Characterization of the endopeptidase PC2 activity towards secretogranin II in stably transfected PC12 cells

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To study the processing of secretogranin II (SgII) by the prohormone convertase PC2 we have generated a stable PC12 cell line which expresses mouse PC2. We here present the characteristics of the PC12/PC2 cell line and demonstrate that the exogenous PC2 is sorted and stored in secretory granules in the PC12/PC2 cell line as efficiently as the endogenous granins. By indirect immunofluorescence with antibodies specific for chromogranin B (CgB) and PC2 we were able to establish that the PC2 is stored in secretory granules in the PC12/PC2 cell line. After subcellular fractionation, followed by immunoblotting, the mature 68 kDa form of PC2 was found co-sedimented with SgII in fractions containing secretory granules. Two-dimensional gel electrophoresis was used to characterize a secretory granule

fraction obtained from the PC12/PC2 cells, and a comparison was done of the electrophoretic pattern obtained from the PC12/PC2 cells with the parent cell line PC12. The products derived from the processing of SgII by PC2 were identified by immunoblotting with a panel of antibodies directed against SgII. Using [³⁵S]sulphate to label the newly synthesized SgII, we performed a time course to monitor the appearance of the lower-molecular-mass fragments of SgII: beginning 15 min after a 5 min pulse of [³⁵S]sulphate we were able to detect the first proteolytic fragment of SgII. Our results demonstrate that SgII is proteolytically processed by PC2 in the immature secretory granule into several lower-molecular-mass proteins, the major ones being an 18 kDa sulphated fragment and a 28 kDa fragment.

INTRODUCTION

Endocrine, neuroendocrine and exocrine cells have evolved to synthesize, store and secrete biologically active proteins and peptides in a regulated fashion. Cells with a regulated pathway of secretion store hormones, enzymes and peptides within secretory granules until an external signal is received which causes the secretory granule to fuse with the plasma membrane and release its contents. Most peptide hormones are synthesized as precursor molecules which are processed at dibasic amino acid cleavage sites, and further modified to yield a mature active hormone. The processing of prohormones at dibasic amino acid sites is performed by one or more endopeptidase activities [1]. Recently, several candidates for these activities have been identified, and are referred to as prohormone convertases, abbreviated to PC enzymes. To date there are at least five members of this family, including PC2 [2,3]. PC2 has been shown to be identical to the type-II endopeptidase identified in insulinoma cells [4]. PC2 is synthesized in the endoplasmic reticulum as an inactive proenzyme and after exit from the endoplasmic reticulum and transport through the Golgi its N-terminal prosequence is autocatalytically cleaved [5]. After transport through the *trans*-Golgi network (TGN), PC2 is sorted into secretory granules [6]. Both the intracellular transport and cleavage of proPC2 are thought to be regulated through interaction with another endocrine-cell-specific protein 7B2 [7].

The exact intracellular location where the PC enzymes cleave prohormones is still somewhat controversial and may be dependent on the particular substrate and cell type [8]. The current view is that although processing of prohormones might begin at the earliest in the TGN, most processing occurs in the immature

secretory granule [9]. The prohormone processing reactions would be subsequent to the sorting of the substrate and the PC enzymes, together with other regulated secretory proteins, into secretory granules. Sorting of regulated secretory proteins from the TGN to secretory granules is a complex event and most likely involves both the aggregation and condensation of the regulated secretory proteins [10] as well as a signal-mediated mechanism [11].

A major constituent of endocrine and neuroendocrine secretory granules are the granins. The granins are a family of secretory proteins of which there are presently five members related to one another by amino acid sequence homology and similar physical properties. All the granins are highly charged acidic proteins with a high proportion of glutamic acid residues, and they contain numerous pairs of dibasic amino acid residues. They are a major soluble component of most endocrine and neuroendocrine secretory granules [12,13]. However, in contrast to most other peptide hormones and biologically active molecules, which have a well defined function outside the cell, the function of the granins has remained elusive. Currently, there are two hypotheses: one that the granins play a role in the sorting of bioactive peptides, for example by initiating the formation of an aggregate in the TGN and allowing aggregation to proceed efficiently [12]; the second being that they are precursors for biologically active peptides [13].

In further contrast to the peptide hormones, and despite numerous dibasic amino acid cleavage sites, the granins are not efficiently processed within all endocrine or neuroendocrine cells, and the extent of the proteolytic processing of each granin varies between different tissues. Several granin-derived peptides which are generated by processing from chromogranin A (CgA) and

Abbreviations used: CgA, chromogranin A; CgB, chromogranin B; DMEM, Dulbecco's modified Eagle's medium; hsPG, heparan sulphate proteoglycan; pi, proteinase inhibitors; PNS, post-nuclear supernatant; SgII, secretogranin II; TGN, *trans*-Golgi network.

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chromogranin B (CgB) have been identified, and these have been the subject of recent reviews [13,14]. However, so far only one biologically active peptide derived from secretogranin II (SgII) has been identified. Following the observation that SgII is efficiently processed in brain tissues (greater than 90 % complete processing) and the identification of a peptide from frog brain derived from SgII [15], a neuropeptide referred to as secretoneurin has recently been identified [16]. Secretoneurin is a 33-amino-acid peptide corresponding to amino acids 154 to 186 of bovine SgII, and is believed to cause an increase in dopamine secretion from the caudate putamen [17]. One other SgII-derived peptide (AMENM) has been identified in the adrenal medulla; it is produced by cleavage at the last C-terminal dibasic amino acid site but its biological activity is unknown [18]. The same C-terminal cleavage site in SgII is probably used in β TC-1 cells to generate a similar peptide [19].

Several features of the granins, including their unusual physical properties resulting from the large number of hydrophilic and acidic amino acids (for review see [12]) and their tendency to aggregate at low pH [20], may explain the unusual refractiveness of the granins to the PC enzymes. Alternatively, the granins may simply not be as good a substrate for the PC enzymes as other prohormones, and their processing may therefore be competitively inhibited by the presence of other prohormones.

