Interaction between secretogranin III and carboxypeptidase E facilitates prohormone sorting within secretory granules

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Summary

Secretogranin III (SgIII) and carboxypeptidase E (CPE) bind specifically to cholesterol-rich secretory granule (SG) membranes. We previously showed that SgIII binds chromogranin A (CgA) and targets CgA to the SGs in endocrine cells. We investigated the binding of SgIII and CPE because they frequently localize close to the periphery of SGs, and they bind each other in mouse corticotrope-derived AtT-20 cells. In Cpe^{fat} mouse corticotropes, which have defective CPE, proopiomelanocortin (POMC)-derived adrenocorticotrophin hormone (ACTH)-containing peptides were distributed over the entire surface of the SGs, and displayed a regulated secretion by secretagogues. The Cpe^{fat} pituitary exhibited elevated levels of SgIII and CgA, which suggests that they compensate for a sorting function of CPE for POMC and its intermediates to ACTH. Indeed, both SgIII and CgA were able to bind POMC-derived intermediates. In a competitive pull-down assay, excessive SgIII led to a decrease in CPE-bound POMC-derived intermediate molecules, and SgIII pulled-down by anti-ACTH antibody increased proportionately. We suggest that SgIII and CPE form the separate functional sorting complex by anchoring to cholesterol-rich SG membranes, and POMC-derived peptides are transferred from CPE to SgIII, and subsequently to CgA.

Key words: Secretogranin III, Carboxypeptidase E, Chromogranin A, Proopiomelanocortin, Prohormone sorting

Introduction

The secretory granules (SGs) of neuroendocrine cells contain granin-family proteins in addition to peptide hormones and prohormone convertases. Granin-family proteins, including chromogranin A (CgA), CgB, secretogranin II (SgII), SgIII and 7B2, are prone to aggregate in a weakly acidic, high-Ca^{2+} intragranular milieu, and are thought to condense peptide hormones to SGs with co-aggregation (Glombik and Gerdes, 2000; Taupenot et al., 2003; Meldolesi et al., 2004). They have been shown to sort peptide hormones and intragranular proteins efficiently into the SGs. With CgB-overexpression in AtT20 cells, a 23 kDa fragment of proopiomelanocortin (POMC) was increasingly stored in SGs, suggesting that CgB acts as a helper for sorting the POMC fragment to the SGs (Natori and Huttner, 1996). Recently, we demonstrated that SgIII binds to CgA between the SgIII sequence 214-373 and the CgA sequence 41-109, and that SgIII targets CgA to SGs in pituitary and pancreatic endocrine cells (Hosaka et al., 2002).

Two decades ago, Tooze and Tooze demonstrated that POMC intermediate peptides that bind to anti-adrenocorticotropin hormone (ACTH) antibody localize at the periphery of immature SGs near the trans-Golgi network (TGN) and distribute over the entire SG when they move close to plasma membranes for the fusion event in AtT-20 cells (Tooze and Tooze, 1986). With processing, ACTH and its proform peptides appear to move from the peripheral to the inner region of the SGs. Granin-family proteins such as CgA are also cleaved by processing enzymes, and granin-derived peptides function to modulate the regulated secretion of peptide hormones and neurotransmitters (Taupenot et al., 2003). Indeed, immunoreactive CgA has been demonstrated to distribute from the periphery to the core region of SGs in rat pituitary gonadotropes (Watanabe et al., 1998) and bovine adrenal chromaffin cells (Yoo et al., 2000), and SgII does the same in mouse pituitary gonadotropes (Crawford et al., 2002; Sakai et al., 2003).

By contrast, SgIII tends to localize in the vicinity of SG membranes, as revealed by electron microscopic observation (Sakai et al., 2003). We recently showed that SgIII binds cholesterol-rich liposomes in a cholesterol concentration-dependent manner up to 65 mol% (Hosaka et al., 2004). Consistently, SG membranes has been reported to contain a high cholesterol level, being 65 mol% in SG membranes and 75 mol% in Triton X-100-insoluble membranes from the bovine pituitary neural lobe (Dhanvantari and Loh, 2000), compared with 26 mol% in plasma membranes from human erythrocytes (Lodish et al., 2003) and 32 mol% in Triton X-100-insoluble constitutive pathway vesicles from MDCK cells (Brown and Rose, 1992). At this cholesterol-rich peripheral

membrane of SGs, prohormone-processing reactions appear to take place because prohormone-processing enzymes exhibit a high affinity to cholesterol-rich membranes. The prohormone convertases PC1/3 and PC2 reportedly associate with lipid raft-like cholesterol-rich SG membranes (Blázquez et al., 2000; Arnaoutova et al., 2003). Carboxypeptidase E (CPE), an exopeptidase that removes dibasic residues from the C-terminal end of peptide hormone precursors after cleavage by PC1/3 and/or PC2, reportedly binds to cholesterol-rich membranes through its C-terminal end comprising an amphiphilic helix region (Dhanvantari and Loh, 2000; Zhang et al., 2003).

Since a point mutation in the CPE gene had been found to cause obesity in *fat/fat* mice (Naggert et al., 1995), processing abnormality by defective CPE has been explored to interpret new functions for obesity (Fricker and Leiter, 1999; Che et al., 2005). Loh's group proposed a new function for CPE, as a regulated pathway sorting receptor, by which POMC is targeted to SGs (Cool et al., 1997). They used two systems to support their hypothesis: a Cpe^{fat} mouse having a mutant CPE (CPE^{S202P}), and a mouse neuroblastoma-derived cell line (Neuro-2a) whose CPE was deleted by antisense RNAs. In the Cpe^{fat} mouse pituitary and CPE-deleted Neuro-2a cells, POMC was secreted through a constitutive pathway without regulation by secretagogues. However, their hypothesis has been contradicted by several other lines of evidence. Irminger et al. demonstrated proinsulin sorting to the regulated pathway in the Cpe^{fat} mouse islets and secretagogue-regulated secretion of proinsulin from cultured islets (Irminger et al., 1997). Varlamov et al. immortalized WT and Cpe^{fat} β-cells by expressing simian virus T-antigen and generated a new β-cell line, NIT-1 (WT β-cell-derived), as well as NIT-2 and NIT-3 (Cpe^{fat} β-cell-derived). NIT-2 and NIT-3 expressed a pro-form CPE whereas NIT-1 expressed a mature form CPE. High levels of glucose stimulated a fourfold increase in the secretion of immunoreactive insulin/proinsulin from NIT-1 and a onefold increase (i.e. a 100% increase) from NIT-2 and NIT-3 (Varlamov et al., 1997). Although Loh's group further demonstrated a constitutive secretion of prohormones, including proinsulin and proenkephalin, using CPE-deleted Neuro-2a cells (Normant and Loh, 1998), they showed a regulated secretion of CPE^{S202P} and PC2 from NIT-3 cells by glucagon-like polypeptide-1 (Cawley et al., 2003). Further, the gonadotropins LH and FSH were secreted upon stimulation with GnRH from primary-cultured Cpe^{fat} pituitary cells (Srinivasan et al., 2004). Thus, if the dysfunction of CPE^{S202P} is compensated, a regulated secretion should be observed from the Cpe^{fat} pituitary.

In this study, we examined a compensatory role of SgIII for CPE in Cpe^{fat} pituitary, since both SgIII and CPE bind to cholesterol-rich SG membranes, and act as a sorting receptor for intragranular proteins including peptide hormones (Dhanvantari and Loh, 2000; Hosaka et al., 2004). We further explored intragranular transport mechanisms of POMC molecules, which are supposed to bind initially to sorting receptors at the periphery, then distribute over the entire granule even in the CPE-defective Cpe^{fat} pituitary. We sought to determine (1) whether SgIII and CPE exist exclusively or cooperatively on the SG membranes, (2) whether POMC sorting to SGs is impaired or compensated by any SG-associated proteins in the Cpe^{fat} mouse, (3) whether POMC is secreted from the Cpe^{fat} pituitary in a regulated or constitutive manner, and (4) whether POMC can be transferred from CPE to SgIII, and subsequently to CgA.

