Involvement of the membrane lipid bilayer in sorting prohormone convertase 2 into the regulated secretory pathway

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Prohormone convertase 2 (PC2) is a neuroendocrine-specific protease involved in the intracellular maturation of prohormones and proneuropeptides. PC2 is synthesised as a proprotein (proPC2) that undergoes proteolysis, aggregation and membrane association during its transit through the regulated secretory pathway. We have previously shown that the pro region of proPC2 plays a key role in its aggregation and membrane association. To investigate this further, we determined the binding properties of a peptide containing amino acids 45–84 of proPC2 (proPC2$^{45–84}$) to *trans*-Golgi network/granule-enriched membranes from the AtT20 cell line. Removal of peripheral membrane proteins or hydrolysis of integral membrane proteins did not affect the binding properties of proPC2$^{45–84}$. Rather, proPC2$^{45–84}$ was shown to bind to protein-free liposomes in a pH- and Ca^{2+}-dependent manner. To identify the component of the lipid bilayer involved in this membrane association, we used chromaffin-granule membranes and studied the binding properties of the endogenous PC2. Treatment of the membranes with saponin, a cholesterol-depleting detergent, failed to extract PC2 from the membranes, whereas chromogranin A (CgA) was removed. Treatment of the membranes with Triton X-100 yielded a low-density detergent-insoluble fraction enriched in PC2, but not CgA. The detergent-insoluble fraction also contained glycoprotein III, known to be part of the lipid rafts (membrane microdomains rich in sphingolipids). Finally, sphingolipid depletion of AtT20 cells resulted in the mis-sorting of PC2, suggestive of a link between the association of PC2 with lipid rafts in the membrane and its sorting into the regulated secretory pathway.

Key words: lipid rafts, protein targeting, regulated secretion, sorting mechanisms.

INTRODUCTION

Two different pathways of protein secretion are present in neuroendocrine cells: the constitutive pathway in which secretion occurs at a constant rate, and the regulated pathway in which proteins are stored in secretory granules and secreted in response to a secretagogue (for reviews, see [1–3]). Aggregation of regulated secretory cargo is thought to be a key process for sorting to the regulated pathway. It is induced by the acidic pH and the millimolar Ca^{2+} concentration in the lumen of the *trans*-Golgi Network (TGN) and results in the segregation of regulated and constitutive secretory proteins [4–6]. Two different models have been proposed to explain the sorting of proteins destined for regulated secretion [7]. The 'sorting for entry' model states that regulated secretory proteins become aggregated at the level of the TGN, where they become associated with membranes. After aggregation and membrane association, the secretory proteins are selectively stored in immature secretory granules (ISGs) ready to complete sorting and secretion. The 'sorting by retention' model postulates that all proteins enter the ISGs by default. Proteins destined for the constitutive secretory pathway or for delivery to the lysosomes are removed owing to their soluble nature and/or their association with specific receptors, whereas proteins destined for the regulated secretory pathway are retained owing to the formation of aggregates, possibly in conjunction with their association with a sorting receptor.

Different sorting mechanisms have been postulated for different secretory proteins, suggesting a lack of universality for a sorting receptor. Sorting is thought to be controlled by the recognition of sequences present in the cargo proteins by, and their subsequent interaction with, membrane components in the secretory granules. Regarding the sequences in the cargo proteins, although for most proteins a common structural sorting motif has not been recognized, the N-terminal disulphide-bonded loop of chromogranin B (CgB; [8,9]) and pro-opiomelanocortin (POMC; [10]) has been implicated in the sorting of these peptides to the regulated secretory pathway. As regards the membrane component recognizing this sequence, it has been suggested, on the basis of studies using CPE-deficient (Cpe^{fat}/Cpe^{fat}) mice or a neuroendocrine cell line (Neuro-2a) stably expressing CPE antisense RNA [11,12], that carboxypeptidase E (CPE) is a common sorting receptor for POMC, proinsulin and proenkephalin. However, using the same mutant mice [13] it has been shown that proinsulin does not require CPE for sorting to ISGs, leaving this matter open to controversy [3,14].

Prohormone convertase 2 (PC2) is one of the major subtilisin/kexin-like proprotein convertases, responsible for the formation of small bioactive peptides in neural and endocrine cells. PC2 is synthesized as an inactive zymogen, proPC2, that undergoes autocatalytic cleavage at acidic pH to generate the mature protein [15,16]. Unlike other members of the same family, proPC2 cleavage occurs late in the secretory pathway [16–18] and may require the help of the specific binding protein 7B2 [19]. Using chimaeric proteins, it has recently been shown that the pro region of PC2 contains a transferable aggregation and membrane association signal [20]. Furthermore, the region between amino acids 52–77 in proPC2 has been identified to play a dominant role in membrane association, although other sequences may also be involved [20]. It has been suggested that a region within the P-domain and C-terminal region of proPC2 is important for sorting to the regulated secretory pathway [21], but a C-terminally truncated form of proPC2 is still capable of membrane as-

Abbreviations used: CgA, chromogranin A; CgB, chromogranin B; CGM, chromaffin-granule membrane; CPE, carboxypeptidase E; GPIII, glycoprotein III; ISG, immature secretory granule; PC2, prohormone convertase 2; POMC, pro-opiomelanocortin; TGN, *trans*-Golgi network.

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sociation [22], suggesting that membrane association may not, in itself, be sufficient for sorting. Hence, despite all these studies, the mechanisms involved in sorting PC2 to the regulated secretory pathway are still poorly understood.

The aim of the present study was to characterize the sorting mechanism involved in the secretion of PC2 via the regulated secretory pathway. This was carried out by determining the characteristics of binding of *in-vitro*-synthesized proPC2 to AtT20 membranes and by determining the membrane-association properties of endogenous PC2 in chromaffin-granule membranes (CGMs). The results show that PC2 binds to a lipid component of the membrane, possibly a lipid raft, and inhibition of the formation of this raft results in mis-sorting of PC2, suggesting that this interaction could be responsible for the targeting of PC2 to the regulated secretory pathway.

EXPERIMENTAL

Chemicals and reagents

[^{35}S]Methionine (100 Ci/mmol), ^{125}I (0.5 mCi) and Rainbow *methyl*-^{14}C-labelled protein markers (molecular-mass range 14–200 kDa), ECL® nitrocellulose membranes and ECL® Western-blot detection system were obtained from Amersham International, Little Chalfont, Bucks., U.K. Rabbit reticulocyte lysate was purchased from Promega UK Ltd., Southampton, U.K. SP6 RNA polymerase, BSA (DNase- and RNase-free), nucleotide triphosphates and RNase inhibitor were obtained from Amersham Pharmacia Biotech, St. Albans, Herts., U.K. m^{7}G(5′)ppp(5′)G cap structure was purchased from New England Biolabs Ltd., Hitchin, Herts., U.K. A full-length cDNA encoding human PC2 and a rabbit anti-(human PC2) antibody (Pep4) was provided by Dr. D. F. Steiner, Howard Hughes Medical Institute, University of Chicago, IL, U.S.A. PC2-expressing AtT20 cells were provided by Dr. R. Mains, Neuroscience Department, The Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A. FITC-labelled anti-rabbit IgG antibody was obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, Scotland, U.K. Rhodamine-labelled anti-mouse IgG was obtained from Jackson Immunoresearch, West Grove, PA, U.S.A. Rabbit anti-(glycoprotein III) (anti-GPIII) antibody (IC17α1), recognizing the A-chain of GPIII, was provided by Dr. A. Laslop, Department of Pharmacology, University of Innsbruck, Innsbruck, Austria. Iodo-Gen™ (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) was purchased from Pierce, Rockford, IL, U.S.A. All other chemicals were purchased from Sigma–Aldrich Co. Ltd., Poole, Dorset, U.K.

