Immunoblot of the heat-stable proteins prepared from adrenal gland (Adr, 24–30 µg), pituitary gland (Pi, 17–24 µg), salivary gland (Sa), brain (Br), muscle (Mu), kidney (Ki), lung (Lu), heart (H), thymus (Thy), liver (Li), pancreas (Pa) and spleen (Spl) (100 µg each) of CgB-transgenic (upper panel) and control (lower panel) mice. To document the presence of endogenous CgB in adrenal gland, pituitary gland and brain, an autoradiogram with an overexposed signal of CgB in the transgenic pancreas is shown; arrow, full-length CgB; arrowhead, N-terminally truncated degradation product of CgB.

versus neuroendocrine RSP sorting for the *in vivo* situation remains to be established. A neuroendocrine RSP, growth hormone, has been expressed in pancreatic acinar cells and found to be secreted into the lumen of the pancreatic duct (Ornitz *et al.*, 1985). While the pathway of secretion (i.e. constitutive, constitutive-like or regulated) was not investigated, this study nonetheless documents that the exocrine pancreas of a transgenic mouse can serve as a suitable model to address the conservation of exocrine versus neuroendocrine RSP sorting *in vivo*.

In the present study, we have generated transgenic mice expressing chromogranin B (CgB, also referred to as secretogranin I), an RSP found in a wide variety of neuroendocrine cells (Rosa *et al.*, 1985; Huttner *et al.*,

Fig. 3. *In situ* hybridization showing expression of CgB mRNA in the exocrine pancreas of CgB-transgenic mice. In the control pancreas (B and D), islet cells, but not acinar cells are stained with the CgB antisense probe (D), but not the sense probe (B, note unstained islet in the lower left corner). In the CgB-transgenic pancreas (A, C and E), in addition to the staining of some islet cells (C, lower left corner), there is massive staining of acinar cells with the CgB antisense probe (C and E), but not with the sense probe (A). Staining of acinar cells is concentrated in the basal area (see higher magnification in E), consistent with the intracellular distribution of the rough endoplasmic reticulum. (A–D) are the same magnification, the bar in (A) corresponds to 25 μ m; (E) is a higher magnification, the bar corresponds to 10 μ m.

1991; Winkler and Fischer-Colbrie, 1992). Upon placing the CgB gene (Pohl *et al.*, 1990) under the control of the human cytomegalovirus (CMV) promoter, the highest level of expression was detected in the exocrine pancreas. We exploited this observation to investigate the question of whether or not this neuroendocrine RSP is sorted to exocrine secretory granules *in vivo*. Two considerations in addition to the ones delineated above made this investigation worthwhile.

First, the membrane-bound form of carboxypeptidase E (mCPE), a processing enzyme (Fricker, 1988) found in neuroendocrine, but not exocrine cells, has been reported (Cool *et al.*, 1997) to bind to the N-terminal disulfide-bonded loop of pro-opiomelanocortin and to various other neuroendocrine RSPs including chromogranin A (CgA), a protein related to CgB (Benedum *et al.*, 1987; Huttner *et al.*, 1991; Winkler and Fischer-Colbrie, 1992), and has been proposed to have an

essential role in their sorting to secretory granules (Cool *et al.*, 1997; Shen and Loh, 1997). Since both CgA and CgB also contain N-terminal disulfide-bonded loops which are highly homologous to each other (Benedum *et al.*, 1987) and essential for sorting (Chanat *et al.*, 1993; Krömer *et al.*, 1998), an answer to the above question would provide information as to a possible role of mCPE in the sorting of the chromogranins to secretory granules.

Second, in certain endocrine cells, CgB has been found to be enriched in a subpopulation of secretory granules containing a distinct set of hormones (Bassetti *et al.*, 1990). Moreover, from the analysis of endocrinologically silent pituitary adenomas, it has been concluded that the granins alone are sufficient to form a secretory granule matrix (Rosa *et al.*, 1992). If CgB should turn out to be sorted to secretory granules in pancreatic acinar cells, an ultrastructural analysis would

Fig. 5. Parallel enrichment of CgB and amylase upon purification of zymogen granules from the pancreas of CgB-transgenic mice. **(A)** CgB immunoblot of the heat-stable protein fraction prepared from equal amounts (1 mg protein) of the initial homogenate (I), crude zymogen granule fraction (C) and purified zymogen granule fraction (P). For reference, the heat-stable protein fraction prepared from a homogenate (500 μ g protein) of mouse adrenal gland (Adr) is shown. **(B)** Comparison of the CgB immunoreactivity (●) and amylase activity (○) per mg of protein in the homogenate and zymogen granule fractions. For CgB immunoreactivity, the bands of full-length CgB and the CgB degradation products shown in (A) were quantified by densitometric scanning and the sum of the absorbance values calculated.

provide insight as to whether or not this reflects the formation of a neuroendocrine-like subpopulation of secretory granules in an exocrine cell or the co-packaging with zymogens into true exocrine secretory granules.

Here, we report biochemical and morphological evidence that CgB expressed in the exocrine pancreas of transgenic mice is sorted to zymogen granules, and discuss the implications of our ultrastructural analysis for current concepts of RSP sorting.