To study the ability of the SgII to act as a substrate for the PC enzymes, and to detect additional possible cleavage fragments of SgII which could potentially have biological activity, we decided to express a PC enzyme in a regulated cell line which contains no endogenous PC enzyme activity, and no abundant prohormone, but which efficiently expresses SgII. The PC12 cell line proved ideal for these experiments, as it contains no PC enzyme activity [6] or abundant prohormone, but synthesizes and sorts two granins, CgB and SgII, into secretory granules [21].

EXPERIMENTAL

Materials

PC12 cells, clone 251, were originally obtained from Professor H. Thoenen, MPI Martinsreid, Munich, Germany. The cDNA encoding PC2 in the expression vector pRC/CMV (Invitrogen, U.S.A.) was obtained from Dr. Nabil Seidah, Clinical Research Institute of Montreal, Canada. The PC2 antiserum (4BF), used at a dilution of 1:1000, was obtained from Dr. Iris Lindberg, LSU Medical Center, New Orleans, U.S.A. The CgB antiserum and monoclonal antibody (219.6; used at a dilution of 1:500) were obtained from Professor W. Huttner, University of Heidelberg, Heidelberg, Germany. Anti-SgII antibody LF19 was provided by Professor H. Winkler, University of Innsbruck, Innsbruck, Austria. All other antibodies used (summarized in Table 1) were raised in rabbits against synthetic peptides by

standard immunization techniques. The antisera CS3, CS4, CS7 and CS8, first described here, have been characterized and shown to be specific for SgII and the lower-molecular-mass fragments derived from SgII, by competition experiments with the synthetic peptide used to raise the antibody. All cell culture materials were obtained from Gibco, U.K., and unless specified all other chemicals are obtained from Sigma, U.K. Radiochemicals were obtained from Amersham, U.K.

Stable transfection of PC12 cells with PC2

PC12 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10 % (v/v) horse serum, 5 % (v/v) fetal-calf serum, and 20 mM glutamine (growth medium), and routinely passaged once a week. One passage after thawing, PC12 cells were plated at a density of 1.1×10^6 cells per 6-cm-diam. dish coated with poly-D-lysine (used at 1 mg/ml diluted in distilled water) and were cultured for 2 days. The cells were transfected with 5 μ g of plasmid (pRC/CMV-PC2) encoding the mouse PC2 cDNA [22] and 25 μ l of lipofectin in DMEM according to the manufacturer's protocol. After 12 h the lipofectin was removed and replaced with growth medium. On day 3 after transfection the cells were trypsinized and plated at a dilution of 1:2 in growth medium containing 0.5 mg/ml G418. The cells were maintained in the selection medium for 4 weeks before isolation of individual clones. The clones were screened for PC2 expression using indirect immunofluorescence using the anti-PC2 antibody (4BF). One clone, clone 1.2 (referred to as PC12/PC2), was used in all subsequent experiments.

Subcellular fractionation of PC12 and PC12/PC2 cells

PC12 or PC12/PC2 cells grown to confluency in 24 cm $\times$ 24 cm dishes were harvested at 4 °C in TBS containing protease inhibitors (pi: PMSF, final concentration 0.5 mM, and leupeptin, final concentration 1 μ M; Boehringer Mannheim, U.K.) by scraping and centrifugation [23]. Alternatively, the cells were labelled with 0.25 mCi/ml [35 S]sulphate in DMEM without sulphate, containing 2.5 % (v/v) horse serum, 1.25 % (v/v) fetal-calf serum for 16 h before harvesting. Usually nine 24 cm $\times$ 24 cm dishes were used to prepare material for six gradients. All subsequent procedures were carried out at 4 °C and as detailed [23]. Briefly, the cell pellet was resuspended in a homogenization buffer (HB: 250 mM sucrose in 10 mM Hepes, pH 7.2, 1 mM EDTA, 1 mM MgCl₂, containing pi) and homogenized. A post-nuclear supernatant (PNS) was prepared and loaded on to a velocity sucrose gradient (0.3–1.2 M sucrose in 10 mM Hepes, pH 7.4) and centrifuged for 15 min at 100 000 *g*. Fractions, typically 13 $\times$ 1 ml, were collected from the top of the gradient and an aliquot was removed for SDS/PAGE, or SDS/PAGE followed by immunoblotting. For additional purification and fractionation of the secretory granules fractions 4–7 were pooled and loaded on to an equilibrium sucrose gradient (0.5–2.0 M sucrose in 10 mM Hepes, pH 7.2) and centrifuged to equilibrium at 100 000 *g* for at least 5.5 h. Thirteen fractions, each 1 ml, were collected from the top of the gradient and again an aliquot was analysed by SDS/PAGE, or SDS/PAGE followed by immunoblotting. The fractions containing the secretory granules (fraction nos. 8–12) were pooled and used for two-dimensional gel electrophoresis and immunoblotting.

[35 S]Sulphate-labelling experiments

PC12 cells or PC12/PC2 cells were plated at a dilution of 1:2 in 35-mm-diam. dishes for each time point and used 2 days after passaging. For overnight labelling the cells were incubated in

Table 1 Summary of rat SgII specific antisera

Name	Peptide	Working dilution	References
100	QRNQLLQKEPDLRLNV	1:250	[19]
LF19	LKHMRFPLMYEENSRENPF	1:250	[16]
CS3	KLGTGPSNQ	1:100	This paper
CS4	KIETQTQEEVRDS	1:250	This paper
CS7	LDSQSIYQLIEISRLN	1:500	This paper
CS8	KSSSEDDLQEEELQEI	1:500	This paper
175	KRAMENM	1:1000	[19]

0.25 mCi/ml [35 S]sulphate as above. For pulse-labelling conditions the cells were first depleted of sulphate by incubation in labelling medium (DMEM without sulphate, 0.1 % dialysed horse serum, 0.05 % dialysed fetal-calf serum, 20 mM glutamine) for 20 min, then incubated with 1 mCi/ml [35 S]sulphate in labelling medium for the 5 min pulse. The [35 S]sulphate-labelled proteins were chased out of the TGN by replacement of the labelling medium with DMEM containing 0.1 % horse serum, 0.05 % fetal-calf serum and 20 mM glutamine for various periods. After collection of the chase medium, the cells were washed at 4 °C in PBS, and solubilized with TNTE (20 mM Tris, pH 7.4, 150 mM NaCl, 0.3 % Triton X-100, 5 mM EDTA) containing pi plus 2 μ M pepstatin A and 30 μ M E64. To stimulate the cells to secrete in a regulated manner, the cells were washed twice for 2 min at 37 °C with BSS (125 mM NaCl, 4.8 mM KCl, 1.4 mM MgCl_2 , 10 mM glucose, 25 mM Hepes, pH 7.4) and then incubated with BSS with or without 3 mM BaCl_2 for 10 min at 37 °C [24]. The chase medium, and the stimulation medium were spun for 5 min to remove debris, and then with the cell lysate was boiled to prepare a heat-stable fraction [25] before any further concentration by precipitation with either 80 % acetone or 10 % trichloroacetic acid, or solubilization in sample buffer or lysis buffer for electrophoresis (see below).

