

Modulation of PC1/3 Activity by Self-Interaction and Substrate Binding

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Prohormone convertase (PC)1/3 is a eukaryotic serine protease in the subtilase family that participates in the proteolytic maturation of prohormone and neuropeptide precursors such as proinsulin and proopiomelanocortin. Despite the important role of this enzyme in peptide synthesis, how PC1/3 activity is regulated is still poorly understood. Using ion exchange chromatography and two-dimensional gel electrophoresis we found that natural PC1/3 present in AtT-20 cells and bovine chromaffin granules, as well as recombinant PC1/3 secreted from overexpressing Chinese hamster ovary cells, exists as multiple ionic forms. Gel filtration and cross-linking studies revealed that protein oligomerization and aggregation contribute greatly to variability in surface charge. The most acidic forms of PC1/3 contained both inactive aggregates as well as oligomerized 87-kDa PC1/3 that exhibited stable activity which was partially latent and could be revealed by dilution. No such latency was observed for the more basic, 66/74-kDa forms of PC1/3. Fractions containing these species were stabilized by preincubation with micromolar concentrations of either fluorogenic substrate or peptides containing pairs of basic residues. In addition, the most active form of 87-kDa PC1/3, a probable homodimer, was activated by preincubation with these same peptides. Cleavage by PC1/3 is often the initiating step in the biosynthetic pathway for peptide hormones, implying that this is a natural step for regulation. Our data suggest that enzyme oligomerization and peptide stabilization represent important contributing factors for the control of PC1/3 activity within secretory granules. (*Endocrinology* 152: 1402–1411, 2011)

Peptide hormones and neuropeptides are initially produced as inactive prohormones that must be cleaved and posttranslationally modified before acquiring bioactivity. One of the first steps of modification involves endoproteolytic events that are mediated by a family of subtilisin-like serine proteases in the secretory pathway. This family includes prohormone convertase (PC)1 (also known as PC3 and PCSK1, and here referred to as PC1/3), PC2, PC4, PC5/6, PC7/8, PC9, PACE4, and furin. PC1/3 and PC2 are neuroendocrine-specific PCs (reviewed in Refs. 1 and 2) and were first described 20 yr ago (3, 4). One or both of these calcium-dependent enzymes are found in the regulated secretory pathway of nearly all neuroendocrine tissues, where they cleave peptide precursors pre-

dominantly following a pair of basic residues, most often KR and RR. It is now clear that these two enzymes are responsible for the initiation of biosynthesis of the majority of neuropeptides and peptide hormones, *e.g.* ACTH and β -lipotropin from proopiomelanocortin (POMC) (5) in the hypothalamus and pituitary, or insulin from proinsulin in β -cells (6).

Although no genetic defects associated with PC2 have been described to date, increasing numbers of genome-wide association studies have shown that mutations and single-nucleotide polymorphisms of PC1/3 are associated with increased body weight, insulin sensitivity and processing, and variations in blood glucose (7–14). The first human PC1/3 mutation to be described was a compound heterozygote re-

sulting in a null phenotype; this mutation resulted in extreme obesity, intestinal dysfunction, and highly increased levels of circulating prohormones (7). Since then, two other human cases have been reported with similar clinical phenotypes (8, 9). In addition, a mouse strain bearing a mutation in the PC1/3-encoding gene also exhibits an obese phenotype (14). PC1/3 is highly conserved, with 92.6% homology between human and mouse sequences; the mouse and human enzymes have virtually identical characteristics in terms of chromatographic behavior, pH dependency, Ca^{2+} dependency, substrate specificities, and molecular forms (15). Despite the broad range of effects that PCs exert on biological processes through their production of peptide hormones and neuropeptides, regulatory mechanisms for PC activity remain poorly understood.

Like the peptide hormones they process, PCs are also initially produced as precursors. Unlike other convertases, PC2 is known to transit the secretory pathway as a zymogen and is converted to mature PC2 only within secretory granules (16). All other convertases, including PC1/3, undergo propeptide cleavage in the endoplasmic reticulum; whether the PC1/3 propeptide continues to remain associated with PC1/3, as is the case for furin (17), is not yet known. The removal of the PC1/3 prosequence yields a mature 87-kDa form. In the acidic environment of the granules, this 87-kDa form is further intermolecularly cleaved, resulting in the formation of a 66-kDa form that is highly active, but also highly labile (18–20). It is unclear whether the 10-kDa C-terminal tail interacts with PC1/3 as an inhibitor at certain concentrations (21) or performs a more complex function (20, 22).

In addition to autocatalytic maturation, PCs can be regulated by their binding partners. For example, pro-PC2 maturation is directly tied to the expression of its bifunctional binding partner 7B2. During transport, the N-terminal domain of 7B2 acts to block unproductive zymogen aggregation, which leads to the formation of inactive enzyme (16), whereas the 7B2 C-terminal peptide is a potent inhibitor of active PC2 (23). PC1/3 also possesses a binding partner, proSAAS (24). Unlike 7B2, proSAAS expression is not obligatory for the production of active PC1/3 (25), but its C-terminal peptide is a potent inhibitor of PC1/3 *in vitro* (24, 26–29).

We recently reported that pro-PC2 and PC2 have a large propensity to aggregate, a process that the PC2 escort protein 7B2 helps to block (30). Like PC2, recombinant PC1/3 is also susceptible to inactivating aggregation (31); whether or not aggregation occurs intracellularly has not yet been investigated. In the report below, we present evidence that both recombinant and natural PC1/3 forms exist as multiple ionic populations consisting of monomers, oligomers, and aggregates and describe the effects of self-association on enzyme

activity. We further address the activation of PC1/3 by small peptides containing pairs of basic residues.

Materials and Methods

Recombinant PC1/3 ion exchange purification

Chinese hamster ovary (CHO) cells overexpressing PC1/3 (31) were incubated overnight with Opti-MEM (Invitrogen, Carlsbad, CA) in roller bottles. The conditioned medium (450 ml) was filtered through a 0.22- μm filter, diluted with two volumes of Buffer A (20 mM BisTris; 2 mM CaCl_2 ; 0.4 mM dodecyl maltoside; 0.02% NaN_3 , pH 7.0), pumped onto a 10 \times 15 cm MonoQ anion exchange column, washed with 30 ml of Buffer A and eluted with the following gradient to Buffer B (1 M sodium acetate; 20 mM BisTris; 2 mM CaCl_2 ; 0.4 mM dodecyl maltoside; 0.02% NaN_3 , pH 7.0): 0–35% Buffer B in 60 min, 35–100% Buffer B in 60 min, and isocratic elution with Buffer B for 10 min. Fractions (4.5 ml) were collected at a flow rate of 2 ml/min.

Gel filtration

Aliquots of PC1/3-containing ion exchange fractions were size fractionated using two Superdex 200 10/300GL columns connected in series and eluted with gel filtration buffer (10 mM BisTris; 150 mM sodium chloride buffer; 6 mM CaCl_2 ; 0.4 mM dodecyl maltoside, pH 6.5). The flow rate was 0.3 ml/min and either 1-ml fractions or 0.4-ml fractions were collected.

Enzyme assay

PC1/3 activity was measured as the rate of cleavage of a synthetic fluorogenic substrate, pERTKR-amc (pyroGlutamate-Arginine-Threonine-Lysine-Arginine-aminomethylcoumarin) in 50 μl of reaction buffer, 0.1 M sodium acetate (pH 5.5), containing 0.1% BSA, 5 mM calcium, and 0.2% octyl glucoside at 37 C for 30–120 min, depending on the assay.