Materials and Methods

Cpe^{fat} mouse

We conducted our animal experiments in accordance with the guidelines for the Care and Use of Laboratory Animals of the Medical Research Council of Gunma University. Heterozygous Cpe^{fat} mice (BKS.HRS-Cpe^{fat}/J strain) were purchased from the Jackson Laboratory. Homozygous fat/fat and wild-type (WT) (+/+) mice were generated by intercrossing heterozygous mice and the genotype of the offspring was determined by amplifying the CPE alleles with PCR, as suggested by the procedure supplied from the Jackson Laboratory. The mice were maintained on a 10-14 hour light-dark cycle and had free access to food and water.

Cell culture

A mouse corticotrope-derived AtT-20 cell line was maintained in DMEM supplemented with 10% fetal bovine serum (FBS). A mouse insulinoma-derived MIN6 cell line was maintained in DMEM supplemented with 15% FBS and 100 μM of 2-mercaptoethanol.

Antibodies

Rabbit polyclonal anti-SgIII (SgIII-C#6) and anti-CgA (CgA-C#101) antisera were prepared and characterized as described in our previous studies (Sakai et al., 2003; Sakai et al., 2004). Monoclonal antibodies to CPE were purchased from three different sources: Research Diagnostics, BD Transduction and Chemicon. The following antibodies to peptide hormones were purchased: anti-insulin (guinea-pig polyclonal, Dako Cytomation A/S), anti-ACTH (rabbit polyclonal, Chemicon; mouse monoclonal, Biogenesis, Poole, UK) and anti-prolactin (mouse monoclonal, QED Bioscience).

Immunoelectron microscopy

Pituitary tissues from Cpe^{fat} mice and wild-type mice were similarly processed for immunoelectron microscopic analyses (Sakai et al., 2003; Sakai et al., 2004). AtT-20 cells grown in 100 mm dishes were fixed first with 0.1% glutaraldehyde-4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 30 minutes at 4°C, and were then rinsed three times in 0.1 M phosphate buffer (pH 7.2) containing 7.5% sucrose at 4°C. After fixation, the cells were gently scraped from the dishes and centrifuged. Pellets of fixed cells were subsequently dehydrated with 70% ethanol and infiltrated into pure LR White resin (London Resin) for 12 hours at 4°C. The pellets were placed in gelatin capsules with fresh LR White resin, and polymerized for 24 hours at 55°C.

Immunogold labeling was performed as described previously (Sakai et al., 2003; Sakai et al., 2004). The primary antisera/antibodies were diluted as follows: anti-rat SgIII, 1:300; anti-CPE (purchased from BD Transduction), 1:10; anti-insulin, 1:1000; anti-prolactin, 1:1000; and anti-ACTH, 1:10,000. For double immunostaining, the two-face technique was applied (Bendayan, 1982). For triple immunostaining, one face of the sections was simultaneously immunostained with a mixture of a rabbit polyclonal antiserum to SgIII and a mouse monoclonal antibody to CPE. The other face of the sections was immunostained for ACTH or prolactin. The intracellular localizations of each protein and hormone were distinguished by labeling with different sizes of colloidal gold particles conjugated to goat anti-rabbit, anti-mouse and anti-guinea-pig IgG (purchased from British Biocell International; particle size 5, 10 or 15 nm in diameter). Following the immunoreaction, the sections were contrasted with saturated aqueous solutions of uranyl

acetate and lead citrate, and examined with the JEOL electron microscope (JEM-1010).

Immunoprecipitations

AtT-20 and MIN6 cells grown in 100 mm dishes were solubilized in 1 ml of 50 mM 2-[N-morpholino]ethanesulfonic acid (MES), pH 5.5, containing 0.15 M NaCl, 10 mM CaCl2, 0.1% NP-40, and a protease inhibitor cocktail (1 μg/ml each of aprotinin, leupeptin and pepstatin A, and 0.4 mM PMSF). After removal of the insoluble materials by centrifugation, an aliquot (1 ml) of soluble extracts was incubated with 10 μl of diluted antisera (1:100) for 1 h at 4°C. Then 30 μl of a 50% (wt/vol.) slurry of protein A or Glutathion-Sepharose was added, and the mixture was further incubated for 12 h at 4°C under continuous rotation. The precipitated immunocomplexes underwent SDS-PAGE, followed by immunoblotting.

Construction of bacterial expression vectors for fusion proteins

GST-fused proteins containing full- or partial-length SgIII and CPE, and full-length CgA were made using pGEX-KG (Guan and Dixon, 1991). The rat SgIII, CgA, 7B2 and CPE fragments were as follows: SgIII 23-471, SgIII 23-186, SgIII 187-373, SgIII 374-471, CgA 1-448, 7B2 27-210, CPE 23-477, CPE 43-477, CPE 23-281, CPE131-365 and CPE 332-477. The GST-fusion proteins were expressed in the *E. coli* BL21(DE3) strain and were purified on glutathione beads.

Carboxypeptidase E enzyme assay

To confirm that the GST-CPE fusion protein has a conformation close to the native one, we assayed CPE activity of GST-CPE using dansyl-Gly-Lys as a substrate by HPLC-fluorophotometry (Yajima et al., 1994). Reaction mixture (100 μl of total volume) contained 25 mM sodium acetate buffer, pH 5.5, 100 mM NaCl, 10 μM dansyl-Gly-Lys in the presence or absence of 1 mM $CoCl_2$. After the addition of GST-CPE (1.0 μg), the mixture was incubated at 37°C for 2 hours. As a positive control source for CPE, we used a rat cortex whose homogenate was made in 50 mM sodium acetate buffer (pH 5.5) containing 200 mM NaCl. The homogenate was centrifuged at 10,000 ***g*** for 60 minutes, and the soluble fraction was used for CPE source. We added the rat cortical supernatant (39 μg), and GST alone (20 μg) to the reaction mixture. The reaction was stopped by heating in a boiling water bath. After centrifugation at 10,000 ***g*** for 5 minutes, 20 μl of sample was applied to HPLC analysis (330/550 nm). To characterize CPE, 1 mM guanidinoethylmercaptosuccinic acid (GEMSA) (Calbiochem, La Jolla, CA) was used as a specific inhibitor.

In vitro binding between CPE and SgIII

AtT-20 cells and MIN6 cells were solubilized in a buffer containing 0.1 M NaCl, 1% Triton X-100, 2 mM EGTA and a protease inhibitor cocktail (1 μg/ml each of aprotinin, leupeptin and pepstatin A, and 0.4 mM PMSF). The buffer was adjusted to pH 5.5 by 50 mM MES, or to pH 7.4 by 20 mM Hepes. The high-calcium solution was made by supplementing $CaCl_2$ at a final concentration of 10 mM. Soluble extracts were incubated for 12 hours at 4°C under continuous rotation with either GST-fused proteins or GST alone immobilized on glutathione beads. The beads were then pelleted by centrifugation. The proteins bound to the GST-fused proteins were analyzed by SDS-PAGE, followed by immunoblotting.

Protein quantification with radiolabeled antibody

Pituitary homogenates from the WT and Cpe^{fat} mice were analyzed by a 7 or 10% SDS-PAGE method (5 μg protein/lane) and immunoblotting on the same gels to adjust for variations at protein transfer. The blots were probed with antibodies to α-tubulin, CPE and SgIII using secondary ^{125}I-antibody detection, and were quantified using a BAS2000 (Fuji film). Five mice of each genotype were analyzed with two different antibodies to ensure reproducibility. To standardize the immunoblotting signals, all blots were simultaneously probed for α-tubulin.

