Dense-core granules: a specific hallmark of the neuronal/neurosecretory cell phenotype

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Summary

Expression of dense-core granules, a typical exocytic organelle, is widely believed to be controlled by coordinate gene expression mechanisms specific to neurones and neurosecretory cells. Recent studies in PC12 cells, however, have suggested the number of granules/cells depends on the levels of only one of their cargo proteins, chromogranin A, regulating the metabolism of the other proteins, and thus the composition of the organelles, by an on/off switch mechanism. In addition, transfection of chromogranin A was reported to induce appearance of dense-core granules in the non-neurosecretory fibroblasts of the CV-1 line. Here the role of chromogranin A has been reinvestigated using not the heterogeneous PC12 line but several clones isolated therefrom. In these clones, investigated as such or after transfection with chromogranin A antisense sequences, the ratio between chromogranin A and its secretory protein mate, chromogranin B, was not constant but highly and apparently randomly variable. Variability of the chromogranin A/chromogranin B ratio was seen by confocal immunofluorescence also among the cells of single clones and subclones and among the granules of single cells. Moreover, stable and transient transfections of chromogranin A in a PC12 clone characterised by a low number of dense-core granules (one fifth of the reference clone) failed to modify significantly the number of the organelles, despite the several-fold increase of the granin. Finally, in three types of non-neurosecretory cells (CV-1, adenocarcinoma TS/A and a clone of PC12 incompetent for secretion) the transfected chromogranin A accumulated mostly in the Golgi/trans-Golgi area and was released rapidly from resting cells (constitutive secretion) as revealed by both immunofluorescence during cycloheximide treatment and pulse-chase experiments. Only a minor fraction was sorted to discrete organelles that were not dense-core granules, but primarily lysosomes because they contained no chromogranin B, and were largely positive for the late endosomal-lysosomal markers, Lamp1 and lamp3. Dense-core granules are therefore true hallmarks of neurones and neurosecretory cells. Their number/cell appears independent of chromogranin A and their composition does not appear to be constant; in particular, they exhibit considerable, and so far unexplained variability in the chromogranin A/chromogranin B ratio.

Key words: Dense-core granules, Neurosecretory cells, Regulated exocytosis, Chromogranin A, Chromogranin B

Introduction

Regulated secretion, i.e. the stimulation-induced, exocytic release of cargo molecules segregated within the lumen of membrane-bound organelles, has classically been envisaged as a property of secretory granules and vesicles, taking place only in professional secretory cells: exocrine, endocrine and neurones. In contrast, exocytic release from non-secretory cells was believed to occur only by constitutive secretion.

Recently, however, these views have been challenged. Regulated exocytosis of lysosomes, believed for a long time to be specific for a subclass of these organelles, the secretory lysosomes expressed in blood leukocytes and a few other cell types, was shown to be ubiquitous (Jaiswal et al., 2002; Reddy et al., 2001), possibly involved in membrane repair. In addition, another widely expressed family of vesicles, also competent for regulated exocytosis, was identified (Borgonovo et al., 2002). Moreover, typical luminal proteins of the neurosecretory dense-core granule (DCG), chromogranins A and B (CgA and CgB), when expressed in non-secretory cells were reported to distribute not only to the Golgi complex/trans-Golgi network (GC/TGN), the area from which constitutive secretion takes place, but also to discrete organelles scattered throughout the cytoplasm (Kim et al., 2001; Rustom et al., 2002; Huh et al., 2003). At least morphologically, some of these organelles resemble typical DCGs of neurones and neurosecretory cells (Kim et al., 2001). Finally, a study of the mechanisms that control the expression of the latter granules suggested, in addition to a still largely undefined master gene(s) programme that coordinates transcription/translation of all proteins participating in regulated secretion (Malosio et al., 1999), also the existence of an on/off switch operated by a single secretory protein, CgA (Kim et al., 2001). A fall in CgA levels was reported to induce the rapid turnover of other proteins of DCGs, with ensuing reduction of the number and preservation of the composition of the organelles (Kim et al., 2001).
Fig. 1. CgA and CgB levels in clones isolated from wild-type PC12 (A) and in subclones isolated from the PC12-15 clone after stable transfection of empty vector (A) or antisense CgA sequences (B). Scatter plot analysis of CgA and CgB western blot bands decorated by the specific polyclonal antisera, GE-19 and PE-11, respectively. The gels were loaded (25 μg of protein/lane) with total cell lysates. The densitometric CgA and CgB signals, normalised to tubulin, are indicated as percentages of the values in the reference PC12-15 clone. (A) Open squares illustrate the CgA/CgB ratios of 9 PC12 clones isolated from wild-type PC12 (Clementi et al., 1992); closed squares the ratios of 3 subclones isolated from the PC12-15 clone after transfection with the empty vector. (B) Twelve subclones of the reference PC12-15 clone, isolated after stable transfection with antisense sequences against rat CgA. A, L, O are the subclones illustrated in the corresponding panels of Fig. 2.

