Chromogranin A Induces the Biogenesis of Granules with Calcium- and Actin-Dependent Dynamics and Exocytosis in Constitutively Secreting Cells

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Chromogranins are a family of acidic glycoproteins that play an active role in hormone and neuropeptide secretion through their crucial role in secretory granule biogenesis in neuroendocrine cells. However, the molecular mechanisms underlying their granulogenic activity are still not fully understood. Because we previously demonstrated that the expression of the major component of secretory granules, chromogranin A (CgA), is able to induce the formation of secretory granules in nonendocrine COS-7 cells, we decided to use this model to dissect the mechanisms triggered by CgA leading to the biogenesis and trafficking of such granules. Using quantitative live cell imaging, we first show that CgA-induced organelles exhibit a Ca\(^{2+}\)-dependent trafficking, in contrast to native vesicle stomatitis virus G protein-containing constitutive vesicles. To identify the proteins that confer such properties to the newly formed granules, we developed CgA-stably-expressing COS-7 cells, purified their CgA-containing granules by subcellular fractionation, and analyzed the granule proteome by liquid chromatography-tandem mass spectrometry. This analysis revealed the association of several cytosolic proteins to the granule membrane, including GTPases, cytoskeleton-based molecular motors, and other proteins with actin- and/or Ca\(^{2+}\)-binding properties. Furthermore, disruption of cytoskeleton affects not only the distribution and the transport but also the Ca\(^{2+}\)-evoked exocytosis of the CgA-containing granules, indicating that these granules interact with microtubules and cortical actin for the regulated release of their content. These data demonstrate for the first time that the neuroendocrine factor CgA induces the recruitment of cytoskeleton- and GTP- and Ca\(^{2+}\)-binding proteins in constitutively secreting COS-7 cells to generate vesicles endowed with typical dynamics and exocytotic properties of neuroendocrine secretory granules. (*Endocrinology* 153: 4444–4456, 2012)
Neuroendocrine cells are specialized in the secretion of hormones and neuropeptides that are stored together with acidic soluble proteins of the chromogranin family in dense-core secretory granules. Aggregation of multiple chromogranins along with peptide hormones at the high Ca\(^{2+}\) and weakly acidic pH environment of the trans-Golgi network (TGN) results in a driving force necessary for the budding of immature secretory granules (1–3). These organelles are routed toward the cell periphery where they mature and undergo exocytosis in response to an increase in cytosolic Ca\(^{2+}\) concentration (4), in contrast to constitutive secretory vesicles, which release their cargo in a Ca\(^{2+}\)-independent manner (5). This controlled secretion of hormones and neuropeptides constitutes the regulated secretory pathway of neuroendocrine cells.

During the last decade, the implication of chromogranin A (CgA), a member of the chromogranin family, in the regulation of secretory granule formation has been demonstrated in several neuroendocrine cell models (6–9). Moreover, expression of CgA in nonendocrine cells led to the formation of secretory granule-like structures able to release their cargo in response to a Ca\(^{2+}\) influx (6, 7, 9). Although this crucial role of CgA gave rise to a fascinating debate about the bona fide nature of the generated granules (10, 11), additional investigations performed in CgA-null mice and transgenic mice expressing CgA antisense RNA confirmed that CgA deficiency is associated with hormone storage impairment in neuroendocrine cells (12–15). This evidence in favor of an important role of CgA in granulogenesis has been complemented by a recent study demonstrating the implication of the secreted CgA-derived peptide serpin in the control of granule biogenesis through the regulation of the protease nexin-1 (16). In a previous study performed in COS-7 cells, we showed that CgA induces the biogenesis of mobile secretory granule-like structures capable of storing and releasing ectopically expressed neuropeptide Y and GH in a Ca\(^{2+}\)-dependent manner (9). This result suggested that CgA could trigger a regulated secretory pathway in nonendocrine cells. However, further characterization of the CgA-induced organelles in terms of their dynamics, and the implication of the cytoskeleton in the regulation of their trafficking, is still required to ascertain CgA granulogenic activity. Indeed, secretion through the regulated secretory pathway relies heavily on the trafficking of secretory granules, which depends on the integrity of the cytoskeleton elements. In particular, microtubules and cortical actin filaments (F-actin) support the transport of secretory granules from the TGN to the cell periphery. Microtubules have been reported to play a pivotal role in the trafficking of both constitutive secretory vesicles (17–20) and secretory granules (21, 22). They mediate anterograde and retrograde transports of secretory granules mainly through the involvement of molecular motors of the kinesin and the dynein families, respectively (23–25). At the cell periphery, F-actin-associated myosin V isoform allows short-range transport of secretory granules and their docking at the plasma membrane (26, 27). After stimulation, cortical F-actin must be partially and transiently reorganized to facilitate secretory granule fusion with the plasma membrane (28, 29). Thus, F-actin-rich cortex behaves as a physical barrier that severely hinders membrane-proximal secretory granules, impeding them from releasing their cargo constitutively (30). In contrast, cortical actin is either not or partially employed in constitutive exocytosis (18–20, 31).

