A Predicted α-Helix Mediates Targeting of the Proprotein Convertase PC1 to the Regulated Secretory Pathway*

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The proprotein convertase PC1 is a protease whose activity is largely confined to the dense core secretory granules of neuroendocrine cells. Efficient processing of PC1 substrates in granules requires a mechanism that will both limit the activity of the enzyme to these organelles and promote its targeting to the nascent secretory granules. In the current study, we provide evidence that targeting of PC1 to secretory granules is mediated by α-helical structures in its C-terminal tail and, at least in part, is dependent on interactions with specific components of the secretory granule membrane.

Proprotein convertases (PCs)1 form a family of serine endopeptidases composed of seven identified members as follows: furin, PACE4, PC1, PC2, PC4, PC5A/B (where PC5-A and PC5-B are two splicing variants of the same mRNA transcript) and PC7, as reviewed previously (1–3). The activity of these enzymes is thought to be crucial for the appropriate processing of numerous proprotein and prohormone precursors before their secretion from synthesizing cells. Whereas some proprotein convertases (furin, PACE4, PC5-B, and PC7) process proteins in the constitutive secretory pathway, others (PC1, PC2, and PC5-A) exhibit their greatest activity in the maturing granules of the regulated secretory pathway (4–7). PCs characteristically cleave their substrates right after a pair of basic amino acids and share a common domain structure that includes an N-terminal signal peptide, a propeptide region, a conserved subtilisin-like catalytic domain, a conserved region named P-domain, which possibly regulates the calcium and pH dependences of the processing activity (8), and a C-terminal region, in which the greatest divergence between PCs occurs.

The expression of PC1 (also known as SPC3) is restricted to endocrine and neuroendocrine tissues where it is responsible for the processing of several prohormones, as is strikingly demonstrated by the recent finding of naturally occurring mutations in the PC1 gene in a patient with severe obesity associated with elevated plasma levels of proinsulin and pro-opiomelanocortin levels (9). Like the prohormones it activates, PC1 is synthesized as an inactive precursor, pro-PC1. Although the prosegment of PC1 is rapidly removed in early compartments of the secretory pathway (10, 11), the additional cleavage of the 135-amino acid-long C-terminal domain of PC1, which occurs specifically in secretory granules, has been shown to increase dramatically the activity of the enzyme for certain substrates (5, 12, 13). This finding has led to a model whereby the C-terminal domain would, at least in part, inhibit PC1 activity in early compartments of the secretory pathway, thereby preventing activation of certain substrates prior to entry in the secretory granules (5). For this model to be valid, however, PC1 (with its C-terminal tail attached) would have to be targeted efficiently to secretory granules where it could subsequently be activated and cleave its substrate. Indeed, the C-terminal domain has been suggested to play a role in proper sorting of PC1 to the secretory granules (12).

The mechanism of protein sorting to the regulated secretory pathway remains a matter of debate, and in contrast to many cellular sorting events that take place throughout the secretory pathway (14–16), a consensus signal sequence has not been identified for sorting of proteins to the secretory granules. The mechanisms that result in protein storage in mature granules are thought to occur at the level of the trans-Golgi network (TGN) and nascent immature secretory granules. Hypotheses for sorting can be summarized by two models that are not mutually exclusive (17). The first model proposes the existence of sorting receptors, capable of specifically binding to a putative structural sorting domain and capable of pulling sorted proteins into immature secretory granules. It was recently proposed that carboxypeptidase E (CPE) was just such a sorting receptor for a wide variety of prohormones including proinsulin (18), but this model has been challenged by the finding that proinsulin storage was not impaired in pancreatic islets from CPE-deficient (CPE<sup>−/−</sup>/CPE<sup>−/−</sup>) mice (19) or in cell lines derived from pancreatic β-cells of these mice (20). The second model predicts non-restricted or bulk-flow entry of proteins into immature granules and subsequent removal of proteins destined to other cellular compartments, paralleled with specific retention of proteins stored in mature granules. Extrusion of proteins from immature granules could occur by a receptor-mediated mechanism or result from capture of soluble proteins in small vesicles budding from the immature granules (21, 22). Several stored proteins have been shown to aggregate under the calcium and pH conditions that prevail in sorting compartments (23, 24) or to associate with phospholipids of the granule membrane (25). These characteristics are postulated to be important in removing regulated secretory proteins from the soluble phase and consequently for efficient retention of these proteins in mature granules.