Fig. 6. Co-fractionation of CgB and amylase upon equilibrium sucrose gradient centrifugation of zymogen granules from the pancreas of CgB-transgenic mice. A crude zymogen granule fraction prepared from the pancreas of CgB-transgenic mice was subjected to equilibrium centrifugation on a 0.5–2 M sucrose gradient and an aliquot of equal volume of each gradient fraction analyzed (fraction 12 = bottom). **(A)** CgB immunoblot. **(B)** Comparison of CgB immunoreactivity (●) and amylase activity (○). For CgB immunoreactivity, the bands of full-length CgB and the CgB degradation products shown in (A) were quantified by densitometric scanning and the sum of the absorbance values calculated.

Results

Generation of CgB-transgenic mice

We generated transgenic mice expressing CgB under the control of a constitutive promoter. For this purpose, a CgB expression vector was constructed in which the mouse CgB gene without its endogenous promoter was placed under the control of the human CMV promoter (Figure 1A). This CgB expression construct proved to be functional because NIH 3T3 cells stably transfected with this vector, but not wild-type NIH 3T3 cells, produced CgB mRNA and protein; the latter showed a

Fig. 4. Immunocytochemistry showing the presence of CgB in pancreatic acinar cells of CgB-transgenic mice. In the control pancreas (A), CgB immunoreactivity is observed in islet cells, but not in acinar cells. In the CgB-transgenic pancreas (B and C), both islet cells (B, left) and acinar cells show CgB immunoreactivity. Staining of acinar cells is concentrated in the apical area and has a granular appearance (see higher magnification in C). No staining was observed with non-immune serum (not shown). (A) and (B) are the same magnification, the bar in (A) corresponds to 25 μ m; (C) is a higher magnification, the bar corresponds to 10 μ m.

Fig. 7. Co-sedimentation of CgB and amylase upon velocity sucrose gradient centrifugation of zymogen granules from the pancreas of CgB-transgenic mice. A crude zymogen granule fraction prepared from the pancreas of CgB-transgenic mice was subjected to a 15 min velocity centrifugation on a 0.3–1.2 M sucrose gradient and an aliquot of equal volume of each gradient fraction including the resuspended pellet (P) analyzed (2+3, pool of half an aliquot of fractions 2 and 3). (A) CgB immunoblot. (B) Amylase activity.

punctate perinuclear localization in immunofluorescence, indicative of targeting of the expressed CgB to the secretory pathway (data not shown). The CMVmCgB insert of the expression vector was microinjected into fertilized mouse oocytes. Nineteen pups were born, of which three showed integration of the CgB transgene into the genome as revealed by Southern blot analysis (Figure 1B). Two of these mice could transmit the CgB transgene to their offsprings in a Mendelian fashion and were used to found CgB-transgenic mouse lines, referred to as 380.6 and 382.6. Both lines showed the massive expression of the CgB transgene in the exocrine pancreas which is documented below and further characterized for the 380.6 line.

Ectopic expression of the CgB transgene in the exocrine pancreas

Northern blot analysis showed that in contrast to control mice, in which the CgB mRNA is known to be present specifically in neuroendocrine tissues (Rosa and Gerdes, 1994) such as brain (Figure 2A, bottom), CgB-transgenic mice contained detectable levels of CgB mRNA also in various non-neuroendocrine tissues (Figure 2A, top). By far the highest CgB mRNA level in CgB-transgenic mice was observed in the pancreas. In CgB-transgenic mice, this tissue also contained the highest level of CgB protein as shown by immunoblot analysis (Figure 2B, top). This was in contrast to control mice (Figure 2B, bottom), in which CgB, though readily observed in immunoblots of tissues rich in neuroendocrine cells such as adrenal, pituitary and brain, is not easily detected in the pancreas because of the low amount of

islet tissue relative to the vast amount of exocrine tissue which physiologically does not express CgB (Rosa and Gerdes, 1994). No significant difference in the pancreatic CgB level was noted between female and male CgB-transgenic mice (data not shown).

The high amounts of CgB mRNA and protein in the pancreas of CgB-transgenic mice suggested that the transgene was ectopically expressed in the exocrine cells of this tissue. *In situ* hybridization showed that this was indeed the case (Figure 3). Staining for the CgB mRNA was obtained with the antisense (Figure 3C–E), but not the sense (Figure 3A and B) probe and was observed in acinar cells of CgB-transgenic (Figure 3C), but not control (Figure 3D) mice. In these cells, staining for the CgB mRNA was concentrated in the basal, perinuclear region (Figure 3E), consistent with the subcellular localization of the rough endoplasmic reticulum. Staining for the CgB mRNA in the pancreatic islets showed a very similar pattern for both CgB-transgenic (Figure 3C) and control (Figure 3D) mice, with a low expression level in many cells and high expression in a few scattered cells.