Perifusion system for ACTH secretion assay

Mice were anaesthetized with sodium pentobarbitone (5 mg/mouse). The whole pituitary was aseptically removed, and cultured in Minimum Essential Medium with 5% fetal bovine serum and 10% normal horse serum for 19 hours. One pituitary from the WT or Cpe^{fat} mouse was placed into a column (total volume including a tubing system: 3.5 ml), and perifused with Krebs-Ringer bicarbonate glucose solution [15 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 24 mM Ma_2HCO_3, 2.8 mM glucose and 0.1% bovine serum albumin] at a flow rate 1 ml/minute for 1 h at 37°. After 1 hour preincubation, each pituitary was stimulated twice with 10^{-9} M corticotropin-releasing hormone (CRH; Calbiochem), and then 60 mM KCl. Perifusate was fractionated to 4 ml aliquot every 4 minutes. After perifusion, the pituitary was subjected to extraction of POMC and its derived peptides in an acid-ethanol solution (70% ethanol and 0.18 M HCl). Immunoreactive (IR) ACTH was measured by the ACTH immunoradioactive assay kit (Mitsubishi Kagaku Iatoron).

Transfer of POMC from CPE to SgIII or CgA

To examine the interaction of SgIII or CgA with POMC-binding capacity of CPE, AtT-20 cell extract was incubated for 12 hours at 4°C with GST-CPE immobilized on glutathione beads at 10 mM Ca^{2+} to fully bind POMC molecules. For preparing recombinant SgIII and CgA, SgIII 23-471 and CgA 1-448 were placed in the pGEX-6P-1 (Amersham), and GST-SgIII and GST-CgA were produced in the BL21(DE3) strain, and then were purified on glutathione beads. Recombinant SgIII and CgA were obtained by digesting with pReScission protease (Amersham) in a buffer containing 50 mM Tris-HCl (pH 7.0), 0.15 M NaCl, 1% Triton X-100, 1 mM EGTA for 5 hours at 4°C under continuous rotation. After digestion, the reaction mixture of SgIII or CgA was dialyzed against the buffer (50 mM MES, pH 5.5, 0.1 M NaCl, 1% Triton X-100 and 2 mM EGTA). The recombinant CgA (0 to 20 μg/mixture) or SgIII (0 to 20 μg/mixture) was mixed with the POMC-bound GST-CPE (2 μg/mixture) as indicated in the Fig. 6C, and further incubated for 12 hours at 4°C. As a control for SgIII and CgA, we used maltose-binding protein (MBP), which is often used for a fusion protein such as GST (Hosaka et al., 2004). The MBP was purified by separation from amylose resin with 15 mM maltose, and dyalyzed against the same buffer used for preparing recombinant SgIII and CgA. GST-CPE or GST-SgIII was precipitated with glutathione beads by light centrifugation. POMC molecules still bound to the GST-CPE or GST-SgIII were analyzed by SDS-PAGE, followed by immunoblotting.

To examine whether POMC molecules are transferred to recombinant SgIII and CgA from GST-CPE, POMC-bound GST-CPE was incubated with either free SgIII or CgA. After removing GST-CPE with glutathione beads, unbound SgIII or CgA in the supernatant was precipitated with anti-POMC antibody. The precipitates by the anti-ACTH antibody were subjected to SDS-PAGE and immunoblotting for SgIII and CgA. As a control IP experiment, preimmune serum was used instead of the anti-ACTH antibody.

Results

Some of SgIII and CPE molecules co-localize in the same secretory granules

We previously demonstrated that SgIII localizes along the periphery of SGs in mammotropes, thyrotropes, corticotropes

and gonadotropes of the rat pituitary, whereas pituitary hormones distribute more widely throughout the entire SG, as observed under the electron microscope (Sakai et al., 2003). We further examined the localization of CPE in the rat pituitary mammotropes. As expected, given the cholesterol-prone feature of CPE (Dhanvantari and Loh, 2000), the CPE molecules localized along the peripheral region of the SGs in the mammotropes as did SgIII (Fig. 1A). Similarly, these two cholesterol-prone molecules localized along the periphery of SGs in the pancreatic β-cells and mouse pituitary corticotrope-derived AtT-20 cells, whereas the peptide hormones, insulin and POMC, distributed without showing any specific spotting in the SGs (Fig. 1B,C, respectively). Localization of SgIII and CPE to the periphery of SGs was confirmed by multiple antibodies recognizing their different epitopes (data not shown). Under close scrutiny, we noted that some SgIII-indicative middle-sized gold particles localized close to CPE-indicative large-sized gold particles, although other SgIII and CPE molecules localized separately in the same SGs (white arrows in Fig. 1A-C). This led us to question whether the two molecules interact with each other.

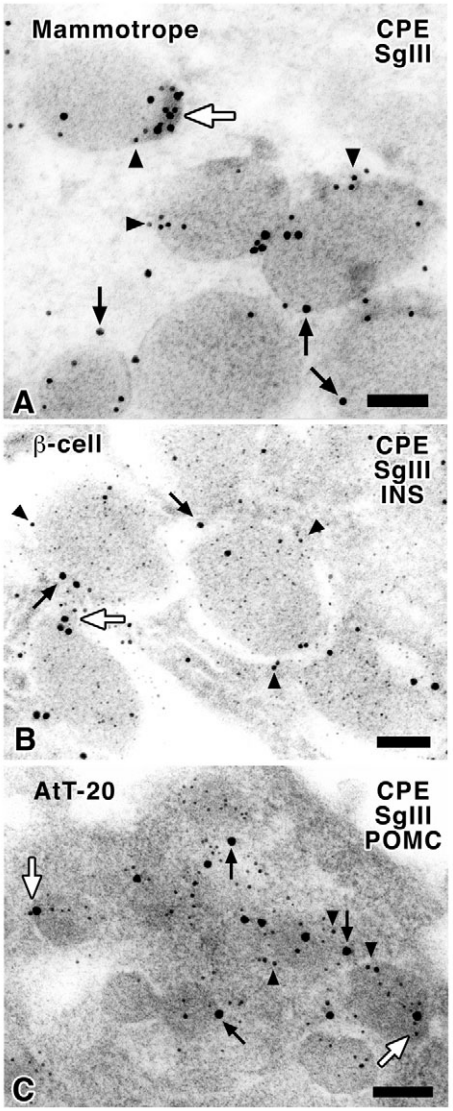

Fig. 1. Intragranular localization of SgIII and CPE in rat pituitary mammotropes (A), rat pancreatic β-cells (B), and mouse AtT-20 cells (C). CPE is visualized with 15 nm gold particles (A-C), SgIII is visualized with 10 nm immunogold particles (A-C). Insulin (B) and POMC-derived ACTH-containing peptides (C) are labeled with 5 nm immunogold particles. Note that SgIII (arrowheads) and CPE (arrows) are preferentially localized in the periphery of the SGs, and the SgIII and CPE are occasionally co-localized at the same sites in the periphery of the SGs (white arrows). Bar, 100 nm.

SgIII and CPE bind each other

Since we observed close localization of some SgIII and CPE molecules in AtT-20 cells (Fig. 1C), we decided to examine the binding between SgIII and CPE biochemically using immunoprecipitation and immunoblot methods. When AtT-20 cell extracts were immunoprecipitated by the anti-CPE antibody and the precipitate was run on the gel, SgIII was recovered on the immunoblot (Fig. 2A, upper left panel). Conversely, by using the anti-SgIII antibody, CPE was detected on the immunoblot (Fig. 2B, upper left panel). Similar SgIII-CPE binding results were obtained when MIN6 pancreatic β-cell extracts were used (Fig. 2A,B, upper right panels). Thus, some SgIII and CPE molecules bind together in the SGs of AtT-20 and MIN6 endocrine cells. Additionally, we examined whether CgA could join the complex to form a trimer of SgIII, CPE and CgA, because SgIII was shown to bind with CgA in pituitary and pancreatic endocrine cells (Hosaka et al., 2002). CgA was immunoprecipitated only by the anti-SgIII antibody but not by the anti-CPE antibody (Fig. 2A,B, lower panels). Since CgA is efficiently processed to 20-21 kDa β-granin in pancreatic β-cells (Hutton et al., 1987), a β-granin band was obtained by the anti-SgIII antibody, but again not by the anti-CPE antibody (Fig. 2A,B, lower right panels). SgIII can bind to CPE and CgA separately, hence a three-molecule complex is not formed in the SGs. Furthermore, with SgII blotting for the SgIII immunoprecipitates, the anti-SgIII antibody did not bring down other aggregation-prone granins such as SgII, but did bring down CgA (Fig. 2B, bottom panels).

