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Overall Lack of Regulated Secretion in a PC12 Variant Cell Clone*

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A stable clone of PC12 neuroendocrine cells, named 27, known from previous studies to exhibit a defect of regulated secretion (lack of regulated secretory proteins, of synaptophysin, of dense granules and of catecholamine uptake and release; Clementi, E., Racchetti, G., Zacchetti, D., Panzeri, M. C., and Meldolesi, J. (1992) *Eur. J. Neurosci.* 4, 944–953) was characterized in detail to clarify the nature of its phenotype and the mechanisms of its establishment. The neuroendocrine nature of the PC12-27 phenotype was documented by specific markers: synapsins, neurofilament subunit H, neuronal kinesin, and α-latrotoxin receptor. Moreover, various intracellular membrane systems of PC12-27, including the endoplasmic reticulum and the Golgi complex, appeared similar to control PC12 in both morphology and marker expression. In contrast, all the investigated markers located either in dense granules (dopamine-β-hydroxylase), in synaptic-like microvesicles (the acetylcholine transporter) or in both these regulated secretory organelles (VAMP2/synaptobrevin-2, synaptotagmin) were missing in PC12-27 cells, and the same was true also for the cytosolic and plasmalemma proteins involved in regulated exocytosis (Rab3, SNAP25, syntaxin). Pulse labeling and *in vitro* translation experiments revealed the defect to consist in a protein synthesis blockade that mRNA studies (reverse transcription-polymerase chain reaction, Northern blotting, and actinomycin D experiments) revealed to take place primarily at the transcriptional level. The secretion defect of PC12-27 cells was modified neither by various types of long term stimulation nor by nerve growth factor treatment. Moreover, when one of the missing regulated secretory proteins, chromogranin B, was expressed by cDNA transfection, it was secreted, however via the constitutive pathway. Our results demonstrate that PC12-27 cells are fully incompetent for both branches of regulated secretion, those of dense granules and synaptic-like microvesicles, possibly because of the impairment of a general expression control system that appears to operate independently of neuroendocrine cell differentiation.

Regulated secretion is the process, active in numerous types of eukaryotic cells, by which quantal release of specific products takes place in response to appropriate stimuli (1–4). In neurons and neurosecretory cells, regulated secretion occurs via two types of organelles: dense granules, formed from the trans-Golgi network (TGN)[1] (Refs. 1–4 and references therein), and synaptic (or synaptic-like) microvesicles, continuously recycled from the plasmalemma and endosomes (Refs. 4–6, and references therein). Both types of regulated secretory processes include two principal steps, the biogenesis of granules and vesicles from their respective donor compartments and their docking/fusion with the plasma membrane (1–9).

Due to its fundamental physiological importance, regulated secretion is among the processes most intensely investigated. Granule biogenesis is known to be initiated in the TGN lumen (for reviews, see Refs. 2 and 3) by the selective aggregation of regulated secretory proteins (10–14). Such an aggregation, which for some regulated secretory proteins depends on the pH and *p*Ca (13, 14), yields dense cores that soon become membrane-enwrapped, possibly because of their binding to specific sites concentrated in discrete areas of the TGN membrane (1, 3, 15). Subsequently, the membrane-enveloped dense cores bud off, thus giving rise to immature secretory granules (Refs. 2 and 3, and references therein). The latter step is regulated, both positively and negatively, by heterotrimeric G proteins (Ref. 15 and references therein) and involves also the ADP-ribosylation factor, a small GTP-binding protein, and the phosphatidylinositol transfer protein (16–18).

Among the secretory cell types in which the above sequence of events has been established is the rat pheochromocytoma line, PC12, where two major regulated secretory proteins, chromogranin B and secretogranin II (CgB and SgII; Refs. 19 and 20), are stored within secretory granules (21, 22) together with various peptides, ATP, and catecholamines (23). PC12 cells also contain the second class of regulated secretory organelles, the synaptic-like microvesicles. These organelles are the neuroendocrine counterpart of synaptic vesicles inasmuch as they contain the same distinct set of membrane proteins (4–6). The classical neurotransmitter taken up, stored, and released upon stimulation by PC12 synaptic-like microvesicles is acetylcholine (24).

Due to the above properties, PC12 cells have been considered since their isolation as a valuable model for neurosecretion

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[1] The abbreviations used are: TGN, trans-Golgi network; ER, endoplasmic reticulum; CgB, chromogranin B; hCgB, human CgB; SgII, secretogranin II; SNAP25, synaptosomal-associated proteins of 25 kDa; PC, proprotein convertase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); RT-PCR, reverse transcription-polymerase chain reaction.

studies. Recently, their use has become even more advantageous because clonal variants have been isolated (25, 26). One of these clones, referred to as 27, was shown in a previous report from one of our laboratories to be devoid of both regulated secretory proteins and secretory granules, and to be also incompetent for catecholamine uptake, storage, and release (26). Up until now, however, it has remained unclear whether these features of the clone were due to an overall de-differentiation, with appearance of properties of pre-neuroendocrine stem cells, or to the selective loss of the regulated secretion activity from cells otherwise maintaining a frank neuroendocrine phenotype. The experimental evidence reported in the present study demonstrates the following conclusions: i) the regulated secretion-defective PC12-27 cells express markers typical of PC12 neuroendocrine cells; ii) their defect consists in the lack of expression of many (if not all) components of secretory organelles (granules and vesicles), of the cytosol, and of the plasmalemma (tSNAREs) participating in regulated exocytosis; iii) their constitutive secretion is fully maintained; and iv) their defect is sustained not by deletion of the genes encoding the granule/vesicle proteins but by an inhibitory mechanism operating primarily at the transcriptional level.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—[^{35}S]sulfate and Trans^{35}S-label were purchased from Amersham International (Buckinghamshire, UK) and ICN Pharmaceuticals (Irvine, CA), respectively; the expression vector pREP4 from Invitrogen (San Diego, CA); fura-2 from Calbiochem, (San Diego, CA); α-latrotoxin was purified to homogeneity as described (27). The monoclonal and polyclonal antibodies against human CgB (hCgB) were raised as described previously (28). Other antisera and monoclonal antibodies used in this study were kindly provided by the following scientists: anti-mannosidase II by M. G. Farquhar (29), anti-TGN38 by G. Banting (30), anti-βCOP by T. Kreis (31); anti-Rab6 and anti-Rab3A by A. Tavitian (32, 33), anti-dopamine-β-hydroxylase by D. Apps (34), anti-vesicular acetylcholine transporter by J. Mallet (35), anti-VAMP2/synaptobrevin-2 and anti-syntaxin 1 by O. Rossetto and C. Montecucco (36, 37), anti-synaptosome-associated protein of 25 kDa (SNAP25) by P. De Camilli (38), anti-synaptotagmin I by A. Malgaroli (39), anti-synapsins (40) by F. Valtorta, anti-proprotein convertases (PC1/3 and PC2) by K. Docherty (41), and anti-neuronal isoform of kinesin by F. Navone and R. Vale (42). The anti-neurofilament H-subunit and the anti-tyrosine hydroxylase antibodies were purchased from Boehringer (Mannheim, Germany) and Chemicon International Inc. (Temecula, CA), respectively. Donkey anti-mouse IgGs coupled to rhodamine, donkey anti-rabbit IgGs coupled to fluorescein, and goat anti-rabbit or anti-mouse IgGs conjugated to peroxidase were obtained from Jackson Immunoresearch Laboratories (West Grove, PA); anti-rabbit IgG-gold particles (5 nm) were purchased from Bio Cell Research Laboratory (Cardiff, UK).