These recent observations raised questions concerning not only the specificity of the expression but also the molecular and functional definition of DCGs. In order to investigate these problems we have performed experiments in both neurosecretory and non-secretory cell lines. Results from many clones of pheochromocytoma PC12 cells failed to confirm the existence of the CgA-dependent, on/off switch control. Rather, they showed that the composition of DCGs can be considerably heterogeneous, both among the cells of single clones and within individual cells. Moreover, the discrete structures induced in non-secretory cells by CgA expression were shown to be not bona fide DCGs but distinct organelles, mostly (possibly all) of lysosomal nature.

Materials and Methods

Cells

Wild-type PC12 and clones isolated therefrom were those described by Clementi et al. (Clementi et al., 1992) and were grown as reported. The reference PC12 clone employed was PC12-15. Those employed in the studies summarized in Fig. 1A were the following: 5a, 10, 11, 12, 16a, 18, 29, 37 and 64 (Clementi et al., 1992). Monkey kidney CV-1 fibroblasts and mouse mammary adenocarcinoma TS/A cells stably expressing human CgA (Colombo et al., 2002) were a kind gift of F. Blasi and A. Corti (San Raffaele Scientific Institute, Milan), respectively.

Antibodies

The anti-CgA antibodies employed were the rabbit GE-19 antiserum (Bauer et al., 2000) and the mouse monoclonal LK2H10 (NeoMarkers, Fremont, CA, USA); the anti-CgBs were the rabbit PE11 antiserum (Kroesen et al., 1996) and the mouse monoclonal CIRO raised in our laboratory. The latter was characterised by western blotting and immunofluorescence and found to be highly specific. Anti-giantin was a gift of H. P. Hauri, anti-lamp3 and anti-lamp1 of C. Valetti; anti-TGN38 was from Affinity Bioreagents (Golden, CO, USA). The monoclonal anti-myc was purified from the 9E10 hybridoma line.

Western blots, immunocytochemistry and image analysis

Western blots, immunofluorescence cytochemistry and confocal microscopy were performed essentially as described by Borgonovo et al. (Borgonovo et al., 2002). Confocal images from antisense PC12 clones shown in the figures were acquired under fixed conditions for all clones with a Leica TCS-SP2 confocal microscope. The laser was set at 30% for CgA and at 10% for CgB. Three-dimensional reconstructions of series of confocal sections were performed with the Leica confocal software.

Electron microscopy was performed on glutaraldehyde-OsO4 fixed cells as described by Gatti et al. (Gatti et al., 1997). Morphometric analysis of the ultrastructural images was performed as recommended by Weibel (Weibel, 1969).

Plasmids, constructs and transfections

The two CgA antisense cDNA sequences, the first targeting the start codon and part of the upstream 5'-UTR, the second the N-terminal coding region of the rat CgA gene, were as described by Kim et al. (Kim et al., 2001). Stable antisense clones were selected in 500 μg/ml and maintained in 300 μg/ml of hygromycin. The transfection of the empty pcDNA3.1 hygro vector served as negative control. The coding region of the human CgA was recloned from pRS1Neo-CgA (Colombo et al., 2002) into pEXp1-puro (Clontech, Palo Alto, CA, USA). For the generation of stable CV-1 and PC12-64 expressing human CgA, cells were transfected with 4 μg of DNA in 10 μl LipofectAmine™ 2000. Transfected cells were selected in 10 μg/ml of puromycin. The coding region including the Kozak sequence of the rat CgA was obtained by RT-PCR from a library of PC12 cells prepared in our laboratory, cloned into pcDNA3.1/myc- His(−) C vector (Invitrogen) and confirmed by sequencing and in vitro transcription/translation (TNT® System, Promega) with Redivue™ L-[35S] methionine (>1000 Ci/mmol; Amersham Biosciences, Aylesbury, UK). Transient transfections of human CgA and rat CgA-myc-His were performed as described above.

