Secretogranin III Binds to Cholesterol in the Secretory Granule Membrane as an Adapter for Chromogranin A*

Received for publication, September 11, 2003, and in revised form, October 31, 2003
Published, JBC Papers in Press, November 3, 2003, DOI 10.1074/jbc.M310104200

Masahiro Hosakaged, Masayuki Suda†, Yuko Sakai†, Tetsuro Izumi†, Tsuyoshi Watanabe§, and Toshiyuki Takeuchi¶

From the †Department of Molecular Medicine, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi 371-8512, Japan and §Department of Anatomy II, Asahikawa Medical College, Asahikawa, Hokkaido 078-8510, Japan

Grain-family proteins, including chromogranin A (CgA) and secretogranin III (SgIII), are secreted to secretory granules (SGs) in neuroendocrine cells. We previously showed that SgIII binds strongly to CgA in an intragranular milieu and targets CgA to SGs in pituitary and pancreatic endocrine cells. In this study, we demonstrated that with a sucrose density gradient of rat insulinoma-derived INS-1 cell homogenates, SgIII was localized to the SG fraction and was fractionated to the SG membrane (SGM) despite lacking the transmembrane region. With depletion of cholesterol from the SGM using methyl-β-cyclodextrin, SgIII was impaired to bind to the SGM. Both SgIII and CgA were solubilized from the SGM by Triton X-100. In contrast to the Triton X-100 insolubility of carboxypeptidase E, SgIII and carboxypeptidase E strongly bound to the SGM-type liposome in intragranular conditions, but CgA did not. Instead, CgA bound to the SGM-type liposome only in the presence of SgIII. Immunocytochemical and pulse-chase experiments revealed that SgIII deleting the N-terminal loop carrying CgA, CgB, and POMC sequences such as the N-terminal lipid-binding region missorted to the constitutive pathway in mouse corticotroph-derived AtT-20 cells. Thus, we suggest that SgIII directly binds to cholesterol components of the SGM and targets CgA to SGs in pituitary and pancreatic endocrine cells.

Peptide hormones and grain-family proteins are sorted to immature-budding granules at the trans-Golgi network (TGN) in neuroendocrine cells. Currently, two models have been proposed for granule protein sorting: 1) sorting for entry and 2) sorting by retention (1, 2). In the sorting for entry model, secretory granule (SG) proteins are sorted at the TGN to immature SGs, which become mature SGs without losing cargo proteins. In the sorting by retention model, sorting takes place in the immature SGs, which become mature SGs after removing nongranule proteins, resulting in the retention of only SG proteins. Because grain-family proteins enter into immature SGs and reside in mature SGs, they must possess an entry and/or retention signal for the SGs.

Grain-family proteins include chromogranin A (CgA), CgB, secretogranin II (SgII), SgIII, and 7B2. They form an aggregation in a weakly acidic high Ca2+-intragranular milieu and display a high capacity for Ca2+ binding (3, 4). This aggregation-prone feature is important for condensing secretory proteins to SGs with co-aggregation (5). However, aggregation itself is not sufficient for SG protein sorting to SGs, because sorting can occur without aggregation and constitutive secretion can occur with aggregation (5, 6). Thus, the sorting of granins may be directed by other factors such as specific protein-to-protein interactions or specific sorting domain structures such as the N-terminal disulfide loop (7). We previously demonstrated that CgA binds to SgIII by the CgA sequence 41–109 and that SgIII targets CgA to SGs in pituitary and pancreatic endocrine cells (8). Thus, CgA is sorted to SGs by the lead of SgIII but the sorting of SgIII to SGs remains to be investigated.