Cell Culture, Subcellular Fractionation, and Immunoblotting—PC12-27 (26) and control PC12 cells (clones 251 (43) and 1 (26)) were grown in Dulbecco's modified Eagle's medium (H-21) supplemented with 10% horse serum and 5% fetal calf serum in 10% CO_2. For the separation of nuclei from the cytoplasm, monolayers were detached and cells homogenized by 10 passages in a Dounce apparatus while resuspended in 0.3 M sucrose. The concentration of sucrose was then brought to 1.8 M by mixing with a 2.5 M solution, and the preparation was layered over a 2 M cushion and covered with a 1.6 M cushion of sucrose in a centrifuge tube (Beckman SW41 rotor), which was filled with 0.3 M solution. After centrifugation for 2 h at 35,000 rpm, nuclei were recovered in the pellet, the cytosol in the loading cushion and microsomal membranes in the 1.6 M cushion, up to its interface with the 0.3 M sucrose. The proteins of various preparations: rat brain total homogenates, Tween 20 extracts of PC12 cells, and Triton X-100 extracts of PC12 cell crude membranes, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting as described (21). Briefly, blotted nitrocellulose filters were incubated for 18 h at 4 °C in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5% fat-free milk powder, followed by an additional incubation for 2 h at room temperature in the same buffer containing 0.3% Tween 20 and the appropriately diluted antibodies. After several washes, the filters were incubated with peroxidase conjugated to goat anti-rabbit or anti-mouse IgG antibodies followed by treatment with the ECL detection reagents (Amersham), carried out as recommended by the manufacturer.

$[Ca^{2+}]_i$ Measurements—Cell monolayers were detached from Petri dishes by a gentle flow of Krebs-Ringer Hepes medium containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose, 25 mM Hepes-NaOH (pH 7.4). Cell suspensions were gently washed three times by centrifugation, resuspended, and incubated for 30 min at 37 °C in Krebs-Ringer Hepes medium supplemented with the Ca^{2+}-sensitive dye, fura-2, administered as acetoxymethylester at the final concentration of 5 μM. Cell aliquots (4×10^6 cells) were finally transferred to a thermostatted cuvette (37 °C) in a Perkin Elmer LS-5B fluorimeter, where they were maintained under continuous stirring, and analyzed as described (44). Traces are representative of results obtained in three different experiments.

RNA Extraction, RT-PCR, Northern Blotting, and Nuclear Run-on—RNA extraction was performed according to the single-step method of Chomczynski and Sacchi (45) on cells grown in standard conditions or treated with 5 μg/ml actinomycin D, for 6, 12, 18, and 24 h. For RT-PCR amplification of cDNA, the PCR primers were as follows (forward and reverse): 5′-GGTGAGTGCCAGTCATCC-3′ (position 528–545 bp) and 5′-GCCACTCCTCGGTCACAT-3′ (1286–1303 bp) for synaptotagmin I (EMBL data base sequence X52772, 1990), 5′-CCAGACCTCAGATTGGAGAA-3′ (148–167 bp) and 5′CCGACCACATCTTCATAGG-3′ (755–773 bp) for SgII (X13618, 1988), 5′-GCTAC-CGCTGCCACCGTC-3′ (27–44 bp) and 5′-GGTGATGGGAACCTCAGGAA-3′ (604–623 bp) for VAMP2 (M24105, 1989), 5′-CCTTCAGGCTGCACCAGT-3′ (244–262 bp), and 5′-GCTGGCTGCCCGTAATCG-3′ (854–871 bp) for synaptophysin (X06388, 1987). First strand cDNA synthesis was conducted using an oligo(dT) primer according to the standard protocol (46). The RT-PCR products were obtained after 35 cycles of the following conditions: denaturation for 1 min at 94 °C, annealing for 30 s at 61 °C, and extension for 45 s at 72 °C using 1 unit of *Taq* polymerase/30 μl (Perkin Elmer, Ampli-*Taq* kit). For Northern blotting, probes were prepared from RT-PCR fragments isolated from polyacrylamide gel by the "crush and soak" method (46), and labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Redivue, Amersham) by random-priming oligolabeling (47). 10 μg of total RNA of each PC12 clone were separated on 1% agarose, 2.2 M formaldehyde denaturing gel and transferred onto nylon membranes (Hybond-N Amersham). Hybridizations were carried out at 65 °C in a mix containing 125 mM Na_2HPO_4, 1 mM EDTA, 250 mM NaCl, 7% SDS, 10% polyethylene glycol, 1% bovine serum albumin, 100 μg/ml denatured salmon sperm DNA, and 10^6 cpm/ml radiolabeled probe. Washes were performed at 65 °C first in 2 × SSC and 0.1% SDS, then twice in 0.1 × SSC and 0.1% SDS for 20 min before film exposure. Northern blot signals were normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA detected as described (48). The transcription rate of the SgII gene was evaluated by run-on experiments carried out with nuclei from PC12-27 and control cells, processed as described in Ref. 49, using the specific cDNA.

Labeling and Stimulation of the Cells—Control cell clones and subclones were grown in 24-well plates until near confluence. The cells were then labeled overnight with 100 μCi/ml [^{35}S]sulfate as described (50). For long term stimulations, before their labeling with [^{35}S]sulfate, the cells were treated for up to 4 days with either 55 mM KCl, 20 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or 1 mM dibutyryl-cAMP. For pulse and pulse-chase experiments the cells were labeled either for 7 min with 500 μCi/ml Trans ^{35}S-label or for 15 min with 300 μCi/ml [^{35}S]sulfate and then chased for 0, 10, 20, and 60 min in a medium containing 1.6 mM Na_2SO_4. For the analyses of regulated secretion, PC12-251 and -27 cells were labeled for either 3 h or 15 min, then washed and transferred to prewarmed medium containing 5 or 55 mM KCl. After a 10-min incubation, the media were collected, cleared by centrifugation in an Eppendorf centrifuge at 13,000 rpm at 4 °C, and stored at −20 °C after the addition of a protease inhibitor mixture (5 μg/ml aprotinin, 4 μg/ml pepstatin, and 2 μg/ml bestatin). The cells were solubilized in ice-cold 20 mM Tris-HCl, pH 7.4, additioned with 10 mM EDTA, 0.3% Tween 20, 150 mM NaCl, and supplemented with 0.5 mM phenylmethylsulfonyl fluoride and with pepstatin plus bestatin (2 μg/ml each). Before use, the cell lysates were centrifuged in an Eppendorf centrifuge at 13,000 rpm for 3 min at 4 °C.

hCgB Expression Vector and Gene Transfer—The cDNA encoding hCgB (51) was cloned downstream of the Rous sarcoma virus promoter inside a pREP4 vector carrying the hygromycin B resistance gene, using standard procedures (46). Transfection of PC12-27 was performed by electroporation using a Gene Pulser transfection apparatus (Bio-Rad Laboratories, Milan, Italy). Cell suspensions (~4×10^6 cells/ml) were incubated at room temperature with 25 μg of hCgBpREP4-DNA and then electroporated with a capacitor discharge of 250 V and 960 micro-

farads. Cells were then diluted to 1×10^5/ml in Dulbecco's modified Eagle's medium supplemented with serum and plated in 4×10-cm dishes (1×10^6 cells/dish). The selection of hygromycin B-resistant cells was started 48 h after the electroporation using 450 μg/ml antibiotic. After 15 days, individual clones were transferred into 24-well plates and grown to sufficient density to screen for hCgB (see below). The stably transfected clones were cultured in the continuous presence of hygromycin B (450 μg/ml).

