The Isoforms of Proprotein Convertase PC5 Are Sorted to Different Subcellular Compartments

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Abstract. The proprotein convertase PC5 is encoded by multiple mRNAs, two of which give rise to the COOH-terminal variant isoforms PC5-A (915 amino acids [aa]) and PC5-B (1877 aa). To investigate the differences in biosynthesis and sorting between these two proteins, we generated stably transfected AtT-20 cell lines expressing each enzyme individually and examined their respective processing pattern and subcellular localization. Biosynthetic analyses coupled to immunofluorescence studies demonstrated that the shorter and soluble PC5-A is sorted to regulated secretory granules. In contrast, the COOH-terminally extended and membrane-bound PC5-B is located in the Golgi. The presence of a sorting signal in the COOH-terminal 38 amino acids unique to PC5-A was demonstrated by the inefficient entry into the regulated secretory pathway of a mutant lacking this segment. EM of pancreatic cells established the presence of immunoreactive PC5 in glucagon-containing granules, demonstrating the sorting of this protein to dense core secretory granules in endocrine cells. Thus, a single PC5 gene generates COOH-terminally modified isoforms with different sorting signals directing these proteins to distinct subcellular localization, thereby allowing them to process their appropriate substrates.

The mammalian subtilisin/kexin-like convertases are proprotein and prohormone proteinases implicated in the processing of numerous precursors. Seven members of this family of enzymes have so far been identified in mammals (for reviews see Seidah et al., 1994, 1996; Van de Ven et al., 1993). These proteins share a conserved catalytic domain, containing the typical Asp/His/Ser triad of subtilisin-like serine proteinases. They also exhibit the presence of a prosegment and a P domain (also called homo B). Apart from these conserved regions, each convertase possesses a distinct COOH-terminal domain exhibiting diverse structural motifs, such as a transmembrane anchor and cytosolic tail, cysteine-rich repeats, or potential amphipathic structures. Furthermore, for three of the mammalian convertases, a differential splicing mechanism leads to the production of COOH-terminally modified isoforms (Seidah et al., 1992; Kiefer et al., 1991; Tsuji et al., 1994; Lusson et al., 1993; Nakagawa et al., 1993a,b; Mbikay et al., 1995). Multiple molecular forms of the convertases are also observed in Aplysia californica (Chun et al., 1994), Hydra vulgaris (Chan et al., 1992), Lymnaea stagnalis (Smit et al., 1992), Caenorhabditis elegans (Thacker et al., 1995), and Drosophila melanogaster (Roebroek et al., 1993).

While functions have been proposed for the pro, catalytic, and P domains of the convertases, the role of the COOH-terminal segment is less understood. It was postulated that the latter could be involved in cellular sorting, or that it could modulate the enzymatic activity of the joined catalytic domain. In this respect, ex vivo coexpression studies demonstrated that the COOH-terminal truncation of furin did not affect the cellular enzymatic activity of this convertase on renin or its mutants (Hatsuzawa et al., 1992). It was also reported that the three isoforms of Drosophila furin1 (dfurin1, dfurin1-CRR, and dfurin1-X) did not display significant differences in either their catalytic activity or cleavage specificity (De Bie et al., 1995). Thus, the proposed involvement of the COOH-terminal segment in cellular sorting remains open to investigations.

To test the hypothesis that the COOH-terminal domain influences the cellular transport of some convertases, we compared the cellular traffic of soluble PC5-A and membrane-bound PC5-B (Lusson et al., 1993; also called PC6-A...
and PC6-B, Nakagawa et al., 1993a,b) (see Fig. 1). These COOH-terminal variant isoforms of the mouse convertase PCS exhibit distinct tissue distributions: while expression of PCS-A is widespread, being especially abundant in intestine and adrenals, that of PCS-B is only detected in these two tissues and lung (Lusson et al., 1993; Nakagawa et al., 1993a,b; Seidah et al., 1994). These isoforms were used as models to determine if both would be sorted to the same organelle, or would reside in different compartments of the secretory pathway. To answer this question, stable AtT-20 cell lines expressing either PCS-A or PCS-B were established. This allowed both the comparison of the respective biosynthesis and subcellular localization of each isoform and the demonstration of the critical role of the COOH-terminal domain on the cellular traffic of these proteins.

Materials and Methods

Cell Culture and Transfection

AtT-20 cells were grown in DME supplemented with 10% FCS. Stable cell lines overexpressing both A and B isoforms of mouse PCS (Lusson et al., 1993) were established using 40-60 μg of RcCMV constructs in which these cDNAs were inserted downstream of the human cytomegalovirus promoter. Cells were transfected using lipofectin (GIBCO BRL, Gaithersburg, MD) according to the manufacturer’s instructions. After transfection, cells were selected with 0.5 mg/ml G418 (neomycin)-containing medium for 2-3 wk. Drug-resistant cells lines were isolated and characterized by Western blotting and immunoprecipitation of radiolabeled PCS. For both A and B isoforms, at least six positive clones were selected and analyzed. Initial experiments were performed using several clones for each cell line. Eventually, single clones were used for all experiments (clones A5 for PCS-A and B1 for PCS-B). Cell lines were maintained in 0.2 mg/ml G418-containing medium, passaged weekly, and were stable for several months. A PCS-A4 construct obtained by PCR (sense primer from bp 2,326-2,348 containing a unique DraIII site, and antisense primer introducing a stop codon and XbaI site at base 2,700 (Lusson et al., 1993)), in which the COOH-terminal 38 amino acids unique to PCS-A were removed, was similarly transferred to AtT-20 cells to compare its sorting with that of the wild-type PCS-A.