Regarding a sorting signal, the N-terminal disulfide loop of CgA, CgB, and pro-opiomelanocortin (POMC) has received much attention (9–12). Disruption of the disulfide loop of CgB resulted in its missorting to a constitutive pathway in PC12 cells (13). However, removal of the N-terminal loop from CgA did not affect its targeting to SGs in GH3 cells (14), and PC12 cells (15). For recognizing the N-terminal loop of POMC, carboxypeptidase E (CPE) has been proposed for its sorting receptor (10) but the sorting of CgA was not disturbed in Neuro-2A cells depleting CPE (16). Thus, the N-terminal loop-carrying CgA, CgB, and POMC appear to depend on distinct mechanisms for their sorting to SGs.

Although CPE is not a common sorting receptor, CPE possesses a specific feature by which CPE is anchored to cholesterol-rich lipid microdomains through its C-terminal domain (17). It has been proposed that cholesterol- and glycosphingolipid-rich microdomains (lipid rafts) exist within the membrane of SGs (7). Cholesterol depletion resulted in the dissociation of CPE from the SG membrane (SGM) (17) and in the block of both regulated and constitutive secretory vesicle formation at the TGN in AtT-20 cells (18). PC2, a prohormone-converting enzyme localized to SGs, is reported to bind to the chromaffin granule membrane via lipid rafts (19). Thus, specialized lipid components may be required for the granule-destined membrane at the TGN to concentrate a number of the granule-
associated proteins as seen in apical sorting (20, 21) and in endocytosis (22).

In this study, we focused on the lipid-binding capacity of SgIII as seen in CPE and examined the interaction of SgIII, CgA, and CPE with lipid components of the SGM. We found that SgIII binds to cholesterol-rich microdomains in the SGM, suggesting that SgIII retains CgA to the SGs with this specific lipid binding.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—A mouse corticotrope-derived AtT-20 cell line was maintained in DMEM supplemented with 10% fetal bovine serum. A rat insulinoma-derived INS-1 cell line was maintained in RPMI 1640 medium supplemented with 5 μM 2-mercaptoethanol and 10% fetal bovine serum (23).

**Linear Sucrose Density Gradients and Granule Fractionation**—A linear sucrose density gradient (20–70%, w/v) buffered with a solution containing 4 mM HEPES (pH 7.4) and 1.5 mM EDTA was formed using a tilt tube in a Gradient Master (BioComp Instruments) for 17.4 min at 58.5° and 25 rpm followed by 0.3 min at 83° and 25 rpm. INS-1 cells were detached from ten plastic plates (diameter, 100 mm) with a 20-min incubation in phosphate-buffered saline containing 10 mM EDTA at 37°C, reticulin light centrifugation, and homogenized in buffer A (250 mM sucrose, 4 mM HEPES (pH 7.4), 1 mM MgCl₂, 0.005% Dnase, and a protease inhibitor mixture (1 μg/ml each of aprotinin, leupeptin, and pepstatin A and 0.5 μM phenylmethylsulfonyl fluoride)). The homogenate was centrifuged at 3,000 × g for 2 min. The resulting supernatant was centrifuged at 5,000 × g for 15 min at 4°C. The postnuclear supernatant was centrifuged at 20,000 × g for 15 min. The pellet, the crude organelle fraction, was resuspended in 0.8 ml of the buffer A with 1.5 mM EDTA. The crude organelle fraction was layered onto the sucrose density gradient and was centrifuged at 113,000 × g for 18 h at 4°C in a swing rotor. Gradients were fractionated by piston displacement from the top to bottom (24) using the BioComp piston gradient fractionator. Samples for gel analysis were precipitated from fractionated samples by adding trichloroacetic acid to 7.5% and bovine serum albumin to 0.01%.

**Preparation of the Secretory Granule Membrane**—Peak fractions with high immunoreactive insulin (IRI) were diluted and homogenized in 25-fold volume of buffer B (20 mM HEPES (pH 7.4), 1 mM MgCl₂, 1 mM dithiothreitol, and the protease inhibitor mixture). The homogenate was centrifuged at 100,000 × g for 2 h at 4°C. The pellet was washed with the buffer B and recentrifuged at 130,000 × g for 2 h at 4°C. The resulting pellet was used as an SGM fraction.