Consistent with the results of *in situ* hybridization, immunocytochemistry showed CgB immunoreactivity in acinar cells of CgB-transgenic (Figure 4B and C), but not control (Figure 4A) mice. CgB immunoreactivity was concentrated in the apical region of the cells and had a punctate pattern (Figure 4C), suggesting a localization of the ectopically expressed CgB in secretory vesicles. CgB immunoreactivity in the pancreatic islets showed a very similar pattern for both CgB-transgenic (Figure 4B) and control (Figure 4A) mice, with significant staining in many cells and high staining in a few scattered cells, consistent with previous observations on non-transgenic animals (Winkler and Fischer-Colbrie, 1992; Rosa and Gerdes, 1994).

CgB ectopically expressed in pancreatic acinar cells is sorted to zymogen granules

Given the ectopic expression of CgB in pancreatic acinar cells of CgB-transgenic mice, we investigated its intracellular localization by subcellular fractionation and immunoelectron microscopy. Upon purification of zymogen granules, the specific CgB immunoreactivity determined by immunoblot analysis (Figure 5A) increased in parallel with the specific activity of amylase (Figure 5B). When a crude zymogen granule fraction was analyzed by equilibrium and velocity sucrose gradient centrifugation, CgB immunoreactivity coincided with amylase activity in the dense fractions of the equilibrium gradient (Figure 6) and the pellet of the velocity gradient (Figure 7). This indicated that the CgB-containing vesicles were indistinguishable from zymogen granules in density and size, and suggested the co-localization of CgB with zymogens in the latter organelles.

Immunogold electron microscopy provided direct evidence for the presence of the ectopically expressed CgB in zymogen granules (Figures 8 and 9). Double labeling for CgB and zymogens (Figure 8) showed CgB immunoreactivity over the matrix of most, if not all, zymogen granules. We did not observe subpopulations of secretory granules containing either mostly zymogens or mostly CgB, nor did we detect secretory granules in

Fig. 8. Double immunogold electron microscopy showing co-localization of CgB with zymogens in the Golgi complex, condensing vacuoles and zymogen granules of the pancreas of CgB-transgenic mice. Ultrathin cryosections prepared from the pancreas of CgB-transgenic mice were double-immunolabeled for CgB (arrows) and zymogens (arrowheads), and immunoreactivity was revealed by 9 and 14 nm protein A-gold. (A, D and E) CgB, 14 nm gold; zymogens, 9 nm gold. (B and C) CgB, 9 nm gold; zymogens, 14 nm gold. Note the co-localization of CgB and zymogen immunoreactivity in the cisternae of the Golgi complex (white G), in the condensing vacuoles (cv) seen in continuity with, or in proximity to, the Golgi cisternae, and in zymogen granules (zg). (B and C) The ratio of CgB to zymogen immunoreactivity is greater in the condensing vacuoles than in Golgi cisternae and in zymogen granules (see also Table I). All panels are the same magnification; the bar in (E) corresponds to 500 nm.

which CgB and zymogens were obviously segregated from each other within the secretory granule matrix, as has been reported for distinct RSPs in neuroendocrine cells (Fumagalli and Zanini, 1985; Ehrhart *et al.*, 1986; Hashimoto *et al.*, 1987; Fisher *et al.*, 1988; Bassetti *et al.*, 1990). Co-localization of CgB and zymogen immunoreactivity was already observed at the level of the Golgi cisternae and single condensing vacuoles (Figure 8). Quantitation of the gold particles for CgB and zymogens revealed that the ratio of CgB to zymogen immunoreactivity over condensing vacuoles/immature granules (for definition see Materials and methods) was 4- to 5-fold greater than that over Golgi cisternae and mature zymogen granules (Figure 8, Table I). This was largely due to the fact that for CgB, the apparent

immunoreactivity per unit area increased from the Golgi cisternae to the condensing vacuoles/immature granules and decreased from condensing vacuoles/immature granules to mature zymogen granules, whereas no such decrease was observed in the case of the zymogens. This was evident from both double labeling for CgB and zymogens (Figure 8) and single labeling for either CgB or zymogens (Figure 9).

Secretion of CgB from the transgenic pancreas

Given the presence of the ectopically expressed CgB in zymogen granules, we investigated the secretion of CgB from pancreatic lobules of CgB-transgenic mice. Following a 5 min pulse labeling with [35 S]methionine/cysteine, the pancreatic lobules were chased for 60 min

Fig. 9. Single immunogold electron microscopy of the pancreas of CgB-transgenic mice showing the decrease in CgB, but not zymogen, immunoreactivity in mature zymogen granules relative to the condensing vacuoles/immature granules. Ultrathin cryosections prepared from the pancreas of CgB-transgenic mice were immunolabeled for either CgB (A and C) or zymogens (B and D), and immunoreactivity was revealed by 9 nm protein A–gold. Note that the CgB immunoreactivity (A and C) is higher in the condensing vacuoles/immature granules (cv; for definition, see Materials and methods) than in the cisternae of the Golgi complex (white G) and in mature zymogen granules (zg, white arrowheads), whereas zymogen immunoreactivity (B and D) is similar in condensing vacuoles/immature granules and in mature zymogen granules. All panels are the same magnification; the bar in (D) corresponds to 500 nm.