Irrespective of the sorting mechanism, the proposed models are compatible with the existence of specific sorting domains in regulated secretory proteins. Thus, in the current study, we
have investigated the function of the C-terminal domain of PC1 in protein sorting to the regulated secretory pathway. Our results suggest that a predicted α-helix in the structure of the C-terminal region is important in protein sorting to secretory granules, possibly by mediating protein binding to membranes in the sorting compartments of the secretory pathway.

EXPERIMENTAL PROCEDURES

Recombinant Plasmid Construction—Numbers in the following constructs correspond to amino acids in native mouse PC1 beginning at the initial methionine. Construction of expression vectors for native mouse PC1 (PC1C) and truncated PC1 (PC1ΔC, truncated on its C-terminal tail) has been described previously (5). Expression vectors containing portions of the C-terminal domain of mouse PC1 fused to a portion of the Fe fragment of the mouse immunoglobulin IgG2b heavy chain (preceded by the signal peptide and 6 amino acids of the prosegment of prorenin (26)) were constructed as follows. The stop codon of the IgG2b heavy chain was deleted by polymerase chain reaction with the following oligonucleotides, 5′-CGAGATCTTTACCCGGGAGAC-3′ antisense primer preceding the stop codon (generation of a BglII site, underlined). The C-terminal portions of mouse PC1 were added in frame to cohesive ligation of BglII/BamHI sites using the following oligonucleotides (generation of a BamHI site is underlined); 5′-CGGGATCCTGTG-GAGCTT-3′ sense primer for BglII (657–673) and 5′-CGGGATCCTGTG-GAGCTT-3′ antisense primer for BglII (657–673); 5′-CGGGATCCTGTG-GAGCTT-3′ sense primer for Fe-PC1 (673–753) and 5′-CGGGATCCTGTG-GAGCTT-3′ antisense primer for Fe-PC1 (673–753); 5′-CGGGATCCTGTG-GAGCTT-3′ sense primer for PC1 (673–753); 5′-CGGGATCCTGTG-GAGCTT-3′ antisense primer for PC1 (673–753) (generation of a stop codon in bold); 5′-CGGGATCCTGTG-GAGCTT-3′ sense primer for PC1 (673–753); 5′-CGGGATCCTGTG-GAGCTT-3′ antisense primer for PC1 (673–753) (generation of a stop codon in bold). Mutations in the Fe-PC1 (Pro427-Pro428) construct were inserted using the following overlapping oligonucleotides: 5′-CATGGACATCCcAAAT-3′ antisense primer and 5′-GGATGTCCATGgGAGCTT-3′ sense primer (mutated nucleotides are in lowercase). All recombinant plasmid constructions were verified by sequencing of double-stranded DNA.

Cell Culture and Transient or Stable Transfections—The rat somatomammotrophic GH4 and mouse corticotrophic AtT-20 cell lines were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in a humidified incubator at 5% CO2 and 10% grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% dialyzed fetal calf serum, labeled with 35S-methionine per well (pulse), and incubated in complete medium for 10 min and mixed. After the 2-h chase period, the neo-synthesized PC1 content of postnuclear supernatants was immunoprecipitated with protein A-Sepharose. Proteins were separated on SDS-PAGE followed by immunoblotting with an anti-mouse IgG antibody coupled to horseradish peroxidase (Amersham Pharmacia Biotech).

Protein Structural Predictions—Protein structure predictions according to the Chou-Fasman and Garnier-Osguthorpe-Robson algorithms were carried out using the Genetics Computer Group sequence analysis program.