**Methyl-beta-cyclodextrin Treatment**—The SGM fraction was resuspended in ice-cold buffer C containing an indicated concentration of methyl-β-cyclodextrin (mβCD). Samples were incubated at 4°C for 30 min at 4°C and were centrifuged at 4°C for 1 h at 100,000 × g. The supernatant and pellet fractions were analyzed by SDS-PAGE for immunoblottting.

**Detergent Lysis and Differential Centrifugation**—The SGM fraction was incubated for 30 min on ice in 500 μl of ice-cold buffer C (150 mM NaCl, 2 mM EGTA, 50 mM Tris (pH 7.3)) containing 0.1% Triton X-100. The cells were incubated with a mixture of guinea pig anti-insulin (1:700) or mouse anti-ACTH and fluorescein isothiocyanate-labeled anti-rabbit IgG (Jackson Immunoresearch). We raised antisera to SgIII (SgIII-C1) by injecting GST-fused rat SgIII (373–471) protein into rats.

**Expression of FLAG-tagged SgIII Fragments**—SgIII fragments fused to FLAG (Stratagene) were constructed in a pDNA3 (Invitrogen) with a putative signal sequence. The SgIII fragments used were as follows: 1) SgIII (1–471); 2) SgIII Δ (40–186) deleting SgIII (40–186); 3) SgIII Δ (187–373) deleting SgIII (187–373). SgIII (373–471) was deleted SgIII (374–471). AtT-20 and INS-1 cells were cultured on an 8-well Lab-Tek chamber slide and were then transfected with FLAG-tagged SgIII fragments with Lipofectamine 2000 reagent (Invitrogen).

**Laser Confocal Microscopy**—AtT-20 and INS-1 cells were fixed with 4% paraformaldehyde and then permeabilized with high salt TPBS (0.1% v/v of phosphate buffered saline (pH 7.3)) containing 0.1% Triton X-100. The cells were incubated with a mixture of guinea pig anti-insulin (1:700) or mouse anti-ACTH and fluorescein isothiocyanate-labeled anti-rabbit IgG and Texas Red-labeled anti-mouse IgG. The coverslips were mounted in 90% glycerol (v/v in phosphate-buffered saline) containing 1% p-phenylenediamine dihydrochloride (Sigma). The stained cells were observed with a laser-scanning confocal microscope (Olympus).

**Radiolabeling and Immunoprecipitation**—After transfection with the SgIII fragments, the AtT-20 cells were radiolabeled with 0.2 μCi of [35S]methionine and cysteine (Amersham Biosciences) for 2 h. After radiolabeling, the medium was changed to DMEM or with 5 mM 8-Br-cAMP as a secretagogue for 1 h. Cells extracts and culture media were immunoprecipitated with anti-FLAG antibody and analyzed by SDS-PAGE for fluorography. The [35S] signals of blots were recorded in BAS2000 (Fuji film). An automatic integration method was used to calculate the intensity of each band, and the relative signals were obtained for each cell extract and medium.

**RESULTS**

*SgIII Is Concentrated to the Membrane Fraction of SGs*—To examine the subcellular localization of SgIII in SGs, we separated subcellular organelles of INS-1 cells into 16 fractions using sucrose density gradient centrifugation (Fig. 1, A and B). Because CgA, the binding partner of SgIII, localizes in the SGs, SgIII is presumed to localize in the same fraction. Insulin activity was used to identify the SG fractions (Fig. 1A), which were also expected to contain SG-associated proteins, SgIII, CgA, and CPE, and these proteins were in fact identified in the same fraction (fractions 11–13) by immunoblotting (Fig. 1B). We used the antibody to CgA (1–128) called betagranin to identify CgA molecules, because CgA is effectively processed to CgA in β-cells (27). Actually, >95% CgA molecules were processed to betagranin in the secretory granules of INS-1 cells (data not shown). Other subcellular organelle fractions were also identified with calnexin for ER, β-COP for cis-Golgi, syn-
aptophysin for synaptic vesicle-like microvesicles, cytochrome c for mitochondria, and cathepsin D for lysosomes. The SG fractions of #11–13 had a buoyant density of 1.178 ± 0.005 g/cm³ (mean ± S.D. of five independent experiments). This value was similar to the previously reported density value for the SGs in PC12 cells (28). The SG fraction was lysed in hypotonic solution and then centrifuged to two fractions, a precipitated SGM fraction and a supernatant soluble fraction. An immunoblot study revealed the accumulation of SgIII, CgA, CPE, and phogrin to the SGM fraction (Fig. 1C), whereas insulin was accu-