Table I. CgB and zymogen immunoreactivity in Golgi cisternae, condensing vacuoles/immature granules and zymogen granules of pancreatic acinar cells of CgB-transgenic mice

Field	Golgi cisternae				Condensing vacuoles/immature granules				Zymogen granules			
	CgB	Zym.	CgB/Zym.	Ratio	CgB	Zym.	CgB/Zym.	Ratio	CgB	Zym.	CgB/Zym.	Ratio
Field 1 (Figure 8A)	5	16	0.31	2.58	3	4	0.75	6.25	18	149	0.12	1
Field 2 (Figure 8B)	6	10	0.60	0.60	41	14	2.93	2.93	38	38	1.00	1
Field 3 (Figure 8C)	12	9	1.33	1.85	85	17	5.00	6.94	33	46	0.72	1
Field 4 (Figure 8D)	3	44	0.07	0.44	n.d.	n.d.	n.d.	n.d.	69	442	0.16	1
Field 5 (Figure 8E)	3	34	0.09	0.75	n.d.	n.d.	n.d.	n.d.	38	309	0.12	1
Field 6	n.d.	n.d.	n.d.	n.d.	26	22	1.18	3.58	24	72	0.33	1
Mean $\pm$ SD				1.24 $\pm$ 0.93*				4.93 $\pm$ 1.97*				1

Gold particles indicative of either CgB or zymogen (Zym.) immunoreactivity over Golgi cisternae, condensing vacuoles/immature granules and zymogen granules were counted in the five fields, parts of which are shown in Figure 8A–E, and in another field (Field 6) of double immunogold-labeled ultrathin cryosections. The ratio of CgB immunoreactivity/zymogen immunoreactivity was calculated (CgB/zym.). For each field, the ratio obtained for zymogen granules was set to 1, and the ratio for Golgi cisternae and for condensing vacuoles/immature granules (for definition, see Materials and methods) was expressed relative to this.

* $P < 0.01$; n.d. not determined.

without stimulation of secretion and then for 30 min in the presence of cerulein, an established secretagog (Vasiloudes *et al.*, 1991). Analysis of the media for the appearance of [35 S]methionine/cysteine-labeled amylase showed that the secretagog, as expected, stimulated the secretion of the zymogen granule contents from the transgenic pancreas (Figure 10A). Addition of cerulein resulted in the secretion of [35 S]methionine/cysteine-labeled CgB along with [35 S]methionine/cysteine-labeled

amylase (Figure 10B, right columns). Interestingly, while the secretion of [35 S]methionine/cysteine-labeled amylase during the first 60 min of chase in the absence of cerulein was low compared with the secretagog-stimulated secretion (Figure 10B, filled columns), more than a third of the [35 S]methionine/cysteine-labeled CgB in the medium was due to unstimulated secretion (Figure 10B, hatched columns), having been released mostly during the 40–60 min chase interval (data not shown).

Fig. 10. Secretion of CgB from the pancreas of CgB-transgenic mice. Pancreatic lobules were pulse-labeled with [35 S]methionine/cysteine for 5 min and chased for 90 min, with 5 ng/ml cerulein being present during the last 30 min of chase. At each of the time points indicated in (A), the medium was collected and replaced with fresh chase medium. Aliquots of the lobules and media were analyzed by SDS-PAGE followed by quantification of amylase and CgB. (A) The amount of [35 S]methionine/cysteine-labeled amylase secreted during the various chase intervals is expressed as a percentage of the total (sum of media plus lobules) and is shown as cumulative secretion. Data are the mean of six observations (triplicate sets of lobules from two CgB-transgenic mice); bars indicate the standard deviation. The arrow indicates the beginning of the period of stimulated secretion. (B) The sum of the [35 S]methionine/cysteine-labeled CgB secreted during the first 60 min of chase in the absence of cerulein (basal secretion) and during the last 30 min of chase in the presence of cerulein (stimulated secretion) was set to 100; basal and stimulated secretion of CgB is expressed proportionally. The stimulated secretion of [35 S]methionine/cysteine-labeled amylase is normalized to that of CgB (regression coefficient 0.996) and the basal secretion of amylase expressed relative to this. Data are the mean of duplicate observations (single set of lobules from two CgB-transgenic mice); bars indicate the variation of the individual values from the mean.

Low pH-induced aggregation of CgB from zymogen granules

To address the mechanism of sorting of CgB to zymogen granules, we isolated the soluble contents of pancreatic zymogen granules purified from CgB-transgenic mice and compared the *in vitro* aggregation of CgB and zymogens at neutral pH and at a mildly acidic pH similar to that thought to exist in condensing vacuoles (Orci *et al.*, 1987). CgB was found to be soluble at pH 7.5 but aggregated upon lowering the pH to 5.5 (Figure 11A). In contrast, at the concentrations used

Fig. 11. Low pH-induced aggregation of CgB in the soluble fraction of a zymogen granule lysate from the pancreas of CgB-transgenic mice. The soluble fraction (≈ 0.8 mg protein/ml) of a lysate of zymogen granules purified from the pancreas of CgB-transgenic mice was incubated at pH 5.5 or pH 7.5, centrifuged, and pellet (P) and supernatant (S) were analyzed. (A) CgB immunoblot of the heat-stable protein fraction prepared from the entire pellet and half of the supernatant. For reference, the heat-stable protein fraction prepared from total pancreatic homogenate (500 μ g protein) of CgB-transgenic mice (TP) and total homogenate (500 μ g protein) of mouse adrenal gland (Adr) are shown. Note the increase in limited proteolysis of CgB after incubation of the soluble fraction of the zymogen granule lysate as compared with the purified zymogen granule fraction (compare Figure 5A). (B) Coomassie Blue staining of an equal proportion of pellet and supernatant. Arrow, amylase.

the zymogens showed only little aggregation (as revealed by their recovery in the pellet after ultracentrifugation) at pH 5.5, the vast majority being recovered in the supernatant at both pH 7.5 and pH 5.5 (Figure 11B).

