Neuropeptide Processing Profile in Mice Lacking Prohormone Convertase-1†

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ABSTRACT: Prohormone convertase 1 (PC1; also known as PC3) is believed to be responsible for the processing of many neuropeptide precursors. To look at the role PC1 plays in neuropeptide processing in brain and pituitary, we used radioimmunoassays (RIA) as well as quantitative peptidomic methods and examined changes in the levels of multiple neuropeptide products in PC1 knockout (KO) mice. The processing of proenkephalin was impaired in PC1 KO mouse brains with a decrease in the level of Met-Enkephalin immunoreactivity (ir-Met-Enk) and an accumulation of higher molecular weight processing intermediates containing ir-Met-Enk. Processing of the neuropeptide precursor VGF was also affected in PC1 KO mouse brains with a decrease in the level of an endogenous 3 kDa C-terminal peptide. In contrast, the processing of proSAAS into PEN was not altered in PC1 KO mouse brains. Quantitative mass spectrometry was used to analyze a number of peptides derived from proopiomelanocortin (POMC), provasopressin, prooxytocin, chromogranin A, chromogranin B, and secretogranin II. Among them, the levels of oxytocin and peptides derived from chromogranin A and B dramatically decreased in the PC1 KO mouse pituitaries, while the levels of peptides derived from proopiomelanocortin and provasopressin did not show substantial changes. In conclusion, these results support the notion that PC1 plays a key role in the processing of multiple neuroendocrine peptide precursors and also reveal the presence of a redundant system in the processing of a number of physiologically important bioactive peptides.

Neuroendocrine peptides are usually synthesized as large precursors and need to undergo limited proteolytic steps as well as other posttranslational modifications to gain biological activity. In most cases the cleavage sites within the peptide precursors contain multiple basic sequences such as Lys-Arg, Arg-Arg, or Arg-X−X-Arg (*1*−*4*). Two members of the subtilisin-like prohormone convertase (PC)[1] family, PC1 (also known as PC3) and PC2, are broadly expressed in brain and neuroendocrine tissues and localized to secretory vesicles along with the neuropeptide precursors (*5*−*7*). It is believed that these two PCs play crucial roles in neuroendocrine peptide precursor processing at dibasic or multibasic sites (*1*, *2*, *4*). Following this endopeptidase cleavage, peptide intermediates with C-terminal basic residue extensions are processed by a carboxypeptidase (CP) such as CPE or CPD (*8*).

The enzymatic activities of PC1 and PC2 as well as the roles they play in neuroendocrine peptide processing have been extensively studied using in vitro techniques including purified recombinant enzymes, vaccinia virus overexpression systems, and neuroendocrine cell lines (*9*−*17*). The studies have suggested that these two enzymes play crucial roles in the processing of several neuropeptide precursors including proenkephalin and proopiomelanocortin (POMC) (*10*, *11*, *14*−*19*). Although both PC1 and PC2 cleave at sites containing multiple basic residues, they each have their own preferences. Moreover, because of the different expression patterns of these two enzymes in neuroendocrine tissue, they are believed to be involved in tissue-specific processing of the same precursor molecule. For example, in the anterior pituitary PC1 alone is expressed at a high level and generates ACTH from POMC. In the intermediate lobe both PC1 and PC2 are expressed at a high level and it appears that the presence of PC2 leads to the further processing of ACTH into α-MSH and CLIP (*20*). In the case of proenkephalin it has been shown that PC1 mainly processes the precursor into higher molecular weight intermediates, while PC2 is more efficient in performing the cleavages that produce the shorter, bioactive products (*9*, *14*, *19*).

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[1] Abbreviations: ACTH, adrenocorticotrophic hormone; α-MSH, alpha-melanocyte stimulating hormone; CLIP, corticotropin-like intermediate lobe peptide; CP, carboxypeptidase; ESI-TOF, electrospray ionization time-of-flight; GHRH, growth hormone releasing hormone; KO, knockout; LC-MS, liquid chromatography mass spectrometry; LPH, lipotropin; Met-Enk, methionine enkephalin; PC1, prohormone convertase 1; PC2, prohormone convertase 2; POMC, proopiomelanocortin.

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Apart from the well-studied precursor molecules, several other less understood neuropeptide precursors have emerged as potential substrates for PC1 and PC2. One example is the peptide precursor VGF which was initially identified as an NGF-induced cDNA clone and is believed to be a neuropeptide precursor (*21*). VGF has a neuroendocrine tissue distribution and is essential for the regulation of energy balance since disruption of the mouse VGF gene produces a lean phenotype (*21*, *22*). The primary sequence of this precursor reveals a number of dibasic or multibasic sites that are consistent with the consensus processing sites recognized by PC1 or PC2, and several of these sites have been shown to be cleaved in GH3 cells expressing ectopic PC1 or PC2 (*23*). Because VGF has a similar tissue and cellular distribution as PC1 and PC2, it is likely that VGF is a physiologically relevant substrate for these enzymes (*21*, *24*).

Another interesting precursor is proSAAS, a neuropeptide precursor that has a potent inhibitory effect on PC1 and a broad neuroendocrine distribution (*25*). The inhibitory region is located in a small region near the C-terminus of proSAAS (*26*−*28*). Cleavage at a Lys-Arg sequence within this region and subsequent removal of the basic residues by a CP eliminates the inhibition of PC1 (*28*). PC1 is able to cleave this site, although it is very slow. Because cleavage at this site is efficient in mouse brain and cell lines (*25*, *29*), it was of interest to see whether PC1 itself is involved in this cleavage under physiological condition.

Although the enzymatic activities of the two PCs have been extensively studied using in vitro biochemical and cell biological methods, it is important to evaluate the roles of the two enzymes in physiological conditions. Mice with a disruption of the PC2 gene (i.e. PC2 KO mice) have a number of neuropeptide processing defects including the processing of proinsulin, proglucagon, as well as prosomatostatin (*30*−*35*). Also, the processing of endogenous opioid peptide precursors, prodynorphin, and proenkephalin are affected (*19*, *36*). The processing of POMC in pituitary is impaired in that the conversion of β-lipotropin into γ-lipotropin and β-endorphin is greatly diminished. The carboxy shortening of β-endorphin is completely abolished, and the processing of ACTH into α-MSH and CLIP is hampered (*32*, *33*, *37*). Therefore, the question to be asked is what role does PC1 play in the processing of these molecules in vivo.