Indirect immunofluorescence labelling

PC12 cells and PC12/PC2 cells, grown overnight on poly-D-lysine-coated coverslips, were fixed at room temperature in 3 % paraformaldehyde in PBS containing 0.1 mM MgCl_2 and 0.1 mM CaCl_2 for 20 min. The cells were washed with PBS and incubated in 50 mM NH_4Cl in PBS for 10 min, washed again with PBS and incubated for 3 min in 0.2 % Triton X-100 in PBS. The cells were washed with PBS, then again with PBS containing 0.2 % gelatin and left at least 20 min. The granins or PC2 were either labelled in separate cells or in the same cell by incubation in the appropriate primary antibody diluted in PBS/0.2 % gelatin for 30 min. After washing with PBS/0.2 % gelatin the primary antibody was detected using a rhodamine- or fluorescein-conjugated secondary antibody (Cappel Laboratories, U.S.A.) specific for either rabbit or mouse IgG at a 1:600 dilution in PBS/0.2 % gelatin. After this antibody incubation the cells were washed in PBS/0.2 % gelatin, followed by PBS and then distilled water, and mounted in Moviol 4-88 (Harco, U.K.). Coverslips were viewed with a Zeiss axiophot, and photographed using standard techniques.

SDS/PAGE and two-dimensional gel electrophoresis

SDS/PAGE was performed according to Laemmli as detailed in [25]. Two-dimensional gel electrophoresis was performed exactly as previously described [25,26]. As stated in the Figure legends for some two-dimensional gel electrophoresis experiments the secretory granule fraction was subjected to boiling [26] to prepare a heat-stable fraction enriched in the granins before solubilization in lysis buffer.

Immunoblotting

Immunoblotting was performed using standard techniques after the proteins were transferred to nitrocellulose (S&S, U.K.) or a PVDF membrane (Millipore, France). The membrane was blocked and washed with 5 % low-fat milk in PBS containing 0.2 % Triton X-100, and the primary and secondary antibodies were diluted in the same buffer. The secondary antibody used was an alkaline phosphatase-conjugated anti-(rabbit IgG) antibody (Bio-Rad, U.K.) at a dilution of 1:2000, which was detected

with a standard alkaline phosphatase reaction using NitroBlue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate as substrates.

RESULTS

Morphological localization of PC2 in secretory granules

We transfected PC12 cells with an expression vector containing the mouse PC2 cDNA [22]. Stable cell lines were selected using standard techniques with G418, and screened for PC2 expression by indirect immunofluorescence. Several clones expressing high levels of PC2 were subcloned and clone 1.2 (referred to as PC12/PC2), which gave the highest level of expression, was used for all subsequent experiments.

As has been shown in both tissues and cell lines [2,3], PC2 is sorted into the regulated secretory pathway and packaged in dense core secretory granules. In PC12 cells SgII is sorted into secretory granules along with CgB [26]. To determine if in the stably transfected PC12/PC2 cells the exogenous PC2 was sorted to secretory granules indirect immunofluorescence labelling was performed. Antibodies to both CgB and PC2 were used in labelling experiments on both PC12 and PC12/PC2 cells (Figure 1). In the PC12 cell line the anti-CgB monoclonal antibody gives a punctate labelling pattern in the peripheral cytoplasm of the cells which represents secretory granules containing CgB (Figure 1b). The same pattern of labelling is seen in PC12/PC2 cells with the anti-CgB antibody (Figure 1a). As expected, when PC12 cells were labelled with the anti-PC2 antibody, there was no detectable signal in the cytoplasm (Figure 1d), while in the PC12/PC2 cells the anti-PC2 antibody gave a punctate labelling pattern virtually identical to that seen with the anti-CgB antibody (Figure 1a), implying that the exogenous PC2 is packaged into dense core secretory granules. Double labelling experiments (results not shown) done with these and other antibodies to PC2, CgB and SgII confirmed that PC2 co-localizes with granins (CgB and SgII) in the PC12/PC2 cell line. These experiments also, of course, establish by morphological techniques that the PC12/PC2 cell line still has dense core secretory granules, and should therefore have a functional regulated secretory pathway.

PC2 co-fractionates with markers of dense core secretory granules in PC12/PC2 cells

To confirm biochemically that the PC12/PC2 cells express PC2 and sort it efficiently to the regulated secretory pathway several different approaches were used. The steady-state distribution of PC2 in PC12/PC2 cells was determined by subcellular fractionation using a protocol designed to separate Golgi and Golgi-derived vesicles from post-Golgi vesicles including secretory granules [23]. The distribution of PC2 was then compared with the distribution of SgII which is found, at steady state, predominantly in secretory granules [27]. A PNS was prepared from the PC12/PC2 cells and applied to a linear velocity sucrose gradient. After velocity-controlled centrifugation fractions were collected from the top of the gradient and analysed by SDS/PAGE followed by Western blotting with either anti-PC2 or anti-SgII antibodies (Figures 2a and 2c). As seen in Figure 2(c), the distribution of SgII immunoreactivity using the antibody 175 (directed against the N-terminal peptide of SgII) peaks in fractions 2–5, as in wild-type PC12 cells (results not shown). The distribution of PC2 immunoreactivity was more complex. In the PC12/PC2 cell line, PC2, which was detected by an antibody directed against a synthetic peptide corresponding to the C-terminal 10 amino acids (4BF, [28]), appears as two major bands: one corresponding to the 75 kDa proPC2 found in the en-