In vitro transcription/translation

PC2 cDNA subcloned into the plasmid SP64-T was linearized with *Bam*HI and used for *in vitro* transcription as previously described [16]. The resulting PC2 mRNA was used for *in vitro* translations in *Xenopus* egg extracts prepared following the method of Matthews and Colman [23]. PC2 mRNA (100 ng) was added to 100 μl of egg extract containing 10 mM creatine phosphate, 0.2 mM spermidine, 10 % (v/v) rabbit reticulocyte lysate and 1 mCi/ml [^{35}S]methionine. Translation was allowed to proceed for 2 h at room temperature. The extract was then resuspended in 10 % (w/v) sucrose in PBS and centrifuged at 10000 ***g*** for 15 min at 4 °C. The supernatant was discarded and the pellet washed, centrifuged again and finally resuspended in water. After three sequential freeze–thaw cycles to lyse the vesicles, the extract was centrifuged at 10000 ***g*** for 15 min at 4 °C. [^{35}S]Methionine-labelled proPC2 was recovered in the supernatant and used for membrane-association assays with TGN/granule-enriched membranes from AtT20 cells.

Preparation of TGN/granule-enriched membranes

TGN/granule-enriched membranes were purified from AtT20 cells by the method of Varlamov and Fricker [24]. AtT20 cells were grown to confluence in 10-cm culture dishes in Dulbecco's modified Eagle's medium (high-glucose medium) supplemented with 15 % (v/v) foetal-calf serum in a 5 % CO_2/air atmosphere. Cells were washed in ice-cold PBS, scraped off the dishes and centrifuged at 800 ***g*** at 4 °C for 3 min. The cells were resuspended in 1.5 ml of 0.25 M sucrose and subjected to ten passes through an ice-cold cell cracker (12 μm clearance diameter; European Molecular Biology Laboratories, Heidelberg, Germany) to break up the cells and liberate the different organelles. The cell lysate was then made up to 1.4 M sucrose, by adding 2 ml of 2.3 M sucrose, layered on top of 1.5 ml of 2 M sucrose, and subsequently overlaid with 5 ml of 1.2 M sucrose and 2 ml of 0.8 M sucrose. All sucrose solutions were made in 10 mM Tris/1 mM magnesium acetate, pH 7.4. The sucrose gradient was centrifuged for 16 h at 120000 ***g*** at 4 °C. After centrifugation, three different layers of membranes could be seen. The top layer containing TGN/granule-enriched membranes, located at the interface between the 0.8 M and 1.2 M sucrose, was diluted with ten times its volume of 0.2 M sucrose and centrifuged for 5 h at 180000 g at 4 °C. The pelleted membranes were resuspended in 200 μl of 0.2 M sucrose in 10 mM Tris/1 mM magnesium acetate, pH 7.4, and kept at −70 °C for further membrane-association and/or binding assays.

Membrane-association assays and immunoprecipitation

Membranes ($\approx$ 50 μg of protein in 100 μl of 0.2 M sucrose in 10 mM Tris/1 mM magnesium acetate, pH 7.4) were sonicated on ice (four bursts of 30 s at 30 s intervals) and incubated in the presence of 900 μl of 0.1 M Na_2CO_3 or 900 μl of 10 mM Tris/1 mM magnesium acetate, pH 7.4 (controls) at 4 °C for 1 h. After incubations, membranes were pelleted by centrifugation at 10000 ***g*** for 15 min, washed twice in a buffer containing 10 mM Tris, 1 mM $CaCl_2$, 1 mM PMSF, pH 7.4, and finally resuspended in 90 μl of a buffer containing 50 mM KCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$, 10 % glycerol (v/v) and 20 mM Mes, pH 5.5, prior to incubation with the radiolabelled proPC2. A 10 μl portion of *in-vitro*-translated [^{35}S]methionine-labelled proPC2 was added to the AtT20 TGN/granule-enriched membranes, and incubated at 4 °C for 1 h. After incubation, the material was made up to 2 M sucrose and buffered by the addition of 100 mM Mes at pH 5.5 or 100 mM Tes at pH 7.0. Samples were sequentially overlaid with 250 μl of 1.5 M, 1.0 M, 0.5 M and 0 M sucrose in 100 mM buffer at pH 5.5 or pH 7.0. The gradient was then centrifuged at 137000 ***g*** for 16 h at 4 °C. Sucrose fractions were collected along with the pelleted material, which was resuspended in a buffer containing 0.1 M NaCl, 1 % (v/v) Triton X-100, 20 mM Tris/HCl, pH 7.6, and 1 mM PMSF. Immunoprecipitation was performed using a sheep antibody raised against amino acids 489–586 of proPC2 coupled to Sepharose beads as previously described [25]. All samples were adjusted to neutral pH by the addition of 2 M NaOH prior to immunoprecipitation.

Brain lipid extract

Tissue from bovine brain was homogenized in 3 vol. of 20 mM Hepes/NaOH, pH 7.4, 1 mM EDTA and 0.3 M sucrose. The homogenate was centrifuged at 1000 ***g*** for 10 min and the resulting supernatant subsequently centrifuged at 100000 ***g*** for

30 min. Lipids from pelleted membranes were extracted with chloroform/methanol following the procedures described by Bligh and Dyer [26].

Liposome preparation

Liposomes were obtained from either bovine brain lipids or from different lipid mixtures by reverse-phase evaporation [27]. Briefly, 50 mg of lipids from bovine brain were dissolved in 3 ml of diethyl ether, mixed with 1 ml of 0.1 % PBS and sonicated at 4 °C (Branson sonifier, amplitude 20, three bursts, 10 min each). The organic solvent was evaporated with rotation at room temperature until a jelly-like consistency was obtained. A 1 ml portion of PBS was added and the mixture vigorously shaken. The remaining diethyl ether was evaporated under a flow of nitrogen. An 8 ml portion of PBS was added to the suspension and the mixture centrifuged at 100000 ***g*** for 30 min. The pellet was finally resuspended in 5 ml of PBS, yielding a liposome suspension with a final concentration of 10 mg/ml. To obtain liposomes from defined lipids, solutions containing 20 mg of the different lipid mixtures were used. The final liposomes contained phosphatidylcholine (from egg yolk, type XVI-E), and variable concentrations of either phosphatidylserine (from bovine brain) or cholesterol (from porcine liver), all obtained from Sigma.