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FIG. 1. Fractionation of SG components in INS-1 cells. A, crude extract of INS-1 cells was fractionated via linear sucrose density gradient centrifugation. 16 fractions were obtained, and an IRI level was measured for each fraction. The IRI peak appeared at fraction 10–13. B, fractionated samples were analyzed for the distribution of the SG-associated proteins, SgIII, CgA, and CPE, by immunoblotting. SgIII, CgA, and CPE accumulated heavily at fraction 10–13. The peak fraction showed a buoyant density of 1.178 ± 0.005 g/cm³ (five independent experiments). C, the SGM was purified from INS-1 cell homogenates as indicated at the top of the panel. One microgram of each purification step sample was analyzed for the presence of SgIII, CgA, CPE, and phogrin. Phogrin, having a trans-membrane region, accumulated on the SGM. Over 80% of SgIII, CgA, and CPE molecules also accumulated on the SGM, although they lacked a trans-membrane region. D, IRI accumulated in a supernatant fraction after hypotonic lysis of the SG. C and D, the INS-1 cell homogenate was centrifuged to obtain the post-nuclear supernatant (PNS). The resultant supernatant was recentrifuged to obtain the crude organelle fraction. This fraction was divided into 16 fractions using sucrose density gradient centrifugation. The SG fraction (fraction 11–13) was hypotonically lysed and divided into supernatant (Sup) and SGM fractions.
mulated to the soluble fraction (Fig. 1D). Thus, SgIII appears to associate with the SGM in INS-1 cells.

**Cholesterol Is Required for the Binding of SgIII and CgA to the SGM**—Since SgIII was pulled down with the SGM despite lacking the transmembrane-spanning region, we attempted to find a partner protein for mediating SgIII binding to the SGM using a yeast two-hybrid system and a pull-down experiment using an antibody to SgIII. However, we were unable to find such candidate proteins (data not shown).

We then hypothesized that SgIII may bind directly to the SGM through lipid components in the SGM. This hypothesis is based on the following four findings. 1) The SGM is composed of a specific lipid composition, which is markedly different from that of other organelles (17). 2) Depletion of cholesterol with lovastatin inhibits the SG formation in AtT-20 cells (18). 3) Lipid raft association of CPE results in its sorting to the secretory granule (17). 4) PC2, a prohormone-converting enzyme, binds to chromaffin granule membrane through lipid rafts (19). We treated the SGM with increasing concentrations of mβCD to examine a binding characteristic of endogenous SgIII (Fig. 2). mβCD is known to complex with cholesterol and deplete it from membranes with high selectivity, leaving phospholipids unaffected (29). When we washed the SGM with mβCD, the three SGM-associated proteins, SgIII, CgA, and CPE, were gradually released from the precipitated SGM fraction to the supernatant soluble fraction (Fig. 2). Both SgIII and CgA were completely released from the SGM at 50 mM mβCD (Fig. 2, lane 5), whereas CPE was still partially retained in the SGM fraction even at 100 mM mβCD (Fig. 2, lane 7). Thus, the binding mechanism of SgIII and CgA to the SGM appears to differ from that of CPE.