In the present study, we show that CgA induces the biogenesis of genuine secretory organelles with dynamics and Ca\(^{2+}\)-sensitivity characteristics that distinguish them from constitutive vesicles in COS-7 cells. A proteomic analysis performed on these granules identified several actin- and Ca\(^{2+}\)-binding proteins which could promote their regulated trafficking and exocytosis. Moreover, the newly formed granules exhibited short-range and directed motions and anterograde and retrograde displacements that depend on cytoskeleton integrity. Therefore, we provide evidence that CgA induces the recruitment of specific cytosolic proteins in nonsecreting COS-7 cells to generate functional granules at the origin of a regulated secretory pathway.

Materials and Methods

Antibodies and plasmids

Primary antibodies used were rabbit polyclonal anti-CgA (9) (1:500 for immunofluorescence, 1:1000 for Western blot); rabbit polyclonal anti-WE14, which recognizes the CgA-derived peptide WE14 (32) (1:500 for immunofluorescence, 1:1000 for Western blot); goat anti-CgA (1:500; Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-ADP-ribosylation factor 6 (ARF6) (Santa Cruz Biotechnology) (1:100 for immunofluorescence, 1:50 for Western blot); rabbit anti-ezrin (Biomedal, provided by M. Malagon, University of Cordoba, Cordoba, Spain) (1:500 for immunofluorescence, 1:1000 for Western blot); rabbit anti-myosin 1b (provided by E. Coudrier, Institut Curie, Paris, France) (1:50 for Western blot); rabbit anti-annexin A11 (Santa Cruz Biotechnology) (1:100 for immunofluorescence, 1:200 for Western blot). For immunofluorescence, secondary antibodies were Alexa 594-conjugated donkey anti-goat IgG, Alexa 594-conjugated goat anti-rabbit IgG, Alexa 488-conjugated donkey anti-rabbit IgG, and Alexa 488-conjugated goat antimouse IgG (Invitrogen, Carlsbad, CA; 1:300). For Western blot, antirabbit and antimouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1,000; Amersham Biosciences, Piscataway, NJ), and goat anti-HRP-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology) were used.
The cDNA encoding full-length CgA was amplified and digested with the appropriate enzymes and then subcloned into the eukaryotic expression vector pIRESneo2 plasmid (BD Clontech, Palo Alto, CA), as previously described (9). The cDNA encoding human CgA was obtained from Origene (Rockville, MD) and cloned in frame to the C terminus end of green fluorescent protein (GFP) in the phrGFP-N1 vector (Stratagene, La Jolla, CA) to generate GFP-tagged human CgA (CgA-GFP). GFP-tagged vesicle stomatitis virus G protein (VSVG) was a gift from D. Toomre (Yale University, New Haven, CT).

Cell culture and transfection
African green monkey kidney fibroblast-derived COS-7 cells (American Type Culture Collection, Manassas, VA; CRL 1651), were maintained in DMEM (Life Technologies, Inc., UK) supplemented with 5% heat-inactivated fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 C in 5% CO₂. Supercoiled plasmid DNA for transfection was purified on Macherey-Nagel columns. Cells were transfected with 0.8 μg (24-well plate) of DNA constructs and 2 μl Lipofectamine 2000 (Invitrogen) per well according to the manufacturer’s protocol. Five hours after the beginning of transfection, the culture medium was replaced by supplemented DMEM, and cells were additionally cultured for 48–72 h. The culture medium for CgA-stably-expressing COS-7 cells was supplemented with 300 μg/ml gentamicin (G-418 sulfate; Life Technologies). After 48 h, ultrathin sections (90 nm, ultracut UCT; Leica, Vienna, Austria) of granules were collected from the top of the tube in 1-ml fractions and analyzed intensively for the presence of the CgA signal.

Subcellular fractionation and secretory granule purification
Cells were collected in PBS and sedimented by centrifugation at 400 × g for 5 min at 4 C. The cell pellet was disrupted by five pulls/pushes through a 21- and then a 25-gauge needle attached to a syringe in ice-cold buffer A [0.32M sucrose, 20 mM Tris-HCl (pH 8), 1 ml/g of cells]. The resulting lysate was centrifuged at 800 × g for 20 min at 4 C. Postnuclear supernatants were centrifuged at 20,000 × g for 20 min at 4 C. Pellets containing dense-core granules were centrifuged on a multistep gradient of 1–2.2 M sucrose (1, 1.2, 1.4, 1.6, 1.8, 2, and 2.2 M sucrose; 5-ml steps), at 100,000 × g for 90 min at 4 C. All gradient steps were collected from the top of the tube in 1-ml fractions and analyzed by Western blotting and by electron microscopy to identify the granule-containing fractions and to verify their quality. The recovered granule fractions were used for liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) analysis.