RESULTS

The C-Terminal Tail of PC1 Contains a Sorting Domain for the Regulated Secretory Pathway—To investigate the role of the C-terminal domain of PC1 in sorting to the regulated secretory pathway, we tested whether its complete deletion would impair the storage of PC1 in secretory granules and alter its stimulated release from cells. GH4 cells were transfected with either native PC1 or PC1ΔC, a C-terminally truncated PC1 construct (Fig. 1). Transfected cells were labeled with [35S]methionine for 2 h, chased for an additional 2 h, and incubated in the presence of NaCl to estimate basal protein secretion and, subsequently, in the presence of KCl to stimulate the release of secretory granule content. Expression of native PC1 in GH4 cells resulted in the secretion of the 87- and 66-kDa forms corresponding to C-terminally uncleaved and C-terminally cleaved PC1, respectively. During the pulse-labeling and chase periods, both constitutive secretion and basal secretion of secretory granules occurs. The C-terminally cleaved PC1, which is generated in the secretory granules, thus appears in the supernatant during these secretion periods. After the 2-h chase period, the neo-synthesized PC1 content of the secretory granules was assessed. The 66-kDa form underwent stimulated release from the cells with a 5.3-fold stimulation, indicating sorting of PC1 to the regulated secretory pathway. This result also indicates exhaustive PC1 C-terminal cleavage within secretory granules since the 66-kDa form represented the only detectable form stored in this compartment. Transfection of GH4 cells with PC1ΔC leads to direct expression of the 66-kDa form, but in contrast to native PC1, the 66-kDa form was no longer sorted to the regulated secretory pathway as judged from the very modest 1.7-fold stimulated secretion. Instead, PC1ΔC appeared to undergo rapid constitutive secretion from GH4 cells. These results indicate that targeting of PC1 to the regulated secretory pathway is dependent on its C-terminal tail, which can thus be considered as the major granule-sorting domain of PC1.

To characterize further the sequences in the C-terminal tail of PC1 that are responsible for its sorting to the regulated secretory pathway...
secretory pathway, various portions of the C-terminal domain were fused to a segment of mouse immunoglobulin 2b heavy chain (corresponding to part of the Fc fragment). We then tested whether the C-terminal tail of PC1 could function in rerouting the constitutively secreted Fc protein to the regulated secretory pathway (Fig. 2). While the Fc fragment alone (Fc) was not apparently sorted to secretory granules in transfected GH4 cells, as evidenced by the weak 1.58-fold increase in its secretion by potassium (Table I), an Fc fusion protein containing the entire C-terminal tail of PC1 (Fc-PC1 627–753) underwent stimulated release from the cells with a mean 3.25-fold stimulation (Table I). Furthermore, when the C-terminal domain was hooked up to a signal peptide followed by the hemagglutinin epitope (HA-1), the fusion protein was also found to undergo stimulated release (not shown). These results demonstrate that the C-terminal region of PC1 can function as a dominant sorting domain in the context of the Fc fusion protein.

Fc fusion proteins containing various portions of the C-terminal domain of PC1 were expressed in GH4 cells in order to identify the minimal sorting domain (Fig. 2). Results indicate that all the fusion proteins were expressed at comparable levels in the transfected cells and generated similar levels of secreted proteins during the pulse-labeling (not shown) and chase periods, suggesting that mis-folding was not a major problem. The mean values of stimulus-dependent secretion demonstrate that the peptide region spanning amino acids 627–670 was inefficient in sorting the Fc fusion protein to secretory granules (Table I). In contrast, portions spanning either amino acids 667–713 or 711–753 of the C-terminal domain resulted in stimulation folds comparable to that obtained with the entire C-terminal tail. Therefore, the C-terminal region of PC1 contains at least two minimal sorting domains (amino acids 667–713 and 711–753) that do not act additively since their combined presence did not augment the sorting efficiency of the fusion protein containing both regions (Fc-PC1-(667–753)).
PC1(Pro745,Pro749) construct, the bulk of the fluorescence was observed in a para-nuclear region consistent with localization of the protein passing through the Golgi apparatus (Fig. 4). However, in AtT-20 cells expressing the mutant Fc-PC1-(711–753) fusion protein (Fig. 3B), the proline mutation did not reduce expression of the fusion protein in those present in the trans-Golgi network (23, 27), and the sorted protein. Although portions of the C-terminal region of PC1 contains one or more peptide sequences predicted to contain an α-helix. After an overnight chase period in labeled AtT-20 cells stably transfected with the appropriate construct. Cells were homogenized in pH and calcium conditions resembling the membranes. As shown in Fig. 6, at least half of the total labeled proteins could still be observed after an overnight chase period (Fig. 3C and data not shown).