Western blots

Samples were fractionated by SDS/PAGE and blotted on to a nitrocellulose ECL® (enhanced chemiluminescence) membrane (Amersham). Membranes were blocked in a solution of 20 % (w/v) low-fat milk in a buffer containing 10 mM Tris/HCl, 0.05 % (v/v) Tween 20 and 0.5 M NaCl. Mouse monoclonal antibody (mAb286), recognizing an epitope in bovine chromogranin A (CgA) C-terminal to residue 60, rabbit anti-GPIII, recognizing the A-chain of GPIII, and rabbit anti-(human PC2) were used as primary antibodies, followed by incubation with horseradish peroxidase-coupled secondary antibody and detection using the ECL® system (Amersham).

Chromaffin-granule membrane purification

CGMs were isolated from bovine adrenal glands following the procedure described by Smith and Winkler [28]. Pelleted membranes from 30 bovine adrenal medullas were resuspended in 50 ml of a buffer containing 20 mM Hepes, pH 7.4, 100 mM NaCl and 5 mM EDTA and lysed by three cycles of freeze–thawing. Membranes were pelleted by centrifugation at 100 000 ***g*** for 30 min, resuspended in 50 ml of the above buffer, pelleted as before and finally resuspended in 20 ml of the buffer. The membrane suspension was sonicated (Branson sonifier, amplitude 20, three bursts, 10 s each) and centrifuged at 100000 ***g*** for 1 h. The pelleted membranes were finally resuspended in 10 ml of the buffer and stored at −70 °C until further use.

Synthesis of PC2 propeptide

On the basis of the amino acid sequence of the human PC2 pro region, a peptide consisting of 40 amino acids from Tyr^{45} to Arg^{84} (proPC2$^{45–84}$), corresponding to the second half of the pro region, was synthesized. The synthesis was performed using a continuous-flow 'Fmoc' (fluoren-9-ylmethoxycarbonyl)-based peptide synthesis protocol on a Pioneer peptide synthesizer and subsequently purified to > 95 % by HPLC. The molecular mass (5015.604 Da) of the obtained peptide was confirmed on a 'MALDI-TOF' (matrix-assisted laser-desorption/ionization time-of-flight) mass spectrometer.

Radioligand binding assay

A 10 μl portion of a 1 mM stock solution of proPC2$^{45–84}$ was mixed with 8 μl of 100 mM sodium borate buffer, pH 8.5, and transferred to a 1.5 ml polypropylene tube previously coated with 50 μg Iodo-Gen™ (Pierce), following the manufacturer's instructions. A 5 μl portion of $Na^{125}I$ (0.5 mCi) was subsequently added to the tube and incubated for 15 min at room temperature. The reaction was stopped by addition of 10 μl of 0.1 M sodium metabisulphite and further incubation for 1 min. The iodinated peptide was then separated from the free iodine on a PD-10 column (Amersham Pharmacia Biotech). Fractions (1 ml each) were collected and those containing the radiolabelled peptide pooled together and frozen at −20 °C until further use. A 100 μl portion of TGN/granule-enriched membranes (≈ 50 μg of protein in 100 μl of 0.2 M sucrose in 10 mM Tris/1 mM magnesium acetate, pH 7.4) was sonicated on ice (four bursts of 30 s at 30 s intervals) and incubated with proteinase K at final concentrations of 10, 50 or 100 μg/ml for 1 h at 37 °C. After incubations, membranes were washed twice in a buffer containing 10 mM Tris, 1 mM $CaCl_2$ and 1 mM PMSF, pH 7.4, centrifuged at 10000 ***g*** for 15 min, and finally resuspended in 100 μl of a buffer containing 50 mM KCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$, 10 % glycerol (v/v) and 20 mM Mes, pH 5.5, prior to incubation with the radiolabelled proPC2$^{45–84}$. Proteinase K-treated TGN/granule-enriched membranes or liposomes (100 μl) were incubated with 1 μl of radiolabelled proPC2$^{45–84}$ (approx. 25 pmol) in the presence (total binding) or absence (non-specific binding) of 10 μl of 10 mM unlabelled proPC2$^{45–84}$ at 4 °C for 1h. After incubation, the TGN/granule-enriched membranes or the liposomes were centrifuged at 245000 ***g*** for 8 min at 4 °C. The radioactivity present in both the supernatants and the pellets, resuspended in a buffer containing 50 mM KCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$ and 20 mM Mes or Tes at pH 5.5, 6.4 or 7.0, was measured using a γ-radiation counter. The final value for the binding was calculated by subtracting the non-specific binding (i.e. binding in the presence of excess, unlabelled proPC2$^{45–84}$) from the total binding.

Detergent extraction and sucrose gradients

CGMs (≈ 0.6 mg of protein/ml) were incubated at 4 °C in a 20 mM Hepes (pH 7.4)/50 mM NaCl/1 mM EDTA buffer, containing 1 % (v/v) Triton X-100 for 30 min or 0.1 % (w/v) saponin for 1 h with gentle agitation. Samples were centrifuged at 126 000 ***g*** for 30 min, supernatants and pellets collected, and analysed by Western blot. To perform the isopycnic sucrose-gradient centrifugation, CGMs were incubated as described above in a Triton X-100-containing buffer. After incubation, the membranes were adjusted to 40 % (w/v) sucrose with 80 % (w/v) sucrose in 1 mM Tris/0.1 M magnesium acetate, pH 7.4, and placed in the bottom of a centrifuge tube. A linear sucrose gradient (5–30 %) was layered on top of the membranes and the material centrifuged for 16 h at 261000 ***g*** at 4 °C in a Beckman SW41 rotor. Fractions (1 ml each) were collected from the top of the gradient and the remaining pellet resuspended in 1 ml of sucrose and collected as the last fraction of the gradient. Fractions were stored at −70 °C until further analysis by Western blot.

Fumonisin treatment and double immunocytochemistry

AtT20 cells stably transfected with PC2 were grown in 8.8 cm^2 plates (Nalge Nunc Int., Naperville, IL, U.S.A.) at an initial density of 50 cells/plate. Fumonisin B1 (Sigma) was dissolved in 20 mM Hepes, pH 7.4, to obtain a 1 mM stock solution. Fumonisin was filter-sterilized and added to AtT20 cells 24 h

after being plated to final concentrations of 0 (control group), 20, 30 and 40 μM. Every 48 h the fumonisin was replaced with fresh fumonisin and treatment continued for 8 days. Cells were then washed with PBS and fixed in ice-cold methanol for 10 min at 4 °C and blocked in a solution containing 6.7 % (v/v) glycerol, 0.2 % (v/v) Tween 20 and 2 % (w/v) BSA in PBS for 15 min at room temperature. For the double-immunofluorescence experiment, cells were incubated at 4 °C overnight with the primary antibodies against PC2 or CgA, washed with a solution containing 6.7 % (v/v) glycerol, 0.4 % (v/v) Tween 20 and 2 % (w/v) BSA in PBS, and incubated with a FITC-conjugated goat antibody against rabbit IgG and a Rhodamine-conjugated goat antibody against mouse IgG for 1 h in the dark. The plates were washed again with the same solution as that described above for 2 h and studied with a confocal laser-scaning microscope and a Bio-Rad confocal image software. Cells were randomly selected and the confocal image adjusted to take a section through the entire body of the cell.