Immunoprecipitation—Cleared cell lysates and media were diluted in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 5 mM EDTA, containing either 0.3% or 1% Triton X-100) and incubated overnight at 4 °C with the appropriate concentrations of specific antisera followed by incubation for 1 h with 15 μl (packaged gel) of protein A conjugated to Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden). The immunoprecipitates thus obtained, as well as aliquots of the cleared cell lysates and media, were analyzed by SDS-PAGE followed by fluorography, as described previously (21). Fluorograms were quantitated by densitometric scanning using an LKB Ultroscan (Pharmacia). Values are expressed as the percent of the hCgB or heparan sulfate proteoglycan present in the cleared cell lysates at the end of the 15-min pulse.

Immunofluorescence and Electron Microscopy—PC12 cells were plated onto poly-L-lysine-coated coverslips, grown for 2–4 days, and then analyzed by indirect immunofluorescence (22). For electron microscopy, cell monolayers were washed in phosphate-buffered saline, fixed for 30 min at 4 °C with 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3), post-fixed in 2% OsO_4, scraped from the dishes, pelleted, dehydrated, and embedded in Epon as described (52). Thin sections, cut using an Ultra-Cut "E" microtome (Reichert-Jung, Wien, Austria), were examined with a Philips 400 electron microscope. For immunoelectron microscopy the cells, fixed with aldehydes as above, were scraped from the dishes and sedimented. The pellets were included in 10% gelatine, fixed for an additional 60 min, and then sliced into small pieces, which were passed through increasingly concentrated sucrose solutions (up to 2.3 M) in phosphate-buffered saline, and finally frozen in liquid nitrogen. Ultrathin cryosections were cut at −100 °C using an Ultra-Cut "E" microtome equipped with an FC4 cryochamber, transferred to Formvar-coated nickel grids, and finally immunolabeled as described previously (28), using an antiserum directed against the C terminus of hCgB revealed by an anti-rabbit IgG conjugated to 5-nm colloidal gold particles.

RESULTS

The Clone 27 Cells Exhibit an Overall Defect of Regulated Secretion—In a previous study (26), the 27 clonal variant of PC12 cells was shown to lack secretory granules as well as CgB and SgII, the major regulated secretory proteins of PC12 cells (21). To study in more detail the extent of the defect, expression of known protein markers for various organelles and functions in neuroendocrine cells was investigated. Fig. 1 shows Western blots of total homogenates prepared from the defective clone PC12-27 and from the control clone 251, which exhibits the typical PC12 phenotype. *Panel A* is focused on proteins active in the Golgi area. βCOP, a well characterized member of the protein complex forming the coats of the vesicles budding from the ER and the Golgi cisternae (see Ref. 7, and references therein), was revealed at similar levels in both the 27 and 251 clones. Likewise, Rab6, a small GTP-binding protein thought to be involved in the regulation of constitutive vesicle transport from the TGN (53), and mannosidase II, an integral membrane protein of Golgi stacks, were immunodetected in both clones, although their levels in clone 27 appeared lower than those detected in clone 251 (Fig. 1*A*). Parallel immunofluorescence experiments, carried out using antibodies against mannosidase II and TGN38, an integral membrane protein of the TGN, suggested a normal organization of the Golgi complex (data not shown). After treatment with brefeldin A, the two markers redistributed similarly in PC12-251 and -27 cells, becoming colocalized with ER and endosomal markers, respectively (data not shown).

Completely different results between clones 27 and 251 emerged when we examined the expression of components specific for one of the secretory organelles (granules or synaptic like microvesicles), common to both these organelles or participating in their exocytosis. As shown in Fig. 1*B*, dopamine-β-hydroxylase, the noradrenaline synthesizing enzyme located at the luminal surface of the granule membrane, and the vesicular acetylcholine transporter, a trans-membrane protein exclusively localized in the synaptic-like microvesicles (35), were both undetectable in PC12-27 cells but clearly expressed in control cells. Since recent results have demonstrated that in PC12 cells (36, 38) the docking and fusion of both synaptic-like microvesicles and secretory granules involves a number of identified proteins that are present in or interact with the vesicles and the plasma membranes (see Refs. 7–9, and references therein), the expression of VAMP2, synaptotagmin I, SNAP25, syntaxin, and the small GTP-binding protein Rab3 was also examined. Fig. 1*B* shows that none of these proteins was detected in clone 27, whereas they were all abundant in rat brain and present in considerable amounts in PC12-251 cells.

Further experiments were focused on the expression of non-granular proteins specific for neurons and neurosecretory cells. Blots shown in Fig. 1*C* demonstrate that tyrosine hydroxylase, the cytosolic rate-limiting enzyme for catecholamine synthesis, is abundant in 251 and scarce, yet clearly appreciable, in PC12-27 cells. Similarly, synapsin I and IIa, the members of a peripheral membrane protein family known to establish a con-