A Potential Structure Involved in Sorting of Fc-PC1 Fusion Proteins to the Regulated Secretory Pathway—Although primary sequence comparison of the two minimal sorting domains of the PC1 C-terminal tail did not reveal an apparent homologous peptide sequence, secondary structure predictions indicated that both the 667–713 and 711–753 regions were expected to contain an α-helix. In addition, nuclear magnetic resonance and circular dichroism spectroscopy of a peptide comprising amino acids 727–753 indicate that this peptide indeed forms an amphipathic α-helix.5 To test whether this structure could be involved in sorting of the fusion protein to the regulated secretory pathway, we altered this structure in Fc-PC1-(711–753) by converting two leucine residues at positions 745 and 749 to proline residues. Structural algorithms predict that this change, in contrast to more subtle changes that we have tested, ensures specific disruption of the predicted α-helix in the C-terminal portion of the resulting Fc-PC1-(711–753) fusion protein (Fig. 3A). As shown in Fig. 3B, the proline mutations did not reduce expression of the fusion protein in GH4 cells but dramatically reduced sorting to secretory granules. Indeed, Fc-PC1(Pro745,Pro749) showed a mean value of stimulus-dependent secretion comparable to the Fc protein alone (Table I), indicating constitutive secretion of the mutated fusion protein. Since long chase periods cannot be performed with GH4 cells because of their high rate of basal secretory granule release as mentioned above, AtT-20 cells were used to verify the altered retention of the fusion protein with the mutated α-helix. After an overnight chase period in labeled AtT-20 cells, Fc-PC1-(711–753) could still be released from the cells upon stimulation (Fig. 3C); however, Fc-PC1(Pro745,Pro749) was undetectable in the supernatant, consistent with the absence of storage of this fusion protein in secretory granules. Immunofluorescence labeling carried out to confirm the intracellular localization of the fusion proteins was consistent with the results obtained from stimulated release assays in transfected cells. In AtT-20 cells transfected with Fc-PC1-(711–753), fluorescence could be detected at the tips of the cellular processes where the secretory granules are known to be localized (Fig. 4). However, in AtT-20 cells expressing the mutant Fc-PC1(Pro745,Pro749) construct, the bulk of the fluorescence was observed in a para-nuclear region consistent with localization of the protein passing through the Golgi apparatus (Fig. 4). Taken together, these results suggest that sorting of Fc-PC1 fusion proteins to the secretory granules is achieved by a mechanism dependent on the presence of an α-helical structure in the sorted protein.

Role of Protein-Protein Interactions in Sorting Fc-PC1 Fusion Proteins to the Regulated Secretory Pathway—Whereas an α-helix appears to be necessary for sorting of the Fc-PC1 fusion proteins to the regulated secretory pathway, it is unclear whether this structure confers some necessary property to the protein itself (such as aggregation) or whether it enables the PC1 C-terminal domain to interact with other proteins that contribute to PC1 entry in nascent secretory granules. To test if specific protein-protein interactions were involved in selective sorting of Fc-PC1 fusion proteins, intracellular cross-linking in transfected GH4 cells was performed. Following a short labeling period, GH4 cells were chased for 1 h at 20 °C to block protein export from the TGN, the intracellular compartment where the sorting mechanism is expected be functional. The cells were then incubated with a membrane-permeable cross-linker (DSS) on ice for 15 min. Following lysis of the cells, the different species of Fc-containing proteins produced from the cross-linking reaction were immunoprecipitated. As shown in Fig. 5, less than 5% of total immunoprecipitated Fc appeared as a protein species of higher molecular weight, indicating little proximal protein association with Fc. In contrast, at least a third of the immunoprecipitated Fc-PC1 proteins were present as protein species of higher molecular weight, corresponding to the sizes expected for dimers of each fusion proteins. Cross-linked Fc-PC1-(667–753) and Fc-PC1-(711–753) proteins were also observed when the chase was performed at 37 °C, indicating the protein complex was stable following entry in secretory granules (not shown). However, at least 30% of Fc-PC1-(627–670), which is not efficiently sorted to secretory granules (Table I), also appeared incorporated in cross-linked protein species (Fig. 5), indicating that this characteristic could not discriminate between fusion proteins sorted to the constitutive or regulated secretory pathways. Although portions of the C-terminal region of PC1 mediate close protein interactions, most probably the formation of dimers, these interactions do not correlate with protein sorting to the secretory granules.