The PC1 KO mice have recently been generated, and they display impairments in the processing of a number of neuroendocrine peptide precursors including hypothalamic growth hormone releasing hormone (GHRH), POMC in the anterior pituitary lobe, pancreatic proinsulin and islet amyloid polypeptide precursor, intestinal proglucagon, hypothalamic CART, and cholecystokinin in several brain regions (*38*−*43*).

In the present study RIA was used to study changes in the processing of three peptide precursor molecules (proenkephalin, VGF, and proSAAS) in PC1 KO mice. In addition, a quantitative peptidomic method was used to analyze changes in the levels of a large number of pituitary peptides in the PC1 KO mice.

MATERIALS AND METHODS:

Western Blot Analysis of PC1 Protein. Brains from the PC1 KO mice or control littermates were frozen in isopentane chilled with dry ice right after sacrificing. For extraction brains were put into liquid nitrogen and ground to a fine powder. The powder was homogenized in 50 mM Tris-Cl pH 7.4 with 1% Triton X-100, 10% glycerol, and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The homogenate was kept on ice for 30 min and then centrifuged at 12 000*g* for 30 min at 4 °C. The protein concentrations of the supernatants were determined using the Bradford method, and approximately 1 μg of solubilized protein was subjected to SDS−polyacrylamide gel electrophoresis. The proteins were then electrotransferred to a nitrocellulose membrane and probed with a 1:1000 dilution of a rabbit antiserum raised against the N-terminal region of PC1 or PC2 as described (*44*). Equal loading was confirmed by probing the blot with anti-tubulin antiserum (Sigma). The PC1 antiserum recognizes a ∼68 kDa protein representing the mature enzyme and a nonspecific band at 78 kDa. The PC2 antiserum recognizes ∼68−71 kDa proteins representing mature forms of PC2 (*44*).

Brain Extraction, Gel-Filtration, and Reverse-Phase-HPLC (RP-HPLC) Procedures. Brains from age- and sex-matched PC1 KO mice or control littermates were frozen in liquid nitrogen and ground into a fine powder. Ten volumes of 0.1 M boiling acetic acid were added. Samples were homogenized and incubated at 100 °C for 15 min. After cooling on ice, samples were centrifuged at 12 000*g* for 30 min at 4 °C. The supernatants were transferred to fresh tubes, dried in a vacuum centrifuge, and stored at −80 °C.

For gel-filtration chromatography the samples were rehydrated in 100 mM sodium phosphate buffer, pH 7.4, containing 0.1% Triton X-100, adjusted to 30% acetonitrile and 0.1% trifluoroacetic acid, and then applied to a Superdex Peptide HR 10/30 gel-filtration column (Amersham Bioscience). The samples were fractionated in 30% acetonitrile and 0.1% trifluoroacetic acid with a flow rate of 0.5 mL/min. One-minute fractions were collected.

For the reverse-phase (RP)-HPLC analysis the gel-filtration fractions containing the immunoreactive peptides (fractions 20−23) were pooled, and the volume of samples was reduced in a vacuum centrifuge. A portion of the material was then subjected to RP-HPLC using a C_{18} column (length = 250 mm; inner diameter = 4.6 mm, VyDac). The samples were fractionated with a linear gradient from 5% acetonitrile/0.1% TFA to 75% acetonitrile/0.1% TFA over 70 min with a flow rate of 0.5 mL/min. One-minute fractions were collected.

Radioimmunoassay (RIA) and Trypsin/Carboxypeptidase B (CPB) Treatment. Gel-filtration fractions were analyzed by RIAs to determine the levels of Met-Enk, the VGF C-terminal peptide AQEE, and the proSAAS-derived peptide PEN. For Met-Enk RIA gel-filtration fractions were either tested directly using antiserum against Met-Enk or subjected to digestion with trypsin/CPB. For this an aliquot (60 μL) of each fraction was treated with 5 μg/mL tosylphenylalanylchloromethyl ketone-treated trypsin (Sigma) for 16 h followed by treatment with 5 ng/mL carboxypeptidase B (CPB, Sigma) for 120 min. The reaction was terminated by boiling for 20 min. Another aliquot of 60 μL ("untreated") was subjected to the same incubation and boiling conditions as above except that enzymes were omitted. This trypsin/CPB treatment releases the Met-Enk moiety from the higher molecular weight precursors without affecting the levels of mature Met-Enk. The Met-Enk antiserum (Bachem Inc. San

Carlos, CA) is specific for the Met-Enk pentapeptide; it exhibits <3% cross reactivity with Leu-Enk and has <1% cross reactivity with proenkephalin or other N- or C-terminally extended peptides. The antiserum used for AQEE RIA was generated against the C-terminal 30 amino acids of the VGF precursor (a gift from Dr. H. Yan, Amgen Inc.). In addition to AQEE, this antiserum recognizes full-length VGF (amino acids 1−617) and a peptide corresponding to the C-terminal 62 amino acids (amino acids 556−617). The antiserum used for PEN RIA was raised against PEN (proSAAS 221−242) and also recognizes C-terminally extended peptides such as big PEN-LEN (proSAAS 221−260) and little PEN-LEN (proSAAS 221−254) (*44*).

Reagents for the Differential Isotopic Labeling Experiment. In this study the compound [3-(2,5-dioxopyrrolidin-1-yloxycarbonyl)-propyl]trimethylammonium chloride containing either nine deuterium or nine hydrogen (D_9- or H_9-TMAB) was used for the labeling of the endogenous pituitary peptides. This compound was synthesized with a procedure originally developed by Regnier and colleagues (*45*). The compound has been characterized and used to label mouse pituitary peptides for comparing the peptide level differences between Cpe^{fat}/Cpe^{fat} and wild-type mice (*46*, *47*).