Figure 1 PC2 in transfected PC12 cells co-localizes with SgII in secretory granules

Indirect immunofluorescence analysis of the PC12/PC2 cells (**a** and **c**) and the PC12 cells (**b** and **d**). Cells were fixed and permeabilized, then labelled with antibodies specific for CgB (219.6) (**a** and **b**) or PC2 (4BF) (**c** and **d**). The transfected PC12/PC2 cell line expresses PC2 (**c**), while the wild-type PC12 cell line expresses no PC2 (**d**). Both the anti-PC2 antibody and the anti-CgB antibody label secretory granules at the periphery of the PC12 and PC12/PC2 cells. Scale bar, 10 μ m.

doplasmic reticulum and Golgi, and the other corresponding to the 68 kDa mature PC2 found in the TGN and secretory granules. The 68 kDa form arises via an autocatalytic cleavage of the N-terminal domain of the 75 kDa proPC2 [5]. Both forms of PC2 had a widespread distribution across the velocity gradient: a substantial amount of the 75 kDa proPC2 was found in fractions 4–12, while the 68 kDa mature PC2 was found in fractions 3–12, with a peak in fractions 3–6 (Figure 2a).

The fractions from the velocity gradient containing the mature secretory granules were pooled as indicated in Figures 2(a) and 2(c), and loaded on to an equilibrium sucrose gradient, and centrifuged to equilibrium. As seen in Figures 2(b) and 2(d) the peak position of PC2 and SgII immunoreactivity, respectively, is found in fractions 9–12. The PC2 immunoreactivity is predominantly detectable as the 68 kDa mature PC2 form. The presence of mature PC2 and SgII in the fractions which contain mature secretory granules [27] confirms the morphological data demonstrating that PC2 is found in secretory granules.

Expression of PC2 in PC12/PC2 cells results in the appearance of lower-molecular-mass [35 S]sulphate-labelled polypeptides

To determine if the numerous pairs of dibasic amino acids in SgII are recognized by the exogenous PC2 in PC12/PC2 cells we

employed metabolic labelling techniques to follow the newly synthesized SgII during subcellular fractionation. Although immunoblotting techniques (Figures 2c and 2d) with the anti-(C-terminus) antibody (175), showed that there was full-length, unprocessed SgII in the secretory granules of PC12/PC2 cells, it was possible that some of the SgII was processed and that the smaller processed fragments were not detected by immunoblotting because, for example, they were not resolved by the SDS/PAGE, not recognized by the antibody, or they were present in very low amounts.

We used [35 S]sulphate to label the PC12 and PC12/PC2 cells because SgII is sulphated on tyrosine residues in PC12 cells [25,26] and an overnight incubation of the cells with [35 S]sulphate results in a very strong signal for SgII. Therefore with this technique any SgII-processing fragments which contain a sulphated tyrosine residue should be labelled and even a low level of putative SgII-processing products should be detected in the PC12/PC2 cells. PC12 and PC12/PC2 cells were labelled overnight with [35 S]sulphate, and then the PNSs were subjected to fractionation by velocity sucrose gradient centrifugation (Figures 3a and 3b). In the PC12 cells the [35 S]sulphate is incorporated into three proteins [21] CgB, SgII and a heparan sulphated proteoglycan (hsPG). Both the CgB and SgII co-fractionate in fractions 3–6 while the hsPG is found primarily in the load and

Figure 2 PC2 and SgII co-localize in secretory granules after subcellular fractionation

A PNS from PC12/PC2 cells was subjected to fractionation on sequential velocity (a and c) and equilibrium sucrose (b and d) gradients (fraction no. 1, top; fraction no. 12, bottom) as described in the Experimental section. Fractions from the velocity sucrose gradient marked by the bar in (a) and (c), enriched in mature secretory granules, were pooled and loaded on to an equilibrium sucrose gradient. Equal volume aliquots of each fraction was analysed by SDS/7.5%-PAGE and immunoblotted with antibodies specific for PC2 (48F) in (a) and (b), and SgII (175) in (c) and (d). The similar distribution of the 68 kDa form of PC2 (arrowhead) and SgII (asterisk) on both the velocity and equilibrium sucrose gradients demonstrates both proteins are present in secretory granules. Molecular masses of the markers are expressed in kDa.

Figure 3 Subcellular fractionation of sulphate-labelled PC12 and PC12/PC2 cells

The PNS obtained from PC12 or PC12/PC2 cells, labelled overnight with [35 S]sulphate, was subjected to velocity and equilibrium centrifugation as in Figure 2. Equal volume aliquots of the fractions obtained from the velocity gradients loaded with a PNS from PC12 (a) or PC12/PC2 cells (b) were analysed by SDS/7.5%-PAGE and autoradiography. Equal volume aliquots of the fractions from the equilibrium sucrose gradients, loaded with the secretory granule pool shown by bar in (a) and (b), obtained from the PC12 (c) and PC12/PC2 cells (d) were analysed by SDS/7.5%-PAGE and autoradiography. Full-length sulphate-labelled SgII (<) in both PC12 and PC12/PC2 cells co-fractionates in the position of mature secretory granules (fractions 9–12) in (c) and (d). Vertical bar indicates position of heparan sulphate proteoglycan, CgB (arrowhead). Molecular masses of the markers are expressed in kDa.

fraction 2 (Figure 3a). In the PC12/PC2 cells, some SgII is detected in the same fractions (fractions 3–6) as in the PC12 cells. However, lower-molecular-mass [35 S]sulphate-labelled fragments, including some migrating in the dye front of the gel, are also detected in these fractions (Figure 3b). In addition, there are

Figure 4 Lower-molecular-mass sulphated proteins fractionate with secretory granules

PC12/PC2 cells were labelled and fractionated exactly as described in Figure 3. Aliquots of equal volume from each fraction of the velocity sucrose gradient (a) and equilibrium sucrose gradient (b) were analysed by SDS/12.0%-PAGE and autoradiography. Several lower-molecular-mass bands detected (●) co-fractionate with SgII (<) in the position of secretory granules. Molecular masses of the markers are expressed in kDa.

differences in the amount of other labelled proteins such as the hsPG and CgB in the two cell lines.