Pulse-chase experiments

CV-1 cells expressing human CgA were seeded at 0.75x10⁶ cells per 6 cm ø petri dishes. Subconfluent PC12-15 clones were transiently transfected with the human CgA plasmid in 10 cm ø dishes as described above (20% efficiency), and after 5 hours 1x10⁶ cells were seeded in 3.5 cm ø petri dishes. Both CV-1 and PC12-15 expressing human CgA were pulse labelled for 1 hour in met/cys-free DMEM supplemented with 10 mM Hepes, pH 7.4 and 166 μCi Redivue™ L-
Results and Discussion

Dense-core granules of neurosecretory cells

In order to investigate whether, in a neurosecretory cell, DCGs are all characterised by a similar composition, we focused our attention on CgA and CgB, two cargo proteins released by PC12. Because, however, the PC12 line (wild-type cells) is known to be highly heterogeneous (e.g. Bitler et al., 1986; Clementi et al., 1992; Grundsohner et al., 2002; Pance et al., 1999; Shoji-Kasai et al., 1992), composed by cells largely different from each other also in terms of granule number, these studies were performed in 10 clones isolated therefrom and extensively characterised (Clementi et al., 1992), one of which, PC12-15, whose CgA and CgB levels are close to the heterogeneous wild-type PC12 line, was used as the reference clone. Fig. 1A summarizes the quantitative western blot results obtained with the clones, normalised to the reference clone 15. As can be seen, in only four clones the levels of both granins were close to the reference, whereas in one more they were similarly decreased (~15-20%). In the other four clones the levels of CgA were the highest of two granins, whereas in the others richer in CgB, not only in the reference PC12-15 clone (not shown) but also in the subclones transfected with the empty vector (Fig. 2V, V'). At high magnification it was clear that even within single cells only some of the granules (or groups of them) exhibited similar levels of the two granins, whereas in the others a predominance of either one occurred (Fig. 2V'). In the antisense-transfected subclones A, L and especially O of Fig. 2, the low, or even very low level of CgA observed in most cells was not accompanied by a parallel low level of CgB. Therefore, the CgA/CgB ratio approached that of controls in only few cells (compare the cell marked by an arrowhead in Fig. 2L to the cells in Fig. 2V), whereas in the majority of the cells this ratio was clearly lower, with some showing only traces of CgA together with CgB at or above the levels of the reference clone (arrows in Fig. 2L and Fig. 2O). From these data we conclude that in the various CgA antisense-transfected clones the levels of CgA and CgB appear largely unrelated to each other.

A second issue investigated was whether a rise of CgA could induce an increase of DCG number in neurosecretory cells comparatively poor of these organelles. For these experiments we used one of the PC12 clones, clone 64, known to share the typical properties of the parental line, however, with a density of granules (0.46/μm², as revealed by ultrastructural morphometry; number of analysed cell profiles: 42; total cytoplasmic area: 740 μm²) and levels of granins (Fig. 3D) only one fifth of the wild-type PC12 and the reference clone 15 (see also Clementi et al., 1992). Transfection experiments were performed using the cDNA of human CgA, taking advantage of an antibody (LK2H10, a monoclonal anti-human CgA) recognizing the human protein without any cross-reaction with the endogenous rat protein. In addition, experiments were performed by transfecting a myc-tagged rat CgA, recognized by both the GE-19 antiserum and the antmyc 9E10 antibody. In the PC12-64 clone the stable expression of exogenous CgA resulted in considerable increases of the total granin (~4-fold or more), approaching the levels of the reference clone PC12-15. However, neither the levels of CgB (Fig. 3D) nor the number of DCGs (0.5/μm²; number of analysed cell profiles: 51; total cytoplasmic area: 1090 μm²) were increased in the transfected cells. Similar results were obtained in a population of PC12-64 cells transfected transiently with the myc-tagged rat CgA (transfection efficiency ~50%). The dissociation between CgA and DCG number is not surprising because in PC12 cells the granin is not the major cargo component of the organelle (Grumolato et al., 2003). Unless accompanied by the parallel increase of other
granule proteins, even large increases of CgA were not able to induce appreciable changes in the granule mass and number.