Extraction and Labeling of Pituitary Peptides with Isotopic Tags. An initial optimizing experiment was done using wild-type mouse pituitary extracts to determine whether identical endogenous mouse pituitary peptide samples labeled with H_9-TMAB and D_9-TMAB show the expected 1:1 ratio upon LC/MS analysis. In the experiment six wild-type mouse pituitaries were sonicated in 600 μL of 10 mM HCl, incubated at 70 °C for 20 min, and combined with 400 μL of 0.4 M Na_2HPO_4 to bring the pH to 9.5. The extracts were centrifuged at 50 000*g* for 30 min at 4 °C, and the supernatant was divided into two tubes. One sample was labeled with H_9-TMAB, and the other was labeled with D_9-TMAB reagent. For the labeling reaction 6 μL of 1.5 M H_9- or D_9-TMAB dissolved in DMSO was added to the supernatant of the extract from three mouse pituitaries. After 10 min 1.0 M NaOH was added to adjust the pH back to 9.5. The addition of TMAB and NaOH was repeated 7 times; then 30 μL of 2.5 M glycine was added to the reaction mixture followed by incubation at room temperature for 40 min to quench any remaining labeling reagents. The two samples were combined, the peptide pool was centrifuged at 50 000*g* for 30 min at 4 °C, and the supernatant was filtered through a Microcon YM-10 unit (Millipore, Bedford, MA). The pH of the supernatant was adjusted to 9.0 with 1 M NaOH and 5 μL of 2.0 M hydroxylamine (in DMSO). This was repeated a total of 3 times (to help remove any labels from the hydroxyl side chains of tyrosine, serine, or threonine residues). The resulting sample was concentrated to 100 μL using a vacuum centrifuge and subjected to LC MS analysis.

For studies comparing peptide levels in PC1 KO vs wild-type mouse pituitaries a total of 12 PC1 KO and 12 wild-type pituitaries were used. For each experiment six pituitaries from PC1 KO mice and six from wild-type mice were extracted in groups of three pituitaries, as described above. Each group was labeled with either D_9-TMAB or H_9-TMAB, as above. After labeling and quenching of the reaction, the extract from PC1 KO mice was pooled with extract from wild-type mice labeled with the complementary TMAB label. This resulted in two pools of extracts that were labeled in reverse directions from each other; one pool contained wild-type tissue labeled with H_9 and PC1 KO labeled with d_9, the other contained wild-type tissue labeled with d_9 and PC1 KO labeled with H_9. This entire procedure was done twice for a total of four pools of PC1 KO and wild-type mouse pituitaries. Each of these four pools was analyzed separately by LC-MS as described below.

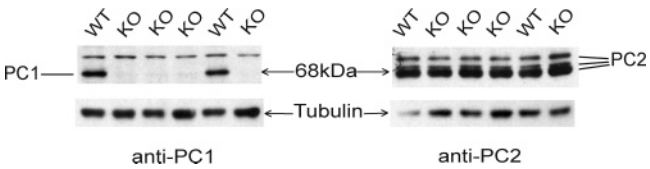

FIGURE 1: Western blot analysis of PC1 and PC2 in PC1 knockout (KO) and wild-type (WT) mice. Brain extracts were fractionated on a denaturing 8% polyacrylamide gel. The proteins were electrotransferred to the nitrocellulose membranes and probed with antiserum against PC1 or PC2 as described in Materials and Methods. The genotype of each mouse is shown above each lane. The positions and sizes of PC1 and PC2 are indicated. The α-tubulin signal shows that comparable amounts of protein were loaded in each lane.

Mass Spectrometry and Data Analysis. To detect and quantify the labeled peptides from PC1 KO and wild-type mouse pituitaries, aliquots (30%) of each sample were subjected to LC-MS analysis on an electrospray ionization-time-of-flight (ESI-TOF) mass spectrometer (Mariner, PerSeptive Biosystems, Foster City, CA). After data collection relative levels of peptides in PC1 KO versus wild-type mouse pituitaries were determined by calculating the ratio of peak intensity of the H_9-TMAB- and d_9-TMAB-labeled peptide pairs, as described (*46*). The identification of the peptides was performed in a separate study (*47*). Briefly, MS/MS spectra for TMAB-labeled peptides were analyzed manually. The criteria for considering a peptide identified from the MS/MS analysis was (i) a parent mass within 40 parts per million (ppm) of the theoretical value and (ii) 80% or more of the collision-induced-dissociation fragments observed in MS/MS matched predicted fragments (minimum five matches).

RESULTS

To confirm the absence of the PC1 protein in the PC1 KO mice Western blot analyses were carried out using antiserum raised against the N-terminal region of PC1. We find a band at ~68 kDa in wild-type mouse brain which is consistent with the size of the mature PC1 enzyme; this band is absent in PC1 KO mouse brain (Figure 1, left). To check whether PC2 expression is changed due to the deletion of PC1 Western blot analyses were done with the same preparation of brain extracts using an antiserum raised against PC2. A protein band at ~75 kDa and a doublet at ~68 kDa were detected; the sizes of these bands are consistent with the sizes of the pro- and mature PC2, respectively (Figure 1, right). The level of PC2 in PC1 KO mice was found to be comparable to that of wild-type mice (Figure 1, right), suggesting that no compensatory up-regulation of PC2 expression has been elicited due to the deletion of PC1.

Previous in vitro studies have suggested that PC1 is involved in the processing of proenkephalin (*11*, *15*). We directly examined this in PC1 KO mice since they serve as an excellent system to test this idea. There is a substantial decrease in the level of mature ir-Met-Enk in PC1 KO mouse