Antibodies

The NH2- and COOH-terminal mouse PCS polyclonal antibodies were obtained by immunization of rabbits using an octopus branched synthetic peptide approach already used for the PCS NH2-terminal antibody (Basak et al., 1995; Benjannet et al., 1993). The peptides chosen consisted of the sequences Asp-Tyr-Asp-Leu-Ser-His-Ala-Gln-Ser-Thr-Tyr-Phe-Ant-Asp- Pro-Lys, representing residues 116-132 consisting of the PCS NH2-terminal sequence after the potential activation site Arg-Thr-Lys-Arg, and Pro-Gly-His-Tyr-His-Ala-Asp-Lys-Lys-Arg-Cys-Arg-Lys, representing residues 677-690 of mouse PCS.

Other antibodies used were as follows: anti-TGN38 raised in rabbit with those raised in guinea pig (Schäfer et al., 1995), guinea pig anti-ACHT (Peninsula Laboratories, Inc., Belmont, CA), rabbit anti-cathepsin B (Lee et al., 1995), rabbit anti-PC2 (Basak et al., 1995; Benjannet et al., 1993), and rabbit anti-glucagon (Incat Co., Stillwater, MN).

Biosynthetic Labeling, Immunoprecipitations, and SDS-PAGE Analyses

Biosynthetic analyses were performed as previously described (Benjannet et al., 1993). Briefly, cells that had reached 80% confluence were washed with PBS, and then switched for 1 h to a methionine- or sulfate-free medium (RPMI 1640) (GIBCO BRL) supplemented with 0.5% FCS. Subsequently, cells were either labeled with [35S]methionine (100 μCi/ml) or [35S]sulfate (sodium sulfate) (200 μCi/ml) (Mandel Scientific Co., Ontario, Canada), temperature-blocking experiments, cells were preincubated in absence of methionine at 37°C, and then labeled with [35S]methionine for 2 h at either 37°C or 20°C. In experiments performed with brefeldin A (Cedarlane Laboratories, Ltd., Ontario, Canada), the drug was used throughout the preincubation and labeling period at a final concentration of 5 μg/ml, as described before (Benjannet et al., 1993). Stimulation of secretion was performed by adding 8Br-cAMP (Sigma Chemical Co., St. Louis, MO) at a final concentration of 5 μM to the incubation medium. At the end of the incubation period, the media were removed and cells were disrupted in lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 20 μg/ml PMSF) by incubation on ice for 20 min. The media and cell lysates were precleared in two steps using normal rabbit serum and protein A–agarose, and then immunoprecipitated. All immunoprecipitations were performed as described before (Benjannet et al., 1993). Endoglycosidase H (Oxford Glycosystems, Ltd., Rosedale, NY) digests were performed according to the manufacturer’s instructions. The immunoprecipitation products were resolved by electrophoresis on 8% polyacrylamide gels (SDS-PAGE) followed by treatment with Entansify (Du Pont-New England Nuclear, Wilmington, DE) and autoradiography. For preparative purposes, immunoprecipitated proteins were resolved on 6% SDS-PAGE gels, which were sliced(1 mm). The eluted radiolabeled proteins were subjected to microsequence analysis on a sequenator (model 470A; Applied Biosystems, Foster City, CA) as described (Benjannet et al., 1993).

Electron Microscopy

Electron microscopy was performed on thin sections for immunocytochemistry at 6°C. Tissue sections were first transferred for 30 min on a drop of 0.15 M PBS, pH 7.2, containing 0.1% ovalbumin, and then incubated overnight at 4°C with the following anti-
bodies at a dilution of 1:100: rabbit anti-PC2, rabbit anti-PC5, and rabbit anti-glucagon (Incstar Co.). Sections were then rinsed with PBS and incubated for 30 min at room temperature with the protein A-gold complex prepared with 10 nm gold particles (OD520 = 0.5), as previously described (Bendayan, 1989; Park and Bendayan, 1992). The grids were then washed with PBS and distilled water, dried, and stained with uranyl acetate before examination. For double immunogold labeling, both sides of the tissue sections were used, according to Bendayan (1982). Combinations of anti-PC2/anti-PC5, anti-PC2/anti-glucagon, and anti-PC5/anti-glucagon antibodies were performed on different faces of the grids in conjunction with protein A-gold complexes formed with 5, 10, or 15 nm gold particles. Several control experiments were carried out to assess the specificity of the labeling obtained. For these controls, incubation was performed with normal serum or specific antibodies preabsorbed overnight with an excess of their corresponding antigens.