Electron microscopy
Purified secretory granules from CgA-stably-expressing COS-7 cells were fixed with 2.5% glutaraldehyde and postfixed with 1% osmium tetroxide. Granules were centrifuged at 14,000 × g for 5 min in 2% of agarose heated at 37 C and put on ice to obtain granules in agarose blocks. These blocks were dehydrated at 4 C in ethanol and embedded in London Resin White. Polymerization was performed at 4 C under UV light for 48 h. Ultrathin sections (90 nm, ultracut UCT; Leica, Vienna, Austria) of granules were collected on carbon-formvar-coated nickel grids and processed for immunogold labeling as previously described (9). Briefly, sections were incubated with the primary WE14 antibody (1:2000) for 2 h. After washing, grids were incubated (1 h) with the rabbit secondary antibody conjugated to 10-nm gold particles (Tebu-British Biocell International, Le-Perray-en-Yvelines, France; 1:20) and fixed with 2% glutaraldehyde for 10 min. Finally, ultrathin sections were doubly stained with uranyl acetate and lead citrate and then observed under a FEI Tecnai 12 Biotwin transmission electron microscope operating at 80 kV, with E5300W Ergalshen CCD camera (Gatan Inc., Pleasanton, CA).

Sample preparation and LC-MS/MS analysis
Purified secretory granules were lysed by freeze thawing. Proteins were subjected to one-dimensional SDS-PAGE (10% polyacrylamide). With a dedicated automated system (MultiPROBE II; PerkinElmer, Courtaboeuf, France), gel-separated proteins were reduced at 50 C (1 h) using 10 mM dithiothreitol (GE Healthcare, Piscataway, NJ), alkylated (1 h) with 55 mM iodoacetamide (Sigma), dried, and digested by a solution of trypsin (6 μg/ml) in 50 mM ammonium bicarbonate overnight at 37 C. The samples were subsequently incubated twice for 15 min in 3% acetonitrile and for 10 min in 1% trifluoroacetic acid to allow extraction of peptides. All analyses were conducted using a nanolC1200 system coupled to a 6340 Ion Trap mass spectrometer equipped with a HPLC-chip cube interface (Agilent Technologies, Massy, France). Peptides were separated on Agilent HPLC-chip (40-nl enrichment column; LC separation channel of 150 mm long × 75 μm inner diameter) packed with C18 reversed-phase matrix (Zorbax 30 Å pore size, 5 μm particle size) by applying a 46-min binary gradient (solvent A, 0.1% formic acid; solvent B, 0.1% formic acid/90% acetonitrile) ranging from 3–85% solvent B at a flow rate of 450 nl/min. Full MS scans from 300-2200 m/z were recorded every 2 sec, and the five most intense peaks were subjected to MS/MS with dynamic exclusion after two spectra. Single charged ions were excluded from collision-induced dissociation selection. Peak list for database searching was created using DataAnalysis (version 6.1; Agilent Technologies). MS/MS spectra with signal/noise above 5 and intensity above 10,000 were picked. Protein identification was performed by comparing MS/MS peak list to Swissprot database using MASCOT Daemon (version 2.2.2; Matrix Science, London, UK) for automatic submission of data files to the local MASCOT server (CRIHAN, St Etienne-du-Rouvray, France). All searches were done with variable modification for carbamidomethylation and oxidation, a maximum of one missed trypsin cleavage, and a mass tolerance of 1.6 Da for ion precursor and 0.6 Da for ion fragment.

Drug treatments
Microtubules were depolymerized by incubating cells in blocking buffer (PBS 1X, 1 mM CaCl₂, 0.5 mM MgCl₂) supplemented with 10 μM nocodazole (Sigma-Aldrich) for 2 h at 37 C, 5% CO₂. The cell shape and adhesion to the glass slide remained satisfactory (data not shown). Nocodazole treatment selectively disrupted the microtubule network and did not induce Golgi stack fragmentation (data not shown). Microtubules repolymerized within 15 min after cells were washed twice in culture medium. Actin filaments were depolymerized using 2.5 μM latrunculin B (Calbiochem, La Jolla, CA) in blocking buffer for 45 min at 37 C, 5% CO₂. Under these conditions, microtubules were not significantly affected (data not shown).
**Immunofluorescence microscopy**

Cells cultured onto coated glass coverslips were transfected as described above and fixed with 4% paraformaldehyde in PBS at room temperature for 10 min. Cells were permeabilized for 20 min with 0.3% Triton X-100 in PBS containing normal goat serum or normal donkey serum (1:50) to reduce nonspecific antibody labeling. Cells were then incubated for 2 h at room temperature with one or two primary antibodies and for 1 h with one or two secondary antibodies. Fluorescein isothiocyanate (FITC) (Invitrogen; 500 nM) was used to detect actin filaments. Nuclei were stained with 4',6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR; D3571, 1 μg/ml). Cells were observed on a Leica SP2 upright confocal laser scanning microscope (DM-RAX-UV) equipped with the Acousto-Optical Beam Splitter system and with ×63 oil immersion objective (Leica Microsystems, Reuille-Malmaison, France). For confocal images, Alexa 488, FITC, and GFP were excited at 488 nm and observed in a 505- to 540-nm window. Alexa 594 was excited at 594 nm and observed in a 600- to 630-nm window. Overlay and maximum projection of the z-stacks files were performed with postacquisition Leica confocal software functions to obtain the corresponding snapshots. To verify the specificity of the immunoreactions, the primary or secondary antibodies were substituted with PBS.

