Lipid Raft Association of Carboxypeptidase E Is Necessary for Its Function as a Regulated Secretory Pathway Sorting Receptor*

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Membrane carboxypeptidase E (CPE) is a sorting receptor for targeting prohormones, such as pro-opsin, to the regulated secretory pathway in endocrine cells. Its membrane association is necessary for it to bind a prohormone sorting signal at the trans-Golgi network (TGN) to facilitate targeting. In this study, we examined the lipid interaction of CPE in bovine pituitary secretory granule membranes, which are derived from the TGN. We show that CPE is associated with detergent-resistant lipid domains, or rafts, within secretory granule membranes. Lipid analysis revealed that these rafts are enriched in glycosphingolipids and cholesterol. Pulse-chase and subcellular fractionation experiments in AtT-20 cells show that the association of CPE with membrane rafts occurred only after it reached the Golgi. Cholesterol depletion resulted in dissociation of CPE from secretory granule membranes and decreased the binding of prohormones to membranes. In vivo cholesterol depletion using lovastatin resulted in the lack of sorting of CPE and its cargo to the regulated secretory pathway. We propose that the sorting receptor function of CPE necessitates its interaction with glycosphingolipid-cholesterol rafts at the TGN, thereby anchoring it in position to bind to its prohormone cargo.

Endocrine and neuroendocrine cells synthesize and secrete peptide hormones and neuropeptides in a highly regulated manner. Peptide hormones are initially synthesized as larger precursors, or prohormones, and may acquire a number of post-translational modifications during transportation through the ER and Golgi stacks. Upon entering the trans-Golgi network (TGN), prohormones are segregated away from constitutively secreted proteins and instead are targeted to the regulated secretory pathway (RSP). It is during the sorting process that prohormones are packaged into budding immature secretory granules. A small subset of constitutive proteins also enters the immature secretory granules and is removed through a process termed constitutive-like secretion (1). The mature secretory granule is the storage compartment of the RSP, from which peptide hormones are released in a Ca2+-dependent manner.

The molecular mechanisms that are involved in the sorting of prohormones to the RSP have been intensively examined. One key step in sorting to the RSP may be selective Ca2+-dependent aggregation, as observed with chromogranins A and B (2). However, aggregation in itself is not sufficient to target proteins to the RSP, since sorting can occur in the absence of aggregation (3), and constitutive secretion can occur in the presence of aggregation (4). Sorting of prohormones to the RSP may therefore be a more active and selective process, requiring the presence of a specific sorting signal and sorting receptor. Sorting signals have been identified in the form of structural motifs, such as the disulfide-bonded loop of chromogranin B (5, 6) and the hydrophobic acidic amino acid motif of pro-opiomelanocortin (POMC) (7). However, it is not clear if CGB itself binds to the membrane of the TGN or if it requires interaction with another protein (8). In contrast, it has been shown that the sorting of POMC, proenkephalin, and proinsulin requires the presence of the membrane form of carboxypeptidase E (CPE) as a sorting receptor (9). A number of studies have provided evidence that CPE functions as a sorting receptor for prohormones. The N-terminal end of POMC, which contains the sorting signal, binds specifically to CPE in secretory granule membranes from bovine intermediate pituitary (9). Antisense-mediated depletion of CPE in Neuro2A cells causes constitutive secretion of prohormones (9, 10). As well, POMC, proenkephalin, and proinsulin all exhibit similar binding kinetics to CPE, in both secretory granule membranes (11) and Sf9 cell membranes expressing recombinant mouse CPE (12). Finally, the binding site on CPE that interacts with the sorting signal of prohormones resides in a domain distinct from the catalytic domain of soluble CPE (12). Therefore, the interaction between the sorting signals of POMC, proenkephalin, and proinsulin and their binding site on CPE is highly specific, indicating that CPE acts as a sorting receptor in the targeting of these prohormones to the RSP.