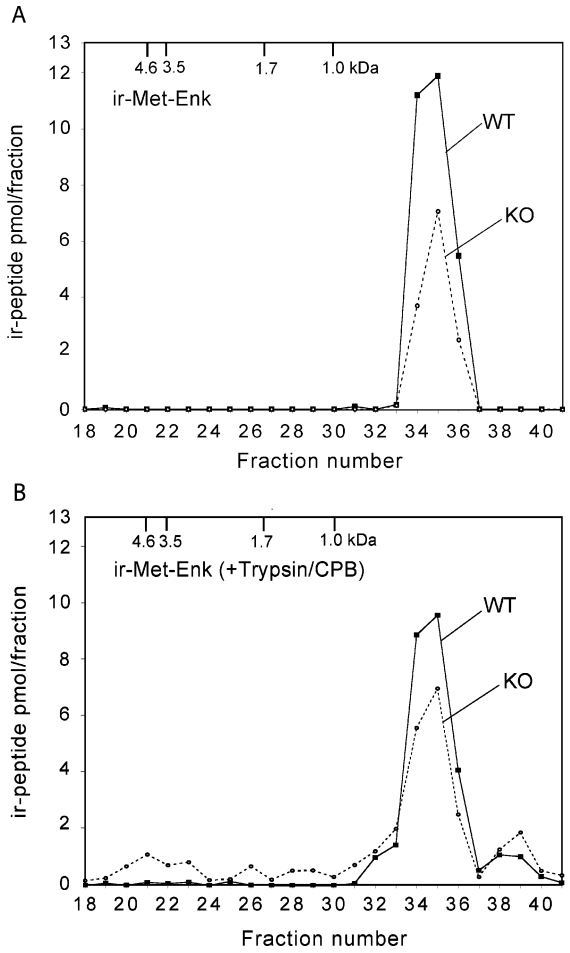

FIGURE 2: Analyses of Met-Enk immunoreactivity in gel-filtration fractions from PC1 KO and wild-type mouse brain extracts without (A) or with (B) trypsin/CPB treatment. Extracts from each brain were subjected to gel-filtration chromatography on a Superdex Peptide HR 10/30 column. As described in Materials and Methods, fractions were either directly analyzed with the antiserum recognizing the Met-Enk pentapeptide or treated with trypsin/CPB and then subjected to RIA analyses with the Met-Enk antiserum. Dashed line: PC1 KO mouse. Solid line: wild-type mouse. The elution positions of the standard peptides are indicated: ACTH, 4.6 kDa; β-endorphin, 3.5 kDa; α-MSH, 1.7 kDa; DynA1−8, 1.0 kDa.

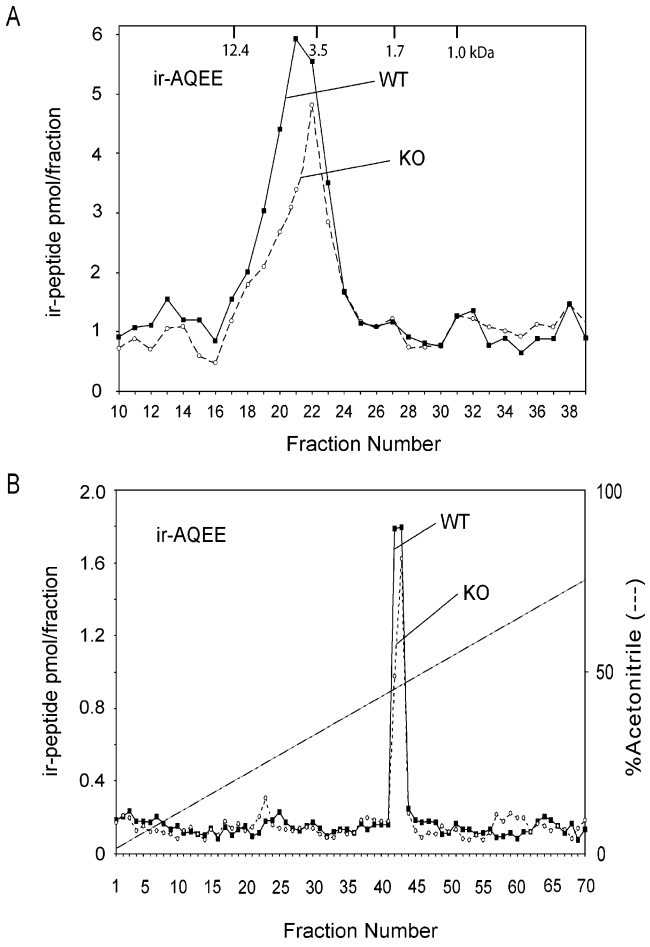

FIGURE 3: Analyses of ir-AQEE levels in gel-filtration and RP-HPLC fractions of the PC1 KO or wild-type mouse brain extracts. (A) Extract from each mouse brain was fractionated using gel-filtration chromatography, and fractions were analyzed with antiserum against AQEE peptide as described in Materials and Methods. (B) Gel-filtration fractions containing ir-AQEE peptides were further resolved using RP-HPLC. The fractions were analyzed for ir-AQEE as described in Materials and Methods. Molecular weight standards: cytochrome C, 12.4 kDa; β-endorphin, 3.5 kDa; α-MSH, 1.7 kDa; DynA1−8, 1.0 kDa.

brain compared to wild-type mouse brain (Figure 2A). Trypsin/CPB treatment of the peptides in the gel filtration fractions releases the ir-Met-Enk from larger peptides that do not react with the Met-Enk-specific antisera, thus allowing the detection of these peptides by RIA (*15*). The PC1 KO mice contain detectable levels of these ir-Met-Enk-containing peptide intermediates, whereas in the wild-type mice the higher molecular weight ir-Met-Enk-containing peptides are not detectable (Figure 2B). These results suggest that the processing of higher molecular weight ir-Met-Enk-containing peptide intermediates has been compromised in PC1 KO mouse brain.

The peptide precursor VGF is richly expressed in neuroendocrine tissues and contains a number of multibasic sites that could be processed by PC1 (*21*, *23*). To investigate whether VGF processing is carried out by PC1 in vivo, extracts from PC1 KO and wild-type mouse brains were fractionated by gel filtration chromatography and the fractions analyzed by RIA using an antiserum raised against the C-terminal 30 amino acids of the VGF protein (AQEE). This analysis revealed a peak of ir-AQEE-containing peptides eluting between 3 and 10 kDa in wild-type mouse brain (Figure 3A). There is a 25% decrease in the level of the immunoreactivity for this peptide in PC1 KO mouse brain compared to wild-type mouse brain (Figure 3A). The gel-filtration fractions containing the ir-AQEE were further analyzed by RP-HPLC. This analysis showed a single peak of ir-AQEE that elutes at the same position as that of the synthetic AQEE peptide (Figure 3B). Furthermore, the level of ir-AQEE in PC1 KO brains was substantially decreased, suggesting a role for PC1 in the generation of the AQEE peptide (Figure 3B).