RESULTS

ProPC2 binds to TGN/granule-enriched AtT20 membranes

It has previously been shown that proPC2 associates with membranes derived from *Xenopus* oocytes in a pH-dependent manner [20,22]. In the present study we used TGN/granule-enriched membranes from the mouse corticotrophic cell line, AtT20, known to contain both the regulated and the constitutive secretory pathways [29]. *In-vitro*-synthesized proPC2 also binds to these membranes in a pH-dependent manner (results not shown). After incubation with AtT20 membranes at pH 5.5, approx. 75 % of the *in-vitro*-synthesized proPC2 was found in the pellet, representing material that had aggregated at this acidic pH; 25 % of proPC2 floated with the membranes and was found in the 1.0 M sucrose layer (Figure 1). After treatment of the AtT20 membranes with Na_2CO_3, no difference was seen in the extent of membrane association, with approx. 25 % of the proPC2 found floating with the membranes in the 1.0 M sucrose layer and 75 % in the pellet (due to aggregation). Hence, removal of peripheral membrane proteins with Na_2CO_3 did not affect the binding properties of proPC2, suggesting that a proteinaceous membrane component was not involved in the interaction of proPC2 with AtT20 TGN/granule membranes.

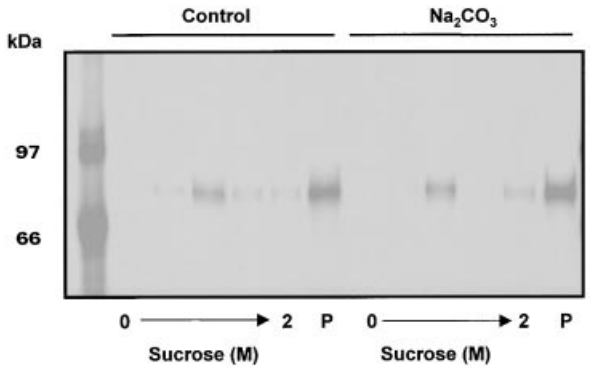

Figure 1 Effect of Na_2CO_3 on the association of proPC2 with TGN/granule membranes from AtT20 cells

Membranes were incubated for 1 h with 0.1 M Na_2CO_3 at 4 °C. After washing, the membranes were further incubated at 4 °C for 1 h with [^{35}S]methionine-labelled proPC2 (10 μl) previously translated in *Xenopus* egg extract. After incubation, the material was adjusted to 2 M sucrose, buffered by the addition of 100 mM Mes at pH 5.5, and subjected to sucrose-gradient centrifugation as described in the Experimental section. Gradient fractions were immunoprecipitated using a sheep antibody raised against amino acids 489–586 of proPC2 and analysed by SDS/PAGE and fluorography. Results are representative of those from three different experiments.

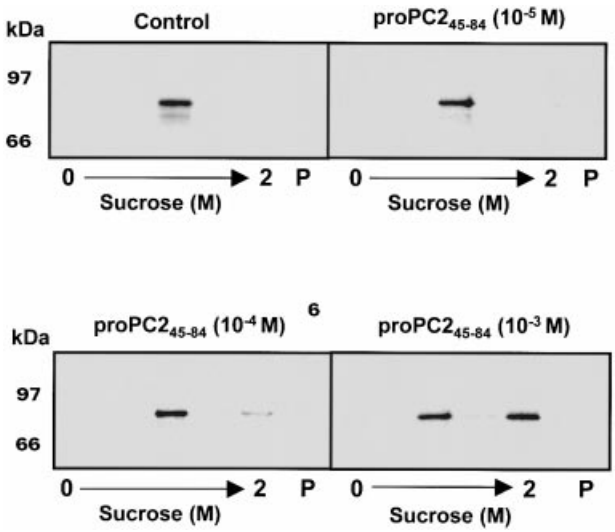

Figure 2 $proPC2^{45–84}$ inhibits the binding of pro-PC2 with TGN/granule membranes from AtT20 cells in a dose-dependent manner

Membranes were incubated for 1 h in the absence (controls) or presence of different amounts of a synthetic peptide ($proPC2^{45–84}$) corresponding to amino acids 45–84 of proPC2 ('$proPC2_{45–84}$'; final concns. 10^{-3} M, 10^{-4} M and 10^{-5} M). Membranes were further incubated at 4 °C for 1 h with [^{35}S]methionine-labelled proPC2 (10 μl) previously translated in *Xenopus* egg extract. After incubation, the material was adjusted to 2 M sucrose, buffered with 100 mM Mes at pH 5.5, and subjected to sucrose-gradient centrifugation as described in the Experimental section. Gradient fractions were immunoprecipitated using a sheep antibody raised against amino acids 489–586 of proPC2 and analysed by SDS/PAGE and fluorography. Data are representative of those from two different experiments.

Table 1 pH-dependent binding of $proPC2^{45-84}$ to liposomes from bovine brain lipids

A synthetic peptide corresponding to amino acids 45–84 of proPC2 was iodinated as described in the Experimental section and incubated with liposomes made from bovine brain lipids for 1 h at 4 °C in a buffer consisting of 50 mM KCl, 6 mM $MgCl_2$, 10% (v/v) glycerol, and either 20 mM Mes or 20 mM Tes at the pH indicated and with $CaCl_2$ at the concentration indicated. The liposomes were pelleted by centrifugation and radioactivity associated with the pellet and supernatant counted in a γ-radiation counter. Binding was determined as the radioactivity recovered in the pellet, after subtraction of the non-specific binding, expressed as a percentage of the total radioactivity added. Results are means $\pm$ S.E.M. of triplicate determinations from three different experiments.