Membrane-binding Properties of Fc-PC1 Proteins Sorted to Secretory Granules—To determine if sorting of Fc-PC1 fusion proteins involved specific membrane association of the stored proteins, a crude membrane fraction was prepared from GH4 cells stably transfected with the appropriate construct. Cells were homogenized in pH and calcium conditions resembling those present in the trans-Golgi network (23, 27), and the post-nuclear supernatants were sonicated in the presence of saponin, to favor complete disruption of vesicles, before pelleting the membranes. As shown in Fig. 6, at least half of the total immunoprecipitated Fc-PC1-(667–713) and Fc-PC1-(711–753) proteins, which are sorted to secretory granules (Table I), were found associated with the membrane pellet. In contrast, Fc-PC1-(627–670) and Fc-PC1(Pro745,Pro749), which are not sorted to the secretory granules (Table I), were mostly found in the supernatant with approximately 20% of the total immunoprecipitated proteins in the membrane pellet. These results indicate that the portions of the C-terminal region of PC1 capable of targeting an Fc fusion protein to the secretory granules also mediate binding of the fusion protein to membranes within the sorting compartments of the secretory pathway.

DISCUSSION

The present results provide clear evidence that the C-terminal region of PC1 contains one or more peptide sequences capable of acting as sorting domains to the regulated secretory pathway. These results are in agreement with a previous report in which a C-terminally truncated form of PC1 was shown

### Table I

| Construct                  | Stimulus-dependent secretion |
|----------------------------|-----------------------------|
| Fc                        | 1.58 ± 0.33                |
| Fc-PC1-(627–753)           | 3.25 ± 0.38                |
| Fc-PC1-(627–670)           | 1.88 ± 0.50                |
| Fc-PC1-(667–713)           | 3.53 ± 0.73                |
| Fc-PC1-(667–753)           | 3.39 ± 0.43                |
| Fc-PC1-(711–753)           | 4.12 ± 0.63                |
| Fc-PC1(Pro745,Pro749)      | 1.89 ± 0.59                |

p < 0.001 relative to the stimulated release value of Fc using Student’s t test.

2 C. Lazure, personal communication.
to undergo constitutive secretion from transfected AtT-20 cells (12). Although mutations in the conserved RRGDL motif in the P domain of the PC1 convertase also result in impaired sorting of the protein (28, 29), these mutations lead to substantial degradation of the mutant PC1 proteins in the endoplasmic reticulum, suggesting that the mutant proteins are improperly folded. In contrast, several studies have shown that C-terminal deletions do not lead to improper folding of PC1 but rather to efficient expression of a form of the enzyme with enhanced proteolytic activity (5, 12, 30). Thus, the inefficient sorting of PC1 missing its C-terminal tail is not likely due to improper folding of the protein.

We have previously shown that the C-terminal domain of PC1 partially inhibits the activity of PC1 in the constitutive secretory pathway, a mechanism that could explain the observed secretory granule-specific processing of certain proproteins by PC1 (5). The C-terminal domain thus ensures targeting of PC1 activity to the regulated secretory pathway by two mechanisms both presumed to occur in the TGN as follows: first, by partially inhibiting its catalytic activity prior to PC1 entry in the secretory granules, and second, by mediating sorting of the protein to the secretory granules.