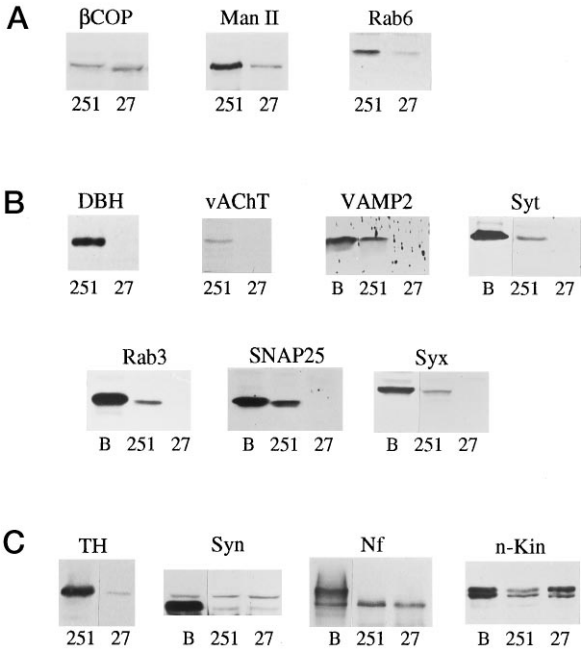

FIG. 1. **Expression of various markers in PC12 clones.** *A*, Western blots of proteins (40 μg each) from total homogenates of PC12-251 (*251*) and PC12-27 cells (*27*) were immunolabeled using antibodies against βCOP (ascites, diluted 1/1000), mannosidase II (*Man II*, diluted 1/1,000) or Rab6 (diluted 1/1,000). *B*, Western blots of proteins from total homogenates of rat brain (*B*, 16 μg), homogenates (*Rab3*, 40 μg) and extracts of crude membrane fractions prepared from PC12-251 and -27 cells (all other markers, 50 μg) were immunostained using the following antisera: anti-dopamine-β-hydroxylase (*DBH*, diluted 1/1,000), anti-vesicular acetylcholine transporter (*VAChT*, diluted 1/15,000), anti-VAMP2 (*VAMP2*, diluted 1/500), anti-synaptotagmin I (*Syt*, diluted 1/1,000), anti-Rab3 (*Rab3*, diluted 1/1,000), anti-SNAP25 (*SNAP25*, diluted 1/250), and anti-syntaxin 1 (*Syx*, diluted 1/500). *C*, the proteins from total homogenates of rat brain (*B*, 16 μg) or PC12-251 and -27 cells (40 μg) were immunolabeled with anti-tyrosine hydroxylase antiserum (*TH*, diluted 1/2,000) anti-synapsin antiserum (*Syn*, diluted 1/5,000), anti-neurofilament H subunit antibodies (*Nf*, 5 μg/ml); and anti-neuronal kinesin antibodies (*n-Kin*, diluted 1/300). The Western blots shown are representative of at least three consistent experiments.

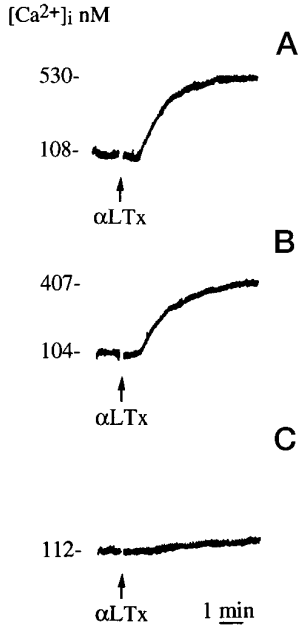

FIG. 2. **Cytosolic *p*Ca effects of α-latrotoxin in PC12 clones and chromaffin cells.** The traces (representative of two experiments) were recorded from suspensions of PC12-251 (*A*), PC12-27 (*B*), and bovine chromaffin (*C*) cells loaded with fura-2 and incubated in the medium containing 2 mM Ca^{2+}. α-Latrotoxin (*αLTx*, *arrows*) was added at the final concentration of 1 nM.

nection between synaptic vesicles and the cytoskeleton (54), were clearly detectable in both PC12 clones, although at levels lower than in the brain. Of the two neurofilament H subunit bands, one corresponding to the fully phosphorylated and the other to the dephosphorylated form (55), PC12 cells exhibited the latter, with no major difference between the two investigated clones. Similar results were obtained with the neuronal isoform of kinesin (42), whose doublet of 120–130 kDa was abundant in the brain and clearly expressed in the two clones (Fig. 1*C*). Another specific marker investigated was the receptor for α-latrotoxin, present in the plasma membrane of presynaptic terminals and found also on typical PC12 cells, whereas other cells, including bovine chromaffin and non-nerve cells, are devoid of it (56). Among the effects of receptor activation is a robust rise of the cytosolic *p*Ca sustained by Ca^{2+} influx, which begins after a few seconds of delay and persists thereafter. Fig. 2 shows that the *p*Ca responses induced by α-latrotoxin in the two PC12 clones, 251 and 27, were very similar, whereas bovine chromaffin cells remained unresponsive.

To further characterize the defect of clone 27, cells were labeled with either [^{3}H]tyrosine or [^{35}S]sulfate, and the total homogenates or the heat-stable supernatants (a preparation known to be enriched in CgB and SgII; Ref. 21) were analyzed by fluorography of SDS-PAGE (Fig. 3*A*). As a whole, the pattern of total proteins labeled with [^{3}H]tyrosine appeared similar in the 251 and 27 clones. However, only clone 251 revealed the typical bands of the two granins that were enriched in the heat-stable supernatants and constituted the major [^{35}S]sulfate-labeled proteins. In contrast, and consistent with the previous study carried out by Western blotting (26), clone 27 cells, even when exposed overnight to the radioactive precursors, remained negative for CgB and SgII (Fig. 3*A*). At variance with the granins, various sulfated macromolecules were detected in the fluorograms of [^{35}S]sulfate-labeled PC12-27 cells, documenting the presence of sulfotransferases in this clone and indicating that the absence of [^{35}S]sulfate-labeled granins was not due to the absence of the relevant enzymatic activity.

In order to investigate whether the above negative results in PC12-27 cells were due entirely to a block of protein synthesis or whether an accelerated turnover contributed to a rapid degradation of newly synthesized proteins, short (7 min) pulse labeling experiments with [^{35}S]methionine were performed (Fig. 3*B*). Even under these conditions both SgII (Fig. 3*B*) and CgB (data not shown) were prominent in the immunoprecipitates from the 251 clone but remained inappreciable in those of clone 27. Similar results were obtained by *in vitro* translation experiments, in which equal amounts of mRNA extracted from the two PC12 clones were expressed in the rabbit reticulocyte system. Immunoprecipitation of the lysates using anti-SgII antibodies revealed an abundant synthesis of the granin with the clone 251 mRNA, whereas with the clone 27 mRNA no synthesis was detected (data not shown).

In previous studies on various neuroendocrine cell lines and chromaffin cells (*i.e.* the cells from which PC12 originate), granin expression was shown to increase after long term treatment with stimulants such as dibutyryl-cAMP, depolarizing concentrations of KCl and the phorbol ester, TPA (57, 58). Experiments were therefore carried out to investigate whether expression of CgB and SgII could be induced in PC12-27 cells when these treatments were prolonged for up to four days. With KCl (55 mM) and TPA (20 nM), the intracellular levels of [^{35}S]sulfate-labeled CgB and SgII did increase in PC12-251 cells. In contrast, the two secretory proteins remained inappreciable in PC12-27 cells (Fig. 3*A*). Similar results were obtained when dibutyryl-cAMP (1 mM) was employed as the stimulant. In separate experiments, the cells of the two clones were exposed to nerve growth factor (50 ng/ml, for 4 days), the treatment that induces the classical neuron-like differentiation of PC12 cells. Also in this case, no trace of [^{35}S]sulfate-labeled granins did appear in PC12-27 cells (data not shown). Undetectability of granins in clone 27, no matter whether resting or long term stimulated, was confirmed by immunofluorescence (data not shown).