The binding between SgIII and CPE was further confirmed by an in vitro pull-down assay using three GST-fused constructs immobilized on glutathione beads: GST-SgIII 23-471, GST-CgA 1-448 and GST-CPE 23-477. SgIII was pulled down by both GST-CgA and GST-CPE from the AtT-20 cell extract (Fig. 2C, top panel), while CgA was pulled down by GST-SgIII (Fig. 2C, middle panel), and CPE was pulled down by GST-SgIII (Fig. 2C, bottom panel). Similar SgIII-CPE binding was observed when MIN6 cell extracts were used (data not shown). Thus, SgIII can bind to either CPE or CgA, but CgA and CPE cannot bind each other even in the presence of SgIII.

Because GST-fusion proteins are produced in bacteria, it is essential that a disulfide bond is formed properly for catalytic activity by correct protein folding in the case of GST-CPE. Thus, enzyme activity of the GST-CPE was assayed using dansyl-Gly-Lys as a substrate by HPLC-fluorophotometry (Yajima et al., 1994). The GST-CPE displayed reasonable exopeptidase activity, which was enhanced by $CoCl_2$ and inhibited by GEMSA in a similar manner to that exhibited by rat cortex supernatant used as a positive control for CPE

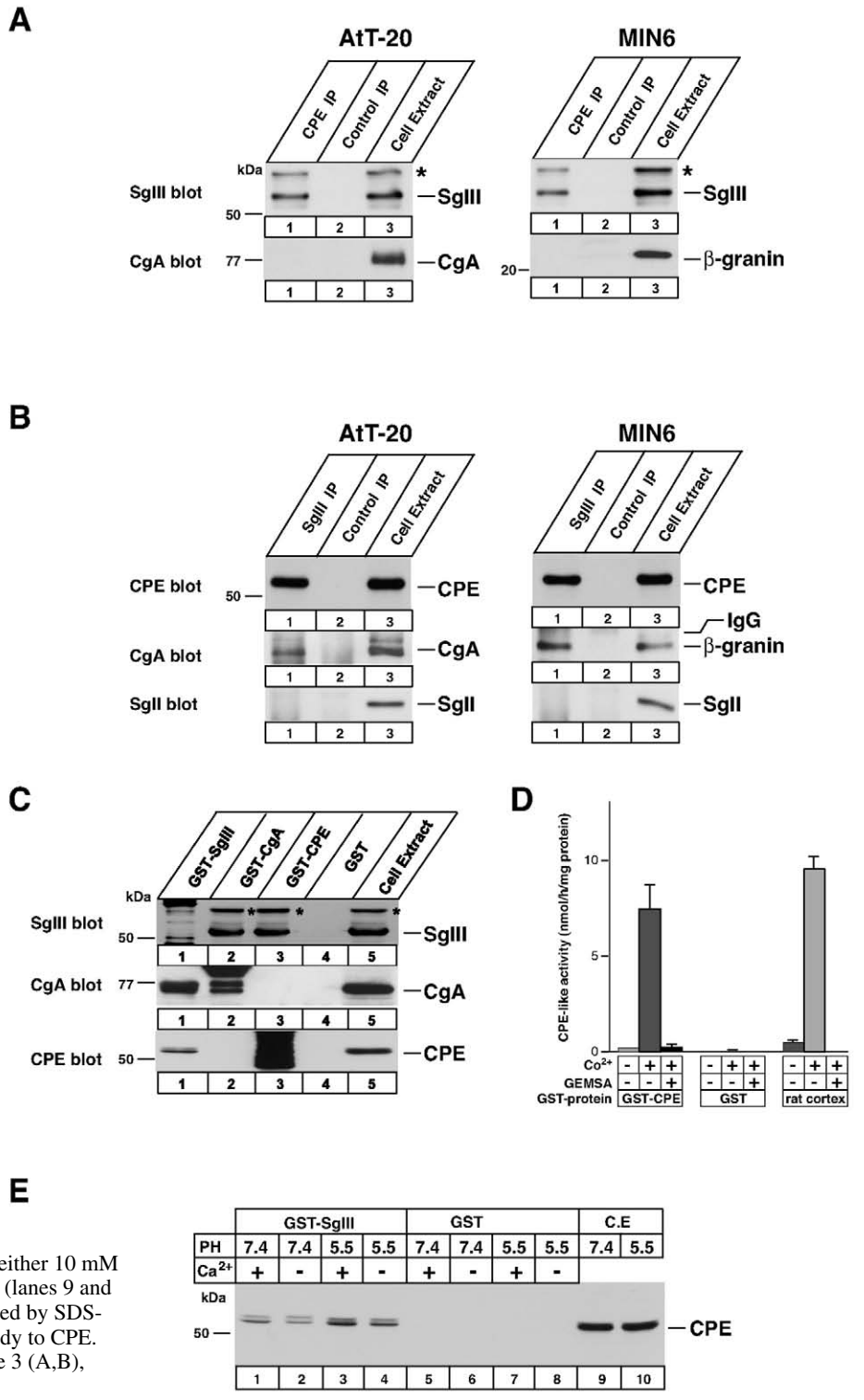

Fig. 2. Co-immunoprecipitation of CPE and SgIII. (A,B) AtT-20 and MIN6 cell lysates were subjected to immunoprecipitation using rabbit antibody to CPE (A) or SgIII (B). In the CPE immunoprecipitation experiment (A), control immunoprecipitation was performed with monoclonal antibody to insulin receptor, a non-SG-residential protein, because the antibody to CPE was monoclonal. In the SgIII immunoprecipitation experiment (B), preimmune serum was used for control immunoprecipitation, because the antibody to SgIII was polyclonal. The starting fraction (lane 3) and immunoprecipitates (lanes 1, 2) were analyzed by SDS-PAGE and immunoblot using antibodies to SgIII, CPE, CgA, β-granin and SgII. SgII blot was performed for the SgIII immunoprecipitation experiment to show whether aggregation-prone granins such as SgII are co-precipitated or not (B, bottom panels). As shown in Fig. 2B, CPE and CgA were pulled-down but SgII was not in both AtT-20 and MIN6 cells. Asterisks indicate the pro-form of SgIII. Both A and B (n=7)-10. (C) In vitro pull-down by GST-SgIII, GST-CgA and GST-CPE. GST-fused proteins, SgIII 23-471, CgA 1-444 and CPE 23-477 (20 μg each), were immobilized on glutathione beads and incubated with AtT-20 cell extract (1.0 ml) for the pull-down experiments. A fifth of each pull-down product was analyzed by SDS-PAGE and immunoblotting using antibodies to SgIII, CgA and CPE. Asterisks indicate the pro-form of SgIII (n=5). (D) Enzyme assay of the GST-CPE fusion protein. CPE activity was assayed using dansyl-Gly-Lys as a substrate by HPLC-fluorophotometry. Rat cortex supernatant was used as a positive control and GST alone was used as a negative control for CPE enzyme activity. The enzyme activity was characterized for CPE by 1 mM $CoCl_2$ and 1 mM GEMSA (n=3). (E) Binding between SgIII and CPE at pH 7.4 or 5.5 with or without 10 mM Ca^{2+}. GST-SgIII (lanes 1-4) and GST (lanes 5-8) (20 μg each) were incubated with AtT20 cell extract (1.0 ml) at either pH 7.4 or 5.5, and either 10 mM Ca^{2+} (+) or Ca^{2+} (–). Next, the cell lysates alone (lanes 9 and 10) and cell lysates with GST-SgIII were analyzed by SDS-PAGE, and were immunoblotted with the antibody to CPE. One-twentieth of the cell extract was run on lane 3 (A,B), lane 5 (C) and lanes 9 and 10 (E). n=3.

activity (Fig. 2D). Thus, we think that the GST-CPE exists in a conformation that is close to the native one.