SDS-PAGE and Western blotting

Aliquots of purification fractions were denatured with Laemmli sample buffer containing 6 M urea, boiled and separated on 7.5% Tris-HCl SDS-PAGE gels (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins were then transferred onto nitrocellulose membranes and Western blotted using polyclonal rabbit antiserum raised against the N terminus of PC1/3, 2B6 (32).

In vitro cross-linking

Recombinant PC1/3 aliquots obtained from gel filtration fractions (0.03 mg/ml) were treated with 0.1% glutaraldehyde for 30 min at 4 C. The reactions were quenched with the addition of 200 mM ethanolamine for 15 min at room temperature and subjected to reducing gel electrophoresis on 7.5% gels.

Preparation of chromaffin granules

Forty bovine adrenals were dissected and homogenized in 0.3 M sucrose solution and subjected to a series of centrifugation steps. Briefly, the sucrose homogenate was centrifuged at 9000 rpm for 20 min to remove microsomes and then at 10,000 rpm for 20 min to pellet granules and mitochondria. The resulting pellet was then resuspended in 0.3 M sucrose, layered over a 1.6 M sucrose layer, and centrifuged at 35,000 rpm for 1 h. The

resulting eight pellets were rinsed with 1.6 M sucrose and stored at -20°C until use. One fifth of one pellet was extracted with 500 μl of Opti-MEM (Invitrogen), clarified by centrifugation, and used for isoelectric focusing experiments.

Isoelectric focusing

Cell and granule lysates were prepared in Opti-MEM medium (Invitrogen) to provide a same-solution comparison with secreted PC1/3 obtained from CHO cells; aprotinin (100 $\mu\text{g}/\text{ml}$) as well as a separate inhibitor mix was included (one tenth volume of a $10 \times$ stock, 20 μM E-64, 20 μM pepstatin, and 20 mM phenylmethylsulfonyl fluoride). Samples were applied to 11-cm IPG strips (pH 5–8) (Bio-Rad) and actively rehydrated at 50 V for 12 h at 10 $^{\circ}\text{C}$ in rehydration buffer containing 8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 10 mM dithiothreitol (DTT), 0.2% Bio-Lyte (Bio-Rad). Proteins were focused at 8000 V for 35,000 kVh, with a current limit of 50 $\mu\text{A}/\text{strip}$. Strips were then reduced with 50 mM Tris-HCl (pH 8.8), 6 M urea, 2% SDS, 30% glycerol, 1% DTT for 10 min, alkylated with the same buffer but containing 1.5% iodoacetamide substituted for DTT, and then transferred to 10% Tris-HCl (Bio-Rad) gels for SDS-PAGE (second dimension) and Western blotted for PC1/3, as described above. After blotting, membranes were stripped, reblocked, and blotted for transferrin as an internal standard for direct comparison of different runs. Two-dimensional gel electrophoresis was performed on each sample at least twice, and ionic strength in each sample was kept constant by diluting each sample 10-fold in rehydration buffer.

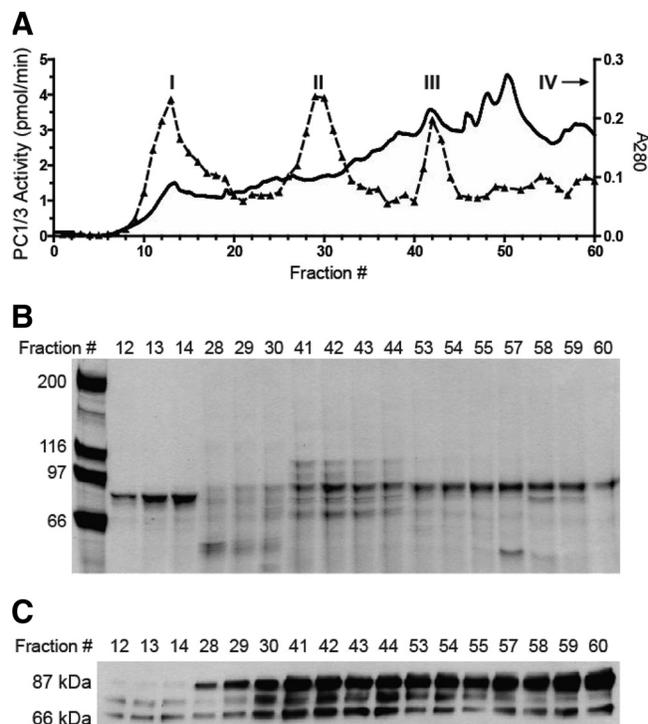


FIG. 1. Recombinant PC1/3 exists as multiple ionic forms. Conditioned medium from CHO/PC1/3 cells was filtered, diluted, loaded onto the MonoQ column, and eluted with a sodium acetate gradient as described in *Materials and Methods*. UV absorbance was monitored at 280 nm (—) and aliquots of each fraction (5 μl of 4.5-ml fraction) were assayed for PC1/3 activity ($-\blacktriangle-$) (panel A). Activity peaks were stained with Coomassie blue (panel B) or Western blotted using N-terminal PC1/3 antiserum 2B6 (panel C).

Radiation inactivation experiments

Recombinant human PC1/3 was partially purified from the medium of GH4C1 cells infected with hPC1/3-expressing vaccinia virus (15). For the determination of the target size (TS), hPC1/3 was labeled with 1 mCi of L-[^{35}S]methionine, and the fragmentation of ^{35}S -labeled hPC1/3 was monitored by SDS-PAGE. Band intensities of intact PC1/3 were quantified using imaging software, and the logarithm of the protein band intensity was plotted as a function of radiation dose. D_{37} (where D_{37} represents the dose necessary to decrease band intensity to 37% of its initial value) was obtained by linear regression analysis and was used to calculate the target size using the following equation, based on a previously determined set of protein standards (33): $\text{TS} = 1.77 \times 10^6/D_{37}$. For the determination of the radiation inactivation size (RIS), 2 U of PC1/3 (where 1 U releases 1 pmol/min of amc from acetyl-RSKR-amc) were rapidly frozen in 1.5-ml tubes and subjected to 1.2 Mrad/h for varying lengths of time in a γ -Cell model 220 ^{60}Co source (Nordion International, Kanata, Ontario, Canada). The temperature was kept at -78°C by the addition of crushed dry ice. The solutions were then thawed, and the residual enzyme activity was determined using the pERTKR-amc substrate; each assay was performed in triplicate and repeated with two independent enzyme preparations. D_{37} (where D_{37} represents the dose necessary to diminish enzyme activity to 37% of its initial value), was obtained by linear regression analysis of residual PC1/3 activity as a function of radiation dose. The RIS was then obtained using this D_{37} , follow-