Because the insolubility of the proteins in detergents is a requisite of lipid raft-associated proteins (30), we investigated whether SgIII is associated with lipid rafts using a variety of detergents. When the SGM from INS-1 cells was treated with 1% Triton X-100 for 30 min at 4°C, both SgIII and CgA were solubilized to the supernatant fraction (Fig. 3A, S), whereas with the same treatment, most of the CPE molecules remained in the precipitated membrane fraction (P). We further attempted to characterize the difference in solubility between SgIII and CPE using non-ionic detergents including Lubrol WX, Brij 35, octylglucoside, Tween 20, and the zwitterionic detergent CHAPS (31). However, both SgIII and CPE were solubilized in the same fashion (Fig. 3B, S). CgA also showed a similar solubility pattern to these detergents (data not shown). Thus, SgIII and CgA appear to use cholesterol for their binding to the SGM instead of lipid rafts, whereas CPE binds to the SGM through Triton X-100-resistant lipid rafts.

**Binding Specificity of SgIII to the SGM**—To further prove the existence of the direct binding of SgIII to lipid components of the SGM without mediator proteins, we made GST-fused SgIII, CgA, and CPE and assessed their direct binding to 3H-labeled liposome incorporating [3H]PC ([3H]liposome) with specific membrane lipid similarity. We immobilized each GST fusion protein onto glutathione beads for incubation with [3H]liposome under an indicated condition. When total lipids from bovine brain was used for liposome components (Fig. 4A),...
SgIII and CPE bound to the brain-type liposome without Ca\(^{2+}\)/H\(_{11001}\) dependence and pH preference (Fig. 4 A). CgA bound to the liposome weakly but more so than did GST alone. We then employed the SGM-type liposome composed of 6.2 mol % PC, 17.7 mol % PE, 3.6 mol % PS, 1.0 mol % PI, 7.5 mol % SM, and 64 mol % cholesterol (17). This SGM-type liposome constituted of highly enriched mol % cholesterol. By microscopic observation, however, the SGM-type liposome was similar to the brain-type one as well as to a standard liposome (76% PC, 24% PS) (data not shown). When the GST-SgIII and GST-CgA were incubated with the SGM-type liposome, only SgIII bound to the liposome at pH 5.5 irrespective of the presence of Ca\(^{2+}\), whereas CgA did not under any of the four sets of conditions. In contrast, CPE bound to the SGM-type liposome more in the presence of 10 mM Ca\(^{2+}\)/H\(_{11001}\) than in its absence and more at pH 5.5 than at pH 7.4 (Fig. 4 B). Thus, the binding characteristics of SgIII, CgA, and CPE to the SGM-type liposome differed from each other.

Because the cholesterol-rich SGM-type liposome displayed the highest binding to the GST-SgIII at pH 5.5, we examined binding of a liposome with 0–64 mol % cholesterol to the GST-SgIII. The \[^{3}\text{H}]\text{liposome increased in binding to the GST-SgIII with the increase of a cholesterol ratio, and the binding to the GST-SgIII was plateaued at 60 mol % cholesterol composition.}

**Fig. 5. Characteristics of SgIII binding to the SGM liposome.**

A, equivalent amounts of GST-fused SgIII-(23–471) (full-length), SgIII-(23–186) (N-terminal region), SgIII-(187–372) (binding region to CgA), and SgIII-(373–471) (C-terminal region) were immobilized to glutathione-agarose beads and were then used for the SGM-type liposome binding at pH 5.5 without Ca\(^{2+}\). B, effect of pH on the dissociation of SgIII from the SGM liposome. GST-SgIII-(23–186) or GST alone was immobilized on glutathione-agarose beads and was then incubated with the SGM-type liposome at pH 5.5 and washed with pH 5.5 or 7.4 buffer.

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beads was incubated with the SGM liposome in the presence of MBP-SgIII or MBP alone at pH 5.5 and with 10 mM Ca\(^{2+}\).