We then examined the pH- and Ca^{2+}-dependent binding efficiency between SgIII and CPE. Pro- and mature-forms of CPE were pulled down from the AtT-20 cell extract by GST-SgIII more efficiently at pH 5.5 than at pH 7.4 (Fig. 2E). The presence of Ca^{2+} slightly enhanced the binding at either pH. Thus, it is reasonable that the binding of SgIII and CPE was more efficient under the intragranular pH and calcium milieu.

Binding domain analysis of SgIII and CPE

To determine a specific binding domain between SgIII and CPE, we separated each sequence into three parts for SgIII, and

four parts for CPE, and we made GST-fused constructs. Four GST-SgIII constructs were SgIII 23-186, SgIII 187-373, SgIII 374-471 and full-length SgIII 23-471; and five GST-CPE constructs were a mature form CPE 43-477, CPE 131-365, CPE 23-281 and CPE 332-477, and a pro-form CPE 23-477. The AtT-20 cell extract was incubated with each GST-fused construct immobilized onto glutathione beads at pH 5.5 and 10 mM Ca^{2+}. CPE was pulled down by the GST-SgIII 374-471 but not by either SgIII 23-186 or SgIII 187-373 (Fig. 3A). Thus, the C-terminal region of SgIII is essential for binding to CPE. Next, SgIII was pulled down by each GST-fused CPE construct. Fragmented CPE did not pull down SgIII whereas both pro-form and mature form CPEs did pull down SgIII (Fig. 3B). Thus, the tertiary configuration of mature CPE appears to be a prerequisite for the binding to SgIII.

SGIII functions compensatory to CPE in the Cpe^{fat} mouse

In the Cpe^{fat} mouse, whose CPE is mutated from Ser to Pro at residue 202 (CPE^{S202P}) (Naggert et al., 1995), CPE^{S202P} is degraded quickly and a minor fraction of CPE^{S202P} that escapes degradation stays in a pro-form without maturation in the SG (Normant and Loh, 1998). Because SgIII is shown to bind CPE, we questioned whether SgIII can compensate for a sorting function of CPE to target POMC to SGs in the Cpe^{fat} pituitary. We initially examined the localization of CPE, SgIII and pituitary hormones prolactin (PRL) and POMC in the pituitary mammotropes and corticotropes of WT and Cpe^{fat} mice. Both CPE and SgIII were localized close to the periphery of the SGs, whereas PRL distributed more intragranularly in the SGs of the WT mammotropes (Fig. 4A, 10 nm particle-stained CPEs indicated by arrowheads), as shown in the rat mammotropes in Fig. 1A. As expected, CPE was not detected in the Cpe^{fat} mammotropes, while SgIII was localized along the periphery and PRL distributed intragranularly as seen in the WT mammotropes (Fig. 4A,B, large 20 nm particles for SgIII, small 5 nm particles for PRL). As with the Cpe^{fat} mammotropes, CPE was barely detected, but SgIII localized along the periphery of the SGs in the Cpe^{fat} corticotropes, whereas POMC distributed intragranularly from the peripheral to the central region of the SGs (Fig. 4C,D, small 5 nm particles for POMC). Thus, PRL and POMC can be sorted to SGs even

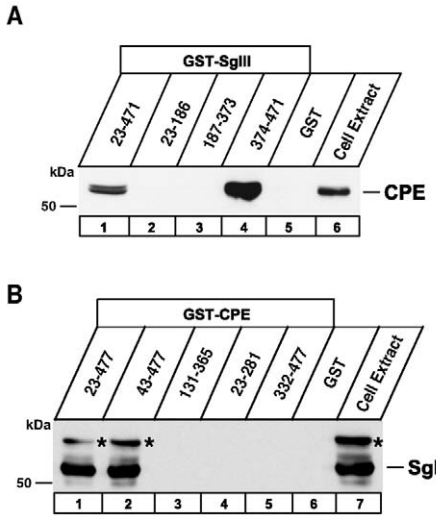

Fig. 3. Analysis of binding domains between SgIII and CPE. To investigate the specific binding domains of SgIII (A) and CPE (B), four GST-SgIII fusion proteins, full-length SgIII 23-471, SgIII 23-186, SgIII 187-373 and SgIII 374-471, were tested for binding to CPE; and five GST-CPE fusion proteins, pro-form CPE 23-477, mature form CPE 43-477, CPE 131-365, CPE 23-281 and CPE 332-477, were tested for binding to SgIII. GST-fusion proteins immobilized on glutathione beads were incubated with AtT-20 cell extract at pH 5.5 with 10 mM Ca^{2+}. The precipitates were detected by antibody to CPE (upper panel) or antibody to SgIII (lower panel) antibody. Asterisks indicate the pro-form of SgIII. One-twentieth of the cell extract was run on lane 6 (A) and lane 7 (B) n=4 (A) and n=3 (B).

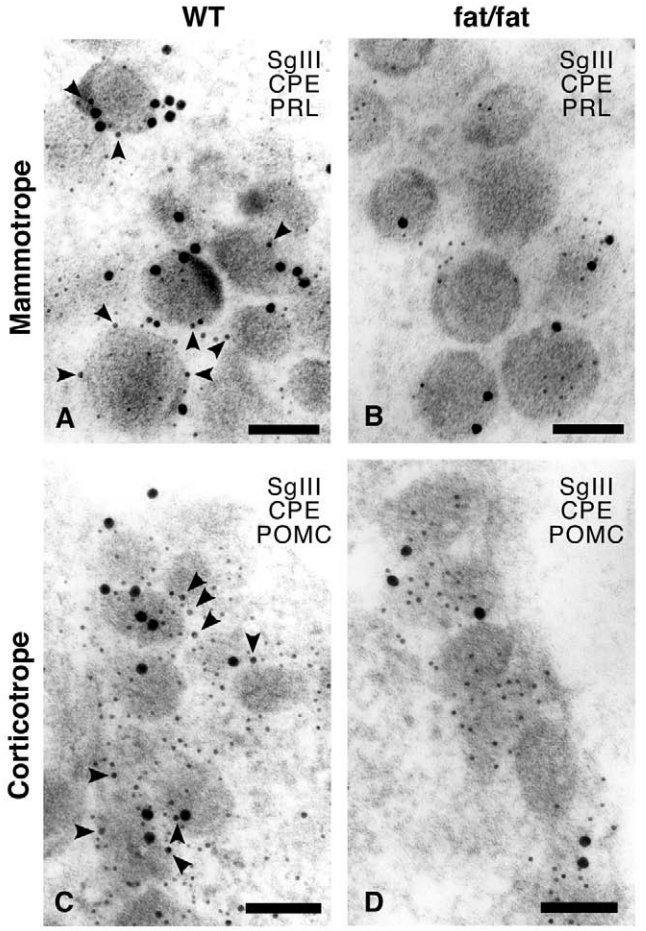

Fig. 4. Intragranular localization of SgIII, CPE and peptide hormones PRL and POMC. (A) WT mouse (WT) mammotropes, (B) Cpe^{fat} mouse (fat/fat) mammotropes, (C) WT corticotropes and (D) fat/fat mouse corticotropes. SgIII and CPE were visualized with 15 and 10 nm gold particles, respectively. PRL (A,B) and POMC-derived ACTH-containing peptides (C,D) were labeled with 5 nm gold particles. CPE observed in the WT mammotropes and corticotropes (A,C, respectively) was not found in the fat/fat mammotropes and corticotropes (B,D, respectively), although these ultrathin sections were immunostained with a less-diluted antibody to CPE (1:10 at dilution). By contrast, SgIII was detected as in Fig. 1. Note that anti-ACTH-antibody-reacting POMC-derived peptides are distributed from the peripheral to the intragranular region of the SGs (C,D). Bar, 100 nm.