Molecular Mechanisms of the Clone 27 Defect—In order to establish whether in PC12-27 cells expression of the mRNAs coding for the missing proteins was completely absent, as expected for deletions of the corresponding genes, non quantitative RT-PCR analyses were carried out. In these experiments, we focused on the mRNAs of one secretory protein (SgII) and three membrane proteins that in control cells are located in both granules and vesicles (VAMP2, synaptotagmin I, and synaptophysin). The results obtained exclude gene deletions, inasmuch as in all four cases clearly amplified cDNA signals were recorded (Fig. 4*A*). The levels of expression of the corresponding mRNAs were established quantitatively by Northern blot analysis. As can be seen in Fig. 4*B*, some degree of variability (<50%) appeared already between two granule-containing control clones, 251 and another clone, denominated 1 (Ref. 26). With PC12-27 cells, however, the differences with respect to controls were very large. Among the mRNAs analyzed in the defective clone, that of synaptotagmin I was barely appreciable while that of VAMP2 accounted for the 11% of control clone 251. In the case of synaptophysin and SgII, the values approached 10 and 5% of the control, respectively (Fig. 4*B*).

The latter two mRNAs were further investigated with the aim of clarifying the mechanisms of their reduction and lack of translation in clone 27 cells. Sucrose gradient centrifugation experiments, carried out in parallel in clones 27 and 251, failed to reveal any difference in the nuclear and cytoplasmic distribution of the two mRNAs (not shown). These results exclude the possibility that in clone 27 the lack of translation of the SgII and synaptophysin mRNAs is due to an acceleration of the mRNA transport across the nuclear envelope. Another possi-

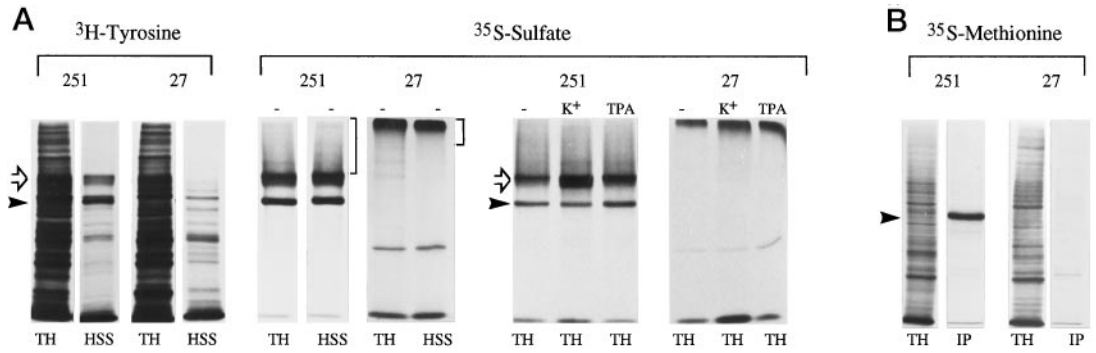

FIG. 3. **Metabolic labeling of PC12 cells.** *A*, *right panel*. PC12 cells from clones 251 and 27 were labeled overnight with [^{3}H]tyrosine. Equal volumes (15 μl) from the cell lysates (*TH*, *right panels*) and the heat-stable fractions (*HSS*, *left panels*) were analyzed by SDS-PAGE followed by fluorography. *A*, *left panel*. PC12-251 and 27 cultured for 4 days in the conventional medium (−) or in the medium supplemented with high KCl (55 mM, *K*) or TPA (20 nM, *TPA*) were labeled overnight with [^{35}S]sulfate. Equal amounts of proteins of cell lysates (3 μg) and equal volumes of the heat-stable fractions (*HSS*) were analyzed as above. Note the absence of the granins in clone 27, no matter whether incubated without any treatment or after long term treatments with high KCl or TPA. *B*, PC12 cells from clones 251 and 27 were labeled for 7 min with the Trans^{35}S-label (*^{35}S-Methionine*) and proteins from the cell lysates were either analyzed by SDS-PAGE followed by fluorography (1 μg of protein, *TH*) or subjected to immunoprecipitation (30 μg of protein, *IP*) using an anti-rat SgII antiserum, before SDS-PAGE. *Open arrows*, rat CgB; *arrowheads*, SgII; *brackets*, heparan sulfate proteoglycans. Results shown are representative of at least two consistent experiments.

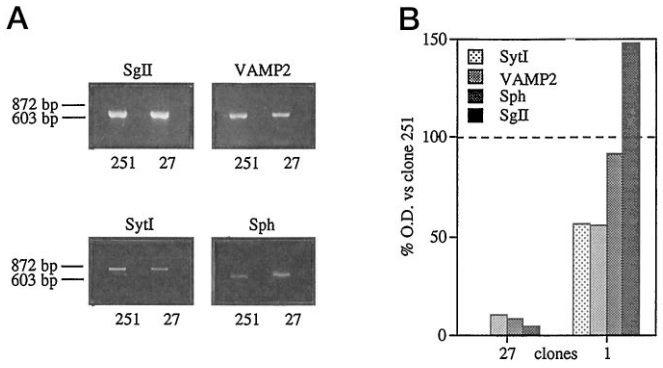

FIG. 4. **RT-PCR (*A*) and Northern blot (*B*) analyses of the control 251 and the defective 27 clones.** *A*, experiments were carried out with the PCR primers designed for amplification of SgII, VAMP2, synaptotagmin I (*SytI*), or synaptophysin (*Sph*) cDNAs, under the conditions specified under "Experimental Procedures." One μg of total RNA extracted from PC12-251 or -27 cells was used as starting material for each reaction. For each sample two lanes are shown: one obtained in the presence (*left*) and the other in the absence (control, *right*) of RT. Results shown are representative of three experiments. *B*, total RNAs (10 μg) prepared from two control clones, 251 and 1, and from the defective clone 27 were analyzed for Northern blotting using specific probes for SytI, VAMP2, Sph, or SgII mRNAs prepared as described under "Experimental Procedures." The densitometric data, normalized based on glyceraldehyde-3-phosphate dehydrogenase data obtained in the same nylon membranes, are expressed as percent of those obtained for the 251 control clone (*broken line*). Results shown are representative of five experiments.

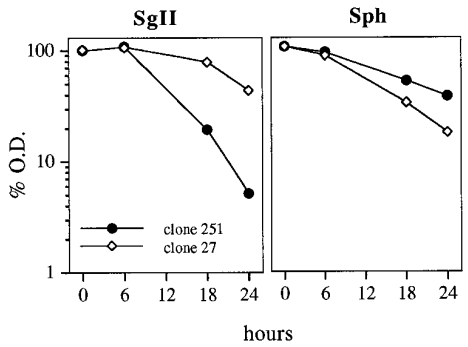

FIG. 5. **mRNA turnover in PC12-251 and -27 clone cells.** Cells were cultured for 0, 6, 12, 18, or 24 h in the presence of actinomycin D (5 μg/ml). Total RNA (10 μg for each sample) was extracted from PC12-251 and -27 cells and analyzed by Northern blot as described in Fig. 5. Values, expressed as percent of those found in each clone at time 0, are averages of three experiments. For relative values see Fig. 4*B*.

bility, an acceleration of mRNA degradation, was investigated by exposing the cells to actinomycin D, an antibiotic known to block transcription. The results obtained demonstrate that in PC12-27 degradation of the SgII mRNA is not faster but rather slower than in the control 251 clone, while that of synaptophysin mRNA is only moderately different in the two clones (Fig. 5). Finally, preliminary data, obtained from nuclear run-on experiments carried out with the rat SgII cDNA, revealed transcription rate ratios of ~ 1:10 between PC12-27 and 251 clones (data not shown).