Discussion

We generated transgenic mice expressing CgB under the control of the human CMV promoter. Ectopic expression of the CgB mRNA was detected not only in the exocrine pancreas, which contained high levels of CgB mRNA, but also, though at lower levels, in numerous other tissues. In contrast to the expression of the CgB mRNA, storage of the ectopically expressed CgB protein was detected only in the exocrine pancreas and not in the other ectopically expressing tissues, presumably because most protein secretion from the latter occurs via the constitutive pathway in which secretory proteins do not undergo significant intracellular storage. By analyzing the storage of the ectopically expressed CgB in the pancreatic acinar cells, we provide biochemical and morphological evidence that CgB is sorted to zymogen granules. Our findings therefore complement and extend previous studies by others (Burgess *et al.*, 1985; Castle *et al.*, 1992, 1997), who had shown that exocrine secretory proteins are sorted to secretory granules when expressed in neuroendocrine cell lines. First, we demonstrate that the converse is also the case, i.e. that a neuroendocrine secretory granule protein can be sorted to exocrine secretory granules. Second, given that our observations were made in a transgenic animal model, our data provide *in vivo* evidence for the notion (Burgess *et al.*, 1985; Castle *et al.*, 1997) that the mechanisms underlying the sorting of secretory proteins to secretory granules are conserved between neuroendocrine and exocrine cells.

What may be the mechanism underlying the sorting of a neuroendocrine secretory protein into exocrine secretory granules? By immunogold electron microscopy, CgB immunoreactivity in condensing vacuoles/immature gran-

ules was markedly increased as compared with that in the cisternae of the Golgi complex. In addition, CgB from zymogen granules exhibited low pH-induced aggregation *in vitro*. Given that the luminal pH of condensing vacuoles in exocrine cells is acidic (Orci *et al.*, 1987), these two observations suggest that low pH-induced aggregation contributed to the concentration of CgB in condensing vacuoles. We find it unlikely that this concentration was due to co-aggregation with zymogens, for two reasons. First, at the concentrations used the zymogens exhibited only little low pH-induced aggregation, in contrast to CgB. Second, CgB immunoreactivity relative to zymogen immunoreactivity increased ≈ 4 -fold from the Golgi cisternae to the condensing vacuoles, and in many condensing vacuoles the zymogen immunoreactivity appeared to be too low to account for the concentration of CgB immunoreactivity. We therefore conclude that the aggregation of CgB in condensing vacuoles and its packaging in concentrated form into immature granules occurred independently of the zymogens. In other words, upon expression in exocrine cells, CgB retained a key feature of its sorting in the TGN of neuroendocrine cells, i.e. its aggregation-mediated concentration in the course of immature secretory granule formation, although secretory protein sorting in exocrine cells is thought to occur largely in the course of secretory granule maturation (Arvan and Castle, 1992).

Immunogold electron microscopy revealed the co-localization of CgB and zymogen immunoreactivity in single condensing vacuoles and zymogen granules. However, relative to the zymogen immunoreactivity, the CgB immunoreactivity was ≈ 5 -fold higher in condensing vacuoles/immature granules than in mature zymogen granules. This may reflect a reduction in mature zymogen granules of the accessibility of the C-terminal epitope of CgB due to condensation of the secretory granule matrix, or the loss of this epitope due to partial proteolysis of CgB which occurred in zymogen granules as revealed by immunoblotting. In addition, a portion of the CgB molecules present in condensing vacuoles/immature granules may be removed in the course of zymogen granule maturation and may undergo constitutive-like secretion (Arvan and Castle, 1992). Our observation that CgB was not only secreted along with amylase upon addition of a secretagog but, in contrast to amylase, also exhibited a significant degree of unstimulated secretion is consistent with this possibility. With respect to a possible constitutive-like secretion of the neuroendocrine protein CgB from immature granules of exocrine cells, it is interesting to note that a precedent for the converse has been reported. Upon expression in the neuroendocrine cell line AtT-20, two exocrine secretory proteins (proline-rich protein and amylase) were found to be sorted to immature secretory granules along with the endogenous peptide precursor pro-opiomelanocortin (POMC), but to be partly removed from secretory granules during maturation followed by constitutive-like secretion (Castle *et al.*, 1997).