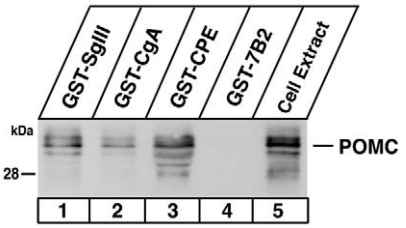

Fig. 5. In vitro pull-down of POMC-derived peptides by GST-CPE, GST-SgIII and GST-CgA. The GST-fused proteins, SgIII 23-471, CgA 1-448 and CPE 23-477 (20 μg each), were immobilized on glutathione beads and incubated with AtT-20 cell extract (1.0 ml) for pulling down POMC-derived peptides. For GST pull-down control, although GST alone did not bring down POMC-derived peptides, GST-7B2 27-210 was used as a negative control to show that particular SG-residential proteins are able to bring down POMC-derived peptides. POMC-derived peptides in the pulled-down precipitates were analyzed by SDS-PAGE and immunoblotting using the antibody to ACTH. One-twentieth of the cell extract was run on lane 5 (n=5).

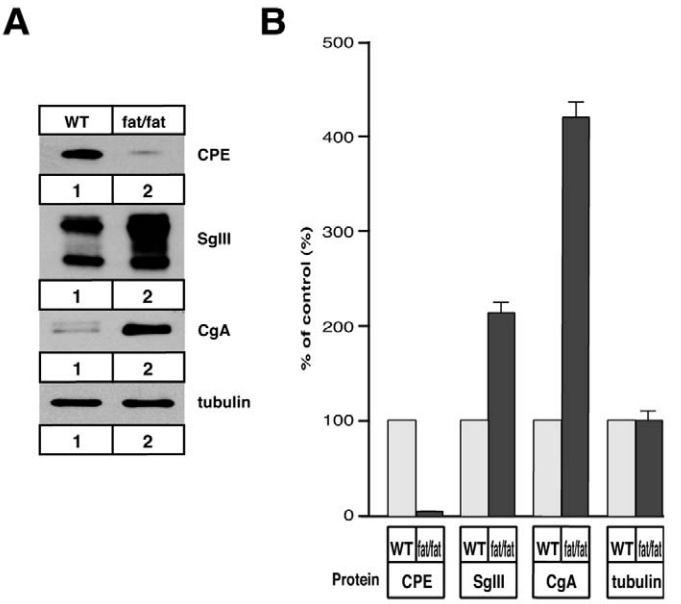

Fig. 6. Compensatory expression of SgIII and CgA for CPE in the Cpe^{fat} mouse. (A) Pituitary homogenates from wild-type mice (WT, lane 1) and the Cpe^{fat} mouse (fat/fat, lane 2) were analyzed by immunoblotting and probed with antibodies to CPE, SgIII, CgA and α-tubulin. The blots were visualized using the ECL kit. (B) The relative levels of CPE, SgIII, CgA and α-tubulin were determined by quantitative radio-immunoblotting with pituitary gland homogenates from five WT mice and five Cpe^{fat} mice. The blots were reacted with polyclonal or monoclonal antibody to CPE, SgIII, CgA and α-tubulin (see upper panel) and were probed with ^{125}I-labeled secondary antibody. The signals were measured using a BAS2000 and were normalized by the signal obtained from an α-tubulin blot as an internal control. The levels of CPE, SgIII, CgA and α-tubulin in the Cpe^{fat} pituitary were expressed by assuming those in the WT pituitary as 100%.

if CPE^{S202P} is dysfunctional in the Cpe^{fat} pituitary mammotropes and corticotropes.

Since POMC distributed intragranularly in the SGs of Cpe^{fat} corticotropes (Fig. 4D), we suspected that both granins compensate for CPE functions, such as a sorting receptor for POMC. To investigate this possibility, we examined the binding of SgIII and CgA to POMC using an in vitro pull-down assay with three GST-fused constructs immobilized onto glutathione beads: GST-SgIII 23-471, GST-CgA 1-448 and GST-CPE 23-477. As expected based on the previous report (Cool et al., 1997), POMC and POMC-derived ACTH-containing peptides of approximately 30 kDa were pulled down by the GST-CPE from the AtT-20 cell extract (Fig. 5, lane 3). To our surprise, POMC and POMC-derived peptides were also pulled down by both GST-SgIII and GST-CgA (Fig. 5, lanes 1 and 2), although their pull-down capacity appeared to be lower than that by the GST-CPE. By contrast, 7B2, a granin-family member, did not bring down POMC (Fig. 5, lane 4). Thus, POMC-binding is not a general feature of granin-family proteins.

To further examine the compensatory functions of SgIII and CgA for CPE, we compared the expression of CPE, SgIII and CgA in the pituitary between WT and Cpe^{fat} mice. CPE^{S202P} remained at a low level as a proform because its position is a little higher than that of WT CPE, as reported previously (Berman et al., 2001; Cawley et al., 2003) (Fig. 6A). Surprisingly, the expression of SgIII and CgA is highly elevated in the Cpe^{fat} mouse pituitary (Fig. 6A). Quantification of the immunoblot intensity, using radioisotope-labeled antibodies to CPE, SgIII and CgA, revealed that SgIII and CgA increased 2.1-fold and 4.2-fold, respectively, compared with those in the WT pituitary, while CPE decreased to 5% of the control level (Fig. 6B). Therefore, it is likely that SgIII and CgA compensate for the reduced sorting function of CPE^{S202P} by their overexpression in the Cpe^{fat} pituitary (Fig. 4B,D).

Immunoreactive ACTH is secreted in a regulated manner

If SgIII and CgA compensate for dysfunctional CPE^{S202P} by their overexpression in the sorting of POMC to SGs of Cpe^{fat} pituitary corticotropes, POMC and POMC-derived ACTH-containing peptides should be secreted in a regulated manner with secretagogues. To explore this question, we performed a perifusion of pituitaries. Immunoreactive (IR)-ACTH content in the Cpe^{fat} pituitary was less than half of that in the WT pituitary (Fig. 7A), although pituitary weight was similar between the two groups (approximately 2.0 mg). The decrease in IR-ACTH in the Cpe^{fat} pituitary may reflect the decreased cross reactivity of the anti-ACTH antibody to ACTH-Lys-Arg accumulated in the Cpe^{fat} pituitary (Che et al., 2001), although we did not assess the cross reactivity of the antibody.

