A unique feature of the eukaryotic subtilisin-like proprotein convertases (SPCs) is the presence of an additional highly conserved sequence of approximately 150 residues (P domain) located immediately downstream of the catalytic domain. To study the function of this region, which is required for the production of enzymatically active convertases, we have expressed and characterized various P domain-related mutants and chimeras in HEK293 cells and α-TC1–6 cells. In a series of C-terminal truncations of PC3 (also known as PC1 or SPC3), PC3-Thr\textsuperscript{594} was identified as the shortest active form, thereby defining the functional C-terminal boundary of the P domain. Substitutions at Thr\textsuperscript{594} and nearby sites are crucial for activity. Chimeric SPC proteins with interspersed P domains demonstrated dramatic changes in several properties. Compared with truncated wild-type PC3 (PC3-Asp\textsuperscript{616}), both PC3/PC2Pd and PC3/FurPd had elevated activity on several synthetic substrates as well as reduced calcium ion dependence, whereas Fur/PC2Pd was only slightly decreased in activity as compared with truncated furin (Fur-Glu\textsuperscript{683}). Of the three active SPC chimeras tested, all had more alkaline pH optima. When PC3/PC2Pd was expressed in α-TC1–6 cells, it accelerated the processing of proglucagon into glicentin and major proglucagon fragment to release GLP-1 and tGLP-1, similar to wild-type PC3. Thus, P domain exchanges generated fully active processing of proglucagon into glicentin and major proglucagon fragment; GLP-1, glucagon-like peptide 1, t, truncated; pGlu, pyroglutamic acid.

Recently, a new family of serine proteases that process a wide variety of proprotein substrates has been identified in eukaryotic cells. Based on the similarity of their catalytic domain to the subtilisins, these proteases have been named subtilisin-like proprotein convertases (SPCs)\textsuperscript{1} (Refs. 1–4; for terminology see Ref. 5). To date, in addition to kexin, a yeast homologue (6, 7) and a number of SPCs found in lower species, seven SPCs have been identified in mammals as follows: furin\textsuperscript{2} (PACE or SPC1), PC3 (SPC3 or PC1), PC2, PC4, PC5/PC6, PC7/PC8 (or LPC), and PACE4 (1, 8, 9).

Each convertase has a distinct but overlapping substrate specificity and a distinctive tissue distribution, subcellular location, and maturation process, consistent with its unique role in some aspect of proprotein processing. Well characterized examples regarding these aspects are furin (expressed ubiquitously in almost all tissues), PC3, and PC2 (both restrictedly distributed in neuroendocrine tissues). We have only a limited understanding of the structural determinants which differentiate the various SPCs from each other in their function and properties. Their basic domain structure includes (Fig. 1) a signal peptide, a partially conserved propeptide, a highly conserved catalytic domain (40–50% identity among the SPCs and 25–30% to the subtilisins) followed by a relatively well conserved region called the P, homoB, or “middle” domain. Studies in recent years have clarified the role of propeptide cleavage in furin activation and the function of the propeptide as an intramolecular inhibitor which prevents early activation (10).

After the P domain, various C-terminal extensions occur; these extensions seem to contain mainly routing/trafficking determinants. For instance, furin is primarily located in the trans-Golgi network, anchored by its transmembrane domain, and mutations in its cytoplasmic domain result in dramatic changes in its subcellular location (11, 12). Attachment of the P domain and C-terminal region of PC2 to the catalytic domain of furin resulted in a chimera which behaved similarly to PC2 in that it was now targeted to regulated pathway vesicles (13), whereas a truncated furin without a transmembrane domain was secreted via a non-regulated (constitutive or basal) secretory pathway. Deletion of residues from the C terminus of PC3 gave a mutant (terminating at Asp\textsuperscript{616}) that was secreted in increased amounts via the constitutive pathway, whereas lesser amounts were routed into regulated secretory granules in A/T-20 cells (14). The C-terminal region after Asp\textsuperscript{616} of PC3 may also function as an inhibitor (15).

Little is known about the function of the P domain. Due to functional differences between the subtilisins (degradative) and kexin (proteolytic processing), the conserved region after the catalytic domain of kexin was named the “P domain” when it became evident that it was required for processing activity (16). Although there are a few examples of bacterial subtilisins with extensions after the catalytic domain, a search of the protein data bank does not reveal any significant sequence similarities.
The P domain participates in the regulation of the pH and calcium dependence, as well as the substrate specificity of kexin and furin, a partial C-terminal deletion of the P domain, and/or non-endocrine mammalian cell lines. Our findings provide evidence that in addition to stabilizing the catalytic domain, the P domain participates in the regulation of the pH and calcium dependence, as well as the substrate specificity of these enzymes.