The observations that CgB, rather than being packaged into a subpopulation of secretory granules devoid of zymogens or being segregated from zymogens within the secretory granule matrix, was co-localized and mixed with zymogens in condensing vacuoles and zymogen granules, have implications for the interaction of CgB with the

zymogen granule membrane. Since CgB apparently entered the regulated secretory pathway of exocrine cells independently of the endogenous zymogens, these observations suggest that during exocrine secretory granule formation and maturation, CgB interacted with the same membrane components as the zymogens. The nature of these granule membrane components remains to be elucidated.

Loh and colleagues (Cool *et al.*, 1997; Shen and Loh, 1997) recently proposed that mCPE, an enzyme expressed in neuroendocrine cells that is involved in the proteolytic processing of peptide precursors, is a granule membrane component involved in the sorting of RSPs to secretory granules. Specifically, mCPE is thought to bind to the N-terminal disulfide-bonded loop of POMC and to a number of other RSPs including CgA (Cool *et al.*, 1997), which also contains an N-terminal disulfide-bonded loop (Benedum *et al.*, 1987). CgB contains an N-terminal disulfide-bonded loop which is highly homologous to that of CgA (Benedum *et al.*, 1987) and has been shown to be essential for the sorting of CgB from the TGN into immature secretory granules (Chanat *et al.*, 1993; Krömer *et al.*, 1998). The disulfide-bonded loops of CgA (Kang and Yoo, 1997) and CgB (Yoo and Kang, 1997) have been reported to mediate the interaction of soluble chromogranins with the secretory granule membrane. Together with these previous observations, our present findings showing sorting of CgB to secretory granules in exocrine cells, which lack CPE, imply that the essential role of the disulfide-bonded loop in the sorting of CgB reflects the interaction of the loop with membrane components that are distinct from CPE and common to both exocrine and neuroendocrine cells.

Materials and methods

Generation of CgB-transgenic mice

For the construction of the CgB expression vector, the 15.8 kb *SacI*–*Bgl*III fragment from the cosmid clone mcSgI-9c (Pohl *et al.*, 1990), containing the entire mouse CgB gene except for the promoter and 33 nucleotides of 5'-untranslated sequence of exon 1, was assembled 3' to the 0.6 kb *EcoRI*–*Hind*III fragment from pRC/CMV (Invitrogen), containing the enhancer/promoter sequence of the immediate early gene of human CMV, in pBluescript (Stratagene). In the course of this construction, a *Hind*III linker was added 5' to the *SacI* site of the CgB gene. This allowed us to distinguish the CgB transgene from the endogenous CgB gene (Figure 1). The final construct in pBluescript is referred to as pCMVmCgB (Figure 1). The 16.4 kb CMVmCgB fragment to be injected into fertilized mouse oocytes was released from pCMVmCgB by *Clal* and purified by electroelution. Microinjection of the DNA into fertilized mouse oocytes, breeding of transgenic mice and Southern blot analysis of tail DNA for the integration of the transgene into the genome followed standard protocols (Gordon *et al.*, 1993). Two CgB-transgenic lines of mice (C57BL/6J), referred to as L380.6 and L382.6, were generated. Except for the data shown in Figure 10, which were obtained with the L382.6 line, the data shown were obtained with the L380.6 line of CgB-transgenic mice; all CgB-transgenic mice and control mice (littermates) used in the experiments were verified for the presence and absence, respectively, of the CgB transgene by Southern blot analysis. Differences between transgenic and control mice with respect to overall phenotype and morphology of various tissues (adrenal, brain, gastrointestinal tract, heart, kidney, liver, lung, pancreas, parotid, pituitary, skeletal muscle, skin, spleen, testis, thymus, thyroid, urinary bladder) were not obvious (data not shown).

Southern and Northern blot analyses

Southern blot analysis of tail genomic DNA prepared using the Quiagen kit and digested with *EcoRI* and *Hind*III was performed following

standard protocols (Ausubel *et al.*, 1997), using the [³²P]dCTP-labeled 1.5 kb *SacI-XbaI* fragment of the mouse CgB gene (Figure 1A) as a probe. Northern blot analysis of total RNA extracted from various tissues of CgB-transgenic and control mice was performed according to standard protocols (Ausubel *et al.*, 1997), using the [³²P]dCTP-labeled 1.6 kb *EcoRI* fragment of the mouse CgB gene containing most of exon 4 (Figure 1A) as a probe.

In situ hybridization

The pancreas was fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin after dehydration with ethanol followed by chloroform replacement. Antisense and sense digoxigenin-labeled cRNA probes were synthesized by *in vitro* transcription using the DIG RNA labeling kit (Boehringer Mannheim) and 1 µg of a linearized plasmid with the 1.6 kb *EcoRI* fragment of the mouse CgB gene containing most of exon 4 (Figure 1A) as template DNA. The transcripts were alkaline-hydrolyzed (Schad *et al.*, 1996) and the resulting ≈500 nucleotide fragments used for hybridization. Sections (3 µm) mounted on silan-treated glass slides were deparaffinized and treated with 10 µg/ml proteinase K for 10 min at 37°C. After acetylation using 0.25% acetic anhydride and 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature, the sections were hybridized with the antisense or sense probe for 16–18 h at 50°C. The sections were then treated with RNase, washed, incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) for 30 min at room temperature, and the phosphatase activity visualized with NBT BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate).