We then measured IR-ACTH in fractionated perifusates from the pituitary. Proportion of basal IR-ACTH secretion from the Cpe^{fat} pituitary was approximately twofold higher than that from the WT pituitary, suggesting that there is some dysfunction of hormone sorting to SGs (Fig. 7B). IR-ACTH was stimulated by CRH, and the increase from the basal secretion appeared similar in the WT and Cpe^{fat} pituitaries (Fig. 7B). Upon KCl stimulation, an IR-ACTH level formed a similarly high peak between the two pituitaries, which was much higher than a CRH-stimulated peak. Although IR-ACTH content was diminished in the Cpe^{fat} pituitary, IR-ACTH

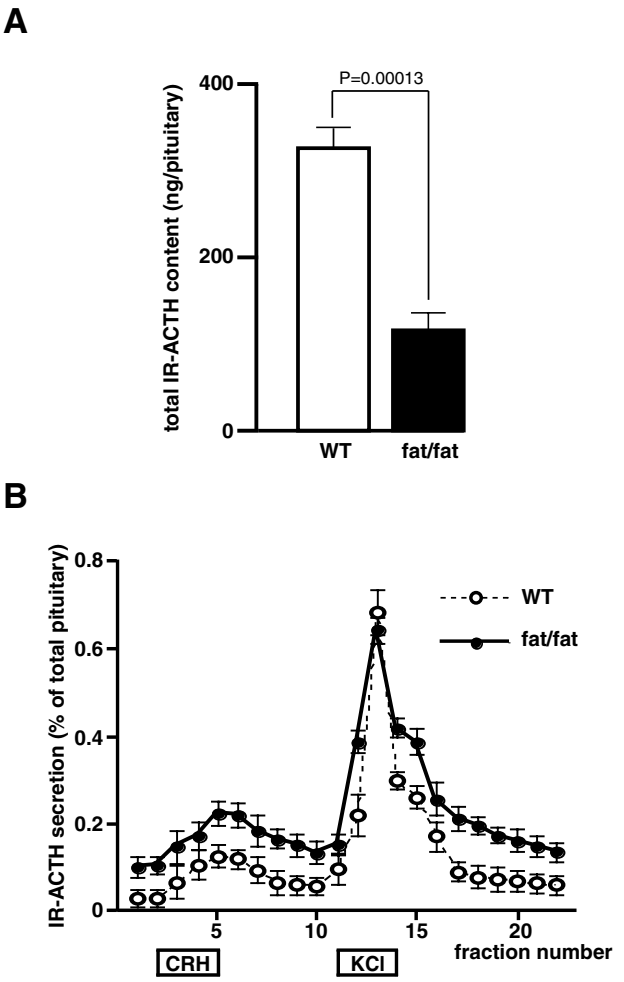

Fig. 7. Secretion of IR-ACTH from the WT and Cpe^{fat} pituitaries. (A) IR-ACTH content in the WT and Cpe^{fat} (fat/fat) pituitaries. The IR-ACTH content is expressed as ng/pituitary. Values are mean±s.e.m. (n=4). (B) IR-ACTH secretion from the WT and Cpe^{fat} pituitaries. A pituitary placed in a column was perifused with the buffer described in the Materials and Methods. The buffer was run at a flow rate of 1 ml/minute, and perifusate was collected every 4 minutes, thus each fraction number indicates 4 ml of perifusate aliquot. IR-ACTH is measured in each fractionated perifusate. Relative IR-ACTH (% of total pituitary content) released from the WT (○) and Cpe^{fat} (fat/fat) (●) pituitary (n=4). IR-ACTH secretion was stimulated with 10^{-9} M CRH and then 60 mM KCl at an indicated position (fr#2-5, CRH; fr#11-14, KCl).

secretion was clearly regulated by CRH and KCl, unlike the previous report (Cool et al., 1997).

Transfer of POMC from CPE to SgIII or CgA

Since SgIII and CPE are localized along the periphery and POMC distributes from the peripheral to intragranular region of the SG (Fig. 4C,D), we suspected that POMC and its intermediates may be transferred from CPE to SgIII, and perhaps further to CgA, for their wide distribution inside the SGs, which makes efficient release of ACTH-containing peptides into the blood stream. Initially, we precipitated POMC down with GST-CPE from the AtT-20 cell extract, then mixed POMC-bound GST-CPE with increasing doses of recombinant CgA or SgIII for incubation at the intragranular milieu. GST-CPE was then precipitated with the glutathione beads for SDS-PAGE and immunoblot analyses. The amount of POMC bound to GST-CPE was barely affected by the addition of recombinant CgA up to 20 μg/mixture compared with 2 μg of GST-CPE (Fig. 8A, upper panel lanes 1 to 4), but it was clearly decreased with increasing doses of SgIII (Fig. 8A, upper panel, lanes 5 to 8). Because CPE can bind to SgIII, but not to CgA (Fig. 1A,B, lane 1 in both AtT-20 and MIN6 panels), we presume that POMC transfer may require direct interaction between donor and acceptor molecules. POMC molecules bound to GST-CPE were not affected by the maltose-binding protein (MBP), a cytosol protein frequently used for fusing other proteins, similarly to GST (Fig. 8A, lower panel, lanes 1 to 4) (Hosaka et al., 2004). We further examined the POMC transfer from SgIII to CgA. POMC molecules bound to GST-SgIII decreased slightly but not substantially with increasing doses of recombinant CgA (Fig. 8A, lower panel, lanes 5-8). This may reflect a weak acceptor function of CgA for POMC as shown in Fig. 5.

We then examined whether POMC molecules are transferred to recombinant SgIII and CgA from GST-CPE. After incubation of POMC-bound GST-CPE with SgIII or CgA, free SgIII or CgA in the mixture with GST-CPE was precipitated by anti-POMC antibody for immunoblotting with SgIII or CgA. The precipitate using anti-POMC antibody did not contain GST-CPE on the immunoblot using anti-GST antibody after treating glutathione beads to absorb GST-CPE (data not shown). The precipitate by the anti-POMC antibody contained a notable amount of SgIII molecules with an increase in recombinant SgIII in the mixture (Fig. 8B, upper left panel). By contrast, anti-POMC antibody pulled down only a small amount of CgA (Fig. 8B, lower left panel). Thus, POMC molecules were efficiently transferred from CPE to SgIII.

Discussion

We demonstrated that SgIII and CPE can bind each other in AtT-20 cells and MIN6 cells, that POMC is contained in SGs of Cpe^{fat} mouse corticotropes and secreted in a regulated manner, that POMC binds to both SgIII and CPE, and that CPE-bound POMC can be transferred from CPE to SgIII. We suggest that SgIII and CPE function for prohormone-processing bases at the periphery of SGs, and POMC-derived peptides at the periphery are transferred from CPE to SgIII, and further to CgA for their wide intragranular distribution, which enables POMC-derived peptides to be released efficiently at exocytosis.

Although SgIII has not been as extensively studied as other granins, Martens and colleagues demonstrated its secretory function using the *Xenopus* pituitary intermediate lobe. The mRNA levels of both SgIII and POMC increased more than 30-fold when *Xenopus* was moved to a black background from a white background, resulting in an increase in melanophore-stimulating hormone for color adaptation (Holthuis and Marten, 1996). Recently, such coordinated induction, as shown with SgIII and POMC messages, has been demonstrated for a wide range of SG-associated proteins. Knock et al. have identified polypyrimidine tract-binding protein (PTB), which binds and stabilizes mRNAs encoding SG-associated proteins,

as an essential factor for the biogenesis of SGs in pancreatic β-cells (Knoch et al., 2004). Knockdown of PTB expression by RNA interference resulted in a simultaneous decrease in SG-associated proteins including PC1/3, PC2, CgA, SgII, synaptobrevin 2 and synaptophysin. Furthermore, our observation that SgIII binds to either CgA or CPE, but CPE and CgA do not bind each other, and both SgIII and CPE bind POMC molecules suggests a finely regulated interaction between SG-associated proteins and hormones. Thus, SG-associated proteins are regulated not only at the gene transcription and mRNA translation levels, but also at the protein-protein and protein-lipid interaction levels.