**MATERIALS AND METHODS**

**Construction of Vectors Encoding Mutant SPCs**

cDNA templates for rat PC3 (PC1), PC2, and PC3-Asp616 (PC1ΔC) (14) were kindly provided by Dr. Richard Mains at the Johns Hopkins University; human furin template was from Dr. Kazuhisa Nakayama at the University of Tsukuba, Japan. pCMV6b/6c (b and c: identical except for the variable C-terminal extensions) were cloned into either the pCMV6b or pCMV6c vector for expression in mammalian cells. pCMV6b or pCMV6c vector for expression in mammalian cells.

**Truncation and Substitution Mutants—** Three truncation mutants, PC3-His592, PC3-Gly593, and PC3-Thr594, were created by adding a stop code and a restriction site immediately after the desired amino acid, respectively. In the substitution study, Thr594 in mutant PC3-Thr594 was replaced with serine, aspartic acid, and asparagine, respectively. Asp616 and/or non-endocrine mammalian cell lines.

**Domain Swapping Mutants—** Three chimeric proteins, PC3/PC2Pd, PC2/PC3Pd, and PC3/FurPd, were generated by using the "gene splicing by overlap extension" (SOE) technique (23). PC3/PC2Pd consists of amino acids 1–453 of PC3 and amino acids 456–638 of PC2. PC2/PC3Pd was a fusion protein with amino acids 1–455 of PC2 and amino acids 454–616 of PC3. Mutant PC3/FurPd consists of amino acids 1–453 of PC3 and amino acids 454–583 of furin. Mutant Fur/PcPd (18) was a kind gift from Dr. John Creemers (Katholieke Universiteit, Leuven, Belgium).

**Wild-type controls**—Three chimeric proteins, PC3/PC2Pd, PC2/PC3Pd, and PC3/FurPd, were generated by using the "gene splicing by overlap extension" (SOE) technique (23). PC3/PC2Pd was created by fusing the amino acids 1–453 of PC3 and amino acids 456–638 of PC2. PC2/PC3Pd was a fusion protein with amino acids 1–455 of PC2 and amino acids 454–616 of PC3. Mutant PC3/FurPd consists of amino acids 1–453 of PC3 and amino acids 454–583 of furin. Mutant Fur/PcPd (18) was a kind gift from Dr. John Creemers (Katholieke Universiteit, Leuven, Belgium).

A truncated PC3 (Ref. 14; PC1ΔC, designated PC3-Asp616 in this study) and a truncated furin (Ref. 18; furin 374–476-end, designated Fur-Glu583 in the present study) were used as wild-type controls in this study.

All mutants and wild-type control SPCs were cloned into either the pCMVb6 or pCMVb6 vector for expression in mammalian cells.

**Transfection and Cell Culture**

HEK293 cells (human embryonic kidney tumor cells) and α-TC1–6 cells (mouse pancreatic alpha cells) were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). SPC mutant vectors were transiently transfected into HEK293 cells using the calcium phosphate method (Promega). When stable transfection was desired, as indicated under “Results,” cells were transfected using the Lipofectin method. Plasmid pSV2neo was co-transfected with the SPC mutant-carrying vectors to provide drug resistance. Transfected cells were then cultured in G418-containing medium (0.5 mg/ml); G418-resistant colonies were screened for the expression of interested SPC protein by biosynthetic labeling/immunoprecipitation, Western blotting, or immunofluorescence staining.

**Metabolic Labeling and Analysis of SPC Proteins**

Processing of transfected SPC protein was characterized by biosynthetic labeling. For transiently transfected cells, the labeling was performed 48 h after transfection. Briefly, cells were first incubated in methionine-deficient media for 30 min and then pulse-labeled with [35S]methionine (1 mCi/ml, 1000 Ci/mmol, Amersham Pharmacia Biotech) with or without a subsequent chase incubation in nonradioactive complete media. Upon termination of incubation, media were collected, and cellular proteins were extracted with a TES buffer containing 20 mM TES, pH 7.4, 10 mM mannitol, and 1% Triton X-100 (24). For immunoprecipitation, samples were dialyzed with an immunoprecipitation buffer (50 mM Tris, pH 8.0, 250 mM NaCl, 0.5% Nonide P-40, 0.02% NaN3) (25). Appropriate antibodies (as specified under “Results”) and protein A (for polyclonal antibodies) or protein G (for monoclonal antibodies) (Pierce) were added. Protease inhibitors (26) were present throughout the sample collection and immunoprecipitation procedures. Immunoprecipitated SPC proteins were fractionated on SDS-PAGE slab gels (7.5% or 10%) and detected by fluorography.