		Binding (%)		
$[Ca^{+2}]$ (mM)	pH...	7.0	6.4	5.5
0		4.5 $\pm$ 1.33	22.3 $\pm$ 0.16	14.4 $\pm$ 0.08
1		1.5 $\pm$ 1.20	26.0 $\pm$ 0.56	24.5 $\pm$ 0.97
10		10.8 $\pm$ 2.22	15.5 $\pm$ 1.16	28.0 $\pm$ 1.00

Previous studies have shown that the pro region of proPC2 was involved in membrane association [20]. A synthetic peptide corresponding to amino acids 45–84 of pro-PC2 (pro-$PC2^{45–84}$) was synthesized and its ability to displace proPC2 from the AtT20 TGN/granule membranes was determined. After in-

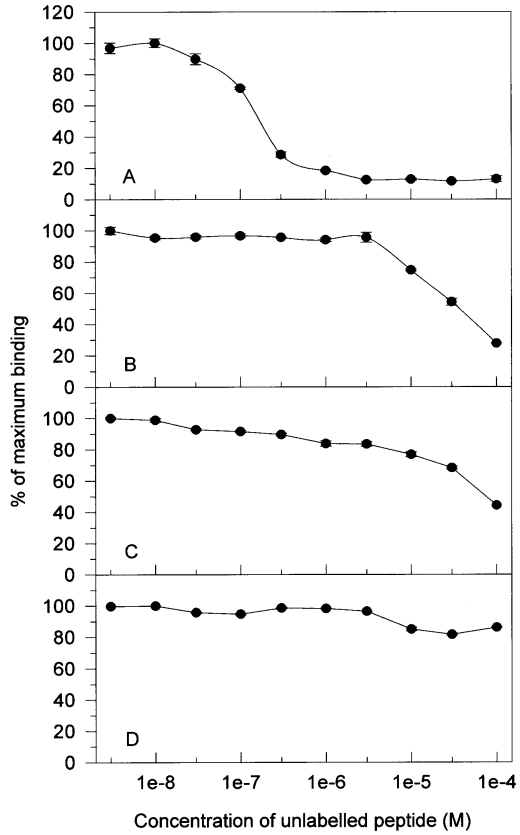

Figure 3 PC2 binds to the lipid bilayer through the region comprising amino acids 45–84 of the propeptide (proPC2$^{45–84}$)

Liposomes made from bovine brain lipids were incubated for 20 min at 4 °C with 10^{-4} M ^{125}I-proPC2$^{45–84}$ in the presence of different concentrations (range 10^{-4}–10^{-8} M) of competing unlabelled proPC2$^{45–84}$ (**A**), proPC2$^{6–42}$ (**B**), CgA$^{1–60}$ (**C**) and CgA$^{406–431}$ (**D**). The radioactivity recovered in the pellet (proPC2$^{45–84}$ bound to the liposomes) is expressed as percentage of maximum binding. Results are the mean ± S.E.M. for triplicate determinations from three different experiments.

cubation with the membranes at pH 5.5, a dose-dependent decrease in the percentage of proPC2 present in the 1.0 M sucrose layer was induced with increasing concentrations of proPC2$^{45–84}$ (Figure 2).

proPC2$^{45–84}$ binds to liposomes

^{125}I-labelled-proPC2$^{45–84}$ bound to AtT20 TGN/granule membranes in a pH-dependent manner. At pH 7.0, specific binding was 10.2 ± 0.46 %, whereas at pH 5.5, specific binding was significantly increased ($P < 0.05$) to 30.1 ± 0.52 % (results not shown). In addition, digestion of the extra-membranous domains of integral membrane proteins after treatment of AtT20 TGN/granule membranes with proteinase K resulted in a significant increase ($P < 0.05$) in the specific binding of the synthetic propeptide, with approx. 31.1 ± 2.4 % bound to the membranes in the absence of treatment, and 46.3 ± 1.3 % or 47.3 ± 0.53 % bound to the membranes after proteinase K digestion (50 μg/ml or 100 μg/ml respectively; results not shown). This result suggests once again that a proteinaceous membrane component was not involved in the interaction of proPC2 with AtT20 TGN/granule membranes. To determine whether binding was due to an interaction with a lipid component of the membrane, binding assays were performed using the synthetic peptide and liposomes

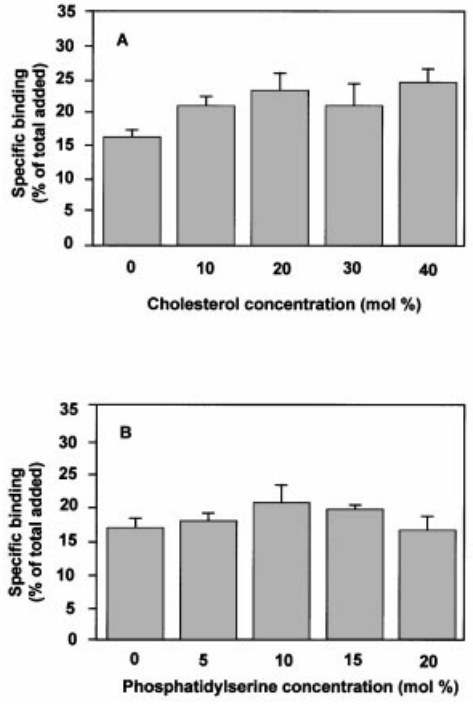

Figure 4 Binding of proPC2$^{45–84}$ to liposomes does not depend on cholesterol or phosphatidylserine

Liposomes containing equal molar amounts of total lipids consisting of the indicated mol% of cholesterol (**A**) or phosphatidylserine/phosphatidylcholine (3:1 molar ratio; **B**) were incubated with 10^{-6} M ^{125}I-proPC2$^{45–84}$ for 20 min at 4 °C. After subtracting the non-specific binding, the radioactivity recovered in the pellet was expressed as the percentage of total added radioactivity. Results are the means ± S.E.M. for triplicates from three different experiments.

derived from bovine brain membranes. The results demonstrate that proPC2$^{45–84}$ also binds to liposomes in a pH- and Ca^{2+}-dependent manner. The highest binding occurs at pH 5.5, and at 10 mM Ca^{2+}, typical for the secretory granules (Table 1). The specificity of the binding assay was assessed by competition of the radiolabelled proPC2$^{45–84}$ with unlabelled proPC2$^{45–84}$ (Figure 3A), proPC2$^{6–42}$, corresponding to the first half of the PC2 pro region (Thr^{6}–Tyr^{42}; Figure 3B), the N-terminal part of CgA (CgA$^{1–60}$; Figure 3C) and a C-terminal peptide of CgA (CgA$^{406–431}$; Figure 3D). A dose-dependent decrease in the binding of proPC2$^{45–84}$ to bovine brain liposomes was seen with increasing concentrations of unlabelled proPC2$^{45–84}$ ($EC_{50} = 2 \times 10^{-7}$ M), whereas none of the other peptides was able to compete with radiolabelled proPC2$^{45–84}$ for binding ($EC_{50} = 3 \times 10^{-5}$ M, 8×10^{-5} M and no inhibition for proPC2$^{6–42}$, CgA$^{1–60}$ and CgA$^{406–431}$ respectively).

To investigate the nature of the lipid component involved in the association with proPC2$^{45–84}$, synthetic liposomes were made with different amounts of cholesterol or phosphatidylserine and a constant amount of phosphatidylcholine. Increasing concentrations of cholesterol (Figure 4A) or phosphatidylserine (Figure 4B) did not result in a significant change in the binding of proPC2$^{45–84}$ to liposomes.