Results

Expression of PC5-A and PC5-B in Stably Transfected AtT-20 Cells

To test the hypothesis that the PC5 isoforms (Fig. 1) could be sorted to different compartments of the secretory pathway, stable transfectants of PC5-A or PC5-B were established in the mouse corticotroph AtT-20 cell line. This cell line was chosen since it has been extensively studied in multiple investigations on the sorting of proteins within the constitutive and regulated pathways (Burgess and Kelly, 1987; Matsuuchi and Kelly, 1991).

The PC5 forms in transfected AtT-20 cells were initially examined by labeling for 2 h with [35S]methionine followed by immunoprecipitation. As shown in Fig. 2, PC5-A-expressing cells produce a major immunoreactive 117-kD protein detected in cell extracts and medium. Immunoprecipitates of the PC5-B–expressing cells revealed the presence in the cell lysate of two PC5-B–specific proteins migrating with an apparent Mr of 210 and 170 kD, the latter form also being detected in the medium (Fig. 2). A similar result was obtained after a 4-h pulse in which the 210-kD form of PC5-B was more evident in the cell extracts and was not detected in the medium (data not shown). These data suggest that the sulfated 210-kD form that reached the TGN (Hart, 1992; Baeuerle and Huttner, 1987) is not secretable, indicating that it represents membrane-bound PC5-B. The presence of a shed 170-kD immunoreactive form of PC5-B is reminiscent of the fate of another type I membrane-bound convertase, furin (Rehemtulla et al., 1992), suggesting that COOH-terminal cleavage of the membrane-anchored PC5-B occurs along the secretory pathway. The two bands detected in the cell extracts of an Mr of ~95–105 kD are considered nonspecific, as they are also detected in control AtT-20 cells transfected with the RcCMV expression vector alone.

Precursor–Product Analysis of PC5-A

The proprotein convertases are initially synthesized aszymogens and must undergo excision of their prosegment before they can be activated (see Fig. 1). To define the organelle in which prosegment cleavage of pro-PC5-A to PC5-A occurs, pulse-chase analysis of the biosynthetic fate of PC5-A in AtT-20 cells labeled with [35S]methionine was undertaken. As shown in Fig. 3, after a 1-min pulse, two immunoreactive PC5 products migrating with an apparent Mr of 126 and 117 kD are detected intracellularly. Progressively upon chase, both bands diminish in intensity with the appearance of a 65-kD form 60 min later. At the same time, the secretion of a major 117-kD protein is observed in the medium, as well as that of a relatively minor 65-kD product that becomes more visible after a 120-min chase. Since an NH2-terminally directed PC5 antibody is used to perform immunoprecipitations, the 65-kD form represents a COOH-terminally shortened fragment of PC5-A. Quantitative scanning of the autoradiogram revealed that in the medium, the ratio of 65 to 117 kD progressively increases from 0.30 to 0.45 during 2–4-h chase periods, respectively (data not shown).

Protein sequencing was then performed to unequivocally establish the identity of the PC5-A protein forms. As shown in Fig. 4, [3H]Tyr3 and [3H]Tyr2,U protein sequences were deduced for the 126-kD and the 117-kD forms, respectively. Based on the reported cDNA sequence and the predicted PC5 primary structure (Lusson et al., 1993), it can be concluded that the 126-kD form is

Figure 1. Schematic representation of mammalian proprotein convertase isoforms PC5-A and PC5-B. Legend to protein domains is depicted at the bottom. Amino acid length is given for each convertase. The sequence at the end of the COOH-terminal region common to PC5-A and PC5-B and the one unique to each isoform is given at the top. A schematic representation of the mutant PC5-AA construct used in this work is also shown.
Figure 2. Sulfate-labeling of PC5-A- and PC5-B-encoded proteins. PC5-A- or PC5-B-transfected cells were labeled for 2 h with [35S]sulfate, followed by immunoprecipitation of both cells and media with an anti-NH2-terminal PC5 antiserum and resolution on 8% SDS-PAGE gels. Control cells transfected with the ReCMV vector alone were similarly treated. Molecular masses are given in kD.

pro-PC5-A, the sequence of which begins after the predicted signal peptidase cleavage site CysArgThr↓ArgValTyrThrAsnHis. The 117-kD protein represents PC5-A, which is produced after cleavage of the 82-amino acid prosegment at the sequence ValValLysLysArgThrLys↓Arg↓AspTyrAspLeuSerHisAlaGinSerThr↓Tyr↓PheAsnAsp-ProLys. Sufficient amounts of the 65-kD form could not be accumulated to obtain an unambiguous sequence. However, since this fragment is immunoreactive to the NH2-terminal antibody that was raised against the above 15-amino acid peptide starting at the ↓AspTyrAsp↓↓↓↓ sequence, it likely represents a COOH-terminally truncated product of the 117-kD PC5-A form.