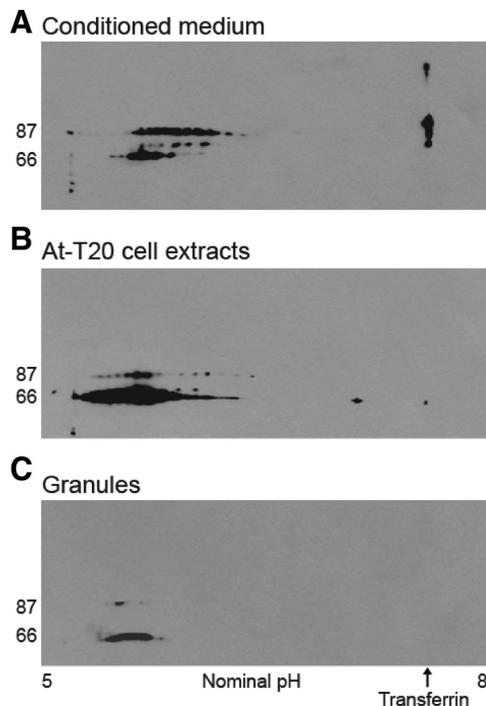


FIG. 2. Natural PC1/3 also exists in multiple ionic populations. Two-dimensional gel electrophoresis of secreted recombinant PC1/3 collected from CHO cells (panel A); AtT-20 cell lysates (panel B); and bovine chromaffin granules (panel C). Proteins were first separated by isoelectric points on pH 5–8 IPG strips followed by separation by size on 10% SDS-PAGE. After the second dimension, proteins were Western blotted with antiserum 2B6 for PC1/3, followed by Western blotting for transferrin as an internal standard. For each run, a parallel experiment was performed with pI markers (Bio-Rad) and stained for reference points.

ing a previously determined experimental relationship between enzyme activity and radiation dose (34): $RIS = 1.29 \times 10^6/D_{37}$.

Dynamic light scattering

Solutions (0.1–0.2 mg/ml) of ion exchange fractions were subjected to dynamic light scattering on a Malvern Zetasizer Nano at 4 C. Late ion exchange fractions from two independent preparations of PC1/3 were diluted either with ion exchange Buffer B, or enzyme assay buffer lacking BSA; the experiment was repeated twice for each preparation.

Substrate stability studies

PC1/3 was preincubated in reaction buffer at 37 C for 2 h together with various peptides and/or the fluorogenic substrate at the concentrations indicated. At the time of assay, pERTKR-*amc* was added to a final concentration of 200 μ M, and PC1/3 activity was measured and compared with identical, nonpreincubated reactions. All peptides except RTKR (Arginine-Threonine-Lysine-Arginine) were synthesized at more than 85% purity at the University of Maryland, Baltimore Biopolymer Core Facility; RTKR was synthesized at Louisiana State University Health Science Center Core Laboratories.

Results

Recombinant and natural PC1/3 exist as multiple ionic forms

To purify recombinant PC1/3, we collected conditioned medium from DHFR-amplified CHO cells overexpressing mouse PC1/3 (in which the major secreted protein is PC1/3; see Ref. 31) and performed anion exchange chro-

matography (31). Figure 1A depicts absorbance at 280 nm and the enzymatic activity of a representative purification. This figure shows multiple PC1/3 activity peaks (labeled I–IV) that elute along the entire salt gradient; coupled with Coomassie staining (Fig. 1B) and Western blotting (Fig. 1C), these data suggest the presence of distinct PC1/3 populations with different surface charges. This general profile was reproduced at least five times with different batches of conditioned medium, although there was variability in the amounts of the last two peaks. Activity peak I exhibited high activity, most likely due to the presence of the more active 66-kDa PC1/3 form, as shown by Western blotting (Fig. 1C); however, Coomassie staining (Fig. 1B) and Western blotting (data not shown) confirmed that the major protein in these early fractions was 74-kDa transferrin, likely obtained from the Opti-MEM collection medium. In contrast, activity peaks III and IV were richest in the 87-kDa form (Fig. 1C). To confirm the presence of multiple ionic PC1/3 populations, we separated an aliquot of the conditioned medium using two-dimensional gel electrophoresis. Again, we observed multiple PC1/3 populations that differed in surface charge (Fig. 2A).

Tissue PC1/3 also exists as multiple ionic populations

We then investigated whether natural tissue PC1/3 also exists in multiple ionic populations. In Fig. 2, B and C, we prepared AtT-20 cell lysates (a mouse anterior pituitary cell line) and bovine chromaffin granules and separated proteins

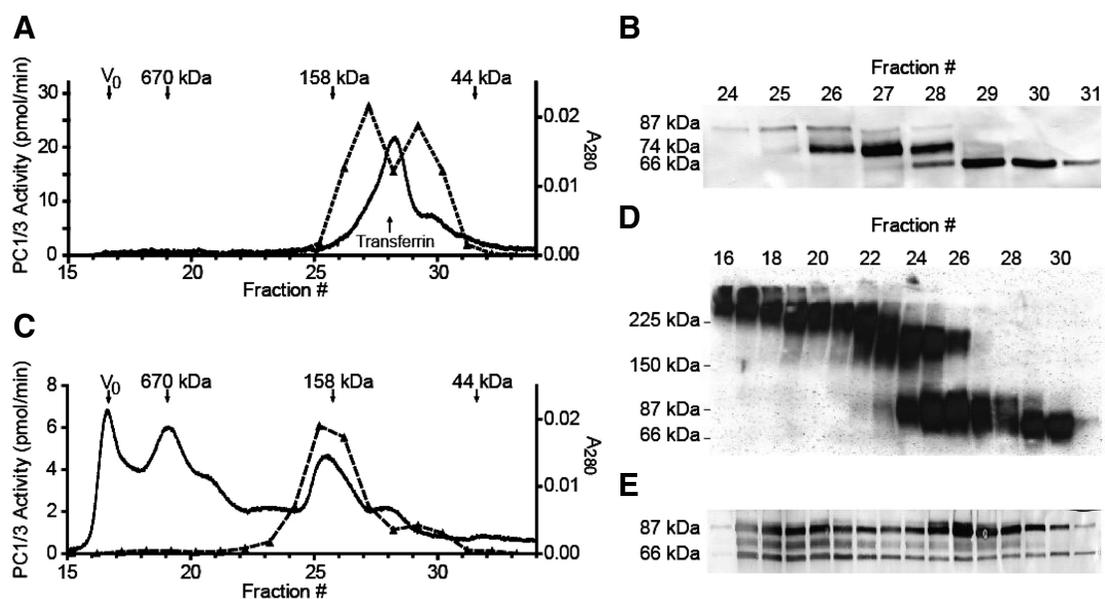


FIG. 3. More acidic PC1/3 forms represent defined oligomers and aggregates, whereas basic PC1/3 forms are monomeric. A, Activity peak I from Fig. 1A was concentrated and loaded onto two Superdex columns connected in series. Protein elution was monitored by at 280 nm (—) and 10 μ l of each 1-ml fraction were assayed for activity (— \blacktriangle). The void and elution volumes of molecular weight standards (Bio-Rad gel filtration standards) are indicated along the top of the panel. B, Activity peaks obtained from gel filtration in panel A were Western blotted for PC1/3. C, Gel filtration of the activity peak IV from Fig. 1A. Each fraction (10 μ l) was assayed for enzyme activity (— \blacktriangle). D and E, Of each 1-ml fraction obtained from panel C, 25 μ l were treated with 0.1% glutaraldehyde (panel D); or reduced in Laemmli sample buffer (panel E) and Western blotted using N-terminal PC1/3 antiserum 2B6.

by two-dimensional gel electrophoresis. We confirmed the presence of multiple PC1/3 populations exhibiting net surface charges between 4.5 and 6 (Fig. 2, B and C) in both cell samples, similar to our results with recombinant CHO cell PC1/3.