Binding of PC2 to CGMs does not depend on the presence of cholesterol

The binding characteristics of endogenous PC2 was assessed using CGMs, which are a rich source of secretory-granule

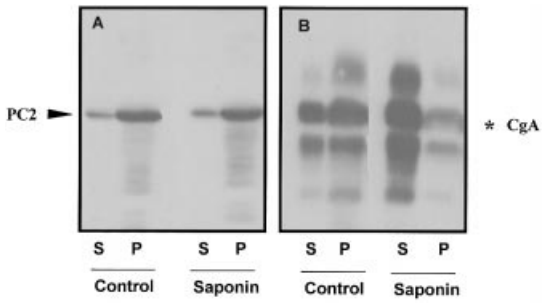

Figure 5 Saponin does not extract PC2 from CGMs

CGMs (0.6 mg of protein/ml) were incubated in a 20 mM Hepes (pH 7.4)/50 mM NaCl/1 mM EDTA buffer containing 0.1% (w/v) saponin for 1 h at 4 °C with gentle agitation. Supernatants (S) and pellets (P) obtained after centrifugation were resolved by SDS/PAGE and analysed by western blot using sequential antibodies to PC2 (**A**), and CgA (**B**). The asterisk (*) indicates the band corresponding to full-length CgA. Results are representative of those from two different experiments.

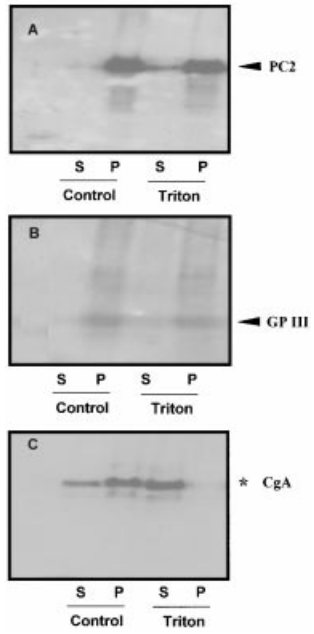

Figure 6 Effect of Triton X-100 on the extraction of PC2, GPIII and CgA from CGMs

CGMs were incubated in a 20 mM Hepes, pH 7.4, 50 mM NaCl, 1 mM EDTA buffer containing 1% (v/v) Triton X-100 for 30 min at 4 °C with gentle agitation. Supernatants (S) and pellets (P) obtained after centrifugation were resolved by SDS-PAGE and analysed by western blot using sequential antibodies to PC2 (A), GPIII (B), and CgA (C). Asterisk indicates the band corresponding to full-length CgA. Data are representative from two different experiments.

components, including PC2 and CgA [30]. Removal of membrane cholesterol by treatment with saponin did not extract PC2 from CGMs. The percentage of PC2 present in the membrane-bound form (pellet) of control and saponin-treated CGMs was 65.8 versus 67.4% respectively (Figure 5A). However, saponin treatment did result in the extraction of CgA from the membranes. About 51.1% of the total CgA was present in the pellet of untreated CGMs, whereas only 10.4% remained in the pellet after treatment with saponin (Figure 5B). This again suggests

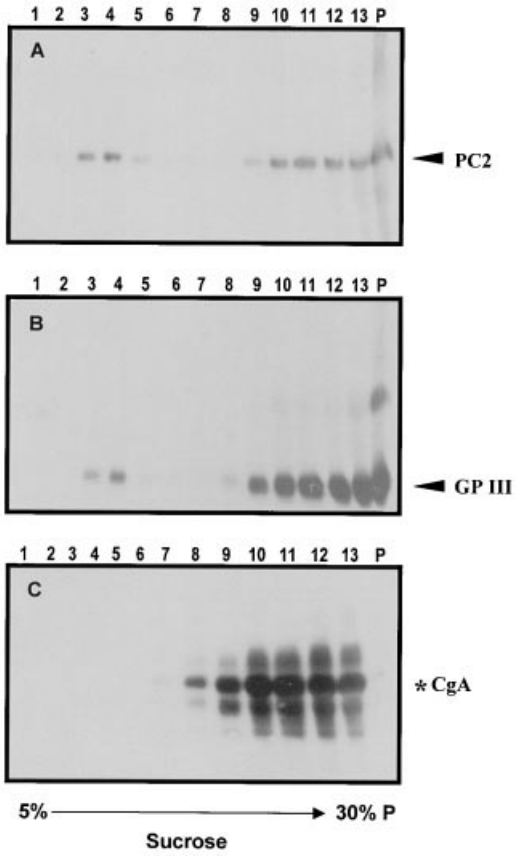

Figure 7 PC2 and GPIII, but not CgA, associate with a detergent-insoluble fraction of CGMs after Triton X-100 extraction

CGMs were incubated at 4 °C in a buffer containing 1% Triton X-100 for 30 min with gentle agitation. The membranes were adjusted to 40% sucrose, layered with a linear sucrose gradient (5–30%) and centrifuged for 16 h at 261000 ***g***. Fractions (1 ml) sequentially collected from the top to the bottom of the gradient (fraction 1–fraction 13) and the remaining pellet (P) were separated by SDS/PAGE. Western-blot analysis were performed using sequential antibodies to PC2 (**A**), GPIII (**B**) and CgA (**C**). The asterisk (*) indicates the band corresponding to full-length CgA. Data are representative of those from two different experiments.

that cholesterol is not the major determinant of proPC2 association with membranes.

Detergent-resistant PC2 associates with membrane lipid rafts

The solubility of endogenous PC2 was determined by Western-blot analysis after extraction of CGMs with Triton X-100 at 4 °C (Figure 6). The results showed that Triton X-100 was able to extract only 24% of the total membrane-bound form of PC2 (89% in the pellet of control CGMs versus 65% in the pellet of treated membranes; Figure 6A). Similarly, 27% of the membrane-bound form of GPIII, a protein known to associate with detergent-insoluble complexes [31], so-called 'rafts' [32], was also extracted with Triton X-100 (100% in the pellet of control CGMs versus 73% in the pellet of Triton-X-100-treated membranes; Figure 6B). However, the same treatment resulted in a complete extraction of CgA from the membranes (62% in the pellet of control CGMs versus 0% in the pellet of Triton-X-100-treated membranes; Figure 6C). These results suggest that PC2, like GPIII, is present in Triton-X-100-insoluble lipid rafts. To investigate this further, CGMs were subjected to extraction with Triton X-100 at 4 °C, followed by equilibrium centrifugation