To define whether intracellular zymogen cleavage of the 126-kD pro-PC5-A is an early event, the protein was blocked in anterior secretory pathway compartments using the fungal metabolite brefeldin A (BFA). This agent causes the disassembly of the Golgi complex and fusion of the cis-, medial-, and trans-GolgI (but not the TGN) with the ER (Lippincott-Schwartz et al., 1989, 1991). In the presence of BFA, the conversion of the 126-kD pro-PC5-A into the 117-kD PC5-A is still observed (Fig. 5 a), but COOH-terminal truncation generating the 65-kD product is no longer detected. Thus, NH2-terminal prosegment processing can occur in early compartments of the secretory pathway, but not COOH-terminal truncation. Furthermore, the absence of sulfation of the 126-kD form (Fig. 2) is taken as evidence that pro-PC5-A does not reach the TGN, where sulfation is known to take place (Hart, 1992; Bauerle and Huttner, 1987). Endoglycosidase H digestions were also performed and demonstrated that the 126-kD pro-PC5-A was digested by endoglycosidase H during chase times of up to 4 h, while the intracellular and

Figure 3. Endoproteolytic transformations of PC5-A. PC5-A-transfected cells were pulse labeled with [35S]methionine for 1 min (p1), and then chased (c) for 30, 60, or 120 min, followed by immunoprecipitation with a polyclonal rabbit anti-NH2-terminal PC5 antiserum and resolution on 8% SDS-PAGE gels. A more contrasted exposure is given to show the intracellular production of the 65-kD fragment. Molecular masses are given in kD.

1. Abbreviations used in this paper: BFA, brefeldin A; POMC, pro-opiomelanocortin; TMD, transmembrane domain.
secreted 117- and 65-kD forms were resistant to this treatment after a 1-h chase (data not shown).

Since the COOH-terminal truncation generating the 65-kD product does not occur in the presence of BFA (Fig. 5 a), this cleavage must occur in a late compartment of the secretory pathway. To further substantiate this hypothesis, an experiment at a restrictive temperature of 20°C, which traps secretory proteins at the level of the TGN (Matlin and Simons, 1983), was performed. The formation of the 65-kD product was not observed at 20°C (Fig. 5 b). Therefore, COOH-terminal truncation of PC5-A occurs after egress of the 117-kD form out of the TGN.

**Cellular Processing of PC5-B**

As already shown in Fig. 2, in AtT-20 cells stably transfected with PC5-B, the sulfated protein forms that reach the TGN have an apparent \( M_r \) of 210 and 170 kD. Since only the 170-kD form is secreted in the medium, this suggests that the 210-kD sulfated protein is retained intracellularly via the transmembrane domain (TMD). In a similar time course to PC5-A (Fig. 3), pulse labeling of these cells with \(^{35}\)S)methionine for 1 min followed by chase times of up to 2 h demonstrated that the 170-kD COOH-terminally truncated form is first detected in the cell extracts and medium after a 60-min chase period (Fig. 6 a). Since the 170-kD product is detected in the cell extracts, COOH-terminal truncation of PC5-B therefore occurs intracellularly. To determine whether the production of the 170-kD form is an early or late event along the secretory pathway,

**AtT-20 cells expressing PC5-B were labeled with \(^{35}\)S)methionine for 2 h in the presence or absence of BFA. Results shown in Fig. 6 b demonstrate that the 170-kD form is not detected in the presence of BFA, suggesting that loss of the TMD occurs in a late secretory pathway compartment.

**PC5-A Enters Secretory Granules While PC5-B Is Localized in the Golgi**

**Biosynthetic studies.** Since both 117- and 65-kD PC5-A (Fig. 3) and the 170-kD PC5-B (Fig. 6) are secreted in the medium, it was essential to determine whether each of these forms was released through the same secretory pathway. The basal and stimulated release of PC5-A and PC5-B in several of our clonal cell lines was thus compared by labeling of the cells for 2 h with \(^{35}\)S)sulfate in the presence (+) or absence (−) of 8Br-cAMP. In Fig. 7 a, the release of the 117-kD PC5-A is seen to increase up to threefold in the presence of the secretagogue. Since the 65-kD PC5-A product is not sulfated, the experiment was
repeated using [35S]methionine labeling. Results shown in Fig. 7 b demonstrate that the release of the 65-kD PC5-A form is also stimulated in presence of 8Br-cAMP. In contrast, the level of the secreted 170-kD PC5-B remains virtually unchanged upon this treatment (Fig. 7 a). The ratio of stimulated over nonstimulated bands was calculated by scanning and quantification of the autoradiograms, and it was found to be 0.90 for PC5-B and 2.5 for PC5-A (average values, see Table I). This suggests that while the 117- and 65-kD forms of PC5-A can enter secretory granules, the shed form of PC5-B (170 kD) exits from the cell via the constitutive secretory pathway (Burgess and Kelly, 1987).

To provide further evidence that the PC5-A sorting signal to secretory granules resides in its COOH-terminal region, a mutant PC5-A construct was produced, in which the last 38 amino acids unique to PC5-A were deleted (see Fig. 1). This mutant PC5-AΔ exhibits a biosynthetic pattern similar to that of PC5-A in pulse-chase experiments, with the presence of 122- and 113-kD forms in the cell extracts (Fig. 8). A notable exception is the production of the 65-kD fragment that, at the 2-h chase period in the medium, is reduced by half as compared with wild-type PC5-A (compare Figs. 3 and 8 a). The release of this PC5-AΔ mutant was not stimulated by 8Br-cAMP to the same extent as that of the wild-type PC5-A, showing only a 1.2-fold increase, while PC5-A release could be augmented by up to threefold in the presence of this secretagogue (Fig. 8 b and Table I).