Several peptides derived from proSAAS have been found to exist in the wild-type mouse brain including PEN, LEN, and big and little PEN-LEN (*44*). To investigate whether PC1 is involved in the generation of PEN the gel-filtration fractions were analyzed using an antiserum against PEN. We find that the majority of ir-PEN eluted as a peak of approximately 2−4 kDa (Figure 4). This major peak corresponds to the size of PEN (2.3 kDa). A small shoulder around 4 kDa could correspond to little PEN-LEN (3.6 kDa) and/or big PEN-LEN (4.4 kDa). A comparison between the PC1 KO and wild-type brain extracts showed no major differences

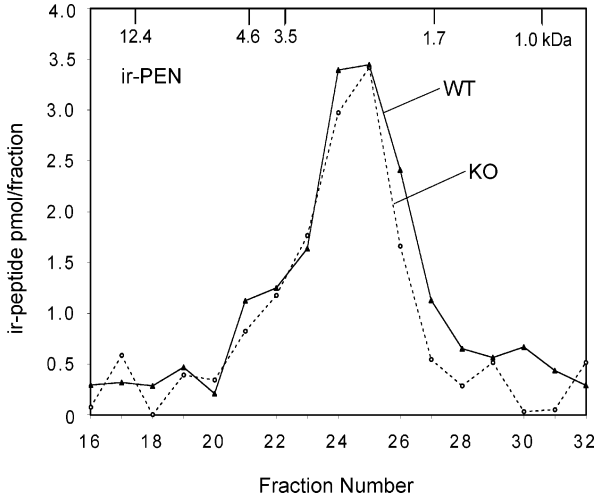

FIGURE 4: Analyses of ir-PEN levels in the PC1 KO and wild-type brain extracts. Brain extracts were fractionated using gel-filtration chromatography as described in Materials and Methods. The fractions collected were analyzed with antiserum against PEN. Molecular weight standards: cytochrome C, 12.4 kDa; ACTH, 4.6 kDa; β-endorphin, 3.5 kDa; α-MSH, 1.7 kDa; DynA1−8, 1.0 kDa.

in the levels of ir-PEN, suggesting that PC1 is not required for the generation of PEN (Figure 4).

Although RIA has a reasonably high sensitivity, this method is restricted to known peptides. To gain insight into changes in the processing of a larger number of peptides in PC1 KO mouse pituitary, we used a quantitative peptidomics method. Unlike RIA, such analysis is not confined to known peptides. Furthermore, peptidomic approaches can detect the exact forms of the peptides and reveal the presence of posttranslational modifications. Since PC1 is present in all three lobes of the pituitary gland, this serves as an ideal system for such a study. Differential isotopic labeling and mass spectrometry were used to examine the relative levels of neuropeptides in PC1 KO vs wild-type mouse pituitaries. In a single experiment six PC1 KO mice and six age- and sex-matched wild-type mice were divided into two groups, with three KO and three wild-type mice in each group. In one group the extracts from three pituitaries of the same genotype were pooled, labeled with the H_9-TMAB reagent, and combined with extracts from three pituitaries of the opposite genotype that were labeled with the D_9-TMAB reagent. In the other group the labeling was reversed to minimize any potential artifacts arising from different reactivities of the two labels.

Upon LC−MS analysis, 59 peptides with different masses were detected (Table 1). The identities of 35 of these peptides were determined by MS/MS analysis, and another three peptides were tentatively identified based on the similarity of their parent mass to known pituitary peptides. Five of the identified peptides were found to be sodium adducts of pituitary peptides (Table 1). The vast majority of the identified peptides are derived from POMC, and a small number are derived from provasopressin, prooxytocin, chromogranin A, chromogranin B, and secretogranin II (Table 1).

The majority of the peptides observed in the present study were found in at least three of the four separate analyses comparing peptide levels in the PC1 KO mouse pituitaries to wild-type mouse pituitaries. Among the peptides identified, some showed consistent decreases in the PC1 KO compared to wild-type pituitaries. Such peptides include oxytocin (CYIQNCPLG-amide) (Figure 5), a chromogranin A peptide (AYGFRDPGPQL), two chromogranin B fragments (LLDEGHYPV and LGALFNPYFDPLQWKNSDFE), a secretogranin II fragment (QELGKLTGPSNQ), and several unidentified peptides (Figure 5 and Table 1). In the case of identified peptides, examination of the cleavage sites involved in generating these peptides revealed that at least one end of these peptides is the result of processing at the consensus PC cleavage site (either KR or RR sites). For example, the C-termini of the secretogranin II fragment, the chromogranin A fragment, and oxytocin require the sequential action of a PC and a carboxypeptidase. One of the chromogranin B fragments (LLDEGHYPV) is generated by a PC-like cleavage at the N-terminus and cleavage at a single Arg at the C-terminus (either by direct endopeptidase cleavage of the Val-Arg bond or by endopeptidase cleavage after the Arg and then removal of this residue by a carboxypeptidase). The other chromogranin B fragment (LGALFNPYFDPLQWKNSDFE) contains PC consensus cleavage sites on both N- and C-termini. The decrease in the levels of these peptides implies that PC1 plays a major role in the processing at these PC consensus sites. However, because low levels of the mature forms of these peptides are detected in the pituitaries of PC1 KO mice, there is some redundancy in the processing pathway. Also, we find that despite the large 80% decrease in oxytocin level in the PC1 KO mouse pituitaries (Figure 5 and Table 1), the level of vasopressin was not decreased (Table 1 and discussion).

Peptides derived from POMC represent a large portion of the total number of peptides observed. Among them there are known peptides including α-melanocyte stimulating hormone (α-MSH) with no acetyl group (desAc-α-MSH), one acetyl group (α-MSH) and two acetyl groups (di-Ac-α-MSH), joining peptide (J-peptide), acetyl β-endorphin 1−26 and 1−27, corticotropin-like intermediate lobe peptide (CLIP), Ac-α-endorphin, Ac-γ-endorphin, and γ-lipotropin (γ-LPH). In addition, several other fragments of POMC-derived peptides were found, including α-MSH fragments lacking one or two N-terminal residues but with an acetyl group on the amino-terminal residue, three CLIP fragments lacking one, two, or three residues from the C-terminus, Ac-β-endorphin 1−15, and several γ-LPH fragments (Table 1). Most of the POMC derived peptides, however, did not show a significant difference in levels when comparing the PC1 KO to the wild-type mice (Table 1). These results suggest that some adaptive changes exist to maintain the levels of the POMC derived peptides.