Immunocytochemistry

The pancreas was fixed in 4% paraformaldehyde overnight and embedded in paraffin. Deparaffinized sections (3 µm) were incubated with a rabbit antiserum (1:100–1:200 dilution) against a synthetic peptide corresponding to the C-terminal 23 amino acids of human CgB (hCgB_{635–657}) (Rosa *et al.*, 1992). Non-immune serum at the same dilution was used as negative control. Immunoreactivity was revealed by the labeled streptavidin–biotin (LSAB) method using the LSAB kit (Dako) and diaminobenzidine tetrahydrochloride.

Immunoelectron microscopy

Pancreatic tissue was fixed by immersion in 8% paraformaldehyde/0.1% glutaraldehyde at room temperature and kept in fixative for a few hours at 4°C. Ultrathin cryosections were prepared according to the Tokuyasu method (Griffiths, 1993). The fixed tissue was infiltrated with 30% (w/v) polyvinylpyrrolidone/1.61 M sucrose and frozen in liquid nitrogen. Ultrathin cryosections were cut at –100°C with a Reichert FCS cryoultramicrotome (Leica). The sections were collected on Formvar/carbon-coated grids and incubated in blocking medium 1 [10% fetal calf serum (FCS)/0.15% glycine in phosphate-buffered saline (PBS)]. For single immunogold labeling, the sections were incubated with the primary rabbit antiserum followed by protein A coupled to 9 nm gold, all in blocking medium 2 (5% FCS/0.15% glycine in PBS). For double immunogold labeling, the sections were incubated with the first primary rabbit antiserum followed by protein A coupled to 9 nm gold in blocking medium 2, treated with 1% glutaraldehyde in PBS, incubated in blocking medium 1 and then with the second primary rabbit antiserum followed by protein A coupled to 14 nm gold in blocking medium 2. The sections were washed with PBS and H₂O, incubated with 0.3% uranyl acetate/1.8% methylcellulose on ice, and air-dried. The immunogold-labeled sections were examined in an electron microscope (EM 10, Zeiss). The primary antisera used were a rabbit antiserum (1:3 dilution) against rat CgB (secretogranin I) purified from PC12 cells (Rosa *et al.*, 1985) and a rabbit antiserum (1:250 dilution) against zymogens of rodent pancreatic juice (kindly provided by Dr John Tooze). For the quantitation of gold particles, the term ‘Golgi cisternae’ refers to Golgi cisternae without the condensing vacuoles in continuity with the cisternae, the term ‘condensing vacuoles/immature granules’ refers to both condensing vacuoles seen in continuity with the Golgi cisternae and granules with a content of relatively low electron density seen in proximity to the Golgi complex, and the term ‘zymogen granules’ refers to granules with a contents of relatively high electron density.

Purification of zymogen granules

Zymogen granules were prepared according to previous reports (De Lisle *et al.*, 1984; Fukuoka *et al.*, 1991). Briefly, the pancreas was homogenized in homogenization buffer containing 250 mM sucrose, 5 mM MOPS-NaOH (pH 7.0), 0.1 mM MgSO₄ and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at

150 g for 15 min and the resulting postnuclear supernatant at 1300 g for 15 min. The resulting crude zymogen granule pellet (≈8 mg protein per pancreas) was resuspended either in homogenization buffer and subjected to equilibrium or velocity sucrose gradient centrifugation as described below, or in 40% Percoll, 250 mM sucrose, 50 mM MES-NaOH (pH 5.5), 0.1 mM MgSO₄, 0.1 mM PMSF and 2 mM EGTA, and centrifuged for 20 min at 100 000 g in the self-forming Percoll gradient to purify the zymogen granules further. The purified zymogen granules, which formed a dense white band near the bottom of the Percoll gradient, were collected, washed twice with homogenization buffer to remove the Percoll, and resuspended in 0.5 ml of homogenization buffer or in pH 11 carbonate buffer as described below. Aliquots of the homogenate, the crude zymogen granules and the purified zymogen granules resuspended in homogenization buffer were used for the preparation of the heat-stable protein fraction and the determination of amylase activity as described below.

Equilibrium and velocity sucrose gradient centrifugation

Equilibrium and velocity sucrose gradient centrifugation were performed as previously described (Tooze and Huttner, 1990, 1992). For the equilibrium centrifugation, 2 ml of the resuspended crude zymogen granule pellet prepared from the pancreas of one CgB-transgenic mouse were centrifuged on an 8 ml linear 0.5–2 M sucrose gradient. For the velocity centrifugation, 1 ml of the resuspended crude zymogen granule pellet prepared from the pancreas of one CgB-transgenic mouse was centrifuged on a 9 ml linear 0.3–1.2 M sucrose gradient. For both gradients, 0.8 ml fractions were collected from top to bottom. In the case of the velocity gradient, the pellet was resuspended in 0.8 ml of homogenization buffer. Aliquots of each fraction were used for the determination of amylase activity (50 µl) and the preparation of the heat-stable protein fraction (300 µl) followed by immunoblot analysis of CgB.