**Fig. 1.** Domain structure of SPCs and mutants used in this study. The numbers in parentheses indicate the percent identity of each indicated domain among mouse furin, PC3, and PC2. The comparison was performed using the GeneWorks program (IntelliGenetics, Inc., Mountain View, CA). The variable C-terminal extensions include serine/threonine-rich region (kexin), cysteine-rich region followed by a transmembrane domain (kexin, furin, PC6b, and PC7), cysteine-rich region only (PACE4), or C-terminal amphipathic helix (PC3 and PC2). PC3-Asp616 and Fur-Glu583 were truncated forms of PC3 or furin prepared for previously published studies (14, 18). They were used as wild-type controls in the present work.

**Fig. 2.** The shortest active form of PC3 terminates at Thr594. HEK293 cells stably transfected with recombinant PC3-Asp616, PC3-Gly593, and PC3-Thr594 were labeled with [35S]methionine for 30 min and then chase incubated for 3 h in non-radioactive medium. At the end of chase incubation, media were collected, and cells were extracted as described under “Materials and Methods.” Samples were then immunoprecipitated with anti-PC3 antibody and analyzed by SDS-PAGE and fluorography. (Polyclonal antibody RS20 was raised against a peptide comprising the propeptide/catalytic domain junction of PC3 (hSPC3.4). It recognizes both unprocessed and processed PC3 proteins.) P, pulse-labeled cell extracts; C, cell extracts after chase incubation; M, chase media. Four independent experiments with identical results were performed.
Analysis of Enzymatic Activity on Synthetic Substrates

HEK293 cells expressing various SPCs were incubated in complete serum-free Dulbecco’s modified Eagle’s medium for 15–20 h. Collected medium was concentrated 20–30-fold using a 30K Ultrafree concentrator (Millipore Corp.). Enzymatic activity assays followed the protocol described by Vindrola and Lindberg (27) using pGlu-Arg-Thr-Lys-Arg-MCA as the substrate. In the substrate specificity assay, Boc-Arg-Val-Arg-Arg-MCA, Boc-Leu-Ser-Arg-MCA and Boc-Ala-Gly-Pro-Arg-MCA (where Boc is t-butoxycarbonyl) were the test substrates used (Peninsula Laboratories).

In experiments aimed at comparing the activity levels of various SPC chimeras, cells were cultured in duplicate plates with equal cell numbers. One plate was used to collect medium and analyze its activity. Cells in the second plate were pulse-labeled with [35S]methionine for 1 h and then chased incubated as described previously (28). After immunoprecipitation with the appropriate antibodies (see “Results”), subjected to SDS-PAGE slab gel analysis. Upon loading the SDS-PAGE gel, each sample was mixed with non-radioactive rainbow molecular weight markers. Fractionated samples were electrophoretically blotted onto Immobolin-P membrane (Millipore); blot areas corresponding to the appropriate molecular weight range of the processed SPCs were excised, treated with a stripping buffer (62.5 mM Tris, 2% SDS, 100 mM β-mercaptoethanol, pH 6.7) at 50 °C for 30 min, followed by scintillation counting. Preliminary tests established that loaded radioactivity could be quantitatively recovered by this procedure. Based on the radioactivity recovered from each immunoprecipitation/SDS-PAGE, after correction for the number of methionine residues in each SPC protein, the amount of medium collected from the duplicate plate used for assay of activity was adjusted so that equimolar amounts of SPC were used in each activity assay.

Analysis of Proglucagon-related Peptides in α-TC1–6 Cells

Wild-type or transfected α-TC1–6 cells were labeled with either [35S]methionine, as described above, or [3H]leucine (0.4 μCi/ml; Amersham Pharmacia Biotech). Cellular protein extraction and immunoprecipitation were performed as described previously (28). After immunoprecipitation with the appropriate antibodies (see “Results”), immunoprecipitated samples were fractionated on a gradient SDS-PAGE slab gel; [3H]leucine-labeled samples were analyzed by SDS-PAGE tube gels. Tube gels were sliced and eluted (29), followed by scintillation counting. Non-radioactive rainbow molecular weight markers were added with samples as internal standards.