Thus far, protein-protein interactions have been the sole mechanism used to describe the process of regulated sorting from the TGN. However, a role for membrane lipids in this process has not been examined. It has been proposed that cholesterol-glycosphingolipid microdomains, or rafts, exist within the plasma membrane of cells (13, 14). The organization of membrane proteins into rafts may be required for a diverse number of cellular functions, including apical sorting (15, 16), endocytosis (17), and intracellular signaling (18, 19). Similarly, lipid rafts could also play a role in sorting to the RSP in endocrine cells. We therefore hypothesized that rafts exist within the membranes of the TGN and secretory granules and that CPE must be anchored to rafts in its function as an RSP.

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1 The abbreviations used are: ER, endoplasmic reticulum; TGN, trans-Golgi network; CPE, carboxypeptidase E; POMC, pro-opiomelanocortin; N-POMC, N-terminal 26 amino acids of POMC; RSP, regulated secretory pathway; SGM, secretory granule membrane; CGA, chromogranin A; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; LPDS, lipoprotein-deficient FBS; ISG, immature secretory granule; HPTLC, high performance thin layer chromatography; TNE, Tris-NaCl-EDTA; GD1a, disialoganglioside 1a; GM1/3, monosialoganglioside 1/3; GB4, globoside.
sorting receptor. Our previous work has shown that CPE is not extracted from pituitary secretory granule membranes treated with Triton X-100 or with 1 m NaCl (11), suggesting that CPE may interact with detergent-resistant membranes. Here we show that CPE, a sorting receptor for the regulated secretory pathway, associates with cholesterol-glycolipid rafts in secretory granule membranes and that interaction with rafts is necessary for its function as a sorting receptor.

**EXPERIMENTAL PROCEDURES**

**Materials—**AtT-20 cells were obtained from the American Type Culture Collection (Manassas, VA). Dulbecco’s modified Eagle’s medium (DMEM) was from Biofluids (Rockville, MD). Fetal bovine serum (FBS) was from Life Technologies, Inc. Lipoprotein-deficient FBS (LPDS) was prepared by Intracel (Issaquah, WA). Tris-glycine gels and nitrocellulose membranes were purchased from Novex (San Diego, CA), and horseradish peroxidase-linked anti-rabbit IgG was from Amersham Pharmacia Biotech. For pulse-chase studies, [35S]methionine and protein A-Sepharose beads were from Amersham Pharmacia Biotech, and carrier-free [35S]sulfate was from ICN (Costa Mesa, CA). For lipid analysis, all phospholipid standards and cholesterol standards were purchased from Sigma, and glycolipid standards were prepared by Matryce, Inc. (Pleasant Gap, PA). High performance thin layer chromatography (HPTLC) plates were from Merck. Sephadex A-25 was from Sigma, and Iatrobeads were obtained from Bioscan (Washington, D. C.). For cholesterol depletion experiments, methyl-β-cyclodextrin and mevastatin were bought from Sigma, and lovastatin was obtained from Calbiochem.

**Detergent Extractions and Western Blotting for CPE—**Highly purified secretory granules and microsomes (P4 fraction) were prepared from bovine pituitary neural and intermediate lobes as described previously (20). In order to prepare granule membranes, granules were lysed by six rounds of freeze thawing in Tritis-succinate, pH 5.9, and then washed in three changes of 10 mM Tris-EDTA, pH 7.4. Membranes were solubilized in cold 1% Triton X-100 in TNE (50 mM Tris-Cl, 150 mM NaCl, 5 mM MgCl2, 2 mM CaCl2, 1 g/liter glucose, 20 mM HEPES, pH 7.2). The [35S]sulfate (ICN) for 4.5 min for TGN localization and chased for 15 min for ISG localization. Cells were washed and scraped in ice-cold homogenization buffer (1 mM EDTA, 1 mM magnesium acetate, 1.6 mM Na2SO4, 250 mM sucrose, 10 mM HEPES, pH 7.15). Cell pellets were homogenized using an EMBL cell cracker. The resulting postnuclear supernatant was loaded onto a linear 0.3–1.2 M sucrose density gradient and centrifuged for 15 min at 110,000 × g in a Beckman SW 28.1 rotor. Fractions of 1 ml were collected; half of each fraction was used for polyacylamide gel electrophoresis analysis of sulfated POMC. Fractio 14 (114) and fraction 15 (ISGs) were pooled with 1 volume of homogenization buffer, and centrifuged at 110,000 × g for 15 min to isolate TGN or at 130,000 × g for 30 min to isolate ISGs. Both TGN and ISGs were lysed by three rounds of freeze-thawing and extracted with 1% Triton X-100 for 30 min on ice. Membrane and soluble fractions were analyzed for CPE immunoreactivity by Western blot.