Based on these data we conclude that, at least in terms of granins, the molecular composition of DCGs can be highly variable, apparently independent of the expression levels of CgA. This conclusion emerged both from the study of clones isolated from the parent PC12 line and subclones isolated from PC12-15 following transfection with CgA antisense sequences. In both models the levels of CgA and CgB were highly variable and exhibited no statistically significant correlation.

Expression of CgA and CgB in non-secretory cells
This set of experiments was performed to investigate the intracellular distribution of the granins when transfected in three types of non-secretory cells, i.e. two lines, CV-1 fibroblasts and adenocarcinoma TS/A, and a PC12 clone, PC12-27, completely defective in neurosecretion (see Clementi et al., 1992; Malosio et al., 1999). The latter clone, which still expresses many properties typical of its neurosecretory line of origin (Grundschober et al., 2002; Malosio et al., 1999), was used to complement the results obtained with cells of totally different nature.

In previous studies on transfected CV-1 and Vero cells, the major localisation of CgA and CgB had been reported in the perinuclear GC/TGN area (Kim et al., 2001; Rustom et al., 2002). In addition, also discrete granin-positive puncta were reported to appear, however only in a fraction of the transfected cells. In the case of CgA, and based on general ultrastructure, the corresponding organelles were defined as DCGs (Kim et al., 2001). With CgB, in contrast, the size of the organelles was large, and the DCG definition was proposed in one (Huh et al., 2003) but not in another (Rustom et al., 2002) case. In the present study the concentration of the two granins in the CG/TGN area was confirmed by co-labelling with specific markers, i.e. giantin and TGN38 (not shown). With regard to the discrete CgA-positive organelles, although appearance in transfected cells was convincing, their nature remained unclear. In our opinion, in fact, their identification as DCGs required several issues to be investigated (see also Day and Gorr, 2002). Among these are: (i) whether or not expression of CgA in non-secretory cells switches on the expression of other granule proteins; and (ii) whether the discrete organelles are bona fide secretory granules or have a different specificity.

Experiments were performed to answer the above questions. As can be seen in Fig. 3C, CgA-transfected CV-1 fibroblasts failed to show any signal for CgB. Similar results were obtained with TS/A cells and with the secretion-defective PC12-27 clone (not shown). Thus, expression of CgA in these cells does not switch on the expression of other DCG proteins. Even more interesting (see Fig. 3A,B), a large proportion of

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**Fig. 2.** Distribution of CgA and CgB in the cells of subclones isolated from the PC12-15 clone after stable transfection with CgA antisense sequences. The confocal images of CgA (GE-19 antiserum, red) are shown in the left panels; those of CgB (monoclonal CIRO, green) are in the middle; the merged images are to the right. Subclone V was transfected with the empty vector; V’ is a close-up view of cells showing non-parallel distribution of CgA and CgB in cytoplasmic granules or groups of them. A,L,O were transfected with CgA antisense sequences. Arrows indicate cells very low in CgA where CgB is in contrast at control levels or above. A cell with CgA levels similar to controls and poor in CgB is indicated by the short arrow. Scale bar: 8 μm.
accompanied by a decrease of the endogenous granin. The PC12-64 cells expression of the transfected CgA is (70-63 kDa), whereas LK2H10 recognizes hCgA only. In rCgA (86-100 kDa) and, to a minor extent, also hCgA monoclonal CIRO). Notice that GE-19 recognizes both PE-11 (c; similar results were obtained with the LK2H10 (b); and with the polyclonal anti-CgB antiserum GE-19 (a); with the monoclonal anti-hCgA antibody immunostained with the polyclonal anti-CgA antiserum PC12-64; hCgA CV -1). The same blot was analysed after stable transfection with human CgA (hCgA CV -1) endogenous CgA, some of which were also lines expressing (PC12-15; PC12-64) and not expressing (CV-1) endogenous CgA, some of which were also analysed after stable transfection with human CgA (hCgA PC12-64; hCgA CV -1). The same blot was immunostained with the polyclonal anti-CgA antiserum GE-19 (a); with the monoclonal anti-hCgA antibody LK2H10 (b); and with the polyclonal anti-CgB antiserum PE-11 (c; similar results were obtained with the monoclonal CIRO). Notice that GE-19 recognizes both rCgA (86-100 kDa) and, to a minor extent, also hCgA (70-63 kDa), whereas LK2H10 recognizes hCgA only. In PC12-64 cells expression of the transfected CgA is accompanied by a decrease of the endogenous granin. M	extsubscript{r} of protein standards is indicated to the right in kDa.