RESULTS

The Shortest Active Form of PC3 Terminates at Thr594

Among the known mammalian SPCs, PC3-Asp616, which migrated as a 67-kDa species in the present study (Fig. 2), is the shortest active form. It is normally produced in neuroendocrine cells after the autocatalytic removal of the propeptide in the endoplasmic reticulum and the subsequent deletion of a C-terminal fragment (amino acids 617–736) in the secretory granules (14, 30). Non-endocrine cells usually do not endogenously express PC3. Its relative simplicity, selective distribution, and our greater understanding of its maturation process and function make this truncated form of PC3 an ideal model convertase for studies on the P domain.

Of the mammalian P domains, the last two conserved amino acids in the C-terminal region, presumably marking their junction with the C-terminal extensions, are Gly593 and Thr594 (numbers are for rat PC3). PC3-Asp616 contains an additional 22 amino acids beyond this conserved region. To define more precisely the shortest sequence needed for activity, three truncation mutants ending at or near these conserved amino acids were expressed in HEK293 cells. Their propeptide cleavage activity and secretion were examined by a pulse-chase metabolic labeling paradigm (Fig. 2). PC3-Asp616 undergoes very efficient autocatalytic cleavage to remove its propeptide. During a 30-min pulse-labeling period, the majority of 75-kDa pro-PC3-Asp616 was converted to 67-kDa mature PC3, and most of the latter was secreted into medium during the subsequent 3-h chase incubation. For truncation mutant PC3-Thr594 (Fig. 2, right), the dominant form during the 30-min pulse was a molecule slightly smaller than 75 kDa. Later, it was processed into a 65-kDa protein, an expected size for this truncated PC3 after removal of its propeptide. The conversion rate of pro-PC3-Thr594 to PC3-Thr594 was not as efficient as that of pro-PC3-Asp616 to PC3-Asp616, but after 3 h of chase incubation, a significant amount of the processed PC3-Thr594 was secreted into the medium. In contrast, neither PC3-Gly593 (Fig. 2, middle) nor PC3-His594 (data not shown) was processed or secreted.

To determine whether the lack of propeptide cleavage in PC3-Gly593 and PC3-His594 was due to the absence of threonine at the C terminus of the P domain, Thr594 in PC3-Thr594 was replaced with serine, aspartic acid, or asparagine, respectively. Fig. 3 shows a biosynthetic labeling experiment on HEK293 cells expressing PC3-T594S, T594D, and T594N. After a 30-min pulse and a 3-h chase incubation, a small amount of mature 65-kDa protein was seen in the media of cells expressing PC3-T594S and PC3-T594N, whereas it was not visible...
with mutant PC3-T594D. For mutant PC3-H592T, in which His592 in the PC3-His592 mutant was replaced with threonine, there was no detectable propeptide cleavage activity. These results thus demonstrated that the shortest form of PC3 capable of cleaving its propeptide is PC3-Thr 594. The few conserved amino acids at the C terminus of the P domain region (His592, Gly593, and Thr 594) also seem to play a crucial role in sustaining the autocatalytic activity of PC3. This also confirmed that the P domain of PC3 has a C-terminal well defined boundary.

**Biosynthesis and Processing of Chimeric SPCs with Foreign P Domains**

The relatively high level of conservation of the P domains among various SPCs suggests that these domains must play some essential role. We therefore reasoned that interchanges of P domains might provide information on these possible function(s). Accordingly, P domain-swapped SPCs were prepared and transfected into HEK293 cells (Fig. 1). The autocatalytic activation of each chimeric form was studied by pulse-chase metabolic labeling, and its processing and secretion were compared with that of the parental proteases.

**Activities of Chimeric SPCs**

The successful propeptide cleavage and secretion of the chimeric SPCs suggested that they were able to fold and be transported in HEK293 cells. Next we attempted to determine whether these chimeric enzymes were active in cleaving substrates. Our preliminary experiments established that wild-type HEK293 cells in our culture system secrete negligible amounts of endoproteolytic activity toward MCA-linked synthetic substrates, as tested over a range of pH values and calcium concentrations. For the chimeric SPCs, we found that calcium was required for autoproteolytic activation and secretion, and that the minimum calcium requirement for secretion was in the range of 1–2 mM. This is consistent with previous reports that calcium has an essential role in the autoproteolytic activation of both furin and PC3.