**Subcellular Fractionation of CPE in the ER—**Isolation of ER was carried out using the method of Watanabe et al. (23). Briefly, AtT-20 cells were grown to confluency in 10-cm dishes. After trypsinization, cell pellets were homogenized in an EMBL cell cracker, and the resulting postnuclear supernatant was loaded on a sucrose density gradient consisting of 3.6 ml of 38% sucrose, 1.8 ml of 30% sucrose, and 1.8 ml of 20% sucrose. Samples were centrifuged at 100,000 × g in a Beckman Ti70.1 rotor for 3 h, and eight 1-ml fractions were collected. Half of each fraction was used for Western blot analysis of the ER marker calnexin. Fractions enriched in calnexin immunoreactivity (fractions 2–6) were pooled, diluted with 1 volume of homogenization buffer, and extracted with 1% Triton X-100 on ice for 30 min. Membrane and soluble fractions were analyzed for CPE immunoreactivity by Western blot.

**Lipid Analysis—**Secretory granule membranes (both unextracted and detergent-extracted) from pituitary neural lobe were weighed and homogenized in 1:1 chloroform/methanol while still frozen. Neutral and acidic lipid fractions were isolated by elution from a Sephadex A25 column (24), and separation and detection of phospholipids were carried out according to the quantitative TLC methods of Macala et al. (24). Neutral glycolipids and gangliosides were purified according to Ariga et al. (25) and Brown and Rose (16). Neutral glycolipids were separated on a TLC plate using chloroform/methanol/acetic acid/water (5:2:1:2) and detected with 0-naphthol. Gangliosides were separated by TLC using chloroform/methanol/0.25% KCl (50:45:10) and visualized with 0-naphthol. Densitometric scanning and quantification of all lipids were carried out using NIH-Image version 1.57 and ImageQuant version 1.2 (Molecular Dynamics) software, respectively.

**Cholesterol Depletion Experiments in Vitro—**Intermediate or neural lobe membranes were prepared as described above. 100 μg of membrane protein was added to microcentrifuge tubes containing 250 μl of 0, 10, 20, 40, 80, or 100 mM methyl-β-cyclodextrin and mixed well. Tubes were incubated at 37°C for 20 min with shaking. Centrifugation, trichloroacetic acid precipitation, and Western blotting for membrane CPE were carried out as described above.

**Binding Assays—**Neural lobe membranes were prepared as described above, extracted with 0, 20, or 80 mM methyl-β-cyclodextrin as described above. Binding of 125I-labeled N-POMC or 125I-labeled proinsulin to extracted neural lobe membranes was carried out as described previously (9, 11). Values obtained for specific binding varied from 2732 ± 6 cpm (POMC) and 2365 ± 136 cpm (proinsulin) in unextracted membranes to 655 ± 101 cpm (POMC) and 1173 ± 226 cpm (proinsulin) in membranes extracted with 80 mM methyl-β-cyclodextrin. All values were plotted as percentage of the total from three experiments.

**Cholesterol Depletion Experiments in Vivo—**For immunocytochemical studies, AtT-20 cells were grown in two-wall plastic chamber slides (Lab-Tek) until approximately 60% confluent. To deplete exogenous cholesterol sources and inhibit intracellular cholesterol synthesis, cells were treated with 10 μM lovastatin plus 250 μM mevastatin in 10%
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RESULTS

CPE Association with Lipids in Secretory Granule Membranes—Previous work from our laboratory indicated that membrane CPE could not be solubilized by treatment of pituitary secretory granule membranes (SGMs) with Triton X-100 at 22 °C (11). Since insolubility in Triton X-100 at 4 °C is one of the criteria by which raft proteins are identified (27), we pursued the hypothesis that CPE is associated with lipid rafts in SGMs.