When one of the SG-associated proteins is defective in function, there seems to be compensatory mechanisms to cover its missing function. In our study, the Cpe^{fat} pituitary exhibited elevated levels of SgIII and CgA (Fig. 6). When the POMC-sorting function of CPE is lacking, SgIII and CgA appear to compensate. Indeed, both GST-SgIII and GST-CgA pulled down POMC as did GST-CPE from the AtT-20 cell extract (Fig. 5). In terms of POMC-binding capacity, SgIII was more capable than CgA; however, their capacity was much lower than that of CPE. The majority of anti-ACTH-antibody-binding peptides appeared to be POMC itself and large ACTH precursors, because their size was over 28 kDa (Fig. 5). POMC molecules initially localize at the periphery of the SGs, then move to an intragranular core region after receiving processing, as previously suggested (Tooze and Tooze, 1986). At the periphery, POMC molecules must bind to cholesterol-rich membrane-anchored adapter proteins such as CPE and SgIII (Hosaka et al., 2004). Because CPE has higher binding capacity to POMC than SgIII (Fig. 5), we presume that POMC is transferred from CPE to SgIII. Interestingly, when recombinant SgIII was exogenously added in vitro to POMC-bound GST-CPE solution, fewer POMC molecules bound to GST-CPE (Fig. 8A). POMC molecules bound to GST-SgIII were also released by exogenous recombinant CgA to a less extent. We further found that POMC-derived peptides were transferred to exogenously added SgIII by anti-ACTH antibody pull-down assay (Fig. 8B). This suggests that POMC is transferred to SgIII from CPE, and perhaps to CgA from SgIII. This transfer of prohormones from adaptor to adaptor together with their successive processing reactions at the periphery of SGs is followed by intragranular distribution of mature peptide hormones perhaps with aggregated forms of CgA and/or CgB for their efficient release at exocytosis.

However, even if the sorting function of CPE is well compensated by other SG-associated proteins, its exopeptidase function remains to be defective, resulting in the retention of Lys/Arg-extended prohormone intermediates (Fricker et al., 2000; Che et al., 2001; Bures et al., 2001). Defective CPE exopeptidase function was found to exert an unfavorable influence on well-coordinated prohormone-processing reactions from studies on Cpe^{fat} endocrine cells, by affecting other prohormone-processing enzymes. Berman et al. demonstrated a reduced level of PC1 activity perhaps due to incomplete inactivation of proSAAS intermediate peptide, a potent PC1 inhibitor (Fricker et al., 2000), caused by defective CPE, and an increased level of PC2 activity perhaps due to chronic expression of 7B2 C-terminal PC2-inhibitory peptide, also caused by defective CPE (Berman et al., 2001). Indeed, a decrease in prohormone convertase activity has been

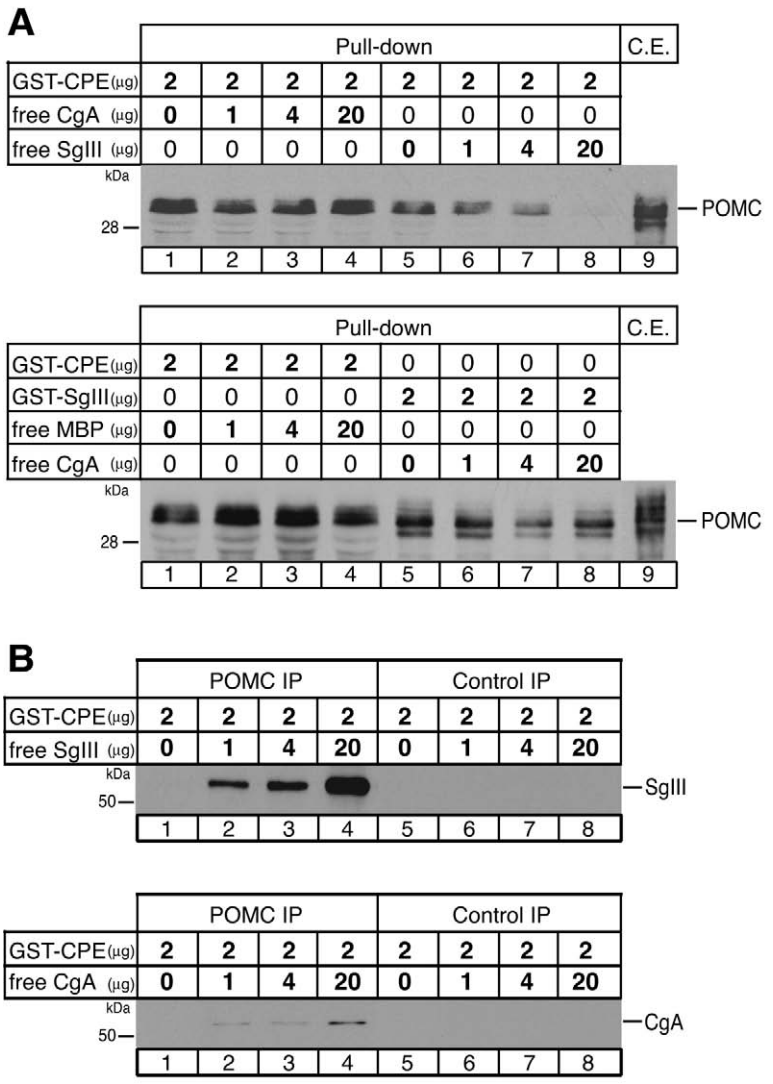

Fig. 8. Transfer of POMC from CPE to SgIII and CgA. (A) Effect of CgA, SgIII and MBP on POMC-binding capacity of CPE or SgIII. GST-CPE 23-477 or GST-SgIII 23-471 was incubated with the AtT-20 cell extract to fully bind POMC molecules. POMC-bound GST-CPE or GST-SgIII was incubated with increasing doses of recombinant CgA, SgIII or MBP, as indicated by bold figures on the top panel. GST-CPE or GST-SgIII was then pulled down with glutathione beads, and subjected to SDS-PAGE for POMC immnoblotting. In lane 9, one-twentieth of AtT-20 cell extract was run as a control. (B) In vitro pull-down of SgIII or CgA by anti-ACTH antibody. After POMC-bound GST-CPE was incubated with recombinant SgIII or CgA, POMC was pulled down by the anti-ACTH antibody. The precipitates were subjected to SDS-PAGE and immunoblotting for SgIII and CgA. Upper panel shows SgIII bound to POMC, and lower panel shows CgA bound to POMC. As a control IP experiment, preimmune serum was used instead of the anti-ACTH antibody (left panels shown as Control IP).

shown by the presence of unprocessed prohormone intermediates such as prodynorphin intermediates and α-melanocyte-stimulating hormone (αMSH) precursors (Berman et al., 2001), pro-thyrotropin-releasing hormone intermediates (Nillni et al., 2002), procholecystokinin intermediates (Lacourse et al., 1998), and proinsulin and cocain- and amphetamine-related transcript (CART) precursors (Cawley et al., 2004) in Cpe^{fat} mice and CPE-knockout mice. Among these hormones, inefficient production of mature αMSH and CART is suggested to enhance food intake and lead to adult-onset obesity in the Cpe^{fat} mouse and CPE-knockout mouse (Zimmermann-Belsing and Feldt-Rasmussen, 2004: Cawley et al., 2004).

Although POMC is not efficiently processed to ACTH by the decrease in prohormone convertase and CPE activity, anti-ACTH-antibody-reacting POMC-derived peptides are distributed correctly over the SG of Cpe^{fat} corticotropes (Fig. 4D). However, the IR-ACTH content in the Cpe^{fat} pituitary was less than a half of that in the WT pituitary. Hormone storage capacity appears to be decreased even by the compensatory functions of SgIII and CgA, perhaps due to an elevated constitutive secretion from the Cpe^{fat} pituitary. As expected by the compensatory mechanism, IR-ACTH was secreted in a regulated manner with CRH and KCl (Fig. 7), unlike the previous report (Cool et al., 1997). Thus, regulated secretion is well-maintained in the Cpe^{fat} pituitary, as previously demonstrated with LH and FSH secretion by gonadotropin-releasing hormone and Ca^{2+} ionophore A23187 (Srinivasan et al., 2004). We propose that the sorting machinery complexes, which contain at least SgIII and CPE, are cooperative for the transfer of peptide hormone precursors, their processing to active forms, their efficient intragranular storage with granins, and efficient release of peptide hormones at exocytosis. Future experiments to downregulate expression of SgIII in cells or in a knockout mouse model will determine whether SgIII is essential for the sorting and secretion of POMC in pituitary cells in vivo.

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