Expression of Transfected hCgB in Clone 27—To study whether the lack of secretory granules in clone 27 cells is the simple consequence of the absence of regulated secretory proteins, transfection experiments with the cDNA for hCgB were carried out. Various stable subclones, isolated from the transfected cell population, were labeled with [^{35}S]sulfate. When the proteins of cells and media obtained from three such subclones (referred to as c4, c5, and c7) were analyzed by SDS-PAGE followed by fluorography, a labeled band of 120 kDa, the expected molecular mass of mature hCgB, was detected accompanied in the media by two bands of lower molecular mass (105 and 90 kDa, respectively) (Fig. 6, *A* and B). These findings suggest a proteolytic processing just before and/or after secretion. Neither the subclones and their parent clone 27 nor the control clone 251 showed detectable levels of the proprotein convertases, PC1/3 and PC2 (data not shown), *i.e.* of the proteases that in other neuroendocrine cells are known to catalyze limited proteolysis of prohormones (see Ref. 3, and references. therein). Thus, other enzymes, *e.g.* furin or a furin-like enzyme, which are more ubiquitous, might be involved in the proteolytic processing of hCgB in the various subclones.

Using an antibody directed against the C terminus of hCgB, the [^{35}S]sulfate-labeled 120-kDa band as well as the smaller bands were immunoprecipitated from the total homogenates and media of the three transfected subclones. In contrast, the preparations obtained from the parental clone 27 remained negative (Fig. 6, *C* and *D*). Treatment of these gels with 1 M HCl at 100 °C for 5 min largely removed the [^{35}S]sulfate labeled from the hCgB indicating that [^{35}S]sulfate was mainly bound to tyrosine residues (data not shown), as is the case with control cells. The amount of hCgB expressed by the various transfected subclones was different. Since c7 cells synthesized more hCgB than c4 and c5 (Fig. 6, *A* and *B*), this subclone was selected for

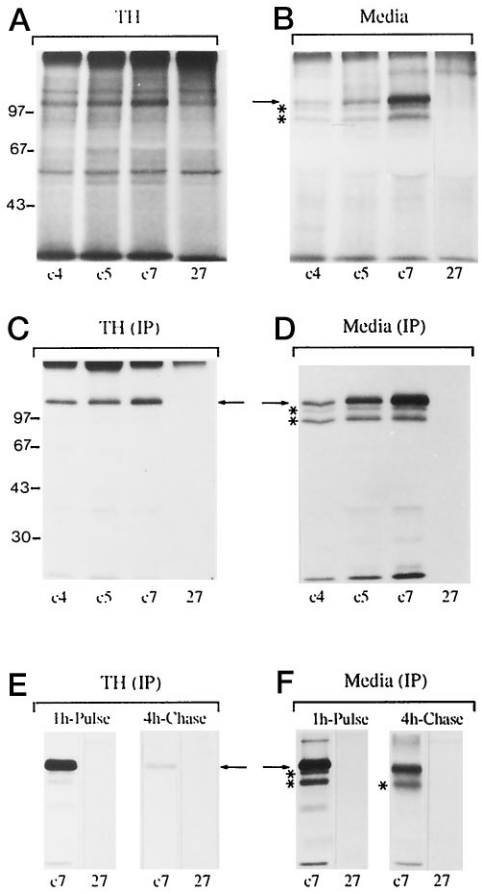

FIG. 6. **Expression of human CgB in transfected subclones isolated from clone 27.** Stably transfected subclones (c4, c5, c7), isolated after electroporation of clone 27 cells with hCgB cDNA, as well as the non-transfected PC12-27 cells, were labeled with [^{35}S]sulfate. *A* and *B*, overnight labeling. Equal amounts of cell lysate proteins (*TH*) and the corresponding proportions of the media samples were analyzed by SDS-PAGE and fluorography. *C* and *D*, overnight labeling. [^{35}S]Sulfate-labeled hCgB was immunoprecipitated from cell lysates (*TH(IP)*) and media (*Media(IP)*) using a polyclonal antiserum directed against the C terminus of hCgB. *E* and *F*, the cells from clones c7 and 27 were labeled for 1 h with [^{35}S]sulfate and then chased for 4 h. Samples normalized as above were subjected to immunoprecipitation (*IP*) using the anti-C terminus hCgB antiserum followed by SDS-PAGE. *Arrows*, hCgB; *asterisks*, degradation products.

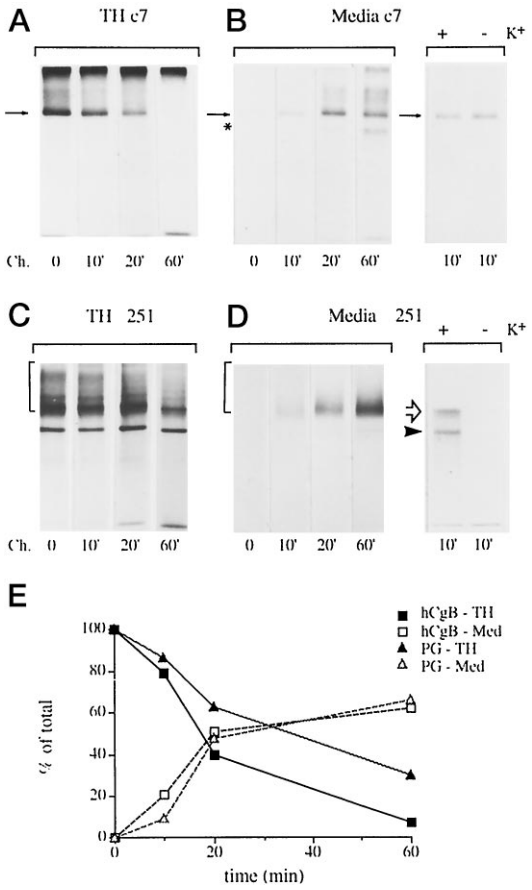

FIG. 7. **Kinetics of hCgB release from the transfected subclone c7 of PC12-27 cells.** Cells from both subclone c7 (*A* and *B*) and the control PC12-251 clone (*C* and *D*) were pulse-labeled for 15 min with [^{35}S]sulfate and then chased for 10, 20, and 60 min (*Ch.*) To investigate stimulated exocytosis, c7 and 251 cells were incubated in the absence (−) or presence (+) of KCl (K^+, 55 mM final concentration) after either 15 (*B*, *right*) or 120 (*D*, *right*) min of labeling (*i.e.* when the majority of [^{35}S]sulfate-labeled hCgB is found intracellularly in subclone c7 and when a pool of [^{35}S]sulfate-labeled granins is stored in the mature secretory granules of the 251 control cells, respectively). Aliquots of the cell lysates and media were then analyzed by SDS-PAGE, followed by fluorography. Since in PC12-251 cells, the rat CgB comigrates with some of the constitutively secreted proteoglycan, this protein and SgII were immunoprecipitated from aliquots of the media after treatment with or without high KCl (*D*, *right*). *E*, the [^{35}S]sulfate-labeled hCgB (arrows in *A* and *B*) and heparan sulfate proteoglycan (*PG*, *brackets* in *C* and *D*) were quantitated in the cell lysates and media and then expressed as percent of those found in the cell lysates at the end of the pulse labeling. The values shown are the means of two independent experiments. *Arrows*, hCgB; *open arrow*, rat CgB; *arrowhead*, SgII; *brackets*, heparan sulfate proteoglycan (*PG*); *asterisk*, degradation products of hCgB.

further experiments.