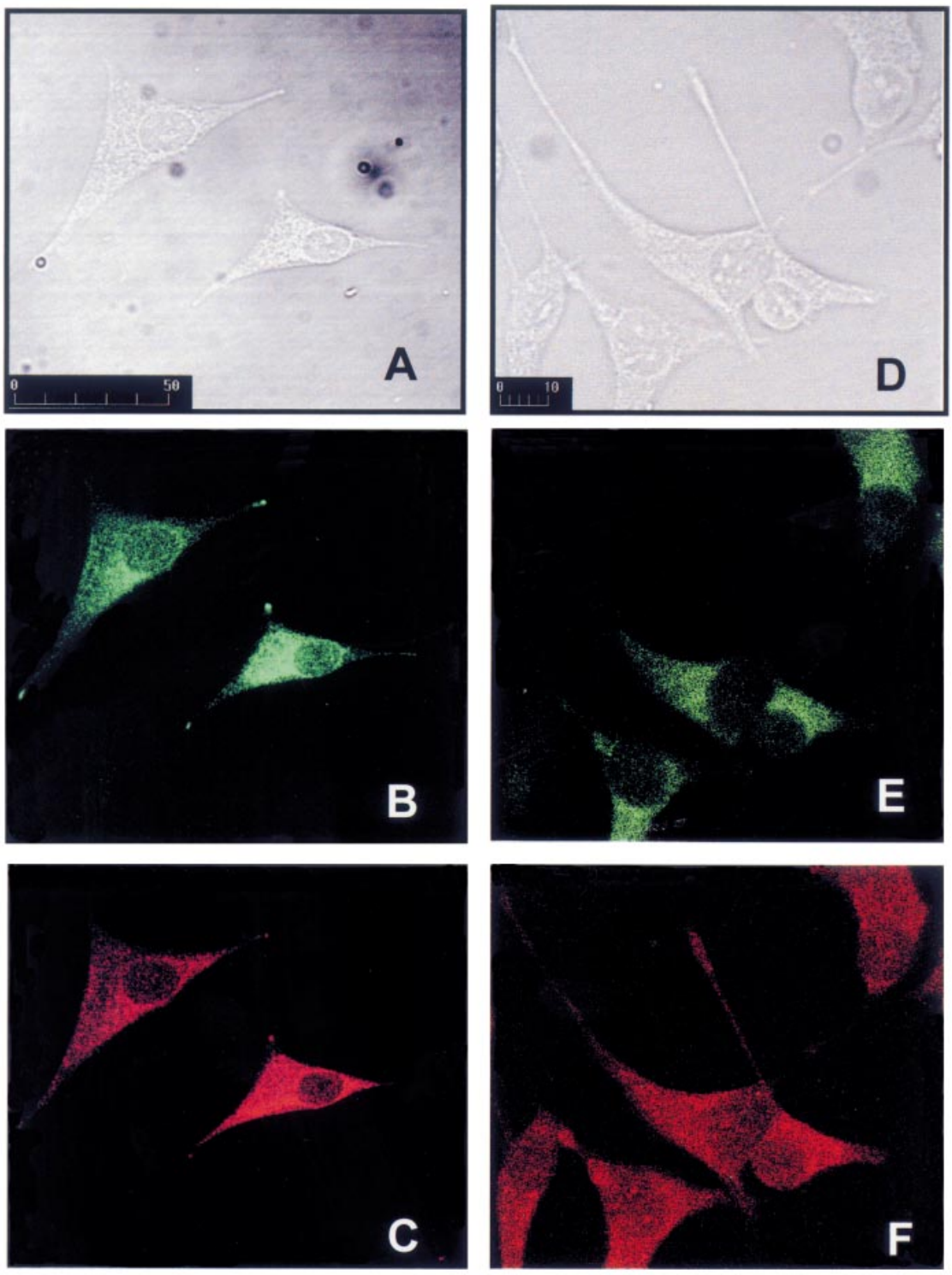

Figure 8 Sphingolipid depletion affects PC2 sorting

AtT20 cells stably transfected with PC2 were incubated in the absence (**A**, **B** and **C**) or presence of 40 μM fumonisin B1 (**D**, **E** and **F**). Every 48 h, the culture medium was replaced by fresh medium containing fumonisin B1 for a total of 8 days. Cells were then washed and processed for double confocal immunofluorescence using antibodies against PC2 (green; **B** and **E**) or CgA (red; **C** and **F**). Phase-contrast images are shown in panels (**A**) and (**D**).

in a sucrose density gradient (Figure 7). The results show that 24% of the total PC2 was present in low-density detergent-insoluble fractions (fractions 3–5), 61% in the high-density fractions (fractions 9–13), with 15% of PC2 remaining in the pellet (Figure 7A). A similar pattern of distribution was found for GPIII, with 10% present in the low-density detergent-insoluble

fractions, 72% in high-density fractions and 18% in the pellet (Figure 7B). In contrast, CgA was not present in the detergent-insoluble fractions or the pellet, but was present exclusively in the high-density fractions of the gradient (Figure 7C).

Sphingolipid depletion affects the sorting of PC2

The sphingolipid-synthesis inhibitor fumonisin B1 was used to assess whether lipid rafts play a role in PC2 sorting. This fungicidal agent inhibits the action of ceramide synthase, a key enzyme in the pathway for sphingolipid biosynthesis and turnover [33]. The pattern of distribution of PC2 in control (untreated) cells (Figure 8A) or cells treated with fumonisin at 20 μM showed perinuclear, Golgi-like localization (Figure 8B). In addition, an accumulation of the protein in the cell processes, typical of a protein sorted to the regulated secretory pathway, was also seen. A similar pattern of distribution was seen for CgA (Figure 8C). Fumonisin treatment at the highest concentrations (30 μM and 40 μM; Figure 8D) resulted in a similar Golgi-type localization of PC2 immunofluorescence, but with a clear absence of signal in the cell processes (Figure 8E). However, no changes in the subcellular localization of CgA were induced by the treatment (Figure 8F).

DISCUSSION

Sorting of proteins into the regulated secretory pathway is essential in neuroendocrine cells and, although subject to intensive study, many questions still remain unresolved [3,14]. Central to the process of sorting is proposed to be an association of the regulated secretory protein with membranes in the TGN or ISG. In order to study the requirements for membrane association of proPC2 and its role in sorting, we investigated both the interaction of *in-vitro*-synthesized proPC2 to AtT20 TGN/granule membranes and the interaction of endogenous PC2 with CGMs. Our results show that proPC2 and a synthetic peptide corresponding to amino acids 45–84 of proPC2 associates with TGN/granule membranes from AtT20 cells in a pH- and Ca^{2+}-dependent manner. Moreover, the synthetic peptide $proPC2^{45–84}$ was able to displace proPC2 from TGN/granule membranes from AtT20 cells in a dose-dependent manner. Further characterization showed that membrane association is not due to interaction with a protein component of the membrane, but to a lipid component, and that endogenous PC2 associates specifically with sphingolipid-rich lipid rafts.