DISCUSSION

The roles of PC1 and PC2 in the processing of neuropeptide precursors have been extensively studied using purified enzyme and neuroendocrine cell lines (*9*−*17*). The data from these studies suggest that PC1 processes the neuropeptide precursors into higher molecular weight peptides while PC2 is more efficient in producing smaller peptides. On the basis of these observations and also on the pH- and Ca^{2+}-dependence of PC1 and PC2, it has been proposed that PC1 functions before PC2 within the secretory pathway. In the case of proinsulin processing, cleavages at both the B-chain−C-peptide junction and C-peptide−A-chain junction are

Table 1: Differential Isotopic Labeling and Mass Spectrometry Analysis of Peptides in PC1 KO and Wild-type Mouse Pituitaries[a]

peptide name	obsd mass	mass (theor)	diff (ppm)	sequence from MS/MS (or predicted)	KO/wt	SEM	n
γ-1-MSH	1337.63	1337.64	−7	KYVMGHFRWD	0.91	0.12	4
J-peptide	1939.87	1939.87	0	AEEEAVWGDGSPEPSPRE-amide	0.86	0.24	4
J-peptide + Na^+	1961.85	1961.87	−10	AEEEAVWGDGSPEPSPRE-amide + 22	0.87	0.22	4
J-peptide fragment	1481.71	1481.70	7	AVWGDGSPEPSPRE-amide	0.72	n/a	1
α-MSH	1663.79	1663.80	−6	Ac-SYSMEHFRWGKPV-amide	1.07	0.40	4
α-MSH (dehydro)	1645.79	1645.79	0	Ac-dehydroS-YSMEHFRWGKPV-amide	1.11	0.42	4
α-MSH (oxidized)	1679.80	1679.80	0	Ac-SYSM/Ox/EHFRWGKPV-amide	1.34	0.56	4
desAc-α-MSH	1621.79	1621.79	0	SYSMEHFRWGKPV-amide	0.82	0.03	4
DiAc-MSH	1705.80	1705.81	−6	(diAc-SYSMEHFRWGKPV-amide)	1.46	0.52	2
α-MSH fragment	1413.71	1413.71	0	Ac-SMEHFRWGKPV-amide	0.64	0.19	3
α-MSH fragment	1576.78	1576.77	6	Ac-YSMEHFRWGKPV-amide	1.46	0.53	3
CLIP	2505.26	2505.26	0	RPVKVYPNVAENESAEAFPLEF	0.90	0.21	4
CLIP + Na+	2527.25	2527.26	−4	RPVKVYPNVAENESAEAFPLEF + 22	0.75	0.17	2
phospho CLIP	2585.22	2585.22	0	RPVKVYPNVAENE-phosphoS-AEAFPLEF	1.39	0.43	4
CLIP fragment	2116.13	2116.06	33	RPVKVYPNVAENESAEAFP	0.98	n/a	1
CLIP fragment	2229.14	2229.15	−4	RPVKVYPNVAENESAEAFPL	0.66	0.17	4
CLIP fragment + phosphate	2309.12	2309.12	0	RPVKVYPNVAENE-phosphoS-AEAFPL	1.33	0.64	4
CLIP fragment	2358.18	2358.19	−4	RPVKVYPNVAENESAEAFPLE	0.78	0.21	4
γ-LPH	4436.12	4436.14	−5	ELEGERPLGLEQVLESDAEKDDGPYRVEHFRWSNPPKD	0.86	0.27	4
Ac-γ-LPH	4478.19	4478.12	16	Ac-ELEGERPLGLEQVLESDAEKDDGPYRVEHFRWSNPPKD	0.81	n/a	1
γ-LPH fragment	1983.96	1983.97	−5	(GPYRVEHFRWSNPPKD)	1.14	0.50	3
γ-LPH fragment	2470.18	2470.18	0	ELEGERPLGLEQVLESDAEKDD	0.69	0.27	2
γ-LPH fragment	3455.67	3455.64	9	LEQVLESDAEKDDGPYRVEHFRWSNPPKD	0.48	0.15	2
Ac-β-endorphin 1-15	1685.81	1685.80	6	Ac-YGGFMTSEKSQTPLV	0.48	n/a	1
Ac-α-endorphin	1786.84	1786.85	−6	Ac-YGGFMTSEKSQTPLVT	0.61	0.04	2
Ac-α-endorphin + Na^+	1808.85	1808.85	0	Ac-YGGFMTSEKSQTPLVT + 22	0.66	0.09	2
Ac-γ-endorphin + Na^+	1921.92	1921.94	−10	Ac-YGGFMTSEKSQTPLVTL + 22	0.85	0.33	3
Ac-β-endorphin 1-26	2899.52	2899.51	3	Ac-YGGFMTSEKSQTPLVTLFKNAIIKNA	0.82	0.11	4
Ac-β-endorphin 1-27	3036.58	3036.58	0	Ac-YGGFMTSEKSQTPLVTLFKNAIIKNAH	1.14	0.52	4
Ac-β-endorphin 1-31	3477.85	3477.84	3	Ac-YGGFMTSEKSQTPLVTLFKNAIIKNAHKKGQ	1.97	1.61	3
β-endorphin 18-26	1017.60	1017.60	0	FKNAIIKNA	0.78	0.15	4
β-endorphin 18-27	1154.65	1154.66	−9	FKNAIIKNAH	0.78	0.30	4
oxytocin	1006.46	1006.44	20	(CYIQNCPLG-amide)	0.19	0.02	4
oxytocin + Na^+	1028.43	1028.44	−10	CYIQNCPLG-amide + 22	0.21	0.02	4
vasopressin	1083.45	1083.44	9	CYFQNCPRG-amide	1.25	0.16	4
provasopressin (151−166)	1712.94	1712.9	23	VQLAGTRESVDSAKPR	2.96	1.76	4
provasopressin (151−168)	1975.06	1975.06	0	VQLAGTRESVDSAKPRVY	0.85	0.22	4
chromogranin A fragment	1219.61	1219.6	8	AYGFRDPGPQL	0.53	0.02	2
chromogranin B fragment	1041.53	1041.51	19	LLDEGHYPV	<0.15	n/a	3
chromogranin B fragment	2400.19	2400.14	21	LGALFNPYFDPLQWKNSDFE	0.68	0.05	3
secretogranin II fragment	1270.65	1270.65	0	QELGKLTGPSNQ	0.51	0.09	3
unknown	739.43				1.11	0.16	4
unknown	831.95				0.89	n/a	1
unknown	929.39				1.00	0.24	4
unknown	946.52				0.50	0.21	2
unknown	1116.60				0.42	n/a	1
unknown	1153.78				0.78	0.09	3
unknown	1178.37				0.30	0.02	2
unknown	1215.67				0.35	0.08	3
unknown	1284.65				1.00	0.29	2
unknown	1295.64				1.09	0.10	3
unknown	1317.76				1.06	0.04	3
unknown	1410.71				0.49	0.25	2
unknown	1565.85				3.21	2.06	4
unknown	1678.81				1.02	0.43	3
unknown	1720.82				1.07	0.40	4
unknown	1789.72				1.66	n/a	1
unknown	1813.76				0.49	n/a	1
unknown	2013.11				0.21	0.00	2