Transfected hCgB Is Secreted Constitutively from Subclone c7—The results described in the preceding section show that the transfected subclones are able to synthesize, post-translationally modify, and secrete hCgB. Pulse-chase experiments, however, demonstrated that in these subclones secretion of the granin is different from that in the control 251 clone. In the latter, newly synthesized, [^{35}S]sulfate-labeled granins are largely retained intracellularly upon chase because these proteins are efficiently (95%) sorted to secretory granules (12). In contrast, when c7 cells were [^{35}S]sulfate-labeled for 1 h and then chased for 4 h without any stimulation, only 7% of the total labeled hCgB was recovered intracellularly. The majority (70%) was found in the chase medium, accounted for by the mature, 120-kDa form together with the degradation products indicated previously (Fig. 6, *E* and *F*). The incomplete recovery observed in these experiments (see also Fig. 7*A*), suggests that a fraction of hCgB is processed to small peptides, undetectable by SDS-PAGE under the employed conditions.

Further pulse-chase experiments were carried out with stimulation of regulated exocytosis. Cells from subclone c7 and the control clone 251 were labeled for 15 min and then chased for 10, 20, and 60 min (Fig. 7). To stimulate regulated exocytosis, some dishes were exposed to depolarizing concentration of KCl immediately after labeling, *i.e.* when the majority of hCgB was still intracellular also in clone c7. As shown in Fig. 7, the kinetics of hCgB release from unstimulated c7 cells was similar to that of heparan sulfate proteoglycan released from PC12-251 cells, a well characterized marker of constitutive secretion (12), except that the recovery of labeled hCgB was incomplete after 60 min of chase (Fig. 7*E*). In control PC12-251 cells, depolarization induced secretion of endogenous CgB via the regulated pathway (Fig. 7*D*), whereas in c7 cells hCgB secretion was not stimulated (Fig. 7*B*). From these data we conclude that in subclone c7 secretion of hCgB occurs constitutively.

Immunocytochemical Localization of hCgB in Control and Subclone c7 PC12 Cells—The intracellular localization of hCgB

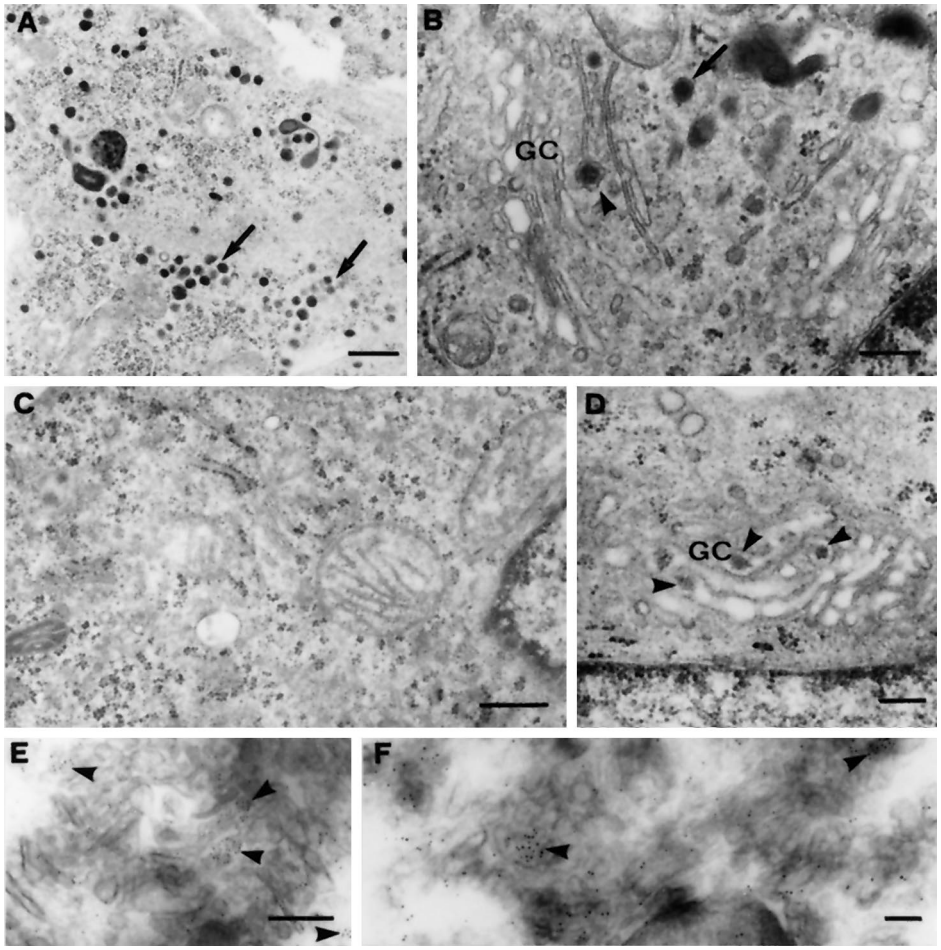

FIG. 8. **Subcellular localization of hCgB in subclone c7 cells.** Epon-embedded ultrathin sections from control PC12-251 cells (*A* and *B*) and subclone c7 (*C* and *D*) illustrate the presence in control cells of many secretory granules scattered in the cytoplasm (*A*, *arrows*), which are completely missing in the c7 PC12-27 cells (*C* and *D*). The Golgi complex of control cells (*GC*, *B*) is highly developed and exhibits granule formation activity with protein condensation in TGN cisternae (*arrowhead*) and a few, probably newly synthesized granules (*arrow*). In a fraction of c7 cells, aggregated material (*D*, *arrowheads*) is also evident within the lumen of the Golgi complex (*GC*, *D*). However, discrete granules never appear even in the Golgi complex area. The hCgB nature of the Golgi luminal protein in c7 cells is revealed by ultrathin cryosection immunolabeling using the anti-C terminus hCgB antiserum decorated with 5-nm anti-rabbit IgG-gold particles (*E* and *F*, *arrowheads*). *Bars*, 500 nm in *A* and *C*; 250 nm in *B*, *D*, and *E*; 100 nm in *F*.

in c7 cells was investigated at the electron microscopy level. In the control PC12-251 cells (Fig. 8, *A* and *B*), a population of dense-core granules was observed throughout the cytoplasm, especially in the proximity of the plasma membrane, whereas in c7 cells no such granules could be seen (Fig. 8, *C* and *D*). Within the TGN lumen of control PC12 cells, the major proteins of the granules, the granins, are known to form aggregates giving rise to moderately electron-dense masses that are later enwrapped by granule membranes (see Fig. 8*B*). Interestingly, aggregates of this type were also observed at the trans side of the Golgi complex in some cells of clone c7, however, without evidence of secretory granule formation (Fig. 8*D*). Moreover, when ultrathin cryo-sections of c7 cells were immunogold-labeled with anti-hCgB antibodies, the signal in the Golgi area was concentrated within a subpopulation of vesicles and short tubules apparently corresponding to TGN elements, with no appearance of granule-like structures (Fig. 8, *E* and *F*). In line with these data, double immunofluorescence results, obtained by the use of anti-hCgB and anti-TGN38 antibodies, revealed in the transfected cells an overall co-localization of the two antigens (data not shown), as was to be expected for a secretory protein released by the constitutive pathway, *i.e.* without storage in a secretory granule compartment.