More acidic forms represent defined oligomers and aggregates, whereas more basic forms are monomeric

To further characterize the ionic populations of PC1/3, we subjected the two most extreme ion exchange peaks (activity peaks I and IV from Fig. 1A) to gel filtration. Gel filtration of activity peak I, *i.e.* the most basic form, yielded activity peaks at fractions 27 and 29 (Fig. 3A), which correlates with the abundance of 74 and 66 kDa PC1/3 in these fractions as observed by Western blotting (Fig. 3B). Coomassie staining, confirmed by Western blotting, showed that gel filtration fraction 28 contained mainly 74-kDa transferrin, explaining the lack of activity despite the large UV-absorbing peak. The fact that the elution volume of PC1/3 is similar to that of transferrin suggests that PC1/3 in activity peak I consists only of monomeric 74- and 66-kDa forms. In contrast, gel filtration of activity peak IV, *i.e.* the most acidic form, resulted in additional UV peaks eluting between the void volume and the 44-kDa marker, suggesting that the PC1/3 species in peak IV consists of a mixture of aggregated, oligomeric, and monomeric PC1/3 forms (Fig. 3C). The large activity peak eluting ahead of the 158-kDa marker (Fig. 3C) exhibited an estimated mass of approximately 216 kDa when calculated using a standard curve constructed from the logarithm of marker protein mass *vs.* elution volume. Interestingly, activity was found only in fractions containing monomeric 87- and 66-kDa PC1/3, and this 216-kDa form of PC1/3, but not in fractions containing highly aggregated PC1/3 forms (Fig. 3C). Cross-linking studies of late ion exchange fractions after gel filtration clearly demonstrated the presence of PC1/3 species with at least four different molecular masses ranging from two species larger than 225 kDa (aggregates); between 150 kDa and 225 kDa (probable dimer; see below); to monomeric 87/66 kDa PC1/3 (Fig. 3D). These aggregates and oligomers consisted mainly of 87-kDa PC1/3, although some 66-kDa PC1/3 was detected in these samples under non-cross-linked, reducing conditions (Fig. 3E). In addition, direct gel filtration of conditioned medium from CHO cells also resulted in PC1/3 eluting in all fractions between void and 44 kDa marker (Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>), indicating that PC1/3 aggregates and oligomers are not artifacts of the purification system. Similarly, cross-linking of live AtT-20 cells, using the cell-permeable cross-linking agent dithiobis(succinimidyl propionate), revealed endogenous oligomeric and aggregated forms of 87-kDa PC1/3 (Supplemental Fig. 2). It is inter-

esting to note that the 87-kDa PC1/3 is the main form that is cross-linked, as evidenced by the preferential disappearance of this band with increasing concentrations of dithiobis(succinimidyl propionate). Lastly, gel filtration coupled with Western blotting of chromaffin granules also supports the idea that natural PC1/3 exists in forms of varying sizes (Supplemental Fig. 3).

Radiation inactivation experiments support the idea that the active PC1/3 species is an oligomer

Radiation inactivation analysis was used to determine both the target size and the enzyme inactivation size of PC1/3. After irradiation of ^{35}S -labeled PC1/3 at different doses, samples were subjected to SDS-PAGE to monitor protein fragmentation. The extent of protein destruction with increasing irradiation is directly related to protein

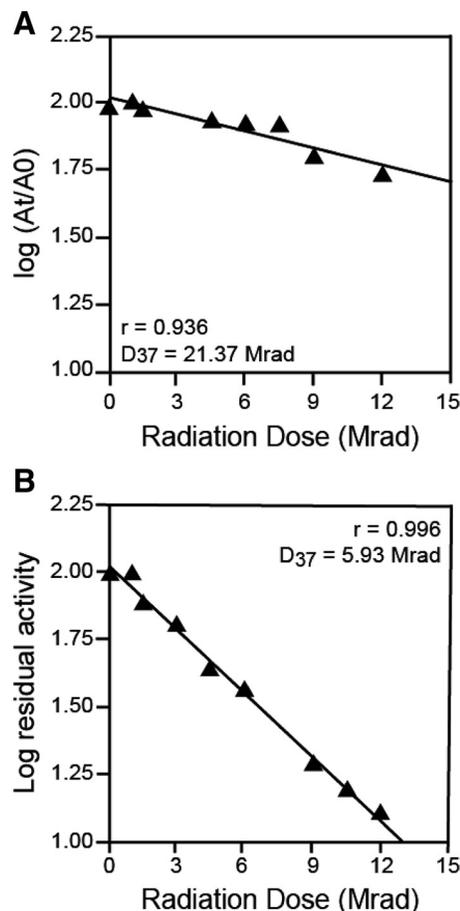


FIG. 4. Radiation inactivation experiments show that the target size of PC1/3 is 89 kDa but the active protein is 218 kDa. A, ^{35}S -labeled hPC1/3 was exposed to increasing amounts of radiation and subjected to SDS-PAGE. The intensity of the intact PC1/3 band was plotted as a function of irradiation dose to obtain D_{37} (the dose necessary to decrease band intensity to 37%), which was then used to calculate the target size. B, hPC1/3 was exposed to increasing irradiation dose and after irradiation, residual PC1/3 activity was measured and plotted against irradiation dose. The D_{37} was obtained from this plot and used to calculate the radiation inactivation size. Calculations were performed using the equations in *Materials and Methods*.

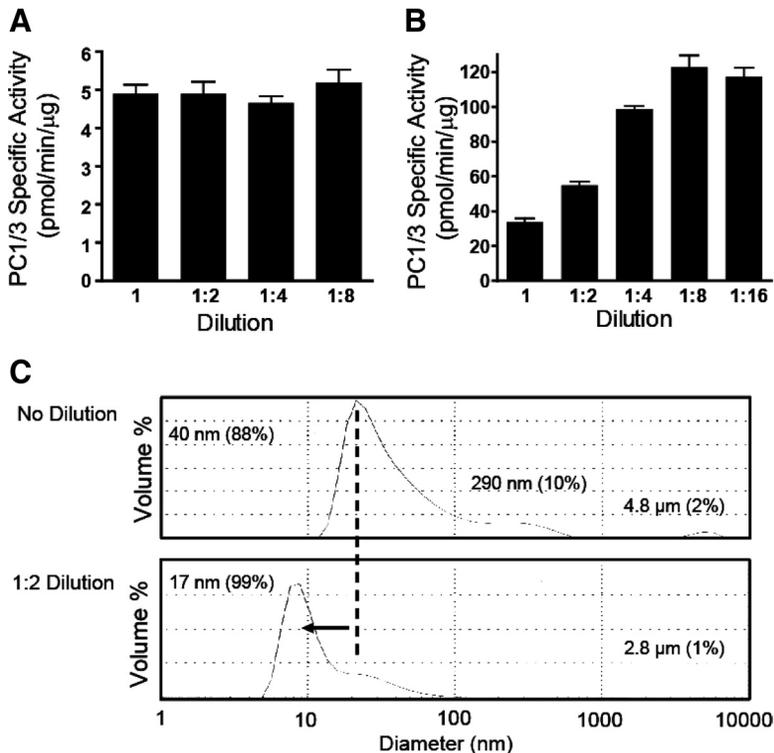


FIG. 5. Dilution of recombinant PC1/3 increases specific activity and decreases apparent size. Activity peak I from Fig. 1A containing only monomeric PC1/3 (panel A) or activity peak IV from Fig. 1A containing aggregates, oligomers, and monomers (panel B) were serially diluted in PC1/3 assay buffer and assayed for activity. Specific activity was calculated by dividing activity by the amount of PC1/3 protein added to the reaction, as measured by Bradford assay. Each bar represent the means \pm SD, $n = 3$. Light scattering experiments reveal that the apparent size of PC1/3 present in activity peak IV decreases upon dilution (panel C).