The search for the minimal sorting domain in the C-terminal region of PC1 revealed that two independent segments were sufficient to target an Fc fusion protein to the regulated secretory pathway. Both of these segments contained regions predicted to form α-helices. Disruption of the predicted α-helix in the sorting domain that composes the last 40 amino acids of the C-terminal region resulted in constitutive secretion of the fusion protein, suggesting that the helical structure is crucially important for protein targeting to the secretory granules and that this α-helix could represent the minimal requirement for granule sorting to occur. Insertion of the L745P and L749P mutations in the entire PC1 resulted in premature cleavage of the C-terminal region in the secretory pathway, making it impossible to judge the role of this α-helix on the sorting of native PC1 because the sorting domain is lost prematurely (data not shown). Data on the three-dimensional structure of PC1 protein will evidently be informative in defining the multiple interactions involving the C-terminal domain as well as the functional importance of these interactions.

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The reaction of Fc-PC1 fusion proteins with cross-linking agents in the TGN did not reveal specific associations with other proteins residing in the TGN such as chromogranins, which have been shown to enhance the storage of endogenous hormones in AtT-20 cells (31). In addition, if a specific sorting receptor contributes to protein entry in nascent secretory granules, an expected Fc-PC1-receptor complex should have been observed with fusion proteins undergoing sorting to secretory granules. In line with this, when a fusion protein containing...
the entire C-terminal tail of PC1 was overexpressed in AtT-20 cells, the sorting to the secretory granules of both the fusion protein and PC1 (which is endogenously expressed in these cells) appeared unaltered, indicating that the sorting mechanism was not easily saturable (not shown). Our results are thus consistent with the sorting of PC1 not occurring by a saturable protein-receptor-mediated mechanism.

Our analysis of membrane preparations demonstrated that the fusion proteins, which had been shown to undergo efficient sorting to secretory granules, were consistently found associated with the membrane pellet. In addition, this feature could clearly distinguish proteins sorted to secretory granules from constitutively secreted proteins. Based on these observations, we propose that the amphipathic α-helix in the C-terminal region of PC1 mediates binding to lipid components of the membrane in the TGN or the immature granules. Association with membranes could serve in removing PC1 from the soluble phase and preventing its entry in constitutive-like vesicles budding from immature granules (17). The interaction of the C-terminal domain with membranes might induce a conformational change, which possibly triggers the C-terminal autocatalytic cleavage and subsequent maximal activation of the enzyme, events known to occur in the secretory granules.

The C-terminal domain has been postulated to be involved in membrane binding of the protein because of the predicted amphipathic helical segment near its extremity (32, 33). Previous studies that have investigated the association of PC1 with the membrane fraction have not found a requirement of the C-terminal domain for membrane association (34, 35). However, it is possible that some of the methods used to prepare membrane fractions lead to incomplete lysis of the secretory granules in which the C-terminally truncated form of PC1 would then remain trapped. Studies with CPE have suggested a link between the capacity of the protein to associate with membranes and CPE targeting to the secretory granules, since the C-terminal region of CPE, which also contains a putative amphipathic α-helix, has been shown to mediate membrane binding of both CPE and an albumin fusion protein (36). Yet the role of membrane binding in targeting CPE to the secretory granules was recently questioned because fusion proteins containing portions of the C-terminal of CPE underwent constitutive secretion even though they appeared to associate with membranes (37). Although the correlation we have observed between the association of Fc-PC1 fusion proteins with membranes and their sorting to secretory granules could be circumstantial, the dramatic effect of the L745P and L749P mutations in disrupting both membrane binding and protein sorting suggests that these interactions are indeed involved in the sorting process. Binding of regulated secretory proteins to the phospholipid constituents of membranes has been shown to occur in an acidic pH-dependent manner (25). Whether the interaction of the C-terminal domain of PC1 with specific lipid microdomains is involved in the retention of PC1 in secretory granules remains to be determined.

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