To clarify the type of release of each PC5 protein, secretion kinetics experiments were performed (Fig. 9). PC5-A−, PC5-AΔ−, and PC5-B−expressing cells were pulsed for...
Table I. Ratio of 8Br-cAMP-stimulated Secretion to Basal Release for PC5 Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ratio of stimulated secretion to basal release (Band intensity + 8Br-cAMP/ Band intensity - 8Br-cAMP)</th>
<th>SD</th>
</tr>
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<tbody>
<tr>
<td>PC5-A</td>
<td>2.50</td>
<td>± 0.17</td>
</tr>
<tr>
<td>PC5-B</td>
<td>0.90</td>
<td>± 0.11</td>
</tr>
<tr>
<td>PC5-ΔΔ</td>
<td>1.21</td>
<td>± 0.19</td>
</tr>
</tbody>
</table>

 Autoradiographs were quantified as described in Materials and Methods. The average ratio of stimulated release over basal release for 2-h labeling periods was calculated for each PC5 protein, taking values of five such experiments.

30 min with [35S]methionine and chased for the times indicated. Their respective accumulation in the medium was calculated for each chase time by scanning and quantification of the autoradiograms as described in Materials and Methods (Fig. 9). While PC5-A demonstrates secretion kinetics representative of a protein released through the regulated pathway (Grimes and Kelly, 1992; Arvan and Castle, 1987; Chavez et al., 1996), PC5-B and PC5-ΔΔ exhibit notably distinct basal secretion patterns.

**Immunofluorescence Analysis:** To define more precisely the subcellular localization of PC5 proteins, AtT-20 transfected cells were analyzed by immunofluorescence microscopy (Marcinkiewicz et al., 1996). The data in Fig. 10 a show that PC5-A immunoreactivity exhibits a pattern of punctate staining observed in the cytoplasm and at the tips of cellular extensions, known to contain secretory granules, to contain secretory granules, and to contain immunostaining at paranuclear positions, which correspond to the Golgi apparatus. Weak immunoreaction could also be seen in the presumptive perinuclear ER. By double immunofluorescence, PC5-A is demonstrated to colocalize with ACTH in secretory granules and Golgi apparatus (Fig. 10, a and b). The PC5-B labeling pattern (Fig. 10 c) is quite different from that observed for either PC5-A or ACTH (Fig. 10, a and b). Immunofluorescence staining of PC5-B (Fig. 10 c) overlaps with that of TGN38 (Fig. 10 d), a TGN-resident protein (Luzio et al., 1990). A mutant PC5-ΔΔ, lacking the PC5-A-specific COOH-terminal segment (PC5-ΔΔ; Fig. 10 e) also colocalizes with TGN38 by double immunofluorescence (Fig. 10 e) and by the displacement of the labeling reaction with excess antigen (data not shown). We note that the labeling pattern of cathepsin B (CB; Fig. 10 f), a marker of lysosomal compartments, is distinct from that observed for PC5 in all our transfected cell lines, although in this case colabeling could not be achieved since both antibodies are from rabbit species.

**Immunocytochemical Evidence of PC5 Localization in Pancreatic Glucagon-secreting Cells by EM**

To demonstrate that the sorting of PC5-A into secretory granules is not fortuitous or the result of overexpression in AtT-20 cells, the cellular localization of PC5 in pancreas was examined by EM. In single-labeling experiments, glucagon immunogold labeling was present over the electron-dense structure of secretory granules, in cells displaying the characteristics of the A cells (Park and Bendayan, 1992) (Fig. 11 a). Using antibodies against the convertases PC2 and PC5 in conjunction with protein A–gold complexes, the gold particles were shown to be associated with the secretory granules of the A cells (Fig. 11, b and c). Double-labeling experiments revealed the colocalization of both PC2 and PC5 within the same granules of the A cells (Fig. 11 d). Double labeling using antibodies to PC2 or PC5 together with a glucagon-specific antibody demonstrated that the majority of glucagon–rich granules also display PC2 or PC5 immunoreactivity (Fig. 11, e and f).

**Discussion**

In this work, the biosynthesis and transport of PC5-A and PC5-B, two isoforms generated from a single gene (Mbikay et al., 1995), were investigated. Specifically, their respec-
Secretion kinetics of PC5 proteins

Figure 9. AtT-20-transfected cells were labeled for 30 min with [35S]methionine and chased for the times indicated. Media were immunoprecipitated and resolved by SDS-PAGE. Autoradiographs of these gels were analyzed as described in Materials and Methods to permit quantification of the accumulation of released proteins in the medium at each time point. The calculated band intensities are given in arbitrary units.

Biosynthetic Fates of PC5-A and PC5-B

Initially, since data were not available on the biosynthesis of PC5, analysis in cells that endogenously express relatively high levels of its mRNA, namely BSC40 cells (Nakagawa et al., 1993a), was attempted. However, detection of sufficient amounts of [35S]methionine-labeled PC5-A above background was unsuccessful. This approach was thus unsuitable to undertake extensive biosynthetic studies on PC5. Hence, the isolation of stable transfectants overexpressing PC5 was preferred, allowing in addition the comparison of the biosynthetic fate and transport of the isoforms PC5-A and PC5-B.