size, and a linear plot of the band intensity of remaining unfragmented, full-length PC1/3 against irradiation dose resulted in correlation coefficients of 0.936 and 0.901 in two independent experiments (one of which is shown in Fig. 4A). Using a previously determined relationship between radiation and protein molecular mass (33), the calculated D_{37} value from this experiment, 19.9 ± 1.5 Mrad, leads to an estimated target size value of 89 ± 7 kDa. This is in good agreement with the observed molecular mass obtained by Coomassie staining of denatured, full-length PC1/3 determined by SDS-PAGE (87 kDa). When PC1/3 solutions were irradiated at different doses and the log of residual activity was instead plotted against irradiation dose, a linear relationship with a correlation coefficient of 0.996 was obtained (Fig. 4B). Two independent activity experiments showed correlations of 0.968 and 0.971, leading to an average D_{37} value of 5.9 ± 0.1 . Using an equation employing known enzyme standards (34), this D_{37} value corresponds to a molecular mass of 218 ± 5 kDa, which is significantly larger than the actual target size. When these data are taken together with cross-linking SDS-PAGE data showing that active fractions contain a molecular species migrating below the position of the 225-

kDa marker (Fig. 3D), these results support the idea that the majority of active PC1/3 in this peak exists as a homodimer composed solely of 87-kDa PC1/3.

Dissociation of recombinant PC1/3 regulates activity; light scattering experiments show activation is accompanied by a decrease in size

The 66-kDa form of PC1/3 is an unstable enzyme that loses activity rapidly after purification (18, 20). We observed that the activity of oligomer-containing fractions consistently remained more stable than fractions containing only monomers. We therefore asked whether a higher molecular weight form was responsible for this prolonged PC1/3 activity, perhaps by slow dissociation to an active species. To promote such dissociation, we diluted aliquots of activity peak IV from Fig. 1A (an oligomer-containing ion exchange fraction) in PC1/3 assay buffer and measured the effect of dilution on PC1/3 activity. Serial dilution resulted in a dramatic increase in the specific activity of PC1/3 (Fig. 5B), whereas dilution of activity peak I (presumed monomeric 66/74 kDa PC1/3) had no effect on specific activity (Fig. 5A). PC2 was similarly unaffected by dilution (data not shown). Because of differences in salt concentration and pH (the PC1/3 assay

buffer contains 0.1 M sodium acetate at pH 5.5, whereas activity peak IV was obtained in 1 M sodium acetate, pH 6.5), we repeated this experiment by diluting the ion exchange fractions in the corresponding higher salt and/or higher pH buffers. All experiments yielded similar results (data not shown), suggesting that dilution *per se* results in increased activity.

To assess whether a molecular size change could be detected upon dilution, we performed light-scattering experiments. Dilution of activity peak IV from Fig. 1A with ion exchange Buffer B or with PC1/3 assay buffer lacking BSA resulted in a greater than 20 nm decrease in the apparent diameter of the major protein population (Fig. 5C). This experiment was repeated two times with similar results. These results support the idea that dissociation of an oligomeric population is associated with increased activity.

Fluorogenic substrate and peptides containing a C-terminal pair of basic residues stabilize/activate various forms of PC1/3

We also investigated whether PC1/3 was subject to other forms of regulation, such as substrate or peptide

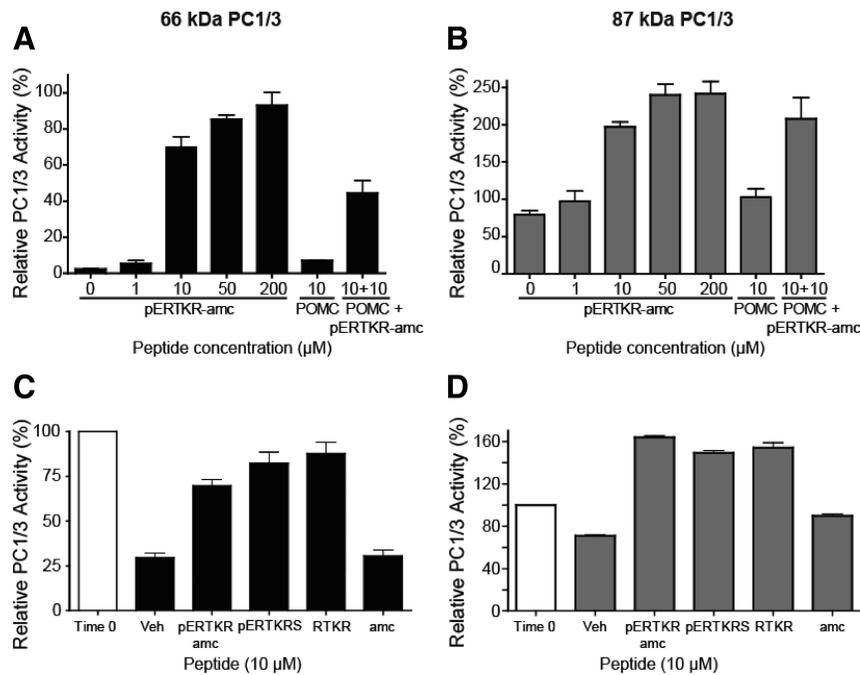


FIG. 6. Substrate-like molecules stabilize/activate various forms of PC1/3 activity in a dose-dependent manner. PC1/3 (66 kDa) (panel A) and 87-kDa PC1/3 (panel B) were preincubated with increasing concentrations of the synthetic substrate, pERTKR-amc, or with POMC, for 2 h at 37°C. Substrate concentration was adjusted to 200 μM immediately before the assay, and activity was compared with PC1/3 not subjected to preincubation. Activity greater than 100% indicates an activation event. PC1/3 (66 kDa) (panel C) and 87-kDa PC1/3 (panel D) were preincubated with 10 μM substrate, substrate-like peptides, or amc for 2 h at 37°C. At the end of the preincubation period, 200 μM substrate was added and PC1/3 activity was measured. Activity of 66 kDa and 87 kDa before preincubation was 1.9 RFU (relative fluorescence units)/min and 0.96 RFU/min (4.3 pmol/min and 2.2 pmol/min), respectively, for dose-dependent studies. For peptide stability assays, PC1/3 activity before preincubation was 2.8 RFU/min (6.4 pmol/min) for both 66- and 87-kDa PC1/3. Results represent mean ± SD of triplicate wells. Veh, Vehicle.

stabilization or activation. These parameters were assessed by preincubating various PC1/3 forms with or without the pERTKR-amc fluorogenic substrate (or other small peptides) at 37°C for 2 h. At the end of the preincubation period, enzyme activity was measured in all samples and compared with the activity obtained without preincubation. After 2 h at 37°C, 66- and 87-kDa PC1/3 lost 98% and 20% of their starting activities, respectively. However, in the presence of substrate, 66-kDa PC1/3 activity was stabilized, and 87-kDa PC1/3 activity was activated, both in a dose-dependent manner (Fig. 6, A and B). At least 10 μM substrate was required to observe these effects.