[a] All masses below 5 kDa are monoisotopic; those above are indicated with average mass. Peptides with sequences shown in parentheses were only tentatively identified based on the similarity of the parent mass. For all other peptides indicated with sequences the identification was confirmed by MS/MS data as described in Materials and Methods. Abbreviations: CLIP, corticotropin-like intermediate lobe peptide; diff (ppm), the difference between observed and theoretical masses in parts per million; KO/wt, the ratio of the peak height of the peptide in PC1 KO mouse pituitary extracts relative to the peak height observed in wild-type mouse pituitary extracts; J-peptide, joining peptide; LPH, lipotropin; MSH, melanocyte stimulating hormone; OacY, *O*-acetyltyrosine; obsd mass (no tag), observed mass (after subtraction of the mass due to the TMAB label and protons); phosphoS, phosphoserine; Mox, oxidized methionine; POMC, proopiomelanocortin; SEM, standard error of the mean; theor mass, theoretical mass; n/a, not applicable.

required for the generation of the bioactive insulin. Using mouse models lacking either active PC2 or PC1 it has been shown that although either enzyme can cleave at both sites in the absence of the other, the efficiency of conversion is greatly enhanced with their coordinated sequential action, and PC1 cleaves prior to PC2 (*30*, *39*). To find out whether this is a universal processing scheme or simply true in particular cases, more extensive studies using the PC1 and PC2 KO mouse models are required. In the present study we have addressed the contribution of PC1 in the processing

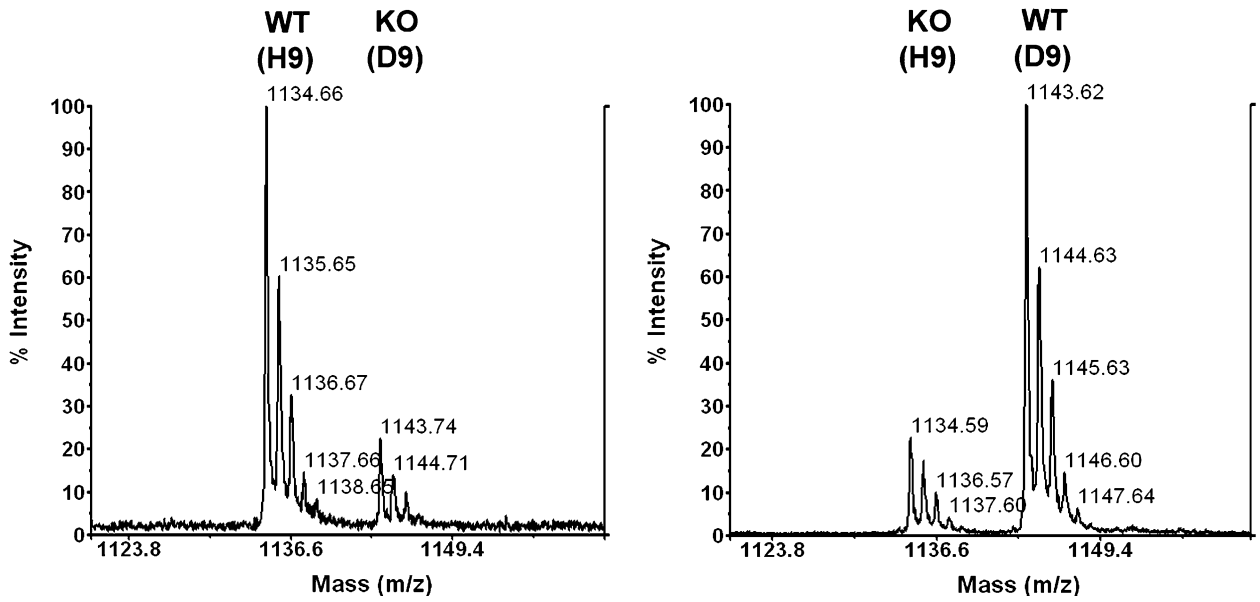

FIGURE 5: MS spectra of the TMAB-labeled oxytocin obtained from LC-ESI-TOF analysis. (Left) Pituitary extracts from wild-type mice labeled with H_9-TMAB were combined with an equal volume of PC1 KO mouse pituitary extracts labeled with D_9-TMAB. (Right) Labeling order was reversed, with H_9-TMAB labeling the pituitary extracts from PC1 KO mice and D_9-TMAB labeling the wild-type extracts. Spectra were selected from two sets of data; similar results were obtained in two additional sets of data. The monoisotopic peak with an *m*/*z* value of 1134.66 (or 1143.62) represents singly charged nonprotonated mono-H_9-TMAB (or D_9-TMAB) labeled oxytocin. Note that each TMAB label imparts a positive charge and a mass increase by 128.117 (H_9-TMAB) or 137.173 (D_9-TMAB) on the peptide.

of proenkephalin using mice lacking the active enzyme. We find an accumulation of Met-Enk containing higher molecular weight peptide intermediates in these mice albeit at low levels as compared to wild-type mice. However, even in the absence of PC1 activity a significant level of ir-Met-Enk is present. This implies that another enzyme (most probably PC2) produces Met-Enk from the intact proenkephalin precursor.

The original study of PC1 KO revealed a growth impairment phenotype in the KO mice (*38*). The phenotype was attributed to the impairment of GHRH processing which led to the lack of growth hormone secretion. Also, in the PC1 KO mouse pancreas the processing of proinsulin was severely blocked, and around 90% of the pancreatic and circulating insulin-related immunoreactivity were represented by proinsulin. Taking into consideration the complexity of the regulation of energy metabolism, it is very likely that the processing of other peptide hormone regulators or modulators is compromised in the PC1 KO mice. Here, we have shown that the processing of VGF, a neuropeptide precursor that contributes to maintenance of the energy balance (*21*, *22*), is impaired in PC1 KO mice. Using an antiserum against the C-terminal 30 amino acids of VGF we identified an immunoreactive peak coeluting from RP-HPLC with the synthetic peptide used for generating the antiserum. A peptide of this size has previously been identified in bovine pituitary (*48*) and rat brain (*23*). A close examination of the VGF sequence shows that the N-terminus of the peptide, AQEE, is generated by a cleavage at the Arg-Arg—a PC consensus site. It is very likely that PC1 is involved in this cleavage in vivo, consistent with the demonstration that PC1 cleavage generates the AQEE peptide in transfected GH3 cells (*23*).