DISCUSSION

In neurons and neuroendocrine cells, regulated secretion is more complex than in other cell types, inasmuch as it includes two distinct, parallel pathways, which, however, share at least some of their molecular components: the pathway of dense granules and that of synaptic-like microvesicles (Refs. 1–4 and 6, and references therein). The mechanisms that govern the acquisition of the dually regulated secretion have never been specifically investigated, and appearance of granules and vesicles has often been conceived as a general differentiation event, occurring in parallel to the acquisition of other typical neuroendocrine properties (59–61).

In a previous study (26) in which 16 permanent clones isolated from the PC12 neurosecretory parental cell population were surveyed biochemically and morphologically, one clone (denominated 27) had already been reported to lack dense granules, two secretory proteins, CgB and SgII, as well as synaptophysin, a membrane protein of synaptic-like microvesicles also present in secretory granules (62, 63). In addition, PC12-27 cells were unable to pick up, store, and regulately release catecholamines (26). In this previous investigation, however, the status of clone 27 cells, *i.e.* whether they are typical PC12 neurosecretory cells just incompetent for regu-

lated secretion or undifferentiated PC12 precursors, was not established, and the effects of long term stimulations (*e.g.* depolarization with high K^+ and activation of protein kinases C or A) were not evaluated. These treatments are important because, on the one hand, they are known to increase considerably the secretory activity of control PC12 cells (58); on the other hand, they have been recently shown to induce the appearance of granules in two clones of ATt20 (another type of neurosecretory cell) that, under resting conditions, had appeared incompetent for regulated secretion (64).

The present detailed investigation of clone 27 cells has yielded answers to the above questions. The neuroendocrine nature of the phenotype was demonstrated by the expression of well known markers, in addition to synapsins, also the neurofilament H subunit, the neuronal isoform of kinesin and the receptor of α-latrotoxin (42, 54–56). Also present, although in low amount, is tyrosine hydroxylase, the cytosolic enzyme that constitutes the specific rate-limiting step in the biogenesis of the catecholamines that in normal cells are destined to be segregated within granules (65). This list does not exhaust all neuroendocrine markers of PC12-27 cells. In preliminary experiments both surface N-type Ca^{2+} channels and the novel G protein XLαs (66) have been detected at levels comparable to those of controls.[2,3] As far as long term stimulations, and at variance with the ATt20 clones, none of the treatments mentioned above was found to induce any reappearance of regulated secretion in PC12-27 cells, and no reappearance occurred after nerve growth factor, the treatment that induces differentiation of PC12 cells toward the neuronal phenotype (23). We conclude therefore that competence for regulated secretion is not a necessary step in the acquisition of the neuroendocrine phenotype but appears to be regulated independently.

Two additional properties of PC12-27 clone cells deserve further discussion. The first is that the molecular defect of the clone appears to concern mainly the two types of regulated secretory organelles, dense granules and synaptic-like microvesicles, as well as the plasmalemma and the cytosolic components that are needed for their exocytic discharge. All these investigated components were in fact undetectable by Western blotting, and regulated secretion was not functional in PC12-27 cells. In contrast, the components of the other investigated structures in the secretory pathway (*i.e.* ER, Golgi cisternae, and TGN) were all appreciable in PC12-27 cells, although some of them (mannosidase II, Rab6) were clearly reduced with respect to controls. Moreover, the ER-Golgi-TGN transport of proteins and the constitutive limb of secretion appeared functional. The concomitant lack of both granules and vesicles does not seem to be a simple consequence of their considerable molecular overlapping, with expression in their membranes of common proteins such as synaptophysin, synaptotagmin I, and VAMP2 (7–9, 37, 62, 63). In fact, specific membrane components, such as dopamine-β-hydroxylase (granules) and the acetylcholine transporter (vesicles), were also missing in PC12-27 cells. Taken together these results strongly suggest that expression within neuroendocrine cells of the two organelle types occurs under the control of common factor(s). The nature of the latter is unknown. At the moment we can only exclude that the lack of the cargo, constituted by the secretory proteins addressed to granules, plays such a role. In fact, when hCgB was expressed at high levels in PC12-27 cells after cDNA transfection, it did not cause the reappearance of secretory organelles. Although some granin aggregates (*i.e.* the initial step of granule formation) could be observed in the TGN lumen of the defective cells, release of the transfected protein did ultimately occur not via the regulated but via the constitutive pathway. Such a diversion of a regulated secretory protein, typical of cells without regulated secretion (1), confirms the full incompetence of PC12-27 cells for the latter process. The possible role of other missing proteins in granule/vesicle acquisition remains to be investigated.

The second information we have obtained concerns the mechanisms sustaining the phenotypic defect of PC12-27 cells. Although not quantitative, the positive RT-PCR results, focused on four components, excluded deletion of the genes for granule/vesicle proteins. On the other hand, the quantitative cellular levels of mRNAs, revealed by Northern blot, were found to be decreased to considerable extents (85–95%) with respect to controls. These results suggest the existence in PC12 cells of a unique transcriptional control system regulating the expression of all the genes for secretory organelle components. The present knowledge about the structure and control of these genes is still fragmentary. In particular, sequences that might account for a common regulation have never been reported. Our present results appear, however, compatible with this possibility.

On the other hand, the reason why in PC12-27 cells the small, yet appreciable levels of specific granule/vesicle mRNAs fail to sustain the appearance of appreciable amounts of the corresponding proteins is unclear. The actinomycin D and the short pulse labeling experiments in intact cells have excluded major roles of accelerated mRNA and/or protein turnover. A possibility is therefore that of a post-transcriptional inhibition. The latter, however, could be due neither to the trapping of mRNAs within the nucleus, since in PC12-27 their distribution appeared the same as in controls; nor to the expression of inhibitory proteins, since (at least in the case of SgII) the blockade was still apparent in the *in vitro* translation experiments, *i.e.* when the purified mRNAs of PC12-27 cells was translated in the reticulocyte system.

In conclusion, the comprehensive investigation of PC12-27, a clone fully incompetent for regulated secretion, carried out by the combination of molecular and cell biological techniques, has revealed new aspects of a process that appears fundamental for neuroendocrine cells, the acquisition of the two types of secretory organelles, dense granules and synaptic-like microvesicles, and thus of regulated secretion. The results demonstrate for the first time the existence of an expression control, probably common to both organelles, and provide initial information on the mechanisms that appear to operate independently of other aspects of cell differentiation.

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Overall Lack of Regulated Secretion in a PC12 Variant Cell Clone

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