We also tested other small peptides, such as the non-fluorogenic peptide pERTKRS and the substrate cleavage product pERTKR, and obtained similar results (Fig. 6, C and D). The other possible cleavage product from the PC1/3-substrate reaction, amc, was inert. The peptides pERTSS and pERT (the final product of the fluorogenic substrate after carboxypeptidase E action) were also inactive (data not shown), suggesting that a terminal KR is required for the PC1/3 stabilization and activation effects. The possi-

bility that activation of the 87-kDa form was effected by increased conversion to the more active 66-kDa form was not substantiated by Western blotting, which showed no increase in the 66-kDa species during the course of the experiment (data not shown).

Because the 87-kDa PC1/3 enzyme used for these experiments contained a variety of PC1/3 forms with varying molecular masses (see Fig. 3, C and D), to determine which enzyme species was most affected by incubation with peptide, we separated activity peak IV PC1/3 from Fig. 1A using gel filtration. Taking small fractions to increase resolution, we measured activity either immediately, or after preincubation with or without 10 μM pERTKR-amc for 2 h at 37°C. Fractions assayed immediately exhibited an activity profile similar to that of Fig. 3C, showing activity peaks at fractions 63 and 67, correlating with the probable dimeric and monomeric forms of 87-kDa PC1/3. After preincubation with 10 μM pERTKR-amc, we observed a pronounced increase in enzyme activity in fractions 62–65 (Fig. 7A). Cross-linking studies revealed that these fractions contain both monomeric and the probable 87-kDa PC1/3 dimer (Fig. 7B). This experiment was repeated three times with different PC1/3 preparations with similar results.

Discussion

PC1/3, most often the first active convertase encountered by neuropeptide and peptide hormone precursors, clearly plays an initiating role in bioactive peptide synthesis. However, little is known about the regulation of its activity beyond its known proteolytic processing to smaller forms in endocrine cells (18–20) and its interaction with proSAAS (29, 26, 35). In the present study, we have identified multiple PC1/3 populations that differ both in charge and in size and have characterized the enzymatic activity of two such forms.

Ion-exchange chromatography demonstrated that recombinant PC1/3 consists of multiple species with varying surface charges. Two-dimensional gel electrophoresis of various samples, *i.e.* medium containing recombinant PC1/3, AtT-20 cell extracts, and chromaffin granule ex-

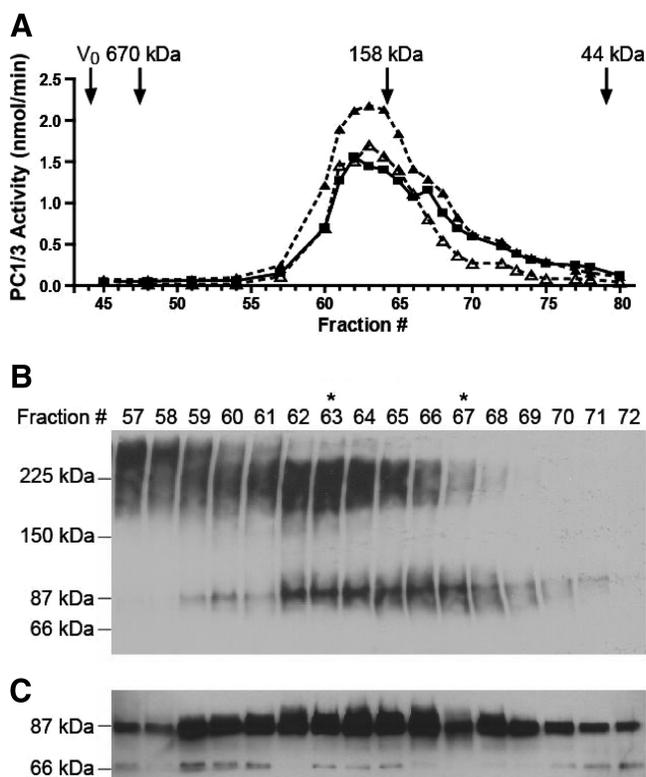


FIG. 7. Only a subset of PC1/3 molecules can be stabilized/activated by pERTKR-amc. To determine the size of the PC1/3 species responsible for the substrate activation, 10 μ l of the 0.4 ml gel filtration fractions shown were either assayed immediately (—■—) or after preincubation for 2 h at 37 C with (—▲—) or without substrate (—Δ—) (panel A). Molecular weight standards for this gel filtration chromatography are indicated at the top of the panel. Each activity point represents the mean \pm SD, $n = 2$. Activity peaks were either cross-linked with glutaraldehyde (panel B) or reduced (panel C) and Western blotted as described in *Materials and Methods*. Activity peaks that were stabilized or activated by the presence of substrate are marked with an asterisk.

tracts (Fig. 2), as well as medium from CHO/PC1/3 cells expressing lower levels of PC1/3 (data not shown), confirmed the presence of multiple ionic forms. These findings rule out the notion that differences in ionic populations arise as an artifact of either overexpression or purification and suggest instead that charge differences are likely to develop from intrinsic properties of this enzyme.

We used gel filtration chromatography to demonstrate that different ionic PC1/3 populations contain PC1/3 species with varying molecular masses. The most basic population of PC1/3 molecules exhibit high levels of enzymatic activity that correlate with the presence of monomeric 66-kDa and 74-kDa PC1/3, known to be much more active than 87-kDa PC1/3 (18, 20, 36). This was unexpected, because the removal of the C-terminal tail for 87-kDa PC1/3, which has a predicted isoelectric point (pI) of 9, results in a 66-kDa form predicted to be much more acidic and therefore better retained by anion-exchange chromatography than 87-kDa PC1/3. Instead, the most acidic PC1/3 forms, peaks III and IV, consisted of

a mixture of aggregated, oligomeric, and monomeric 87-kDa PC1/3 species. Interestingly, the bulk of enzyme activity in these acidic populations eluted slightly ahead of the 158-kDa marker with an estimated size of 216 kDa, indicating that active PC1/3 is an oligomer. Radiation inactivation experiments also supported the idea that active PC1/3 is an oligomer, as did native blue gels (results not shown). Although the estimated size of 216 kDa by gel filtration and radiation inactivation could also support the idea of a trimer, cross-linking experiments of the major peak of activity upon gel filtration confirmed the presence of distinct PC1/3 species between 150 and 225 kDa, indicating that a dimer (174 kDa) represents the more likely possibility. The majority of PC1/3 in this peak consisted of the 87-kDa species, suggesting a possible homodimer; we speculate that the conformation of this dimer may be such that it migrates anomalously by gel filtration.

Although oligomerization appears to account for the majority of the ionic heterogeneity of PC1/3, posttranslational modifications could potentially also account for some of the charge and size variation in different enzyme populations. PC1/3 has been shown to be both glycosylated and sulfated (20, 37). Indeed, treatment of PC1/3 from activity peaks I–IV from Fig. 1A with *N*-glycosidase F resulted in deglycosylation of both 66-kDa and 87-kDa PC1/3, resulting in a downward molecular weight shift (results not shown). However, there was no difference in deglycosylation pattern among the various ion-exchange peaks, suggesting that glycosylation *per se* does not affect PC1/3 self-interaction.