To resolve further the vesicles containing the [35 S]sulphate-labelled proteins, fractions 4–7 from the PC12 or from the PC12/PC2 cells were pooled and loaded on to equilibrium sucrose gradients and centrifuged to equilibrium. The secretory granule peak containing the [35 S]-labelled proteins from the PC12 cells (Figure 3c), enriched now for CgB and SgII, sedimented into the gradient and was found at the bottom in fractions 9–12. Likewise, the secretory granules from the PC12/PC2 cells sedimented to the position of fractions 10–12 (Figure 3d). In the gradients from the PC12/PC2 cells residual proteoglycan-containing vesicles were separated from the secretory granules and found in a less dense region (fractions 5 and 6) of the sucrose gradient (Figure 3d). The secretory granule fractions (fractions 10–12) from the PC12/PC2 cells again contained [35 S]sulphate-labelled lower-molecular-mass proteins which were not detected in the PC12 cells.

To better identify the lower-molecular-mass fragments detected in the PC12/PC2 sucrose gradients SDS/12.0%-polyacrylamide gels were used to analyse the [35 S]sulphate-labelled proteins. As in Figures 3(b) and 3(d), an aliquot of each fraction from both the velocity and equilibrium sucrose gradients was applied to the gels and analysed (Figure 4). In the fractions from the velocity sucrose gradient (Figure 4a), migrating below the full-length SgII band, are at least four major [35 S]sulphate-labelled bands with molecular masses in the region of 38, 26, 18 and 14 kDa. In addition, there are several other minor species detectable. These fragments all co-sediment with full-length SgII in secretory granules in fractions 9–12 on the equilibrium sucrose gradient (Figure 4b).

This result demonstrates that in PC12/PC2 cells, lower-molecular-mass [35 S]sulphate-labelled peptides appear which are not found in the parental PC12 cell line. All of these lower-molecular-mass fragments are probably contained within the secretory granules as shown by co-sedimentation on the equilibrium sucrose gradient. These peptides, therefore, could be derived from either CgB or SgII, as both are labelled by [35 S]sulphate.

Stimulation of PC12/PC2 cells causes release of [35 S]sulphate-labelled proteins

The defining property of cells with a regulated secretory pathway is the ability to store proteins and peptides in secretory granules

Figure 5 Lower-molecular-mass proteins are released in a regulated fashion from PC12/PC2 cells

PC12 and PC12/PC2 cells were labelled overnight with [35 S]sulphate and chased for 4 h. After collection of the chase medium (lanes 2 and 8) the cells were collected for analysis (lanes 1 and 7) or incubated in BSS with or without BaCl_2 . After incubation for 10 min, the stimulation medium either with (lanes 3 and 9) or without (lanes 4 and 10) BaCl_2 was collected, and the cells after incubation with (lanes 5 and 11), or without BaCl_2 (lanes 6 and 12) were harvested. SgII ($\blacktriangle$) is released only after stimulation from both the PC12 and PC12/PC2 cells. Likewise, the additional lower-molecular-mass proteins ($\bullet$) in the PC12/PC2 cells are released only after stimulation. Molecular masses of the markers are expressed in kDa.

until a stimulus is given to the cell which causes depolarization of the plasma membrane and release of the secretory granule content by fusion of the secretory granule with the plasma membrane. Depolarization of PC12 cells can be achieved by incubating the cells in BaCl_2 [24]. To demonstrate that the lower-molecular-mass polypeptides seen in the PC12/PC2 cells are indeed inside secretory granules, PC12/PC2 cells were labelled overnight with [35 S]sulphate, chased and then stimulated with BaCl_2 . To control the depolarization conditions, PC12 cells were labelled and treated identically (Figure 5). After an overnight label and 4 h chase, full-length SgII can be detected in the cell extracts from both the PC12 and PC12/PC2 cells (Figure 5, lanes 1 and 7 respectively). In addition, in the PC12/PC2 cell extracts several smaller polypeptides are detected (Figure 5, lane 7) which have similar molecular masses to those seen in the secretory granule fraction in Figure 4(b). After stimulation of the PC12 cells with BaCl_2 , both full-length CgB and SgII are released (Figure 5, lane 3), resulting in a decrease in the amount of both proteins in the cell extracts (Figure 5, lane 5). A similar behaviour is seen with the PC12/PC2 cells after stimulation with BaCl_2 ; however, the lower-molecular-mass proteins are also detected in the stimulation medium (Figure 5, lane 9) and are decreased in the cell extract (Figure 5, lane 11). Under control stimulation conditions without BaCl_2 , none of the granins or the lower-molecular-mass proteins is released either from the PC12 or PC12/PC2 cells (Figure 5, lanes 4 and 10 respectively).

The data in both Figures 4 and 5 demonstrate that in the PC12/PC2 cell line the expression of PC2 results in the appearance of [35 S]sulphate-labelled lower-molecular-mass polypeptides which are as efficiently released from secretory granules as the granins.

Identification of lower-molecular-mass PC2 processing products by two-dimensional gel electrophoresis

To begin to identify the putative processing products resulting from the PC2 activity we first compared the two-dimensional gel

Figure 6 Two-dimensional gel electrophoretic pattern of secretory granules from PC12 and PC12/PC2 cells reveals additional lower-molecular-mass proteins

Secretory granules were prepared from either PC12 (a) or PC12/PC2 cells (b) using velocity and equilibrium sucrose gradient centrifugation. The fractions from the equilibrium gradient (nos. 8–12) enriched in secretory granules were analysed by two-dimensional gel electrophoresis (2nd dimension with 12.0% gels) followed by silver staining. CgB (arrow) and SgII (arrowhead) are detected in both PC12 and PC12/PC2 cells, while additional lower-molecular-mass proteins (a–g) are detected in the PC12/PC2 cells. (*) denotes the four prominent marker spots present in both PC12 and PC12/PC2 cells. Molecular masses of the markers are expressed in kDa. pH units of the 1st dimension are shown on the axis.

electrophoretic pattern of a secretory granule preparation from PC12 cells and PC12/PC2 cells using silver staining (Figure 6). In the pattern obtained from PC12 cell secretory granules (Figure 6a) the two predominant components are the granins, CgB and SgII. In addition there are four other major spots which are always seen in these preparations and can be used as reference markers. By comparison, the PC12/PC2 cell secretory granule fraction (Figure 6b) has a greatly reduced amount of CgB or SgII, while the four marker spots appear to be present at similar levels (compare Figures 6a and 6b). Importantly, however, in the PC12/PC2 granules several lower-molecular-mass spots with a pI similar to the granins can be easily detected, the major ones are denoted by letters a–g in Figure 6(b).