**Vesicle distribution analysis by Euclidean distance map (EDM)**

Image treatment was achieved using ImageJ software implemented with the visual scripting interface MRI cell image analyzer (33). The method is derived from Ory et al. (34) and was performed according to Ceridono et al. (35). Briefly, confocal images were segmented to isolate vesicles, and the relative position of each vesicle was estimated by transferring the corresponding region of interest (ROI) onto an EDM, which assigned a gray value corresponding to the minimum distance of each pixel from the manually delineated cell boundary. The distribution of the percentage of vesicles along the EDM gradient values (by increments of five pixels) was determined and fitted to a gauss curve using ImageJ curve fitter. EDM lower values correspond to vesicles in the vicinity of plasma membrane, whereas higher values correspond to vesicles located toward the cell center.

**Real-time video microscopy**

For imaging of living COS-7 cells, transfected cells were cultured in Labtek (Nunc, Rochester, NY) for 48–72 h at 37°C in a controlled atmosphere (5% CO2) and examined under an inverted microscope (Leica) equipped with an incubation chamber (Solent Scientific Ltd., Segensworth, UK), computer-controlled motorized stage and illumination shutters. Images were acquired with a ×63 oil immersion objective and a CoolSnap fx camera (Princeton Instrument, Trenton, NJ) with a pixel size of 6.7 × 6.7 μm (2 × 2 binning). The speed of image acquisition was five frames/sec. Images containing a ROI within the cell were streamed to memory on a computer during acquisition and saved to a disk. Mean velocity and distance covered by each vesicle from its origin were analyzed with Single Particle Tracking software (Molecular Devices, Saint-Germain, France). Displacement efficiency of each particle was determined by dividing the distance from its origin by the actual traveled distance during the time course of recording. Each particle was tracked for 50 frames on the 151 frames of the full sequence. For analysis of single events, each acquired sequence (151 frames) was reviewed multiple times on screen at various settings of intensity to pick out all visible events. Measurements were performed using a whole-cell approach, and only a small ROI was used to illustrate vesicle trajectory. Estimation of trajectory lengths was assessed by using the particle-tracking software DIATRACK version 3.0 (Semasopht, North Epping, Australia). Briefly, the average vesicle lifetime (i.e., number of frames along which a vesicle can be continuously followed) was calculated for each cell imaged. This value was used as a filter to eliminate those vesicles displaying short lifespans. The remaining vesicles were classified depending on their short-range or their net movement into anterograde or retrograde.

**SDS-PAGE and Western blot analysis**

Proteins were separated by SDS-PAGE gels (10% polyacrylamide) in denaturing conditions and electroblotted onto nitrocellulose sheets (Amersham Biosciences). Membranes were incubated in a blocking buffer containing 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (Sigma) for 1 h at room temperature, and overnight with primary antibodies, at 4°C. Then, membranes were washed for 45 min with Tris-buffered saline containing 0.05% Tween 20. Blots were subsequently incubated for 1 h with appropriate HRP-conjugated secondary antirabbit antibody (Amersham Biosciences) in blocking buffer. Immunoreactive proteins were detected by chemiluminescence (Amersham Biosciences). The radiographs were analyzed with a computer-assisted image analyzer (Biocom 2000, Les Ulis, France).

**Secretion analysis**

Transiently transfected COS-7 cells cultured in 24-well Costar plates were extensively washed with basal secretion medium [150 mM NaCl, 5 mM KCl, 2 mM CaCl2, 10 mM HEPES (pH 7.4)] and subsequently incubated in this medium (basal release of CgA) or in barium secretion buffer [150 mM NaCl, 5 mM KCl, 2 mM BaCl2, 10 mM HEPES (pH 7.4)] (stimulated release of CgA) for 15 min at 37°C in 5% CO2. Barium was used as a replacement of external Ca2+ to stimulate cell secretion through calcium-sensing receptors with a higher efficacy (36). Secretion media were collected, cleared by centrifugation (5 min at 6000 × g at 4°C) and stored for further analysis. Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, and protease inhibitor mixture (Sigma-Aldrich) and centrifuged (15 min at 20,000 × g at 4°C). Proteins in secretion media and cell homogenates were precipitated using 10% trichloroacetic acid and analyzed by Western blotting.

**Data analysis**

Data are reported as mean values ± SEM from at least three to four independent experiments. Data were analyzed with the Prism program (GraphPad Software, San Diego, CA). To test significance, the nonparametric Mann-Whitney U, Kruskal-Wallis, and χ2 tests were used. P values < 0.05 were considered significant.
Results