Gel filtration analysis confirmed that activity peak IV from Fig. 1A contains a mixture of aggregates, oligomers, dimers, and monomers of 87-kDa PC1. We found that dilution of activity peak IV resulted in increased specific activity. Dynamic light-scattering experiments and cross-linking studies (data not shown) supported the notion that dilution promotes the dissociation of a given oligomeric PC1/3 population into a smaller and more active enzyme species, suggesting that certain PC1/3 forms exist in equilibrium and can interconvert. In addition, this mixture also contained a large amount of inactive, aggregated PC1/3 molecules that could not be reactivated. It is also possible that the removal of inhibitory aggregates by dilution or purification may allow monomers and dimers to function more effectively. More work is needed to distinguish the molecular differences between PC1/3 species that can serve as a reservoir for generation of active PC1/3 enzyme and those that cannot or are, in fact, inhibitory. In secretory granules, where protein concentrations are high, the presence of the known PC1/3-binding protein proSAAS may affect PC1/3 oligomerization and activity; however, we were unable to directly demonstrate any ef-

fects of recombinant proSAAS on PC1/3 oligomerization (results not shown). This is consistent with data obtained using AtT-20 cells in which inactive, aggregated PC1/3 is secreted despite probable expression of endogenous proSAAS (38).

An important finding of the current study is that low concentrations of the standard convertase fluorogenic substrate, pERTKR-amc, can stabilize the 66-kDa form of PC1/3. It should be pointed out that substrate present during preincubation was most likely hydrolyzed by the enzyme; likely stabilization by product rather than by substrate is indicated by the fact that peptide products containing a pair of basic residues were functional. This stabilization could represent either a direct or an allosteric effect. Oddly, the natural PC1/3 substrate POMC was not able to stabilize 66-kDa PC1/3; we speculate that the large size of POMC could render its binding inefficient.

Interestingly, we found that the same peptides that stabilize 66-kDa PC1/3 function as activators for 87-kDa PC1/3. Because the activation effect is only observed for 87-kDa PC1/3, the C-terminal tail that differentiates the 66-kDa from the 87-kDa species of PC1/3 must contribute substantially to activation. It has been shown that the C-terminal tail can act as a PC1/3 inhibitor at micromolar concentrations in a complex, bimodal manner (21); we speculate that binding of activating small peptides alters the conformation of 87-kDa PC1/3 such that tail autoinhibition is lessened. In this regard it is interesting to note that tethering of PC1/3 by its C-terminal tail to membranes results in an apparent increase in the activity of transfected enzyme (22). Alternatively, it is possible that stabilizing peptides shift the equilibrium between different active forms, perhaps between 87-kDa dimers and monomers, or oligomers and dimers, in a C-terminal tail-mediated fashion; interestingly, the PC1/3 C-terminal tail has been shown to induce dimerization of monomeric immunoglobulins (39). The latter hypothesis is supported by the fact that peptide activation of 87-kDa PC1/3 disappears at dilute enzyme concentrations, implying that conditions favoring PC1/3 self-association are required for this effect to manifest. More work is needed to identify the biochemical mechanism underlying enzyme activation, but this study implies that the C-terminal tail could represent a promising pharmacological target for the therapeutic increase of PC1/3 activity.

Our study also has implications for clinically observed PC1/3 polymorphisms. The biochemical basis for the profound effect of most human PC1/3 mutations on body mass and glucose levels remains unclear. For example, the catalytic domain N222D mutation results in an enzyme that is 30–50% less active than wild-type PC1/3 and generates a semidominant phenotype with regard to body mass in N222D heterozygote mice placed on a high-fat

diet (14). If PC1/3 functions as a higher order oligomer, then the presence of mutant enzyme may impair the activity of a large number of wild-type enzyme molecules, thus explaining the aberrant heterozygote phenotype revealed by challenge with a high-fat diet. Experiments to directly test the effects of the N222D PC1/3 on wild-type PC1/3 activity in HEK cells confirm a strong dominant-negative effect of mutant enzyme on the secretion and activity of wild-type PC1/3 (Lindberg, I., and L.A. Pickett, unpublished results). Whether this finding will hold for endocrine cells remains to be determined, but the data presented here showing that PC1/3 can dimerize and oligomerize provide a potential biochemical foundation for effects of PC1/3 mutations on enzymatic activity.

In conclusion, we have here demonstrated that multiple species of recombinant and endogenous PC1/3s exist that efficiently self-associate and that carry varying surface charges. Most interestingly, one 87-kDa PC1/3 population was identified as a likely dimer and exhibited activity that was strongly enhanced by short peptides containing two sequential basic residues. Taken together, our data suggest that oligomerization and peptide activation can be added to intermolecular cleavage and association with proSAAS as potent methods of regulating PC1/3 activity.

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References

1. Steiner DF 1998 The proprotein convertases. *Curr Opin Chem Biol* 2:31–39
2. Seidah NG, Day R, Marcinkiewicz M, Benjannet S, Chrétien M 1991 Mammalian neural and endocrine pro-protein and pro-hormone convertases belonging to the subtilisin family of serine proteinases. *Enzyme* 45:271–284
3. Smeekens SP, Steiner DF 1990 Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. *J Biol Chem* 265:2997–3000