We then determined a binding domain of SgIII to the SGM-type liposome using four sets of GST-fused SgIII fragments: SgIII-(1–23); SgIII-(23–186); SgIII-(187–372); and SgIII-(374–471). SgIII-(1–23), a signal sequence, was deleted from the GST-fused fragments. SgIII-(23–186) was as adherent to the SGM-type liposome as full-length SgIII-(23–471), whereas SgIII-(187–372) (binding domain for CgA) did not adhere to the liposome (Fig. 5A). Thus, GST-SgIII-(23–186) reflects the binding potency of the full-length SgIII-(23–471) to the liposome. The C-terminal domain, SgIII-(373–471), showed a weak but a significant level of binding capacity to the SGM liposome.

When SGs are exocytosed by fusion to the plasma membrane, the intragranular pH milieu shifts from weakly acidic to neutral and granin proteins are released to the extracellular space. We examined the effect of pH on the dissociation of SgIII from the SGM-type liposome. After incubation of GST-fused SgIII with the SGM-type liposome at pH 5.5, the mixture was washed with pH 5.5 or 7.4 buffer. However, the binding of SgIII to the SGM-liposome was unchanged at either pH 5.5 or pH 7.4 (Fig. 5B), suggesting that other mechanisms are involved in the dissociation of SgIII from the SGM.

We previously demonstrated that SgIII binds strongly to CgA in an intragranular milieu at pH 5.5 and 10 mM Ca\(^{2+}\) (8). In this study, we tested whether CgA requires SgIII mediation for the binding to the SGM-type liposome. We expressed SgIII-(23–471) as a MBP fusion protein and isolated a fusion protein with maltose-binding beads. GST-CgA immobilized on GST beads was incubated with the SGM-type liposome in the presence of MBP-SgIII or MBP alone at pH 5.5 and 10 mM Ca\(^{2+}\). GST-CgA bound to the liposome only in the presence of MBP-SgIII (Fig. 6). MBP did not affect the binding of GST-CgA to the SGM-type liposome. These data indicate that CgA requires SgIII mediation for its binding to the SGM.

**Determination of Specific Binding Domain of SgIII to the SGM**—We then determined a binding domain of SgIII to the SGM-type liposome using four sets of GST-fused SgIII fragments: SgIII-(23–471); SgIII-(23–186); SgIII-(187–372); and SgIII-(374–471). SgIII-(1–23), a signal sequence, was deleted from the GST-fused fragments. SgIII-(23–186) was as adherent to the SGM-type liposome as full-length SgIII-(23–471), whereas SgIII-(187–372) (binding domain for CgA) did not adhere to the liposome (Fig. 5A). Thus, GST-SgIII-(23–186) reflects the binding potency of the full-length SgIII-(23–471) to the liposome. The C-terminal domain, SgIII-(373–471), showed a weak but a significant level of binding capacity to the SGM liposome.

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**SgIII Lacking the SGM-binding Domain Did Not Target to SGs**—Because SgIII-(23–186) binds to the SGM effectively (Fig. 5A), this fragment may play an essential role in its targeting to the SGs. We made a series of deletion constructs of SgIII tagged with a FLAG epitope as shown in the left panel of Fig. 7. Their targeting to SGs was evaluated in AtT-20 cells by immunocytochemical staining and a pulse-chase labeling experiment. By immunostaining, we localized SgIII-(1–186) for the SGM binding domain (SgIII-(40–186)) and SgIII-(187–373) containing the binding domain SgIII-(23–186) for the SGM. They were localized densely at the tips of cell processes in addition to the perinuclear Golgi area (Fig. 7, A and C). They were virtually co-localized with ACTH. To further examine the functional significance of the domain 40–186, we made two additional constructs, SgIII-(1–186) and SgIII-(1–186). Their intracellular localizations were strikingly distinct. SgIII-(40–186) was virtually restricted to the Golgi area and failed to target to SGs (Fig. 7B). In contrast, SgIII-(1–186) was properly targeted to the SGs at the tips of cell processes (Fig. 7D). These findings suggest that the N-terminal sequence 40–186 is indispensable for SgIII targeting to SGs in AtT-20 cells. Although we attempted to investigate the functional significance of the C-terminal domain 374–471 of SgIII, we failed to express a construct SgIII-(374–471) in AtT-20 cells for reasons unknown.