Dynamics of CgA-containing vesicles are Ca\(^{2+}\) dependent

We have recently shown that CgA could trigger, in COS-7 cells, the biogenesis of mobile granular structures that are able to store and release peptide hormones along with CgA (9). To determine the trafficking mode of CgA-induced granules, fluorescence video microscopy of living cells was carried out after expression of CgA-GFP in COS-7 cells. Automatic computer tracking was performed to assess the mean velocity as well as the travel distance of each fluorescent organelle. Analysis of the collected data showed that CgA triggers the biogenesis of granular organelles displaying a variable distribution within velocity categories, which is significantly modified after exposure of COS-7 cells to 2 mM barium (BaCl\(_2\)) (\(P<0.01\), Kruskal-Wallis test; Fig. 1A). Moreover, this treatment resulted in a decrease in the mean velocity of CgA-containing granules (0.41 ± 0.02 \(v/s\). 0.57 ± 0.03 \(\mu m/sec\); \(P<0.05\), Mann-Whitney U test) (Fig. 1A). To study the behavior of these CgA-induced structures, the displacement efficiency of each fluorescent granule was calculated (see Real-time video microscopy in Materials and Methods). We observed that BaCl\(_2\) treatment induces a significant increase in the displacement efficiency of CgA granules (\(P<0.05\), Mann-Whitney U test; Fig. 1B), suggesting Ca\(^{2+}\)-dependent interactions of the generated granules with the cytoskeleton, as previously observed for secretory granules in endocrine cells (22). Immunocytochemical experiments, combined with confocal microscopy, revealed that, in COS-7 cells, CgA was targeted to vesicular structures that did not display labeling with the GFP-tagged VSVG, a marker of constitutive secretory vesicles (18, 37) (Fig. 1C), indicating that CgA induces the biogenesis of novel granules distinct from constitutive carriers. Moreover, the analysis of the distribution of VSVG fluorescent structures according to their velocity revealed dynamics distinct from those of CgA-induced granules (Fig. 1, A and D). These results are in line with those obtained in neuroendocrine cells where dynamics of secretory granules and constitutive carriers are quite different (21, 38). In CgA-expressing COS-7 cells, VSVG concentrated into both tubular structures near the TGN exhibiting slow displacements and more peripheral small vesicles displaying fast movements. The mean velocity of total tracked VSVG organelles was 0.64 ± 0.03 \(\mu m/sec\) (Fig. 1D). These observations corroborate the previously reported data showing that VSVG is sorted to slow-moving tubular intermediate carriers that undergo fission to generate smaller vesicles displaying rapid motion (17, 18). Moreover, BaCl\(_2\) treatment did not alter the behavior of the constitutive vesicles (0.60 ± 0.07

FIG. 1. Ca\(^{2+}\)-dependent dynamics of CgA-containing granules in COS-7 cells. A, COS-7 cells were transfected with CgA-GFP, imaged via live epifluorescence microscopy (five frames/sec) and fluorescent vesicles tracked for 30 sec before (n = 10 cells, 227 granules) and after (n = 10 cells, 226 granules) treatment with 2 mM BaCl\(_2\) for 15 min. Velocity profiles were determined by grouping velocities into 0.1-\(\mu m/sec\) intervals and then plotted either in control (gray bar) or Ba\(^{2+}\)-stimulated (black bar) cells. P<0.01, Kruskal-Wallis test. Inset shows the mean velocity. The values represent the means ± SEM from three independent experiments. *, \(P<0.05\), Mann-Whitney U test. B, Mean processivity of vesicles, either in control (gray bars) or Ba\(^{2+}\)-stimulated (black bars) conditions. The values represent the means ± SEM from three independent experiments. *, \(P<0.05\), Mann-Whitney U test. a.u., Arbitrary unit. C, COS-7 cells were cotransfected with the full-length CgA and VSVG-GFP (green), and stained with anti-CgA antibody (red). Insets show higher-magnification views of the boxed region of the single confocal plane (1024 × 1024 pixels) revealing no colocalization of CgA and VSVG-GFP. Scale bar, 10 \(\mu m\). DAPI, 4',6-Diamidino-2-phenylindole. D, COS-7 cells were transfected with VSVG-GFP, imaged via live epifluorescence microscopy (5 frames/sec) and fluorescent vesicles tracked for 30 sec before (n = 10 cells, 384 vesicles) and after (n = 10 cells, 358 vesicles) treatment with 2 mM BaCl\(_2\) for 15 min. Velocity profiles were determined by grouping velocities into 0.1-\(\mu m/sec\) intervals and then plotted either in control (gray bar) or Ba\(^{2+}\)-stimulated (black bar) conditions. P > 0.05, Kruskal-Wallis test. Inset shows the mean velocity. The values represent the means ± SEM from three independent experiments. P > 0.05, Mann-Whitney U test.
Actin- and Ca\textsuperscript{2+}-binding proteins are recruited to the surface of CgA-induced granules

To characterize the proteins associated with CgA-induced granules, we performed a systematic proteome analysis of these granules through mass spectrometry. First, we developed a stable COS-7 cell line that expresses CgA (Fig. 2A) and is able to release CgA in a regulated manner (Fig. 2B). Granular structures were isolated by subcellular fractionation and examined under electron microscopy to verify their integrity and their CgA content (Fig. 2C). A highly enriched granule fraction was obtained from this stable cell line (Fig. 2D). LC-MS/MS analysis of the proteome of purified CgA-induced granules revealed the presence of numerous Ca\textsuperscript{2+}-sensitive proteins, such as actin- and phospholipid-binding proteins (Table 1 and Supplemental Table 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). These include several actin-associated molecular motors, i.e. myosin 1b, myosin 1c, myosin VI, myosin 9, myosin 10, myosin 11, and myosin 14, and membrane-binding proteins, i.e. copine 3 and annexins (A1, A2, A4, A6, and A11), which also interact with actin. We also identified Ras-related GTPases, such as ARF5 and ARF6.