Treatment of SGMs prepared from bovine pituitary intermediate lobes with 1% Triton X-100 for 30 min at 4 °C did not solubilize C-terminal immunoreactive CPE (Fig. 1A). As a positive control, blots were stripped and probed with antibodies to chromogranin A (CGA), a regulated secretory protein that is removed from SGMs by cold Triton X-100 (Fig. 1A). These extractions were repeated in SGMs derived from bovine chromaffin granules with similar results. Quantification of CPE- and CGA-immunoreactive bands indicated that >60% of the membrane CPE stayed in the pellet after extraction with Triton X-100, while >95% of granule CGA was solubilized. SGMs were also treated with β-octyl glucoside, since this detergent solubilizes raft proteins (16). Approximately 70% of total immunoreactive CPE was extracted from SGMs with 60 mM octyl glucoside.

To further determine whether CPE is associated with detergent-insoluble lipids, the density of the Triton X-100-insoluble fraction was examined by centrifugation on sucrose density gradients. As seen in Fig. 1B, a large pool of CPE floated to a lower density fraction (1.12–1.08 g/cm³), and a smaller pool floated to the lightest fractions (1.05–1.03 g/cm³). To this result we compared the density of solubilized CPE by extracting SGMs with octyl glucoside instead of Triton X-100 prior to density gradient centrifugation. Most of the CPE was recovered in the highest density fraction following this treatment (Fig. 1B), as are other raft proteins following solubilization (16).

Pulse-chase experiments were performed in AtT-20 cells in order to determine the kinetics with which CPE acquires Triton X-100 insolubility in vivo. After a 2-min pulse with [35S]methionine, cells were chased for 5, 20, 60, and 180 min; these chase times approximate passage into the ER, Golgi, immature secretory granule, and mature secretory granule, respectively. Cells were then extracted with 1% Triton X-100 in TNE, and CPE was immunoprecipitated from both particulate and soluble fractions. Analysis by polyacrylamide gel electrophoresis shows that CPE was almost entirely detergent-soluble in the first 5 min of chase (Fig. 2). However, CPE began to acquire resistance to Triton X-100 extraction at 20 min of chase, and by 60 min, all of the radiolabeled CPE was resistant to detergent extraction (Fig. 2). In order to precisely determine the compartment in which CPE becomes detergent-resistant, subcellular fractionation of AtT-20 cells was used to isolate vesicle preparations enriched in ER, TGN, or ISGs. The ER resident protein calnexin was used as an ER marker, and ER membranes were prepared from fractions enriched in calnexin immunoreactivity (Fig. 3A). Western blot analysis of soluble and membrane fractions showed that CPE was detergent-soluble in fractions enriched in calnexin (Fig. 3A). Membranes of the TGN and ISGs were prepared from fractions isolated by velocity density gradient centrifugation. Since CPE was not sulfated appreciably (data not shown), [35SO₄]POMC was used as a marker protein (21). After a 5-min pulse, [35SO₄]POMC was present in fractions 4–6, representing its localization in the TGN; after a 15-min chase, POMC had moved into fractions 10–14, which contain ISGs (Fig. 3B). A significant portion of CPE (50%) was Triton X-100-insoluble in both TGN and ISG membranes. These results indicate that CPE associates with detergent-insoluble membranes in TGN and immature secretory granules.

Lipid Composition of the Triton X-100-insoluble Fraction of SGMs—Phospholipid, glycolipid and cholesterol content of Triton X-100-extracted SGMs was analyzed by HPTLC (16, 24, 25). Lipids from three separate pituitary neural lobe SGM preparations were quantified by densitometric scanning of
HPTLC plates and comparison with standards, and the results are shown in Table I. Phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin are the major phospholipids in SGMs. These results are comparable with those reported previously for pituitary and chromaffin SGMs (28, 29). Cholesterol comprised over half of the total lipid content of SGMs (Table I). Extraction of SGMs with Triton X-100 at 4 °C reduced total phospholipid and cholesterol content; 70–90% of phospholipids and 65% of cholesterol were removed by detergent treatment (Table I). On a molar basis, cholesterol was enriched by 20% in detergent-insoluble fractions. Qualitative analysis of phospholipids and cholesterol from pituitary intermediate lobe SGMs yielded similar results (data not shown).