4. Seidah NG, Gaspar L, Mion P, Marcinkiewicz M, Mbikay M, Chrétien M 1990 cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. *DNA Cell Biol* 9:789
5. Zhou A, Mains RE 1994 Endoproteolytic processing of proopiomelanocortin and prohormone convertases 1 and 2 in neuroendocrine cells overexpressing prohormone convertases 1 or 2. *J Biol Chem* 269:17440–17447
6. Smeekens SP, Montag AG, Thomas G, Albiges-Rizo C, Carroll R, Benig M, Phillips LA, Martin S, Ohagi S, Gardner P, Swift HH, Steiner DF 1992 Proinsulin processing by the subtilisin-related proprotein convertases furin, PC2, and PC3. *Proc Natl Acad Sci USA* 89:8822–8826
7. Jackson RS, Creemers JW, Ohagi S, Raffin-Sanson ML, Sanders L, Montague CT, Hutton JC, O'Rahilly S 1997 Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene. *Nat Genet* 16:303–306
8. Jackson RS, Creemers JW, Farooqi IS, Raffin-Sanson ML, Varro A, Dockray GJ, Holst JJ, Brubaker PL, Corvol P, Polonsky KS, Ostrega D, Becker KL, Bertagna X, Hutton JC, White A, Dattani MT, Husain K, Middleton SJ, Nicole TM, Milla PJ, Lindley KJ, O'Rahilly S 2003 Small-intestinal dysfunction accompanies the complex endocrinopathy of human proprotein convertase 1 deficiency. *J Clin Invest* 112:1550–1560
9. Farooqi IS, Volders K, Stanhope R, Heuschkel R, White A, Lank E, Keogh J, O'Rahilly S, Creemers JW 2007 Hyperphagia and early-onset obesity due to a novel homozygous missense mutation in prohormone convertase 1/3. *J Clin Endocrinol Metab* 92:3369–3373
10. Benzinou M, Creemers JW, Choquet H, Lobbens S, Dina C, Durand E, Guerardel A, Boutin P, Jouret B, Heude B, Balkau B, Tichet J, Marre M, Potoczna N, Horber F, Le Stunff CC, Czernichow S, Sandback A, Lauritzen T, Borch-Johnsen K, Andersen G, Kiess W, Körner A, Kovacs P, Jacobson P, *et al.* 2008 Common nonsynonymous variants in PCSK1 confer risk of obesity. *Nat Genet* 40:943–945
11. Chang YC, Chiu YF, Shih KC, Lin MW, Sheu WH, Donlon T, Curb JD, Jou YS, Chang TJ, Li HY, Chuang LM 2010 Common PCSK1 haplotypes are associated with obesity in the Chinese population. *Obesity* 18:1404–1409
12. Kilpeläinen TO, Bingham SA, Khaw KT, Wareham NJ, Loos RJ 2009 Association of variants in the PCSK1 gene with obesity in the EPIC-Norfolk study. *Hum Mol Genet* 18:3496–3501
13. Heni M, Haupt A, Schäfer SA, Ketterer C, Thamer C, Machicao F, Stefan N, Staiger H, Häring HU, Fritsche A 2010 A Association of obesity risk SNPs in PCSK1 with insulin sensitivity and proinsulin conversion. *BMC Med Genet* 11:86
14. Lloyd DJ, Bohan S, Gekakis N 2006 Obesity, hyperphagia and increased metabolic efficiency in P₁ mutant mice. *Hum Mol Genet* 15:1884–1893
15. Jean F, Basak A, Rondeau N, Benjannet S, Hendy GN, Seidah NG, Chrétien M, Lazure C 1993 Enzymatic characterization of murine and human prohormone convertase-1 (mPC1 and hPC1) expressed in mammalian GH₄C₁ cells. *Biochem J* 292:891–900
16. Muller L, Lindberg I 1999 The cell biology of the prohormone convertases PC1 and PC2. In K. Moldave, ed. *Progress in nucleic acids research*, vol 63, Cambridge, MA: Elsevier Science; 69–108.
17. Anderson ED, VanSlyke JK, Thulin CD, Jean F, Thomas G 1997 Activation of the furin endoprotease is a multiple-step process: requirements for acidification and internal propeptide cleavage. *EMBO J* 16:1508–1518
18. Zhou Y, Lindberg I 1994 Enzymatic properties of carboxyl-terminally truncated prohormone convertase 1 (PC1/SPC3) and evidence for autocatalytic conversion. *J Biol Chem* 269:18408–18413
19. Rufaut NW, Brennan SO, Hakes DJ, Dixon JE, Birch NP 1993 Purification and characterization of the candidate prohormone-processing enzyme SPC3 produced in a mouse L cell line. *J Biol Chem* 268:20291–20298
20. Boudreault A, Gauthier D, Rondeau N, Savaria D, Seidah NG, Chrétien M, Lazure C 1998 Molecular characterization, enzymatic analysis, and purification of murine proprotein convertase-1/3 (PC1/PC3) secreted from recombinant baculovirus-infected insect cells. *Protein Expr Purif* 14:353–366
21. Rabah N, Gauthier D, Dikeakos JD, Reudelhuber TL, Lazure C 2007 The C-terminal region of the proprotein convertase 1/3 (PC1/3) exerts a bimodal regulation of the enzyme activity in vitro. *FEBS J* 274:3482–3491
22. Bruzzaniti A, Mains RE 2002 Enzymatic activity of soluble and membrane tethered peptide pro-hormone convertase 1. *Peptides* 23: 863–875
23. van Horsen AM, van den Hurk WH, Bailyes EM, Hutton JC, Martens GJ, Lindberg I 1995 Identification of the region within the neuroendocrine polypeptide 7B2 responsible for the inhibition of prohormone convertase PC2. *J Biol Chem* 270:14292–14296
24. Fricker LD, McKinzie AA, Sun J, Curran E, Qian Y, Yan L, Patterson SD, Courchesne PL, Richards B, Levin N, Mzhavia N, Devi LA, Douglass J 2000 Identification and characterization of proSAAS, a granin-like neuroendocrine peptide precursor that inhibits prohormone processing. *J Neurosci* 20:639–648
25. Fortenberry Y, Hwang JR, Apletalina EV, Lindberg I 2002 Functional characterization of ProSAAS: similarities and differences with 7B2. *J Biol Chem* 277:5175–5186
26. Basak A, Koch P, Dupelle M, Fricker LD, Devi LA, Chrétien M, Seidah NG 2001 Inhibitory specificity and potency of proSAAS-derived peptides toward proprotein convertase 1. *J Biol Chem* 276: 32720–32728
27. Sayah M, Fortenberry Y, Cameron A, Lindberg I 2001 Tissue distribution and processing of proSAAS by proprotein convertases. *J Neurochem* 76:1833–1841
28. Apletalina E, Appel J, Lamango NS, Houghten RA, Lindberg I 1998 Identification of inhibitors of prohormone convertases 1 and 2 using a peptide combinatorial library. *J Biol Chem* 273:26589–26595
29. Qian Y, Devi LA, Mzhavia N, Munzer S, Seidah NG, Fricker LD 2000 The C-terminal region of proSAAS is a potent inhibitor of prohormone convertase 1. *J Biol Chem* 275:23596–23601
30. Lee SN, Lindberg I 2008 7B2 prevents unfolding and aggregation of prohormone convertase 2. *Endocrinology* 149:4116–4127
31. Zhou Y, Lindberg I 1993 Purification and characterization of the prohormone convertase PC1(PC3). *J Biol Chem* 268:5615–5623
32. Vindrola O, Lindberg I 1992 Biosynthesis of the prohormone convertase mPC1 in AtT-20 cells. *Mol Endocrinol* 6:1088–1094
33. Le Maire M, Thauvette L, de Foresta B, Viel A, Beauregard G, Potier M 1990 Effects of ionizing radiations on proteins. Evidence of non-random fragmentations and a caution in the use of the method for determination of molecular mass. *Biochem J* 267:431–439
34. Beauregard G, Maret A, Salvayre R, Potier M 1987 The radiation inactivation method as a tool to study structure-function relationships in proteins. *Methods Biochem Anal* 32:313–343
35. Cameron A, Fortenberry Y, Lindberg I 2000 The SAAS granin exhibits structural and functional homology to 7B2 and contains a highly potent hexapeptide inhibitor of PC1. *FEBS Lett* 473:135–138
36. Zhou Y, Rovere C, Kitabgi P, Lindberg I 1995 Mutational analysis of PC1 (SPC3) in PC12 cells. 66-kDa PC1 is fully functional. *J Biol Chem* 270:24702–24706
37. Benjannet S, Rondeau N, Paquet L, Boudreault A, Lazure C, Chrétien M, Seidah NG 1993 Comparative biosynthesis, covalent post-translational modifications and efficiency of prosegment cleavage of the prohormone convertases PC1 and PC2: glycosylation, sulfation and identification of the intracellular site of prosegment cleavage of PC1 and PC2. *Biochem J* 294:735–743
38. Vindrola O, Lindberg I 1993 Release of the prohormone convertase PC1 from AtT-20 cells. *Neuropeptides* 25:151–160
39. Jutras I, Seidah NG, Reudelhuber TL 2000 A predicted alpha-helix mediates targeting of the proprotein convertase PC1 to the regulated secretory pathway. *J Biol Chem* 275:40337–40343