The presence of ARF6 was confirmed by immunofluorescence and Western blotting analysis of CgA-induced granules (Fig. 3, A and B). Ezrin, which is a component of the ezrin-radixin-moesin complex involved in cortical actin/plasma membrane interaction (39), was also localized in the subplasmalemmal fraction of CgA-containing granules (Fig. 3, A and C). Various proteins with unknown functions in the regulated secretory pathway were also identified in the granular fraction. Among these, we confirmed by immunofluorescence and Western blotting that the Ca\textsuperscript{2+}- and phospholipid-binding protein annexin A11 is associated with CgA-induced vesicular structures (Fig. 3, A and D) and that the actin-based molecular motor myosin 1b is colocalized with CgA granules mainly in the perinuclear area (Fig. 3, A and E).

Microtubules and F-actin regulate intracellular distribution of CgA-induced granules

We took advantage of video microscopy and immunofluorescence quantitative approaches to address the influence of microtubules and F-actin on the cellular distribution of CgA-induced structures. For this, CgA-transfected COS-7 cells were treated or not with cytoskeleton-depolymerizing agents, double-labeled for CgA and microtubules or F-actin, and imaged by confocal microscopy. In control conditions, we observed that a large proportion of CgA-containing granules were associated with microtubules and F-actin (Fig. 4A). Depolymerization of microtubules with 10 \( \mu \text{M} \) nocodazole or disassembly of F-actin-rich cortex with 2.5 \( \mu \text{M} \) latrunculin B caused CgA-containing granules to be clustered toward the center of the cell (Fig. 4B). To quantify these effects, we measured the relative distance of each granule to the cell periphery by the method of EDM according to Ceridono et al. (35) (see Materials and Methods). Disruption of microtubules or F-actin cortex reduced significantly the total number of CgA-containing granules localized to the cell periphery, as indicated by the shift of the curves toward higher EDM values (\( P < 0.01 \), Kruskal-Wallis; Fig. 4C). To assess the impact of these effects on the trafficking of CgA-induced granules, we analyzed their displacements and observed that microtubule depolymerization induced a significant redistribution of granules from directed to short-range displacements (\( P < 0.001, \chi^2 \) test; Fig. 4D).
Dynamics and exocytosis of CgA-induced granules depend on microtubule- and F-actin integrity

We next determined the extent to which cytoskeleton controls the trafficking of CgA granules in living CgA-expressing COS-7 cells. First, to assess the influence of microtubules on the transport of CgA-containing granules, fluorescence video microscopy was carried out in the presence or absence of 10 μM nocodazole in control or 2 mM BaCl₂-stimulated cells. In unstimulated conditions, microtubule depolymerization induced a significant decrease in the mean velocity from 0.57 ± 0.03 to 0.34 ± 0.09 μm/sec (P < 0.01, Mann-Whitney U test; Fig. 5A), with an acute elevation of the proportion of granules displaying a velocity lower than 0.1 μm/sec (Supplemental Fig. 1A). Ca²⁺ influx did not seem to affect the dynamics of CgA-containing granules in nocodazole-treated cells (mean velocity of 0.30 ± 0.03 μm/s vs. 0.34 ± 0.09 μm/s; P > 0.05, Kruskal-Wallis test; Fig. 5A and Supplemental Fig. 1B). Therefore, these data demonstrate that microtubules are actively involved in the transport of CgA-induced granules in COS-7 cells. In addition, nocodazole treatment provoked a significant decrease in the displacement efficiency of CgA-containing granules, and again Ca²⁺ influx did not affect the behavior of CgA-containing granules in nocodazole-treated cells (Fig. 5B). Together with the results described in the previous section, these data indicate that microtubule depolymerization leads to an immobilization of CgA-containing granules in the central region of the cell. This observation prompted us to investigate how microtubules could be involved in Ca²⁺-mediated CgA secretion. BaCl₂ significantly enhanced CgA secretion from 35.8 ± 0.54 to 63.34 ± 9% in control cells, although it had no effect after microtubule depolymerization (P < 0.05, Mann-Whitney U test; Fig. 5C). Altogether, these results suggest that CgA induces the biogenesis of granules interacting with microtubules to allow post-TGN trafficking to the plasma membrane and Ca²⁺-evoked exocytosis.

To examine the influence of F-actin on the dynamics of these granules, the mean velocity of tracked granules was determined in the presence or absence of 2.5 μM latrunculin B in control or BaCl₂-stimulated cells. In unstimulated conditions, F-actin disassembly induced an increase in the mean velocity from 0.57 ± 0.03 to 0.65 ± 0.02 μm/sec (P < 0.05, Mann-Whitney U test; Fig. 6A), with an acute elevation of the proportion of granules displaying a velocity higher than 1 μm/sec (Supplemental Fig. 2A). Exposure of latrunculin B-treated cells to 2 mM BaCl₂ inhibited the latrunculin B-induced increase in the mean velocity from 0.65 ± 0.02 to 0.51 ± 0.05 μm/sec (P < 0.05, Mann-Whitney U test; Fig. 6A), with an elevation of the proportion of granules displaying a velocity lower than 0.3 μm/sec (Supplemental Fig. 2B). Moreover, latrunculin B treatment resulted in a significant decrease in the dis-

**TABLE 1. Identification of cytosolic proteins associated to CgA-induced granules**

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Secretory granule (SG) fraction was subjected to one-dimensional SDS-PAGE, 1-mm gel slices were excised and subjected to trypsin digestion, and the proteins were analyzed by LC-MS/MS. Identification was based on human sequences, as well as those of other mammalian species. Only proteins that were identified with one or more high scoring peptides from Mascot were considered to be true matches. “High scoring peptides” corresponded to peptides that were above the threshold in Mascot (P < 0.05) searches. The table shows a non-redundant list of the proteins associated to CgA-induced SG and the reference to the Uniprot-access number used in database searches (http://uniprot.org).