the discrete CgA-positive puncta observed in transfected CV-1 cells (218 out of 314 observed in 24 cells, i.e. 70%) were also positive for lamp3 (Fig. 3B), a well-known lysosomal/late endosomal marker, and an approximately similar fraction was positive for the lysosomal marker, lamp1 (Fig. 3A). These results strongly suggest that the nature of most CgA-rich organelles is not that of DCGs but of lysosomes. Moreover, the CgA-positive puncta negative for lamp3 and lamp1 could be vesicles destined to be discharged constitutively and/or post-TGN vesicles on their way to the late endosomal/lysosomal compartment.

Because not only granules, but also lysosomes are organelles competent for regulated secretion (Jaiswal et al., 2002; Reddy et al., 2001), stimulated release experiments appeared inappropriate to identify CgA-positive puncta as either one of these organelles. Of great significance, in contrast, were the results of CgA turnover experiments. Within bona fide DCGs of unstimulated PC12, cells granins are stored for long times before release, and this could occur also in lysosomes before proteolysis. In contrast, in constitutive vesicles, turnover is expected to be rapid because of uninterrupted release. The turnover of CgA transfected in non-secretory cells and in bona fide PC12 was investigated by two approaches. First, resting cells were analysed by confocal immunocytochemistry before and after 1-3 hours of incubation with the protein synthesis inhibitor, cycloheximide, applied to prevent accumulation of newly synthesized granins. Wild-type PC12 and clone 15 cells, when incubated with the protein synthesis inhibitor, exhibited no appreciable change of the levels and intracellular distribution of CgA (Fig. 4A,B) and CgB (not shown) as revealed by 3D reconstructions of confocal images. In contrast, clear changes were observed in transfected CV-1. Already after 1 hour the signal of both the GC/TGN area and discrete puncta was greatly decreased (not shown); after 3 hours only a trace remained appreciable (see Fig. 4C,D for CgA). Similar results were obtained with the non-secretory TS/A cells and with the neurosecretion-defective PC12 clone, PC12-27 (not shown).

PC12-15 and CgA-transfected CV-1 cells were also pulse-labelled with 35S-methionine for 1 hour and the release of radioactive CgA into the medium was monitored over a period of 0.5-3 hours. With PC12-15 almost all radioactive CgA (99%) was recovered in the cells throughout the experiment (not shown). In contrast, with CV-1 the recovery in the medium was almost 50% at 30 minutes and over 80% at 3 hours (Fig. 4E). The results of both types of turnover experiments, therefore, concur with those of expression experiments to indicate that release of CgA from transfected non-secretory cells occurs primarily by constitutive exocytosis. The small fraction remaining with the cells could be segregated into lysosomes, destined to be proteolysed with slower turnover.

Although not reported so far, the appearance of granin-positive lysosomes in transfected non-secretory cells might not be surprising. Assembly of granules in PC12 cells is believed to start by Ca2+- and pH-dependent aggregation of granins in the TGN lumen (Chanat and Hutner, 1991; Cowley et al., 2000; Gerdes et al., 1989; Gorr et al., 1989), for which the disulfide bonds of CgB (Chanat et al., 1993) and of the N-terminal domain of CgA (Cowley et al., 2000) appear necessary. From the TGN, aggregate cores might be addressed to granules by a process defined as ‘sorting by retention’ (Dannies, 1999; Thiele et al., 1997; Tooze, 1998). In non-secretory cells a fraction of the transfected granin might be sorted by this or a similar aggregation process (Cowley et al.,
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appear in non-secretory cells after transfection of the proteins appear to be mostly lysosomes in nature, and not bona fide DCGs. The latter, therefore, appear specific of the neurosecretory cell phenotype, generated in the framework of a complex gene programme which seems to regulate the expression of all proteins needed not only for the structure of these organelles, but also for their exocytic release (see Malosio et al., 1999).

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Does CgA have a role in DCG biogenesis?