To characterize granin-specific, lower-molecular-mass fragments, a heat-stable fraction was prepared from a secretory granule fraction obtained from [35 S]sulphate-labelled PC12/PC2 cells and analysed by two-dimensional gel electrophoresis followed by silver staining and autoradiography. This approach allowed us to enrich further the secretory granule preparation for granin-derived processing products and determine which are sulphate-labelled. With the exception of b, all of the spots (a–g) identified in Figure 6(b) in the pI range of the granins were found

Figure 7 Identification by silver staining and autoradiography of the lower-molecular-mass proteins in a heat-stable fraction prepared from secretory granules obtained from PC12/PC2 cells

Secretory granules were prepared from PC12/PC2 cells after [35 S]sulphate labelling as in Figure 6. After preparation of a heat-stable fraction the proteins were subjected to two-dimensional gel electrophoresis, and detected using either silver staining (a) or autoradiography (b). In both (a) and (b) the heat-stable fraction is enriched in CgB (arrow), or SgII (arrowhead). In (a) the lower-molecular-mass proteins labelled as in Figure 6 (a, c–g), can be detected as well as four other spots (h–k). In (b) the major [35 S]sulphate-labelled proteins are CgB (arrow), SgII (arrowhead), and spots h and i. One additional spot (l) is seen in (b). The marker spots (*) are seen in (a) but not (b). Molecular masses of the markers are expressed in kDa. pH units of the 1st dimension are shown on the axis.

in the heat-stable fraction by silver staining (Figure 7a). The relative abundance of spots a and f was reduced, that of spots c and e was unchanged, while that of spot d was increased slightly. Four additional spots, labelled h–k, were detected in the heat-stable fraction by silver staining (Figure 7a). In comparison to the pattern obtained by silver staining (Figure 7a), the autoradiographic pattern of [35 S]sulphate-labelled proteins was much simpler (Figure 7b). In addition to CgB and SgII, three major lower-molecular-mass [35 S]sulphate-labelled spots were detected on the autoradiograph, h and i [which correspond to the visible protein spots (h and i) in Figure 7(a)] and a new spot l (Figure 7b). The spots h, i, and l have molecular masses of 24, 18 and 38 kDa, respectively, and most likely correspond to the sulphate-labelled bands in Figure 4(b) found in the secretory granule fraction. The lowest-molecular-mass [35 S]sulphate-labelled protein (14 kDa) seen in the secretory granule fraction in Figure 4(b) was not detected using two-dimensional gel electrophoresis.

To determine whether the lower-molecular-mass proteins seen by silver staining in Figure 7(a) were specifically derived from

SgII we employed two-dimensional gel electrophoresis followed by immunoblotting with various antibodies to SgII (Figure 8). The positions of the epitopes recognized by the antibodies are distributed across the entire SgII molecule, as shown schematically in Figure 9(a). The antibodies cover all the regions which might be found in potential cleavage products except those between cleavage sites 4 and 5, and 5 and 6. The dibasic amino acid residues at positions 5 and 6 were not considered likely candidates for recognition by PC2 because of their sequence, which is Arg-Lys and Arg-Arg, respectively, and the fact they are not conserved between species [29]. Using these antibodies it was possible to identify several of the lower-molecular-mass spots as being derived from SgII. The fragments recognized by the various antibodies in Figure 8 are illustrated in Figure 9, and summarized in Table 2.

As seen in Figure 8, in addition to SgII and the 78 and 72 kDa products of SgII, several of the spots (d, h, i and k) seen by silver staining in Figure 7(a) can be immunolabelled with various antibodies: the relative abundance of each immunoreactive spot that is also identified by silver staining varies, however. With the exception of antiserum 175, there is a clear correlation between the immunolabelled spots, the putative cleavage sites and the molecular mass of the SgII fragment (compare Figures 8 and 9 and Table 2). In the results obtained with antiserum 175, however, there are two anomalies: antiserum 175 sometimes recognizes a spot which appears to migrate as a 78 kDa spot (see Figure 8g, compare also Figures 2c and 2d), as well as spot k. However, in other experiments using two-dimensional gel electrophoresis (results not shown), and using standard SDS/PAGE, the antiserum 175 only recognizes full-length SgII. The ability of 175 to detect the 78 kDa spot may be due to either the fact that SgII is occasionally detected as a doublet of molecular mass 86/84 kDa [25] and the resolution of the gels is not great enough to distinguish the molecular mass differences, or that SgII is processed at an additional site such as the monobasic site at amino acid number 22. The variability in the detection of spot k with 175 may reflect a minor amount of processing of the unsulphated 38 kDa band at sites 8 or 9.

The two SgII-derived spots d and i are the most abundant SgII-derived proteins detected by the silver stain (Figure 7a), suggesting that they are the major cleavage products. There are several other spots between molecular masses of 69 and 46 kDa which are recognized by the various antibodies but are not visible by silver stain. In addition, spot l which is sulphate-labelled, and strongly labelled by antibody CS4, is not detected at all by silver staining.