**Calcium dependence of chimeric SPC activity**

Medium samples were collected after incubating cells in complete serum-free medium for 16–24 h and then concentrated through Ultrafree Centrifugal Filter Device (cutoff 30 kDa). The assay mixture contained (final concentration) 200 mM sodium acetate, pH 5.8, 200 mM pGlu-Arg-Thr-Lys-Arg-MCA, 10 mM pepstatin A, 100 mM N-tosyl-L-phenylalanine chloromethyl ketone, 1.7 mM phenylmethylsulfonyl fluoride, 10 μl of medium sample, and calcium chloride with various concentrations as indicated in the figure. At 0 mM calcium concentration, 2 mM EGTA or 2 mM EDTA was also added to deplete calcium bound to SPC proteins. The reaction mix was then incubated at 37°C for 16 h, quenched with 1x acetic acid, and subjected to fluorospectrophotography. The activity levels were presented as percentages to the maximum activities within the calcium ion concentration range used in this study. Each point represents mean ± S.E. of 3–10 independent preparations and assays.

**pH dependence of chimeric SPC activity**

Sample collection and activity assays were performed as described in Fig. 5 with 5 mM calcium chloride. pH gradient was created with 200 mM sodium acetate and 100 mM Bis-Tris (56). The activity levels at different pH were demonstrated as percentages of activities at optimum pH. Each point represents mean ± S.E. of 3–4 independent experiments.

PC3/PC2Pd was initially synthesized as a 79-kDa protein, which was then converted into a 69-kDa protein through an intermediate (Fig. 4, top panel). The conversion rate was slower than that of the propeptide processing of PC3-Asp 616. The secretion of 69-kDa PC3/PC2Pd protein, however, was as efficient as the secretion of PC3-Asp 616. The counterpart mutant, PC2/PC3Pd, did not show any propeptide cleavage activity (Fig. 4, bottom panel), even when 7B2 (a unique PC2 helper protein) (31–33) was co-expressed and the chase incubation was extended to 8 h (data not shown).

Mutant PC3/FurPd also displayed propeptide cleavage activity, although it was much less efficient than that of PC3-Asp 616, and the secretion of the processed form was relatively slow (Fig. 4, middle panel). The presence of two forms in the cells after a 3-hour chase presumably represents differences in glycosylation status. During the course of this study, Creemers et al. (13) reported that, in their study on the sorting mechanisms of SPCs, a chimeric protein with the catalytic domain of furin and the P domain of PC2 underwent efficient propeptide cleavage and secretion via the regulated secretory pathway in AtT-20 cells. When we expressed this mutant in HEK293 cells, it also showed efficient propeptide cleavage and secretion, similar to that of Fur-Glu 583 (data not shown).
were compared at their optimum pH and with 10 mM calcium ion concentrations. Collected media samples from HEK293 cells expressing various SPC mutants and wild-type SPCs were analyzed for their activities against fluorogenic substrates. PC3/PC2Pd, PC3/FurPd, and Fur/PC2Pd were all active (see below).

Three major enzymatic properties that distinguish eukaryotic SPCs from bacterial subtilisins are their dependence on calcium for activation and activity, their more acidic pH optima, and their requirement of two or more basic amino acids at the substrate cleavage site. Efforts were made to address each of these properties in the mutants.

Ca²⁺ Dependence—We first tested the calcium dependence of the active chimeras. Between 0 and 20 mM calcium ion concentration, PC3-Asp616 showed a gradual increase of activity. The activity level plateaued above 20 mM calcium ion concentration. Omitting calcium and adding 2 mM EGTA or 2 mM EDTA to the assay completely suppressed the activity of PC3-Asp616 (Fig. 5, top panel). Quite strikingly, PC3/PC2Pd retained 40–55% of its activity in the presence of EGTA or EDTA, and its activity increased only moderately over the range from 0 to 40 mM calcium ion concentration. PC3/FurPd (Fig. 5, top panel), on the other hand, had little activity in the presence of EGTA or EDTA. It gained most of its activity in the lower range of calcium ion concentration (0–5 mM). The activity of Fur/PC2Pd responded to the changes in calcium ion concentration in a pattern similar to that of Fur-Glu583 (Fig. 5, bottom panel), i.e. no activity in the presence of EGTA or EDTA and gaining most of the activity in the low range of calcium ion concentrations.

pH Dependence—Full-size PC3 and C-terminally processed PC3 have different acidic pH optima (34, 35). Similar to other published results, PC3-Asp616 secreted from HEK293 cells showed maximal activity at pH 6.0 within a narrow range (Fig. 6, top panel). Fur-Glu583 had a broader optimum pH, ranging between pH 7 and pH 8 (Fig. 6, bottom panel). The optimum pH for PC3/PC2Pd was shifted to between pH 7.0 and pH 8.0 (Fig. 6, top panel). The optimum pH for PC3/FurPd was pH 7.0 (Fig. 6, top panel). The optimum pH for Fur/PC2Pd shifted to between pH 7.0 and pH 8.0 (Fig. 6, bottom panel). Taken together, all three chimeric SPC proteins had a more alkaline pH optima than their parental wild types.