The glycolipid composition of pituitary SGMs has not been previously documented. Here, we show the major neutral glycolipid to be globoside; visual inspection of HPTLC plates did not reveal any other neutral glycolipid present, even upon overloading the plate (data not shown). Three major gangliosides were present in SGMs: GD1a, GM1 and GM3. Approximately 70% of total glycolipid remained in Triton X-100-insoluble membranes, thereby enriching them 2–3-fold in glycolipid content (Table I). Therefore, the detergent-resistant SGMs in which CPE resides appear to be enriched in neutral glycolipids and gangliosides (i.e. glycosphingolipids) and cholesterol and relatively depleted of all phospholipids.

Membrane Cholesterol Is Required for Binding of Prohormones to CPE—Of all the lipids that comprise detergent-resistant secretory granule membranes, the most abundant is cholesterol (Table I). We therefore assessed the solubility of CPE in cholesterol-depleted membranes using methyl-β-cyclodextrin to remove cholesterol from SGMs. Fig. 4 shows that CPE was removed from SGMs in a dose-dependent manner by methyl-β-cyclodextrin. To show that removal of CPE by depleting membrane cholesterol affects binding of prohormones, binding assays were carried out using cholesterol-depleted SGMs. Treatment of SGMs with methyl-β-cyclodextrin decreased 125I-labeled N-POMC binding in a dose-dependent manner (Fig. 5). The removal of cholesterol and CPE also significantly (p < 0.05) reduced 125I-labeled proinsulin binding in a similar manner, from 2365 ± 138 cpm in control membranes to 1173 ± 253 cpm in membranes extracted with 80 mM methyl-β-cyclodextrin.

Membrane Cholesterol Is Required for Sorting to the RSP in Vivo—In order to correlate the in vitro binding results with the in vivo biological function of CPE, cholesterol depletion experiments were carried out in AtT-20 cells. Cells were depleted of both exogenous and endogenous sources of cholesterol, and the sorting of CPE and its prohormone cargo POMC were assessed by immunocytochemistry and the secretory response to stimulation with 50 mM K+. Figs. 6 and 7 show the effects of cholesterol depletion on the sorting of POMC and CPE, respectively. In control AtT-20 cells, punctate staining of POMC (Fig. 6A) and CPE (Fig. 7A) was evident in the cell processes through to the tips, indicating storage in regulated secretory granules. After cholesterol depletion, punctate POMC and CPE staining was almost completely abolished (Figs. 6B and 7B); instead, localization to the perinuclear area was observed, indicating lack of storage in regulated secretory granules. Following cholesterol repletion with DMEM containing 10% FBS for 12 h, punctate staining of POMC and CPE was recovered (Figs. 6C

**Table I**

Lipid composition of bovine pituitary neural lobe secretory granule membranes and Triton X-100-insoluble membranes

Lipids were extracted from secretory granule membranes and analyzed by quantitative TLC as described under “Experimental Procedures.” mol % was determined by calculating the moles of each lipid present in each membrane preparation and expressing the values as percentages of the total. Values are presented as means ± S.E. from three separate membrane preparations. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin.