Regulation of the two prohormone convertases has been extensively studied. In the case of PC2 the protein 7B2 is coexpressed with PC2 in many neuroendocrine tissues and required for the proper maturation and timing of the activation of PC2 (*49*−*51*). The 7B2 protein possesses a PC2 inhibitory domain, and a PC consensus site in the inhibitory fragment (Lys-Lys) is required for its specific inhibitory action on PC2 (*52*, *53*). This site can be slowly processed by PC2, and the inhibition is released after endopeptidase cleavage and subsequent removal of the Lys residues by a CP (*54*). A similar mechanism has been proposed for the inhibition of PC1 by proSAAS (*27*, *28*). The C-terminal portion of ProSAAS (PEN-LEN) is slowly cleaved in vitro by PC1 into PEN-Lys-Arg and big-LEN, and after removal of the Lys and Arg residues by a CP, the products are no longer inhibitory to PC1 (*27*, *28*). However, our RIA data indicate that there is no difference in the level of ir-PEN in the PC1 KO and wild-type mouse brains. This implies that the cleavage event that converts the PC1-inhibitory fragments of proSAAS into the noninhibitory forms must be due to an enzyme other than PC1.

Although RIA is a powerful technique with relatively high sensitivity, it requires a specific antibody for each peptide, which limits the technique to only known peptides. Also, characterizing the changes in peptide levels on a large scale would be laborious. Here, we applied a quantitative peptidomic method using differential isotopic labeling in combination with LC−MS analysis to characterize the global peptide processing profile change in the PC1 KO mouse pituitaries. Among the peptides observed a big portion have also been previously identified using the Cpe^{fat}/Cpe^{fat} mouse system [(*55*) the peptides observed were all with basic residue extensions] and/or in wild-type mice (*46*, *47*).

One important finding in this study is that the level of oxytocin decreases by about 80% in the PC1 KO mice. This decrease was seen in all four sets of the differential isotopic labeling/MS experiments. Oxytocin functions as the ligand of a seven-transmembrane domain G-protein-coupled receptor, and in mice it is crucial for the milk ejection reflex in the nurturing mother and a number of cognition and social behaviors (for reviews, see refs 55 and 56). The mice lacking

the pro-oxytocin gene fail to recognize familiar conspecifics after repeated social encounters and become more aggressive. The oxytocin KO pups emit fewer ultrasonic vocalizations with maternal separation. It would be interesting to check whether any of these phenotypes are present in the PC1 KO mice.

Another interesting point is that although oxytocin and vasopressin are closely related and their sequences around the cleavage sites are very similar, a comparison of the vasopressin levels between the PC1 KO and wild-type mice revealed no measurable differences. It has been previously shown that both PC1 and PC2 are coexpressed with prooxytocin and provasopressin in certain groups of neurons in the supraoptic and paraventricular nuclei in hypothalamus (*58*). The observation that the vasopressin level in PC1 KO mice does not differ from that in wild-type mice suggests that either PC1 is not physiologically relevant for the processing of provasopressin or the expression level of provasopressin is up-regulated in these neurons to compensate for the inactivation of PC1.

One unexpected finding is that the peptides derived from POMC did not show large changes in their levels in PC1 KO pituitaries. In the original paper describing the PC1 KO mouse an up-regulation of POMC mRNA in the whole pituitary was reported (*38*). Thus, an increase in the production of POMC protein could easily compensate for the decreased processing due to the absence of PC1. In support of this proposal is the large variability in the levels of Ac-β-endorphin-1−31 detected in the present study; in some PC1 KO mice this precursor was elevated 3−5-fold, while in others the levels were no different from wild-type mice. Ac-β-endorphin-1−31 is a precursor of Ac-β-endorphin-1−27 and -1−26; these latter two forms are the major forms in the intermediate pituitary. Because PC2 is expressed at relatively high levels in the intermediate lobe of the pituitary, this enzyme could as well compensate for the absence of PC1 in the production of the POMC-derived peptides in the intermediate pituitary.

Another point worth mentioning is that as the cleavage event generating the C-terminus of γ-3-MSH and the N-terminus of J-peptide has long been thought to be exclusively from PC1 based on the in vitro studies using either purified enzymes and substrates or neuroendocrine cell lines (*10*); a drop in the levels of both peptides is expected to be seen in PC1 KO pituitaries. However, the level of J-peptide in PC1 KO mice is comparable to that in wild type. Thus, in the absence of PC1 the cleavage at this site can be compensated by other PC-like activity. A recent report showing a substantial decrease in plasma ir-γ-MSH level in PC2 KO mice strongly suggests that PC2 plays a more crucial role (*59*).

In this study full-length ACTH was not detected in either PC1 KO or wild-type mice. The lack of detection of this peptide in PC1 KO is consistent with the previous data in PC1 KO pituitaries (*38*). Interestingly, pulse-chase experiments show the presence of a small amount of peptide that appears early during the chase but is quickly cleaved, presumably to αMSH and CLIP since it does not appear in the medium (Zhou, A., and Steiner, D. F. unpublished). This is consistent with the lack of hypocorticalism, suggesting the presence of sufficient peptides with corticotropic activity. The fact that we do not detect ACTH in the wild-type pituitaries in our MS analysis could be because of the relatively large size of the glycosylated form which may be retained on the microcon YM-10 membrane. Although nonglycosylated ACTH should be in the filtrate, the sensitivity of the MS analysis used in this study might not be high enough to detect the signal. Larger peptides are typically more difficult to detect, in part because the signal is divided among multiple charge states.

In summary, the present study provides a better understanding of the role PC1 plays in the processing of a number of important neuroendocrine peptide precursor molecules and also provides insights for understanding novel aspects of the physiological functions PC1 might be involved with.

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