In vitro aggregation assay

In vitro aggregation of the zymogen granule contents was performed according to previous reports with minor modifications (Freedman and Scheele, 1993; Leblond *et al.*, 1993; Colomer *et al.*, 1994, 1996). The pellet of purified zymogen granules obtained after Percoll gradient centrifugation from the pancreas of two CgB-transgenic mice was resuspended at 4°C in 250 µl of 0.1 M Na₂CO₃ (pH 11), 0.1 mM PMSF, 0.1 mg/ml soybean trypsin inhibitor and 20 KIU/ml aprotinin, and kept for 30 min at 4°C to allow for the lysis of zymogen granules. The lysate was centrifuged for 60 min at 220 000 g and the resulting supernatant containing the soluble zymogen granule contents (≈16 mg protein/ml) stored at –80°C until use. Aliquots (100 µl) of the zymogen granule contents were incubated for 30 min at 37°C in a final volume of 2 ml (≈0.8 mg protein/ml) containing either 100 mM MES-NaOH/0.5 mM PMSF (final pH 5.5) or 100 mM Tris-HCl/0.5 mM PMSF (final pH 7.5). After the incubation, samples were cooled to 4°C and centrifuged for 60 min at 220 000 g. The supernatants were collected and (in the case of the pH 5.5 supernatant) adjusted to pH 7.5 with 2 M NaOH. The pellets were resuspended in 0.4 ml of 100 mM Tris-HCl/0.5 mM PMSF/5 mM β-mercaptoethanol (pH 7.5). Aliquots corresponding to 0.5% of pellets and supernatants were subjected to SDS-PAGE and Coomassie Blue staining for the analysis of zymogens, the remainder of the pellets and half of the supernatants were used for the preparation of the heat-stable protein fraction followed by immunoblot analysis of CgB.

Heat-stable protein fraction and CgB immunoblot analysis

For the preparation of the heat-stable protein fraction (Rosa *et al.*, 1985) from various tissues of CgB-transgenic and control mice, the frozen tissues were thawed and homogenized (1:10 w/v) in 20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM PMSF, followed by centrifugation for 30 min at 10 000 g. The supernatant was supplemented with 5 M NaCl to a final concentration of 150 mM, boiled for 5 min and centrifuged as above. The heat-stable proteins in the resulting supernatant were concentrated by acetone precipitation and subjected to immunoblot analysis. The heat-stable protein fraction was also prepared from various subcellular fractions after addition of NaCl following the above protocol. Immunoblot analysis was performed as described previously (Bauerfeind *et al.*, 1993), using the rabbit antiserum (1:200 dilution) against a synthetic peptide corresponding to the C-terminal 23 amino acids of human CgB (hCgB_{635–657}) (Rosa *et al.*, 1992) followed by either [¹²⁵I]protein A (Amersham) and autoradiography or the ECL detection system (Amersham). Immunoblots were quantitated (NIH Image 1.55) after scanning (Adobe Photoshop™ 3).

Secretion from pancreatic lobules

Pancreatic lobules from two CgB-transgenic mice were prepared in methionine- and cysteine-free HEPES-buffered Dulbecco's modified Eagle's medium (DMEM) following previously described methods (Vasiloudes et al., 1991) and equilibrated in this medium for 15 min in the coldroom. For each CgB-transgenic mouse, the pancreatic lobules were distributed into three tissue culture wells, and each set of lobules was labeled in a 10% CO₂ incubator at 37°C for 5 min in 300 µl of methionine- and cysteine-free DMEM containing 500 µCi/ml of [³⁵S]methionine/cysteine (Pro-mix, >1000Ci/mmol, Amersham). The labeling medium was discarded and the lobules were washed thoroughly with ice-cold DMEM containing twice the normal concentrations of unlabeled methionine and cysteine. The lobules were chased in this medium containing 20 µg/ml soybean trypsin inhibitor and 14 µg/ml aprotinin at 37°C for seven consecutive intervals of 20, 10, 10, 20, 10, 10 and 10 min, with 300 µl of fresh chase medium being added at the beginning and collected at the end of each interval. The chase media used for the last three intervals contained 5 ng/ml cerulein (Sigma C9026). At the end of the chase, each set of lobules was solubilized in 500 µl of 1% SDS, 10 mM HEPES-NaOH pH 7.4, 20 µg/ml soybean trypsin inhibitor, 14 µg/ml aprotinin and 0.5 mM PMSF, using a glass-Teflon homogenizer followed by immediate boiling. Aliquots of the media and solubilized lobules were analyzed by SDS-PAGE followed by phosphoimaging and quantification of the amylase and the full-length CgB band. Analysis of the solubilized lobules by two-dimensional PAGE and phosphoimaging showed that full-length CgB was the major labeled spot in the relevant molecular weight range (data not shown).

Miscellaneous

Amylase activity was determined using a commercially available kit (Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan). Protein was determined by the Lowry or Bradford method using bovine serum albumin as standard.

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Note added in proof

Normant and Loh [*Endocrinology*, **139**, 2137–2145 (1998)] recently also reported that the sorting of chromogranin A to secretory granules is not dependent on CPE.