Previous studies indicated that proPC2 undergoes an aggregation and membrane-association event at acidic pH which is not due to the C-terminal amphipathic α-helix [22]. More recently it was shown that the pro region contained a transferable aggregation and membrane-association domain and that amino acids 52–77 may have a dominant influence on both these events [20]. These previous studies were performed with membranes derived from *Xenopus* oocytes, but in the present study it is clear that both proPC2 and amino acids 45–84 of proPC2 also associate with membranes derived from AtT20 cells, a mammalian neuroendocrine cell line with a well-characterized regulated secretory pathway. As shown previously, membrane association is both pH- and Ca^{2+}-dependent. CPE also shows pH-dependent binding to AtT20 membranes, and the results obtained here for PC2 are similar in some respects to those obtained for CPE [34]. However, CPE binding was found to involve a C-terminal amphipathic α-helix, whereas we have previously shown that a similar region is not involved in the membrane association of proPC2 to either oocyte membranes [22] or to AtT20 membranes (results not shown). Also, the binding of CPE to membranes was not inhibited by high concentrations of unlabelled CPE [34], whereas we show that binding of PC2 propeptide is inhibited by unlabelled peptide, suggesting the presence of limited binding sites. The detergent-insolubility of mature PC2 is also reminiscent of the inability of Triton X-100 to extract CPE from membranes [34]. A crucial difference, however, is that CPE can be extracted from membranes at neutral pH, whereas we show here that a proportion of PC2 remains detergent-insoluble at neutral pH. The present study shows that binding is not dependent on either peripheral or integral membrane proteins, as both Na_2CO_3 and proteinase K treatment of membranes did not affect membrane binding. Interaction with a lipid component of the membrane was confirmed by the ability of $proPC2^{45–84}$ to bind to either protein-free liposomes made from bovine brain lipids or to synthetic lipid mixtures. Similar results have been reported for pancreatic zymogen aggregates which bind to phospholipids of the zymogen granule membranes which have been stripped of membrane proteins [35]. The interaction of proPC2 with membranes is not affected by increasing concentrations of cholesterol or phosphatidylserine in the liposomes. It could thus be that a simple non-specific hydrophobic interaction is responsible for the binding of the peptide to the membrane. However the inability of $CgA^{1–60}$, which contains the N-terminal disulphide-bonded loop responsible for the homodimerization of CgA [36] and also for chromogranin sorting [9,37,38], to compete with $proPC2^{45–84}$ indicates that these two proteins use different, possibly specific, mechanisms to associate with membranes. Ionic interactions may be important for the binding of $proPC2^{45–84}$ to membranes, as this region contains a number of charged residues, particularly histidine residues, which would become protonated at acidic pH. This is the first indication that a lipid membrane component is responsible for tethering proPC2 to the membrane.

To investigate the possible involvement of cholesterol in the membrane association of endogenous PC2, we used CGMs, as they are rich in cholesterol [39]. As for $proPC2^{45–84}$, cholesterol does not seem to be involved in binding of endogenous PC2 to CGMs, as low concentrations of saponin, a cholesterol-binding agent, failed to extract membrane-associated PC2. In contrast, the membrane-associated form of CgA was completely extracted by saponin, even at the lowest concentrations. Together, these results reinforce the idea that a lipid membrane component other than cholesterol is responsible for the binding of PC2 to membranes.

It has previously been reported that PC2, along with CPE, becomes associated with GPIII in high-molecular-mass protein aggregates in both membrane and soluble fractions of chromaffin granules [31]. GPIII, which is present in adrenal chromaffin granules, from where it is released via the regulated secretory pathway [31,40,41], is also a component of detergent-insoluble complexes [42], characteristic of lipid rafts [32]. The lipid rafts, which are created by hydrophobic interactions of the long fatty-acyl chains of the sphingolipids with cholesterol and glycophosphatidylinositol-anchored proteins [43,44], are important in the apical sorting of proteins in polarized epithelial and hepatic cells [44,45]. Similarly, axonal transport of synaptic vesicles in neurons has also been shown to involve lipid rafts [46]. The present study showed that, similar to GPIII, PC2 is also associated with a detergent-insoluble fraction. In contrast, CgA became completely solubilized after Triton X-100 extraction, suggesting that either it does not bind to the membrane lipid rafts or that binding, if it exists, is sensitive to detergent treatment and therefore differs from the raft binding of PC2. The form of PC2 that associates with lipid rafts is the mature form, i.e. after propeptide cleavage, whereas our previous results suggested that the pro region directed binding to lipid. This suggests that regions

in addition to the propeptide help tether PC2 to the membrane. In fact, using chimaeric proteins, it has been suggested that sorting of PC2 into the regulated secretory pathway may depend on both its C-terminus and the middle domain [21], although Taylor et al. [47] suggested that if the sorting signal were located in the C-terminus, it had to be restricted to amino acids 563–569. It is possible, therefore, that other sequences in the mature portion of PC2 help to tether it to the membrane. It is clear that only a proportion of the total cellular mature PC2 becomes raft-associated. This is in keeping with the view that aggregation is the primary event in protein sorting and that the aggregated PC2 would be taken into the granules by virtue of being associated with a smaller proportion of membrane-bound PC2. Once the granule has fused to the plasma membrane and the granule contents exposed to the neutral pH of the extracellular medium, PC2 would become soluble and be secreted from the cell.

The importance of the lipid rafts for the sorting of PC2 to the regulated secretory pathway was assessed by inhibition of the sphingolipid biosynthetic pathway using fumonisin B1. No change in the subcellular localization of PC2 or CgA was found at low concentrations of fumonisin (20 μM), both being localized at the TGN and the cell processes. However, at higher concentrations (30 μM and 40 μM) of fumonisin, PC2 appeared to be localized only in the TGN and absent from the cell processes, whereas no apparent effect on CgA localization was observed. This absence of regulated secretory proteins from the cell processes has been previously related to protein mis-sorting [48]. Thus it is possible that reduction of the sphingolipid content in the TGN prevents the protein becoming tethered to the membranes (lipid rafts), which may then prevent its entry into the ISG and thus secretion is abolished. A similar approach was taken by Ledesma et al. [46] to demonstrate that the viral glycoprotein haemagglutinin, and Thy-1, a glycosylphosphatidylinositol-anchored protein, interact with membrane lipid rafts therefore involving the rafts in axonal membrane sorting. The fact that no change was observed in the subcellular distribution of CgA reinforces the idea that CgA does not interact with the sphingolipid component of lipid rafts.

Thus we have shown that amino acids 45–84 of the PC2 pro region bind to a lipid component of membranes and that mature PC2 associates with lipid rafts. Although more experimental evidence is required, on the basis of our results, we could suggest that, induced by the acidic pH and high Ca^{2+} concentrations, proPC2 forms soluble aggregates in a late compartment (the TGN or the ISG) of the secretory pathway. This aggregation could trigger the initial segregation of proPC2 from other constitutively secreted proteins and promote its interaction with the lipid bilayer via the propeptide. After cleavage and removal of the propeptide, mature PC2 would remain attached to lipid rafts, via other sequences in the mature portion of PC2, until the granule fuses to the plasma membrane. Thereafter, exposure to a more neutral environment would result in release from the membrane and secretion of soluble PC2.

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