Relative Activity Levels and Substrate Specificity—The activity levels of various SPC chimeras with fluorogenic substrates were compared at their optimum pH and with 10 mM calcium ion concentrations (Table I, top). The specific activities of PC3/PC2Pd and PC3/FurPd were 3–4-fold higher than the activity of PC3-Asp616, respectively, whereas the activity of Fur/PC2Pd was slightly lower than the activity of Fur-Glu583. PC3-Thr594 had a level of activity similar to that of PC3-Asp616 (data not shown); PC3-T594S and PC3-T594N, although secreted in minor amounts, did not show any detectable enzymatic activity (data not shown).

The substrate specificity of the SPCs is a complex and incompletely understood issue. For a given SPC, specificity may be greatly influenced by the amino acid sequence and conformation of the substrate-binding sites, as well as their accessibility. When the activities of wild-type and chimeric SPCs were tested against two commonly used fluorogenic substrates (at pH 5.8 and in the presence of 5 mM CaCl₂) (Table I, bottom), PC3-Asp616 had lower activity toward pGlu-Arg-Thr-Lys-Arg-MCA as compared with Arg-Val-Arg-MCA, whereas PC3/PC2Pd showed an increased activity toward Arg-Val-Arg-MCA. On substrates with a single basic amino acid only at the P1 position (Leu-Ser-Thr-Arg-MCA and Ala-Gly-Pro-Arg-MCA), none of the convertases tested showed any significant activity.

PC3/PC2Pd Activity in Vivo—To examine the activity of the chimeric SPCs with a natural preprotein substrate, we created α-TC1–6 cell lines stably expressing either PC3/PC2Pd or PC3-Asp616 (as a control).

Our α-TC1–6 cell line endogenously expresses proglucagon and PC2, but no detectable amount of PC3 (28). Previous studies have established specific roles of PC2 and PC3 in proglucagon processing in different neuroendocrine cell lines (28, 36, 37). In wild-type α-TC1–6 cells, in a typical metabolic labeling experiment (Fig. 7A), the major peptides identified with an antiserum against glucagon were 19-kDa proglucagon, 9-kDa glucagon, and a minor amount of 4.5-kDa oxyntomodulin; a GLP-1 antiserum recognized proglucagon, and 8-kDa MPGF, and a minor amount of GLP-1. This result is consistent with published studies. When PC3-Asp616 or PC3/PC2Pd was expressed in α-TC1–6 cells, greater amounts of glicentin (Fig. 7A, band 2) and oxyntomodulin (band 4) peptides were produced and secreted in both cell lines, as compared with that in wild-type cells. The production of glicentin in PC3-Asp616 cells and PC3/PC2Pd cells could be seen even during the pulse period. A more profound change in proglucagon processing in PC3-Asp616 cells and PC3/PC2Pd cells, however, was the production of GLP-1 and tGLP-1, which was barely detectable in wild-type cells (Fig. 7B). The production of these two peptides, normally an indication of the involvement of active PC3, e.g. in intestinal L cells (36–38), was even greater in the α-TC1–6 PC3/PC2Pd cells than in PC3-Asp616 cells. Also noticeable in the α-TC1–6 PC3/PC2Pd cells and PC3-Cys616 cells was a new fragment smaller than MPGF (tMPGF: truncated MPGF), presumably