We then confirmed these morphological data by 2 h of pulse labeling with \(^{35}\)S)methionine cysteine and 1 h of chasing with or without 8-Br-cAMP, a stimulant of ACTH secretion. As shown in Fig. 8, radiolabeled SgIII-(1–471) and SgIII(1–186) were detected over 2-fold more often in the cell extract than in the culture medium in a non-stimulating state. In an 8-Br-cAMP-stimulating state, their radiocontent in the cell extract...
decreased to a third of that in the culture medium, indicating that it was secreted to the culture medium by stimulation. In contrast, there was approximately 2-fold more radiolabeled SgIII(1–471) secreted into the culture medium than in the cell extract, even in a non-stimulating state compared with the stimulating state. Thus, it appears that the SGM-binding domain, SgIII(40–186), is essential for the retention of SgIII inside the cells. Taken together with the immunocytochemical studies in Fig. 7, these data indicate that SgIII requires the SGM-binding domain 40–186 for its targeting to secretory granules in AtT-20 cells.

**DISCUSSION**

In this study, we demonstrated that SgIII is localized to the SGM fraction together with CgA and CPE by subcellular fractionation. Biochemical analyses with liposomes revealed that SgIII specifically binds to cholesterol-rich SGM in a weakly acidic condition via its N-terminal region SgIII-(23–186). Conversely, SgIII(40–186), deleting SgIII(40–186) from its full-length sequence, could not target to the ACTH granules, resulting in its mis-sorting to a constitutive secretory pathway in AtT-20 cells. In the absence of SgIII, full-length CgA could not bind to the SGM-type liposome. We previously reported that SgIII binds to CgA between SgIII-(214–373) and CgA-(48–111) under weakly acidic pH and high calcium conditions similar to the luminal milieu of the TGN and that CgA(48–111) could not target to SGs in AtT-20 cells (8). We further showed that SgIII is localized preferentially at the peripheral region of secretory granules in pituitary endocrine cells (32). Because SgIII(23–186) adheres to cholesterol molecules in the SGM, SgIII appears to serve an adapter function for SG proteins including CgA to form the intragranular protein complex in SGs.

Cholesterol-sphingolipid micromdomain (lipid raft)-dependent transport has received much attention for its role in the correct intracellular trafficking of proteins and lipids to cell surface membrane (20–22, 31). The lipid raft also appears to be essential for the targeting of SG proteins to the SGs (7). CPE was one of the first SG proteins to be shown to bind to the SGM via the cholesterol-rich lipid raft (17). In this study, we demonstrated that SgIII specifically binds to the cholesterol-rich membrane but that SgIII is different from CPE in its resistance to mβCD and Triton X-100 (Figs. 2 and 3A). CPE was revealed to possess a lipid-binding domain in its C-terminal tail four residues, Thr-Glu-Leu-Asn-Phe (33). In contrast, the lipid-binding domain of the SgIII was situated in the N-terminal region SgIII-(23–86), although we must still narrow down the region responsible for lipid binding. Another lipid raft-residential protein, PC2, has been shown to bind to the sphingolipid-rich SGM via its N-terminal region PC2-(45–84) (19). Interestingly, the binding of PC2 to liposomes made from bovine brain lipids was not disrupted by either CgA(1–80) containing the N-terminal disulfide loop or CgA-(406–431) covering the C-terminal end. The interaction between the SG proteins and lipid components of the SGM requires extensive study before a consensus interaction mechanism is obtained.