Time course of appearance of [35 S]sulphate-labelled lower-molecular-mass proteins in PC12/PC2 cells

Processing of prohormones is believed to begin initially in the TGN and continue during the packaging of the hormones into secretory granules. Thus, if the lower-molecular-mass proteins stored in the secretory granules of the PC12/PC2 cell line were generated from SgII by the expression of active PC2, we would expect that these peptides would appear only after SgII had gone through the TGN. To determine when the lower-molecular-mass proteins were first detected in the PC12/PC2 cells a pulse-chase-labelling experiment was performed using [35 S]sulphate. From the data summarized in Table 2, we expect that incubation with [35 S]sulphate will label predominantly the 78 kDa, 38/37 kDa, 26/24 kDa and 18 kDa cleavage forms of SgII. A brief (5 min) pulse of [35 S]sulphate was used to label the proteins in the TGN, and was followed by different chase periods up to 240 min

Figure 8 Identification of SgII-derived lower-molecular-mass proteins using a panel of antibodies

Two-dimensional gel electrophoresis was performed using secretory granules prepared as in Figure 6. After electrophoresis the proteins were transferred to nitrocellulose and immunoblotted with the antisera summarized in Table 1, and Figure 9. (a) 100, (b) LF19, (c) CS3, (d) CS4, (e) CS7, (f) CS8, (g) 175. In (h) PC12 cells were used to prepare secretory granules which were then analysed identically to the PC12/PC2 cells and immunoblotted with antiserum 175. Full-length SgII (arrowhead), and the 72 kDa cleavage product of SgII (—) are shown. The 78 kDa fragment of SgII migrates between the full-length SgII and the 72 kDa band. The spots identified in Figure 6 and 7 are labelled, and the pattern of immunoreactivity is summarized in Table 2. Molecular masses of the markers are expressed in kDa. The (+) indicates the basic end and the (—) the acidic end of the 1st dimension.

(Figure 10). After a 5 min pulse (Figure 10, lane 1) there are no lower-molecular-mass [35 S]sulphate-labelled peptides detectable. After a 5 min pulse and 15 min chase (Figure 10, lane 2) a small

amount of the 78 kDa form of SgII is present. During the next two chase periods, i.e. 30 and 60 min total chase time (Figure 10, lanes 3 and 4) the 78 kDa band increases while the

Figure 9 Schematic illustration of SgII and putative dibasic amino acid cleavage sites

(a) A schematic illustration of the rat SgII sequence with the putative dibasic amino acid cleavage sites numbered from 1 to 9, and the position of the peptide used to generate each antiserum. The position of the tyrosine residue which is thought to be sulphated is shown (●). The amino acid number in rat SgII of the dibasic residues comprising the cleavage sites shown are: 1, 134 and 135; 2, 155 and 156; 3, 190 and 191; 4, 258 and 259; 5, 271 and 272; 6, 290 and 291; 7, 498 and 499; 8, 540 and 541; 9, 585 and 586. (b) Summary of the lower-molecular-mass processed products of SgII. The SgII products identified from the data shown in Figures 6, 7, and 8 are presented by decreasing molecular mass as determined by the migration relative to the molecular-mass markers. The fragments shown correspond to the following spots: the 38/37 kDa band to spot l, the 38 kDa to spot k, the 28 kDa band to spot d, the 26/24 kDa band to spot h, and the 18 kDa band to spot i.

38/37 kDa, and 26/24 kDa forms of SgII become detectable. A small amount of the 18 kDa form can also be seen at these times (Figure 10, lanes 3 and 4). After 120 and 240 min of chase (Figure 10, lanes 5 and 6) the label in the 26/24 and 18 kDa bands increases while the label in the full-length SgII, 78 kDa and 38/37 kDa bands decreases. The 14 kDa sulphate-labelled band appears at 60 min of chase and increases during the last two chase periods of 120 and 240 min (Figure 10, lanes 5 and 6). As expected, none of these proteins is secreted from the cell during these chase periods (Figure 10, lanes 7–12).

From the data presented in Figures 7 and 8 and summarized in Figure 9 we can conclude that all of the sulphated lower-molecular-mass proteins are generated from SgII, except the 14 kDa band which has not been positively identified. The

Figure 10 Lower-molecular-mass proteins appear after exit from the TGN

PC12/PC2 cells were pulse-labelled for 5 min with [35 S]sulphate, and then chased for 0 (lane 1), 15 (lane 2), 30 (lane 3), 60 (lane 4), 120 (lane 5) and 240 (lane 6) min. The chase medium was collected at 0 (lane 7), 15 (lane 8), 30 (lane 9), 60 (lane 10), 120 (lane 11), and 240 (lane 12) min. Both the cell lysate and medium were analysed by SDS/PAGE on 12.0% gels followed by autoradiography. The position of SgII (Δ) and the lower-molecular-mass proteins (●) is shown. Molecular masses of the markers are expressed in kDa.

temporal sequence in which the sulphated cleavage products appear suggests that the first dibasic amino acid site to be recognized by PC2 is the very C-terminal dibasic amino acids at site 8 to generate the 78 kDa band, followed by cleavage at sites 4 and 3 to generate the 38/37 kDa band and 26/24 kDa band respectively, and finally cleavage at site 2 to generate the 18 kDa band (see Figure 9a).

DISCUSSION

We have established a stably transfected PC12 cell line which expresses PC2. The transfected PC12/PC2 cell line retains all of the regulated secretory characteristics of the parent PC12 cell

Table 2 List of SgII-derived proteins identified by two-dimensional gel electrophoresis

For each particular antibody: + + +, strongest signal; + +, good signal; +, detectable; +/–, weak; –, negative.

Name	MW (kDa)	Tyr-SO ₄ ^a	Antibody						
			100	LF19	CS3	CS4	CS7	CS8	175
SgII	84/86	+	+	+	+	+/–	+	+	+
78	78	+	+	+	+	+/–	+	+	+
72	72	+/–	+	+	+/–	+/–	+	–	–
k	38	–	–	–	–	–	+	+	+/–
l	38/37	+	+/–	+/–	–	+	–	–	–
d	28	–	–	–	+	+	+	–	–
h	26/24	+	+	+	+	–	–	–	–
i	18	+	+	+	–	–	–	–	–

^aSignal was positive (+) or negative (–).

line, and is therefore able to sort PC2 accurately and efficiently into secretory granules. The exogenous PC2 was found by indirect immunofluorescence and immunoblotting to co-localize with CgB and SgII. The presence of the 68 kDa PC2 band in the fractions containing the mature secretory granules demonstrates that PC2 is present in mature secretory granules from PC12/PC2 cells. Furthermore, the exogenously expressed PC2 was active, and able to cleave SgII: peptides derived from the endoproteolytic processing of SgII were detected both by silver staining and immunoblotting.