| Phospholipids | Secretery granule membranes | Triton X-100-insoluble membranes |
|---------------|-----------------------------|---------------------------------|
|               | Tissue | Total lipid | n mol/mg | mol % | Tissue | Total lipid | n mol/mg | mol % | Enrichment |
| PC            | 7 ± 1  | 6.3 ± 0.2   | 0.2 ± 0.1 | 0.7 ± 0.1 |
| PE            | 19 ± 2 | 18 ± 0.4    | 3 ± 1     | 8.6 ± 2  |
| PS            | 4 ± 0.3| 3.7 ± 0.1   | 1 ± 0.1   | 3.4 ± 0.5 |
| PI            | 1 ± 0.02| 1 ± 0.1    | 0.2 ± 0.1 | 0.6 ± 1.0 |
| SM            | 8 ± 2  | 7.6 ± 2     | 1 ± 0.5   | 4.4 ± 2  |
| Cholesterol   | 67 ± 5 | 65 ± 2      | 24 ± 3    | 76 ± 2   | 1.2 ± 0.1 |
| Neutral glycolipid | 1 ± 0.1 | 1.2 ± 0.1 | 1 ± 0.1 | 3.1 ± 0.3 | 2.6 ± 0.2 |
| Gangliosides  |   |            |           |        |        |        |           |        |
| GD1a          | 0.7 ± 0.1| 0.7 ± 0.1| 0.5 ± 0.01| 1.6 ± 0.3 | 2.4 ± 0.3 |
| GM1           | 0.2 ± 0.3| 0.2 ± 0.02| 0.2 ± 0.03| 0.5 ± 0.03| 2.5 ± 0.1 |
| GM3           | 0.1 ± 0.01| 0.1 ± 0.03| 0.1 ± 0.01| 0.2 ± 0.01| 2.6 ± 0.2 |
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and 7C). Using the same cholesterol depletion procedure, pulse-chase studies were carried out to assess the extent of regulated secretion. Cells were labeled for 15 min and chased for three consecutive 1-h periods. The regulated secretory response was measured in cells that were treated with 50 mM K^+ during the final 1 h of chase. In control cells, 50 mM K^+ induced a 2.1 ± 0.02-fold increase (p < 0.05) in POMC secretion and a 2.3 ± 0.8-fold increase (p < 0.05) in N-terminal CPE secretion. For both POMC and CPE, there was a lack of secretory response to 50 mM K^+ following cholesterol depletion (0.9 ± 0.2 and 1.2 ± 0.5-fold, for POMC and CPE, respectively). Taken together, both the immunocytochemical and secretion experiments show that the sorting of CPE and its prohormone cargo, POMC, to the regulated secretory pathway is dependent upon membrane cholesterol.

DISCUSSION

Our previous work has shown that the sorting of prohormones to the RSP involves a receptor-mediated mechanism. The sorting receptor was identified as the membrane-associated form of CPE, which binds to a specific sorting signal motif on prohormones. It has been shown that POMC, proenkephalin, and proinsulin are dependent upon CPE for targeting to the regulated secretory pathway. Moreover, only the membrane-associated form of CPE was able to bind the RSP sorting signal contained within N-POMC (12). Since previous studies indicated that membrane CPE was primarily Triton X-100-insoluble, we investigated whether CPE could be directly associated with detergent-insoluble lipid domains, also known as lipid rafts. Raft microdomains have been implicated in the sorting of proteins, such as influenza virus hemagglutinin (15, 30) and placental alkaline phosphatase (16), in polarized epithelial cells to the apical surface of the plasma membrane. Also, GPI-anchored proteins require interaction with rafts for endocytic sorting (17). However, the role of rafts or raft proteins in sorting to the regulated secretory pathway of endocrine cells has not been demonstrated. In this study, we provide evidence that CPE, an RSP sorting receptor, is associated with cholesterol-glycosphingolipid-rich membranes at the TGN and remains associated within these rafts in secretory granule membranes derived from budding off of the TGN.

That CPE is associated with cholesterol-glycosphingolipid-rich rafts is supported by three criteria generally used to define a raft protein (14). First, CPE is resistant to Triton X-100 extraction at 4 °C from purified secretory granule membranes. This detergent resistance of CPE is not an artifact due to heterotypic aggregation, since chromogranin A, a regulated secretory protein known to form large aggregates (31), is solubilized by Triton X-100 treatment. Second, extraction of cholesterol by
methyl-β-cyclodextrin resulted in the solubilization of membrane CPE. Third, sucrose density centrifugation of Triton X-100-insoluble SGMs resulted in the floatation of CPE to low membrane CPE. Third, sucrose density centrifugation of Triton similar to that of placental alkaline phosphatase (1.08 g/cm³) (16), and influenza virus hemagglutinin (1.14 g/cm³) (32), two well characterized raft proteins. The means by which CPE is anchored to rafts may be through a specific domain within the C-terminal end. The last 21 C-terminal amino acids of CPE are predicted to form an amphipathic helix that may serve as a membrane anchor (33). We postulate that these same determinants interact with raft lipids to anchor CPE within rafts in SGMs.