Together, these data indicate that both microtubules and F-actin cortex are involved in the distribution of CgA-induced granules and that their directed displacements are mainly regulated by microtubules, suggesting that their transport toward the cell periphery could be mediated by the recruitment of molecular processive motors, such as the microtubule motor protein kinesin-1 identified in the secretory granule fraction (Table 1). Codistribution of kinesin-1 and CgA granules was confirmed in CgA-expressing COS-7 cells (Fig. 4E).
placement efficiency of CgA-containing granules ($P < 0.05$, Mann-Whitney $U$ test; Fig. 6B). These results suggest that the F-actin-rich cortex may potentiate the directed motions of CgA-containing granules to reach the plasma membrane. Exposure of latrunculin B-treated cells to 2 mM BaCl$_2$ increased the displacement efficiency of CgA-containing granules ($P < 0.01$, Mann-Whitney $U$ test; Fig. 6B). Although F-actin depolymerization with 2.5 mM latrunculin B did not alter significantly basal CgA release, the drug increased the CgA-regulated secretion by approximately 37% ($P < 0.05$, Mann-Whitney $U$ test; Fig. 6C). These data demonstrate that CgA promotes the formation of de novo granules 1) that are distinct from vesicles emanating from the constitutive secretory pathway and 2) whose dynamics are Ca$^{2+}$ dependent, as previously shown for secretory granules in neuroendocrine cells (21, 22, 44). Interestingly, homeostatic regulation of the local cytosolic Ca$^{2+}$ around secretory granules in chromaffin cells has been shown to influence their motion and exocytosis (45).

Both constitutive secretory vesicles and secretory granules are transported from the TGN to the cell periphery through microtubules (18–21, 38, 46–48). With respect to the regulated secretory pathway, microtubules tightly regulate the kinetics of secretory granules accounting for the reserve pool that is involved in the refilling of the readily releasable pool in neuroendocrine cells (21, 22, 47). In the present study, we found that microtubule depolymerization provokes the clustering of CgA-induced granules near the perinuclear area and severely reduces their mean velocity and displacement efficacy, indicating that microtubule tracks allow the trafficking of these newly formed organelles. Furthermore, we observed that CgA-containing granules exhibit short-range and directed anterograde/
retrograde displacements. Thus, CgA induces the formation of granules that likely recruit microtubule-processive motors to ensure their trafficking on the cytoskeleton toward the cell periphery. In support of this hypothesis, we found that kinesin-1 (Table 1) as well as other kinesin isoforms and dynein (data not shown) are expressed in COS-7 cells. Moreover, disruption of microtubules abolished the effect of Ca\textsubscript{2+}/H11001 influx on the dynamics and the secretory activity of CgA-containing granules, indicating that microtubules are involved in the translocation of CgA-containing granules to the plasma membrane.

In neuroendocrine cells, secretory granules are efficiently captured in the cortex by an F-actin-dependent mechanism (30). Cortical F-actin acts as a dynamic network allowing the motion of secretory granules in a restricted space (49–51) but has no effect on constitutive secretion (19, 20, 31). A remarkable finding of our work is the accumulation of CgA-containing granules on cortical F-actin of COS-7 cells. Actin remodeling triggered by latrunculin B induced retention of newly formed granules in the perinuclear region, indicating that F-actin is actively involved in the trafficking and in the capture in the cell