Pulse-chase analysis performed in transfected cells demonstrated that PC5-A is initially produced as pro-PC5-A, and rapidly processed to PC5-A (within a 1-min pulse) in early compartments of the secretory pathway, in a fashion similar to that of the granule-associated convertase PC1 (Benjannet et al., 1993). Pro-PC5-A predominates intracellularly, even after chase periods of up to 2 h (Fig. 3). It is possible that the intracellular predominance of pro-PC5-A over PC5-A at all chase times may be due to its relatively high expression levels in transfectant clones. As assessed by its persistent sensitivity to endoglycosidase H digestion, pro-PC5-A remains in a compartment where transformation and trimming to complex sugar types does not occur (data not shown). With the added observation that pro-PC5-A is not sulfated (Fig. 2), whereas PC5-A is, this suggests that the zymogen remains in the ER, and only the NH2-terminally processed PC5-A is allowed to exit from this compartment. This is in contrast to PC2, which is processed in the TGN/granules (Benjannet et al., 1993). PC5-A also undergoes COOH-terminal truncation into a 65-kD product, presumably within immature secretory granules. This is again similar to the fate of PC1 (87 kD), which is COOH-terminally cleaved into a 66-kD form, also within granules (Vindrola and Lindberg, 1992; Benjannet et al., 1993). Based on its size and the PC5-A sequence (Lusson et al., 1993), and by homology to the re-
cently described COOH-terminal cleavage site of PC1 (Zhou and Lindberg, 1994), the probable cleavage site generating this 65-kD fragment is at the sequence His<sub>646</sub>TyrHisAla-AspLysLysArg<sub>651</sub>Cys.

Pulse-chase analysis of the isoform PC5-B was then undertaken to compare its biosynthetic fate to that of PC5-A. This protein is present in transfected AtT-20 cells as a membrane-bound 210-kD form and a shed 170-kD product (Figs. 2 and 6, a and b). The truncated 170-kD product is detected after a 1-h chase both in the cell extracts and medium (Fig. 6 a), which suggests that COOH-terminal cleavage takes place in an intracellular compartment. Production of the PC5-B 170-kD truncation product is prevented in the presence of BFA, pointing to a late Golgi compartment as the location where COOH-terminal processing takes place. Similarly, the COOH-terminal truncation of membrane-bound furin was demonstrated to take place in the TGN (Vey et al., 1994). It should be mentioned that the extent of cleavage of the 210-kD form into the 170-kD PC5-B is somewhat dependent on protein expression levels. For example, in clones where expression levels are high, the ratio of the intracellular 210-kD to 170-kD form is in favor of the latter species, whereas this ratio is reversed in moderately expressing clones. This observation may be relevant to the different tissues that express this isoform. For example, in the ileum in which PC5-B mRNA is abundant, production of the 170-kD form would be expected to be more substantial than in lower expressing tissues, such as lung. The membrane-bound convertases furin (Molloy et al., 1994; Rehemtulla et al., 1992), the Drosophila furins (De Bie et al., 1995), and kexin (Germain et al., 1992) also exhibit shedding of substrates. A smaller truncation product of 65 kD, such as the one detected for PC5-A, is never observed for PC5-B.

To further demonstrate that the sorting of PC5-A to secretory granules is neither an artifact nor the result of overexpression in AtT-20 cells, we performed EM studies to demonstrate the presence of PC5 in the granules of an endocrine tissue. The data (Fig. 11) clearly demonstrate that PC5 colocalizes with both glucagon and PC2 within the granules of the pancreatic A cells. The presence of PC5 in the Golgi could not be established, as a result of the scarcity of this organelle in these cells. Further undertakings are needed to demonstrate the presence of PC5 in the Golgi of pancreatic A cells at the EM level and to determine which form of PC5 prevails in this cellular compartment.

The pancreatic A cells provide a physiological model for the sorting of PC5 in an endocrine tissue. In agreement, in two glucagon-expressing cell lines, the 65-kD PC5-A product has been detected by Western blotting (Blache et al., 1994). One of these cell lines, namely aTC1-6, is derived from the pancreatic A cells (Hamaguchi and Leiter, 1990). This strongly suggests that PC5-A, and not PC5-B, is the species detected in the A cells of the pancreas.

The principal sorting signal that permits PC5-A to enter secretory granules seems to reside within its unique COOH-terminal 38 amino acids. Indeed, a truncation mutant of PC5-A in which this segment was removed was no longer detected in the secretory granules of AtT-20 cells by immunofluorescence microscopy (Fig. 10 e). Moreover, 8Br-cAMP stimulation of the release of this protein only resulted in a 1.2-fold increase of secretion in the medium, while that of wild-type PC5-A could be augmented by up to three times (Table 1). Therefore, PC5-A<sub>A</sub> seems to have lost its capacity to efficiently enter secretory granules.