We carried out experiments to demonstrate that CPE associates with rafts in vivo. Pulse-chase analysis of detergent insolubility in AtT-20 cells demonstrated that CPE began to acquire detergent resistance at 20 min of chase. Subcellular fractionation determined that membrane CPE was in a detergent-resistant domain at the TGN. GPI-anchored and transmembrane raft proteins trafficked to the plasma membrane first become detergent-insoluble in the Golgi (16, 32, 34). There is some recent ultrastructural evidence of membrane enzymes associating with rafts in the Golgi (35), and raft-enriched vesicles have been shown to originate from the TGN in vivo (36). We have now demonstrated for the first time that endocrine cells can also form rafts in the TGN and that CPE becomes associated with rafts in this compartment.

Analysis of the lipid profile of Triton X-100-insoluble SGMs revealed an enrichment in glycosphingolipids and cholesterol and a depletion of phospholipids, a composition consistent with rafts found in plasma membranes in mammalian cells (16, 37). Therefore, CPE may associate preferentially with the identified glycolipids and cholesterol in the TGN and SGMs. As a luminal protein within the secretory granule (38), we predict that CPE would anchor to glycolipid-cholesterol rafts on the luminal leaflet of the lipid bilayer of SGMs. Analysis of lipid orientation in chromaffin secretory granule membranes indicates that glycolipids are located in the luminal leaflet of the membrane bilayer (39, 40), suggesting that glycolipid-cholesterol rafts exist on the luminal side of SGMs. The lipid orientation is therefore consistent with the anchoring of CPE to rafts on the luminal side of SGMs.

Our results show that cholesterol is the major component of SGMs and SGM rafts. This is in agreement with previous studies, which show that filipin-cholesterol complexes are abundant on the surface of secretory granules in pancreatic β cells (41). It is therefore not surprising that removal of cholesterol in SGMs by methyl-β-cyclodextrin results in the solubilization of CPE. Cholesterol efflux induced by methyl-β-cyclodextrin solubilizes several plasma membrane raft proteins (42) and disrupts the clustering of GPI-anchored raft proteins in microdomains (43, 44). The functional significance of the cholesterol dependence of CPE membrane association is illustrated by the marked decrease in the binding of N-POMC, which contains an DSP sorting signal (45), and proinsulin to secretory granule membranes depleted of cholesterol. These results show that binding of N-POMC to membranes occurs only in the presence, not the absence, of membrane CPE. This is consistent with previous results, which showed that the presence of CPE is required for the binding of N-POMC to SGMs (9, 11) and to SP cell membranes (12). The fact that the binding of N-POMC is not affected by treatment of membranes with Triton X-100 (11) but is affected by cholesterol depletion demonstrates that CPE is available to bind its cargo in phospholipid-depleted, but not cholesterol-depleted, membranes. Therefore, the binding of prohormones to membranes is dependent upon the presence of raft-associated CPE. Consistent with this conclusion is the observation that, in vivo, cholesterol depletion by lovastatin treatment resulted in the missorting of both CPE and POMC, as assessed by immunocytochemistry and pulse-chase experiments. Cholesterol depletion studies have highlighted the importance of membrane cholesterol in intracellular sorting (17, 34, 35) and signaling (46, 47). The present study is the first to show that CPE requires the presence of membrane cholesterol in order to bind and sort its prohormone cargo. However, the in vivo experiments must be interpreted with caution, since cholesterol depletion in cells may have other effects on regulated secretion, including effects on granule formation (48). Nonetheless, our findings indicate a role for the binding of prohormones by raft-associated CPE in the TGN, from where they are sorted to the RSP in a cholesterol-dependent manner.

In summary, this paper shows for the first time that CPE, a sorting receptor for the regulated secretory pathway, is associated with glycolipid-cholesterol rafts within secretory granule membranes. That raft association of CPE occurs at the TGN allows us to expand our hypothesis of receptor-mediated sorting of prohormones to the RSP to include the interactions of CPE with lipid rafts. We suggest that lipid rafts may play a role in the sorting of prohormones by clustering CPE in the TGN, allowing its cargo to bind either as monomers or as aggregates. Rafts may also facilitate the clustering of other proteins destined for the RSP at the TGN, either directly or indirectly through interaction with other raft proteins.

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