![FIG. 4. Distribution of CgA-containing granules is microtubule and F-actin dependent in COS-7 cells. A, COS-7 cells expressing CgA were stained with anti-CgA antibody (red) and anti-\(\alpha\)-tubulin antibody (upper panel) or phalloidin-FITC (lower panel) (green). B, COS-7 cells expressing CgA were treated with 10 \(\mu\)M nocodazole or 2.5 \(\mu\)M latrunculin B and stained with anti-CgA antibody (red) and phalloidin-FITC (green). Maximal projections of z-serial confocal images (0.304 \(\mu\)m thick, 1024 \(\times\) 1024 pixels) are displayed. Scale bars, 10 \(\mu\)m. Insets emphasize representative granule distributions. C, Gauss curve showing the distribution of the percentage of vesicles along the EDM gradient values, in control (gray circles, \(n = 15\) cells, 950 granules), 10 \(\mu\)M nocodazole-treated (white circles, \(n = 9\) cells, 628 granules), or 2.5 \(\mu\)M latrunculin B-treated (black circles, \(n = 9\) cells, 396 granules) COS-7 cells. \(P < 0.01\), Kruskal-Wallis test. D, Stacked histogram comparing the distribution in percentage of each vesicle category (AD, anterograde displacement; RD, retrograde displacement; SRD, short-range displacement, distance travel <0.5 \(\mu\)m) in control (Ctl) (\(n = 6\) cells, 376 granules), 2.5 \(\mu\)M latrunculin B-treated (LB) (\(n = 5\) cells, 154 granules), or 10 \(\mu\)M nocodazole-treated (Noc) (\(n = 4\) cells, 347 granules) COS-7 cells (contingency table, \(\chi^2 = 58.59\), two degrees of freedom). ***, \(P < 0.001\), \(\chi^2\) test. E, Immunofluorescence studies revealed that kinesin-1 (red) localizes on a fraction of CgA granules (green) of CgA-expressing COS-7 cells. Insets show higher-magnification views of the boxed region of the single confocal plane (1024 \(\times\) 1024 pixels). Scale bar, 5 \(\mu\)m. DAPI, 4',6-Diamidino-2-phenylindole.](https://academic.oup.com/endo/article-abstract/153/9/4444/2424183)
periphery of CgA-containing granules. Moreover, actin depolymerization induced a decrease in the displacement efficiency of CgA-containing granules, suggesting that F-actin is involved either in the potentiation of the directed motions on microtubules to reach the plasma membrane or in the caging of these granules. Thus, CgA-induced granules are competent to coordinately interact with both microtubules and F-actin to optimize their recruitment to the plasma membrane. In neuroendocrine cells, regulated exocytosis is accompanied by a focal and transient remodeling of the cortical actin network, F-actin being a target of Ca²⁺-dependent signaling cascades (30). Furthermore, F-actin disassembly enhances Ca²⁺-evoked exocytosis in chromaffin cells (52, 53) via a Ca²⁺-activated myosin, myosin Va (54). In CgA-expressing COS-7 cells, actin re-arrangement may act to facilitate the directed movement of CgA-containing granules and to promote refilling of the cortical vesicle pool, leading to an enhanced Ca²⁺-dependent secretion of CgA. Moreover, F-actin depolymerization does not affect the basal release of CgA, suggesting that the cortical actin meshwork controls specifically the regulated secretion. Besides, the use of a higher concentration of latrunculin B obliterated Ca²⁺-mediated secretion in COS-7 cells (data not shown), suggesting that F-actin may play both an inhibitory and a facilitating role during exocytosis, as previously observed in pancreatic β-cells, chromaffin cells, and PC12 cells (53, 55, 56). Interestingly, the proteomic analysis by LC-MS/MS of newly formed CgA-containing granules led to the identification of numerous membrane-associated proteins that could regulate granule interaction with F-actin in a Ca²⁺-dependent manner at distinct cellular levels. Among these actin-binding proteins, several belong to the annexin and myosin families. Some of them have been identified by the proteomic analysis of neuroendocrine secretory granules like annexins and myosins 1b and 1c, and 9 (57, 58), and others, like annexin A2 or myosin VI, have been shown to be involved in the regulated secretory pathway of neuroendocrine cells in different studies (59–62). Inter
estingly, we also identified the small GTPases ARF5 and ARF6, which are known to interact with secretory granules to sustain their trafficking in neuroendocrine cells (63, 64). Thus, these observations demonstrate that the expression of CgA in COS-7 cells could confer a neuroendocrine-like phenotype in terms of secretion competence. Besides, association of the ezrin-radixin-moesin family member ezrin to CgA-containing granules may involve the N-terminal region of CgA, which is structurally related to ezrin-binding protein 50 and is able to interact with membranes (65). In addition, several Ca^{2+} - and phospholipid-binding proteins such as cofolin or different annexins and myosins were associated with CgA-induced granules and may exert important functions at different stages of the regulated secretory pathway. These observations need to be substantiated in neuroendocrine cells. Of note, our proteomic analysis of CgA-induced granules failed to identify Soluble N-ethylmaleimide-sensitive-factor Attachment protein REceptor (SNARE) proteins that could mediate their exocytosis, probably due to the low expression of these proteins in COS-7 cells making difficult their detection by mass spectrometry. Together these findings suggest that CgA expression triggers the establishment of molecular machineries mediating focal actin polymerization necessary for the recruitment of CgA-containing granules to the plasma membrane for subsequent regulated exocytosis.

In conclusion, the use of a CgA-expressing COS-7 cell model provided important clues for the characterization of the role of CgA in the biogenesis of secretory granules. Newly formed CgA-containing structures exhibited Ca^{2+}-dependent dynamics and exocytosis, which required microtubule integrity and F-actin cytoskeleton remodeling. These properties are most likely due to the ability of CgA to trigger the recruitment of cytosolic proteins at the membrane of neuroformed granules. The actin- and/or Ca^{2+}-binding properties of these proteins confer to CgA-induced granules dynamic characteristics similar to those of neuroendocrine secretory granules. Altogether, these data strengthen the notion that CgA per se promotes the biogenesis of functional granules to establish a regulated secretory pathway. Understanding the molecular mechanisms by which CgA recruits these proteins to form secretory granules is an exciting future challenge.

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