The secretion kinetics of PC5-A<sub>A</sub> are also quite distinct from those of PC5-A. In Fig. 9, where the basal releases of PC5-A, PC5-A<sub>A</sub>, and soluble PC5-B are compared, PC5-A demonstrates a secretion pattern typical of proteins released through the regulated secretory pathway of trans-
Figure 11. EM immunocytochemical detection of PC5 and PC2 in pancreatic glucagon-secreting cells. Pancreatic A cells were immunolabeled with the following antibodies complexed to protein A-gold: anti-glucagon (a, e, and f), anti-PC2 (b, d, and e), and anti-PC5 (c, d, and f). In a, the gold particles complexed to the glucagon antibody are detected over the secretory granules (g). (b) The secretory granules (g) of the A cell are labeled with the PC2 antibody complexed to protein A-gold. (c) Labeling of the A cell with the PC5 antibody complexed to protein A-gold is seen over the majority of secretory granules (g). (d–f) Sections of glucagon-secreting cells were then processed by double immunogold labeling: (d) PC2 (5-nm particles) and PC5 (10-nm particles) are colocalized in the secretory granules (g). (e) PC2 (5-nm particles) is colocalized with glucagon (15-nm particles) in many of the secretory granules. (f) PC5 (5-nm particles) colocalizes with glucagon (15-nm particles) in many granules of the A cell. Bars: (a and b) 0.2 μm; (c–f) 0.1 μm.
formed cell lines, while PC5-ΔA and soluble PC5-B exhibit linear constitutive releases. Tumor-derived endocrine cell lines have been demonstrated to have a high rate of basal, unstimulated release of granule contents. This is especially true of AtT-20 cells (Matsuuchi and Kelly, 1991; Milgram et al., 1994), which release about half of their granule content of ACTH in an unstimulated fashion (Moore et al., 1983). Therefore, Fig. 9 depicts this unstimulated granule exocytosis of PC5-A, which is initially rapid and thought to occur through immature granules, and then reaches a plateau as the protein accumulates in the mature secretory granules.

The basal release of PC5-ΔA and the soluble form of PC5-B, on the other hand, is believed to occur through constitutive vesicles, as their content increases linearly with time in the extracellular medium. The observed rate of release of PC5-B is the lowest of the three PC5 forms, probably because this protein must first undergo excision of its transmembrane domain before being released into the extracellular medium. The fact that the initial rate of release of PC5-A is higher than that of the mutant PC5-ΔA may be due (a) to the fact that the expression levels of PC5-A in the cell lines analyzed are higher than those of PC5-ΔA, and therefore the PC5-A overflow from granules is more important than the constitutive release of the mutant; or (b) to the release of PC5-ΔA from the cell being delayed by the slower rate of exit of this protein out of the ER, as the mutation introduced in the COOH-terminal domain could affect the proper folding of this protein.

The small increase of PC5-ΔA release in the presence of 8Br-cAMP (Fig. 8 b; Table I) could be explained by the entry of this protein in immature secretory granules, as these organelles can release their content upon stimulation (Tooze et al., 1991). This is further supported by the production of a 65-kD truncation product at 37°C, but not at 20°C, by both PC5-A (Fig. 5 b) and PC5-ΔA (data not shown). In comparison, PC5-B is never processed into this smaller truncation product, even though the cleavage site is present within the region common to both isoforms. The differential sorting of PC5-A and PC5-ΔA could therefore occur in immature granules (Arvan and Castle, 1992; Bauerfeind and Huttner, 1993), from which PC5-ΔA could be released through the constitutive-like pathway (Kuliwat and Arvan, 1994). Alternatively, PC5-ΔA could enter the immature granules, but then be recycled from this compartment back to the TGN to be released through constitutive vesicles.

**Sequence-specific Sorting Signals of PC5-A and PC5-B**

PC5-A. In an attempt to uncover a potential granule-sorting signal, the sequence unique to PC5-A was tentatively aligned with those of other proteins known to enter the secretory granules, namely chromogranin B and pro-opiomelanocortin (POMC). A consensus sequence emerged that conformed to the signals already defined for chromogranin B (Chanat et al., 1993) and POMC (Cool et al., 1995). This consensus sequence consists of two cysteines, which in the cases of chromogranin B (Chanat et al., 1993) and POMC (Bennett et al., 1986) have been demonstrated to form a disulfide bond, and conserved hydrophobic residues, especially a central leucine (Fig. 12 a). This motif is also observed in chromogranin A (Benedum et al., 1987) and in the convertase PACE4-A (Kiefer et al., 1991). We may therefore have identified a potential granule-sorting signal for PC5-A, which could also be functional in the

![Figure 12](https://example.com/figure12.png)

**Figure 12.** (a) Alignment of amino acid sequences showing homology between PC5-A, PACE4-A, chromogranin B, chromogranin A, and POMC. Conserved amino acid sequences of mouse mPC5-A, human hPACE4-A, human chromogranin B (hCg B), human chromogranin A (hCg A), and human hPOMC are boxed, and the important (hydrophobic) is boxed. Acidic stretches are topped with an asterisk (*). m, mouse; h, human; y, yeast; r, rat.
convertase PACE4-A. Further studies are needed to demonstrate whether this signal is a true sorting domain and can act on constitutively secreted proteins, as was accomplished with chimeras of the prosegment of anglerfish somatostatin-1 and α-globin (Stoller and Shields, 1989). We conclude that the COOH-terminal 38-amino acid sequence is involved in the sorting of PC5-A to the regulated pathway. This segment may be necessary for the presumed calcium-induced aggregation of PC5-A, allowing its entry into mature secretory granules (Burgess and Kelly, 1987; Tooze et al., 1993). This does not exclude the possibility that additional sorting signals are present in other PC5 domains. Studies with both POMC (Chevrier et al., 1993) and somatostatin (Sevarino and Stork, 1991) demonstrated that multiple sorting signals to the regulated secretory pathway exist in these molecules.