### Table I

| SPC                                | Activity at optimum pH with pGlu-Arg-Thr-Lys-Arg-MCA and 10 mM calciuma |
|------------------------------------|-----------------------------------------------------------------------|
| PC3-Asp616                         | 314 ± 74                                                              |
| PC3/PC2Pd                          | 443 ± 148                                                             |
| PC3/FurPd                          |                                                                       |
| Fur-Glu583                         | 63 ± 31                                                               |
| Fur/PC2Pd                          |                                                                       |

| Percent activity toward pGlu-Arg-Thr-Lys-Arg-MCA (100%) versus Arg-Val-Arg-MCA at pH 5.8 and 5 mM calciuma |
|------------------------------------------------|------------------------------------------------------------------|
| PC3-Asp616                         | 39 ± 8.5                                                          |
| PC3/PC2Pd                          | 178 ± 10                                                          |
| PC3/FurPd                          | 131 ± 18                                                          |
| Fur-Glu583                         | 98 ± 13                                                           |
| Fur/PC2Pd                          | 85 ± 12                                                           |

a Equimolar amounts of PC3-Asp616, PC3/PC2Pd, and PC3/FurPd or Fur-Glu583 and Fur/PC2Pd proteins were used in each assay, as described under “Materials and Methods.” Briefly, cells expressing a particular SPC were cultured in duplicate plates. Cells from one plate were metabolically labeled with [³⁵S]methionine. Radioactive convertases secreted into the media were immunoprecipitated, analyzed by SDS-PAGE, and blotted onto Immobolin-P membrane. Radioactivities in blot areas corresponding to the appropriate molecular weight range of each of the processed SPCs were compared among samples from the expressing cells. Corrections in medium volume used for assay were made on the basis of both recovered radioactivity and the number of methionine residues in each SPC protein.

b p < 0.05.

c For each SPC protein, equal volumes of medium sample were used for the assay of activity toward two different substrates. Activity toward Arg-Val-Arg-MCA was expressed as the percentage of activity toward pGlu-Arg-Thr-Lys-Arg-MCA.
generated by cleavage at the single arginine site within MPGF that gives rise to tGLP-1 (36, 38). To summarize briefly, the chimeric protein PC3/PC2Pd was active in vivo; it cleaved proglucagon in a manner similar to that of wild-type PC3-Asp616.

**Discussion**

The P domains in the proprotein convertases are unique sequences in that they are well conserved and their appearance in evolution parallels the acquisition of the specificity for multiple basic residues and other important properties that distinguish these proteases from the subtilisins. In this study, we describe the creation and expression in mammalian cells of several active chimeric SPC proteins, which have allowed us to address the functional role(s) of the P domain in greater detail.

We first investigated the C-terminal region of PC3-Asp616. A truncation study clearly defined the C-terminal boundary of the P domain of PC3 to be at Thr594. Similar findings have been reported for yeast kexin; a kexin protein terminating at Glu593, which aligns with Thr594 of PC3, was active but also exhibited a relatively slower rate of maturation and secretion (17). In PC3, Thr594 could not be replaced with other amino acids bearing a similar charge or side chain size, nor could this threonine be reserved but placed at another position (PC3-H592T). The decisive role played by Thr594 suggests that the C-terminal end of the P domain of PC3 interacts with other regions in the PC3 protein or is required for its structural integrity. A human PC3 gene mutation has been reported recently in which Gly593 was mutated to arginine, resulting in an inactive PC3 protein (22). This finding is in complete agreement with our results and supports the conclusion that this region of the P domain of PC3 is crucial for the generation of an active enzyme.

The C termini of the chimeric SPCs used in this study did not end precisely at the last conserved amino acids (Thr594 in PC3, Thr594 in PC2, and Thr573 in furin). Instead, mutant PC3/PC2Pd included the entire C-terminal region of PC2, and PC3/FurPd was extended to Glu583 of furin. Creemers et al. (13) reported that a PC2 protein truncated after Thr594 was inactive. The shortest active form of PC2 is not known at this time. The shortest active form of furin as tested in vitro was a truncated form ending at Glu583 (18); another truncated form, ending at amino acid 576, has also been reported but without data on its secretion or activity (39). More recently, it has been demonstrated that a furin protein ending at Thr573 is active when expressed in Pk15 cells (13). The foregoing findings imply that these last two conserved amino acids (Gly and Thr) do not precisely define a commonly shared C-terminal boundary for the P domains in all SPCs. Additional sequence or other structural elements might be needed in a molecule-specific manner.
The regulatory role of calcium on convertase activity is well documented (34, 35, 40–47). On the other hand, although calcium plays a role in stabilizing subtilisin, the enzymatic activity of subtilisin is not regulated by calcium and may not be sensitive to chelators (48–50). Calcium-binding sites in a number of subtilisin-related proteases are known from crystallographic studies (49, 51), whereas the structural basis for the calcium dependence in the SPCs is unknown. In thermotase, Ca1 (calcium-binding site 1, strong, $K_{D} \approx 10^{-8}$ M) was fully occupied without added medium calcium ions, whereas Ca2 (calcium-binding site 2, medium strength) exhibited various degrees of occupancy at 0, 5, and 100 mM calcium (51). Sites homologous to Ca1 and Ca2 have been predicted to be present in the catalytic domain of furin (52). Sequence alignment illustrates the conservation of several amino acids located in these predicted calcium-binding sites in other SPCs as well (4). Other than these, no sequences resembling known calcium-binding motifs (e.g. EF-hands, etc.) have been identified in the convertases. If the calcium-binding sites in the convertase catalytic domains are functionally similar to those in subtilisin or thermotase, then at the calcium concentrations that prevail along the secretory pathway in vivo, one of the sites (Ca1) should be fully occupied, whereas the second site (Ca2) might be playing regulatory roles. It should be mentioned that there are no disulfide bonds in the subtilisins, whereas two disulfide bonds have been predicted in the catalytic domain of the SPCs (52, 53). It is reasonable to postulate that bound calcium may have a dual functional role in the convertases, stabilizing the structure and regulating the activity.