SgIII is localized in the SGs of neuroendocrine cells (34, 35). SgIII mRNA is expressed in particular subsets of central nervous system neurons (36), pituitary cells (32), and various endocrine cells (8). Mice missing the SgIII gene revealed no major obvious effects on viability, fertility, or locomotor behavior, so that SgIII is not required for their survival (37). However, because SgIII is a residential protein in SGs, Martens and colleagues (38) have addressed its secretory function using the Xenopus pituitary intermediate lobe. The mRNA levels of both SgIII and POMC increased >30-fold in the intermediate pituitary when Xenopus was placed on a black background from a white background, resulting in an increase in melanophore-stimulating hormone for color adaptation (38). The coordinated induction of SgIII and POMC messages and the resultant melanophore-stimulating hormone secretion exemplifies a finely integrated secretory system for biological adaptation. Although we did not examine the coordinated expression and binding of SgIII and ACTH in AtT-20 cells, we observed that SgIII is sorted to ACTH-containing granules unless the N-terminal region of SgIII is disrupted.

In addition to the coordinated expression of SgIII and POMC, other granins also coordinate well with POMC and other peptide hormones in SG biogenesis. With CgB overexpression in AtT20 cells, a 23-kDa fragment of POMC was increasingly stored in SGs, suggesting that CgB acts as a helper for sorting the POMC fragment to the SGs (39). CgB appears to condense the POMC fragments efficiently into the SGs, although CPE reportedly acts as a sorting receptor for POMC to the SGs (10). SgII enhanced lutenizing hormone storage into the SGs by co-aggregation in mouse gonadotrophs (40). Because SgII is localized in small granules but not in large granules and because lutenizing hormone is distributed in both types of granules in male gonadotrophs (41), other granin-like proteins may play a role in condensing SgII and lutenizing hormone in small granules. CgA and CgB form a multimer that binds to the inositol 1,4,5-triphosphate (IP3) receptor/Ca2+ channel by which secretory granules retain a high level of Ca2+ for their exocytosis (3). Although we do not know whether IP3 receptor possesses a sorting signal to SGs, IP3 receptor appears to coordinate functional sorting with CgA and CgB for SG biogenesis. Thus, granins are thought to enhance the condensation of peptide hormones and other SG-associated proteins to SGs by co-aggregation or by specific interaction with other SG-associated proteins.

It remains to be determined just how intragranular protein complexes are formed at the TGN and SGs. We believe that at least two SGM-binding proteins, CPE and SgIII, serve as an adapter function for intragranular complex formation via specific lipid binding. Since CPE has been proposed to be a sorting receptor for POMC (10), a number of studies have presented...
data both for and against a sorting receptor function of CPE. The sorting of proinsulin was not impaired in CPE-deficient Cpe<sup>−/−</sup>/Cpe<sup>fast</sup> mice (42, 43), whereas CPE was shown to coaggregate with POMC, prolactin, and insulin at an acidic pH in vitro (44). Although CPE plays an essential role in sorting for a limited number of prohormones, it is not a universal sorting receptor. This is also true for the sorting role of SgIII. SgIII anchors CgA to the SGM, and CgA multimers condense prohormones and SG-associated proteins in the intragranular complex by co-aggregation. Thus, we believe that SG formation depends on at least three mechanisms: 1) specific lipid binding complex by co-aggregation. Thus, we believe that SG formation

hormones and SG-associated proteins in the intragranular anchors CgA to the SGM, and CgA multimers condense pro-

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Acknowledgments—We thank Dr. Claes B. Wollheim for INS-1 cell (University Medical Center Rue Michel-Servet 1) and M. Hossi and M. Kosaki for their technical support.
Secretogranin III Binds to Cholesterol in the Secretory Granule Membrane as an Adapter for Chromogranin A
Masahiro Hosaka, Masayuki Suda, Yuko Sakai, Tetsuro Izumi, Tsuyoshi Watanabe and Toshiyuki Takeuchi

J. Biol. Chem. 2004, 279:3627-3634.
doi: 10.1074/jbc.M310104200 originally published online November 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M310104200

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