**PC5-B.** Potential signals permitting the retention of PC5-B in Golgi compartments were likewise investigated, by comparing its COOH-terminal-specific sequence to those of other membrane-bound proprotein processing enzymes.

Several trafficking signals governing TGN localization have been recently defined to reside within the cytoplasmic tail of the convertase furin (Molloy et al., 1994; Chapman and Munro, 1994; Voorhees et al., 1995; Schäfer et al., 1995; Jones et al., 1995; Takahashi et al., 1995; Bosshart et al., 1995). These include (a) a cluster of acidic amino acids in the sequence CPSDSEDEEG, where the phosphorylation of both serines by a casein kinase II-like enzyme plays an important modulatory role in the retrieval of furin to the TGN (Jones et al., 1995; Takahashi et al., 1995); (b) a Tyr-containing motif, where the tetrapeptide consensus sequence Y-XX-(hydrophobic) is a necessary and sufficient cytoplasmic domain signal to retrieve integral membrane proteins from the cell surface to the TGN (Trowbridge et al., 1993); and (c) adjacent leucine and/or isoleucine residues, termed dileucine signal, which has been described as an internalization motif (Johnson and Kornfeld, 1992).

All three signals are present in the cytosolic tail of PC5-B (Fig. 12 b). Two acidic clusters are seen in mouse PC5-B, within the sequence (Nakagawa et al., 1993b): TMD-28aa-SYLDEDQVIEYRDRDYDEDDEDD-19aa-DETEDDE-LEYDDE-5aa-COOH, with two potential (Ser and Thr) casein kinase II phosphorylation sites. Both acidic clusters and the casein kinase II phosphorylation site at the serine residue are conserved in rat PC5-B (GenBank accession number: P9sk5 V47014). The cytosolic sequence of PC5-B also exhibits four Tyr-containing motifs: TMD-12aa-YEKL-6aa-YSSY-35aa-YRKF-1aa-YGGL-18aa-COOH, which could be potentially functional. Interestingly, PC5-B (LeuLeu, at two positions), furin (LeuLeu), and PC7 (LeuLeu, at two positions) all contain a dileucine motif within their cytoplasmic tail. Future internalization studies aimed specifically at detecting PC5-B at the cell surface and its predicted retrieval to intracellular compartments should assess the implication of the Tyr-containing and/or dileucine signals in this process. Alignment of the known cytosolic tail localization motives of furin, PC7, kexin (Wilcox et al., 1992), and TGN38 (Humphrey et al., 1993; Bos et al., 1993; Wong and Hong, 1993; Ponnambalam et al., 1994; Wilde et al., 1994), all membrane-bound protein residents of the Golgi or TGN, with the motives seen in PC5-B, is shown in Fig. 12 b.

In conclusion, PC5-A and PC5-B constitute the first example for the convertase family of processing enzymes, in which two active forms originating from a single gene exhibit distinct cellular localization. This may represent a mechanism to regulate bioactive enzymes by directing them to different subcellular destinations to process unique sets of precursor substrates. Since the convertases exhibit overlapping tissue distributions as well as in vitro or ex vivo cleavage specificities, the formation of the appropriate convertase/substrate combinations could ultimately be regulated by their particular intracellular localization.

We are grateful to Drs. G. Banting and W. Garten for their gifts of TGN38 antisera, and to Dr. J. Mort for his gift of cathepsin B antiserum.

Synthesis of MAP peptides by Dr. Ajoy Basak is much appreciated, as well as the technical assistance of Edwige Marcinkiewicz, Diane Savaria, Annie Lemieux, Suzanne Benjennett, Andrew Chen, Jim Rockemont, Maya Mamarbachi, Josééle Hamelin, Xue-Wen Yuen, Dr. Robert Day, Diane Gingras, Jean Godbout, and Jean Leveillé. Many thanks to Dr. Andréas Laslop for critical reading of the manuscript, to Dr. J. Scott Munzer for countless encouragements, and to Christian Charbonneau for expert handling of photographic material. The secretarial assistance of Mrs. Sylvie Emoned and Sandra Gauthier is greatly appreciated.

This work was supported by Medical Research Council of Canada program grants (PG1471, PG11474, MT-12686, and MT-7284), a Protein Engineering Network of Centres of Excellence (PENCE) grant, and J.A. De Sève Succession. I. De Bie received support in part from funds of the PENCE Program and from a scholarship from Fonds pour la Formation de Chercheurs et de l'Aide à la Recherche (Québec, Canada).

Received for publication 7 March 1996 and in revised form 10 September 1996.

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The Journal of Cell Biology, Volume 135, 1996 1274
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