The changes in calcium dependence that we have observed in the chimeric SPCs clearly indicate the importance of the P domain in regulation of activity. Since chimeric SPC proteins with identical catalytic domains but different P domains showed altered patterns of calcium dependence, it is likely that additional calcium-binding site(s) may either occur within the P domain or at new sites created by the intersection of the P and catalytic domains. All evidence taken together suggests that multiple calcium-binding sites may be involved in regulating convertase activity.

In vitro analyses indicate that PC3, PC2, and furin have different pH optima within the acidic to neutral range. PC3 has the lowest (pH 5.0), that of PC3 is also more acidic (pH 5.5–6.0), whereas that of furin is nearer neutrality (34, 35, 40–44, 54). It is thus very surprising that the chimeras PC3/PC2Pd, PC3/PC2Pd all demonstrated more alkaline pH optima than the corresponding wild-type SPCs, values ranging from pH 7 to 9. The subtilisins in general have similar neutral to alkaline pH optima. Our results thus strongly suggest that in wild-type SPC, the authentic P domain somehow enables the convertase catalytic domain to function optimally in an acidic environment. An alternative explanation could be that the chimeric enzymes were destabilized at more acidic pH values, possibly due to changes in surface charge in the Glu- and Asp-rich substrate-binding region. In either case, however, these results indicate that structural interactions between the P and catalytic domains are needed to maintain function at a neutral or acidic pH.

In an in vivo system (proglucagon processing in α-TC1–6 cells), PC3/PC2Pd was as active as PC3-Asp161. It maintained a similar cleavage site selectivity to that of PC3-Asp and both enzymes seemed to be able to cleave at the single arginine site (residue Arg27 of proglucagon) required for the production of tGLP-1 and tMPGF. The increased activity of the PC3 chimeras toward synthetic substrates in vitro was also surprising, as was the altered activity of PC3/PC2Pd versus PC3-Asp161 toward certain synthetic substrates (pGlu-Arg-Thr-Lys-Arg-MCA versus Arg-Val-Arg-Arg-MCA). Such findings, however, are not without precedent in other serine proteases, i.e. that alteration in the region following the subtilisin-like catalytic domain would change the substrate specificity. One such example is the Lactococcus lactis proteinase (55). L. lactis proteinase has two isoforms, which differ in only 44 out of 1902 amino acids. Both isoforms have C-terminal extensions (>1200 amino acids) downstream of the catalytic domain. Fragment exchanges between the two isoforms within the C-terminal region resulted in a different pattern of casein cleavage.

The structural relationship between the catalytic domain and the P domain at present is not known; there has been no published crystal structure of any SPC to date. By using predictive methods and computer-assisted modeling, we have found that the P domains are likely to have an eight-stranded β-barrel motif as their core structure. This independently folded domain, in turn, is likely to interact with the catalytic domain in the SPCs through a set of hydrophobic contacts in a defined patch that exists on the surface of the catalytic domain opposite the active site in the SPCs but not in the subtilisins

Presumably, it is through conformational changes in this latter region that these regulatory effects are exerted. An overall conserved intrinsic structure of the P domain would explain why P domain swapping gives rise to functional chimeras. However, the dramatic property changes we have observed in these chimeras are more difficult to explain. In preliminary thermostability experiments and limited endoprotease digestion experiments, no significant differences between PC3-Asp161 and PC3/PC2Pd were observed, implying that domain swapping did not result in significant conformational destabilization. Crystallographic studies and further mutagenesis to map more precisely important sequence determinants in the P domain may shed light on these questions.

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