Rat SgII has six dibasic amino acid sites (all Lys-Arg) which could potentially be recognized by PC2, as well as two additional Arg-Lys sites, one Arg-Arg site, and a single potential monobasic site at position 22. SgII should therefore be an ideal substrate for the PC enzymes in endocrine cells which normally express both the PC enzymes and the granins, and in our transfected cells. Our results show that we could detect significant processing of SgII in our transfected cell line: the major cleavage products of SgII detected by silver staining, and identified by immunoblotting, were a 28 kDa band, derived from the middle of SgII, and a sulphated 18 kDa band, derived from the N-terminus. These processing products are probably produced by the cleavage of the dibasic lysine and arginine amino acid residues at positions 155 and 156 (site 2, Figure 9b) and at positions 498 and 499 (site 7, Figure 9b). The 18 kDa band produced by the expression of exogenous PC2 in PC12 cells is most likely identical to the 21 kDa band previously detected in the anterior pituitary [30], and in bovine adrenal medulla and brain [16]. The 18 kDa band described here is recognized by antiserum LF19 [16], the same antiserum used by Kirchmair and colleagues [16] to identify the 21 kDa protein in bovine adrenal medulla and brain. Furthermore, the 18 kDa protein is recognized by antiserum 100 [19], which was raised against an N-terminal peptide of SgII corresponding to amino acids 2–20 of rat SgII. The 21 kDa protein described by Kirchmair was also recognized by an antiserum directed against a peptide derived from amino acids 1–15 of bovine SgII. Finally, neither the 18 kDa protein nor the 21 kDa protein were recognized by two antisera, {CS3 (see above) and TQ33 [16] used independently by the respective authors} which were raised against sequences derived from, or corresponding to, secretoneurin [16].

We found additional SgII cleavage products present in lower amounts (see Figures 8 and 9), which suggest that the cleavage of SgII by PC2 does not result in a homogenous end product produced in stoichiometric quantities, but rather several different peptides produced in varying amounts. From our data, therefore, it appears as if all of the six Lys-Arg dibasic amino acid residues are recognized to various extents by PC2.

Although we have no direct evidence that the PC12/PC2 cells produce secretoneurin, which would be derived from amino acids 157–189 of rat SgII, it is possible that the 24 kDa band, which we can detect by sulphate labelling and only weakly by silver staining, may be further processed by PC2 at the dibasic amino acid site 155–156 to give rise to the 18 kDa band described above and secretoneurin. Alternatively, secretoneurin could be produced by cleavage of the 28 kDa N-terminal fragment at the dibasic amino acids 190 and 191. Further experiments are needed to determine whether the PC12/PC2 cell line does indeed produce secretoneurin.

Pulse-chase labelling with [³⁵S]sulphate demonstrated that the first processed SgII fragment was only just detectable at 15 min of chase. It is known that in PC12 cells the sorting of SgII from the TGN to immature secretory granules has a half-time of 5 min, and after 15 min of chase all of the pulse-labelled SgII is found in immature secretory granules, while the subsequent

maturation of an immature secretory granule to a mature secretory granule has a half-time of 40 min [27]. The time lapse between the 5 min pulse and the appearance of the first lower-molecular-mass [³⁵S]sulphate-labelled bands, which is at least as long as 15 min, proves that the first cleavage of SgII by PC2 does not occur until the labelled proteins have exited the TGN and are present in the immature secretory granule. In other words, in the PC12/PC2 cell line sorting precedes cleavage.

The delay between the sulphation of the SgII and the appearance of the first cleavage product, and therefore the first detectable PC2 activity, could be influenced by two factors; the first being the activation of the proPC2 by autocatalytic cleavage to PC2, and the second being the conformation of the substrate, SgII. Recent experiments by Seidah and co-workers [31] which examine the conversion of [³⁵S]sulphate-labelled proPC2 to PC2 in GH₃C₁ cells suggest that the predominant form after a 15 min pulse is proPC2. Therefore, it is possible that the absence of processing during the first 15 min of chase in the PC12/PC2 cells might reflect the relative low amount of mature PC2 in the immature secretory granule. Experiments using the isolated enzyme have demonstrated that the optimal conditions for PC2 activity are obtained at a pH of approximately 5.5 in the presence of approximately 5 mM Ca²⁺ [1,4,32]. The autocatalytic maturation of proPC2 requires conditions which are identical to those required for activity of the mature enzyme [33]. The fact that processing occurs between 15 and 30 min suggests that the conditions for optimal PC2 activity might only be reached at these times after pulse labelling. In light of all these data it is tempting to speculate that the optimal pH and Ca²⁺ concentration may only be achieved in the immature secretory granule approximately 15 min after it has budded from the TGN.

Very little is known about the post-TGN conformation of the SgII in the aggregated core of the immature secretory granule, or the changes which occur as aggregation continues in the maturing secretory granule, which might influence the ability of SgII to act as a substrate for PC2. Except in brain tissue SgII is not efficiently processed by the PC enzymes [16]. One possible explanation of why the granins are inefficiently processed *in vivo* may be that they are competed out by endogenous prohormones which are better substrates. Our results allow us to eliminate this possibility as the PC12/PC2 cells have no endogenous prohormones. A second reason why SgII, and the other granins, might be poor substrates for the PC enzymes is their intrinsic tendency to form aggregates in the the low-pH, high-Ca²⁺ environment of the TGN [34]. The aggregation of the granins, which will increase as the secretory granule matures, might either obscure the dibasic amino acid sites present, or physically exclude the PC enzymes. In tissues, or cell lines such as the PC12/PC2 cells, where it is observed that the granins are efficiently processed, it may be that a sufficiently high concentration of PC enzymes is available to ensure that the dibasic sites in the granins are processed either before they are obscured, or that the PC enzymes are contained in the granin aggregate.

The fact that the physical properties of the granins may simply combine to make them poor substrates for the PC enzymes could have interesting implications on the entire processing reaction which occurs in secretory granules which contain one or more granin and one or more prohormone. As suggested [35] perhaps the granins function as modulators of the PC activity in the secretory granule. As the granins are very abundant proteins this would imply that they might act as low-affinity substrates for the PC enzymes, and the amount of the granins present in a secretory granule, which could be controlled at the level of transcription or translation, could therefore increase or decrease the amount of processed hormone produced by the cell.

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