Biological Processing of the Cocaine and Amphetamine-regulated Transcript Precursors by Prohormone Convertases, PC2 and PC1/3*

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Cocaine and amphetamine-regulated transcript (CART), a neuropeptide peptide influencing reward, feeding/appetite, and stress responses is derived from two peptide precursors of 129 and 116 amino acid (aa) residues that arise via alternative splicing from a single Cart gene in rats and mice. The signal peptide constitutes the first 27 aa resulting in pro-CART molecules of either 102 or 89 aa. In the present study, we have shown that pro-CART is a substrate for the neuroendocrine subtilisin/kexin-like prohormone convertases, PC2 (SPC2) and PC1/3 (SPC3). By using different neuroendocrine cell lines, with or without endogenous expression of either PC2 or PC1/3 or both enzymes, we have demonstrated through transient transfection studies that long pro-CART gives rise to an intermediate peptide, residues 33–102, and the two major bioactive CART forms, residues 55–102 (I) and 62–102 (II), respectively. Likewise, short pro-CART also generates three peptides, an intermediate, residues 10–89, and the two identical bioactive CART forms. We have confirmed the identities of the bioactive and intermediate CART molecules by microsequencing and/or high performance liquid chromatography and mass spectrometry. We have shown that PC2 is more efficient in generating bioactive CART I compared with PC1/3, whereas the production of the smaller bioactive CART II is exclusively carried out by PC2. PC1/3 is predominantly responsible for generating the intermediate CART fragments, 33–102 and 10–89, from long and short pro-CART, respectively. To compare in vitro and in vivo processing of pro-CART, we have examined its processing in PC2, 7B2, and PC1/3 knock-out mouse hypothalamic extracts and demonstrated that, as in vitro, PC2 is more potent than PC1/3 in generating bioactive CART I whereas bioactive CART II is solely generated by PC2. Also, in vivo, we have shown that PC1/3 is predominantly active in liberating the two intermediate CART fragments, 33–102 and 10–89. These findings confirm the key roles of PC2 and PC1/3 acting together or separately to carry out CART processing in selected sites in vivo.

Cocaine and amphetamine-regulated transcript (CART) encoding a hypothalamic neuropeptide precursor protein was identified and characterized in rat brain and later in human brain by Douglass et al. (1, 2), Spiess et al. (3) in 1981, had identified a CART peptide fragment from ovine hypothalamus but its functional significance was not explored further. Characterization studies have demonstrated that CART protein, first shown to be produced by psychomotor stimulants, has both short and long isoforms present in rats and mice while in humans, only the short form is present. The long pro-CART, generated because of alternate splicing, is composed of 102 aa and has an extra 13-aa stretch located within the protein coding region (1) whereas the short pro-CART consists of 89 aa following an N-terminal hydrophobic signal sequence of 27 aa. Both isoforms can give rise to two bioactive CART peptides, I (55–102/42–89) and II (62–102/49–89) that are expressed abundantly in hypothalamus (4), mainly in the arcuate nucleus (5) among several other hypothalamic nuclei (6, 7) (Fig. 1). In a recent study in rats, Kristensen et al. (5) have demonstrated that intracerebroventricular injections of bioactive CART fragments cause a significant block of normal and starvation-induced feeding as well as totally inhibiting neuropeptide Y-augmented feeding responses. They have also shown that following intracerebroventricular injection of anti-CART antisem, food intake is increased significantly in rats. CART has also been shown to act downstream of leptin in the signaling pathway controlling obesity (5, 8). Other studies have also confirmed that intracerebroventricular injections of the 47-residue bioactive CART fragment (residues 55–102 of the long isoform or 42–89 of the short isoform) in normal rats (9) as well as in lean and obese Zucker (fa/ft) rats (10), inhibit feeding and cause weight loss. Recently, Thim et al. (11) have shown that two bioactive forms of CART, I and II, are the most abundant forms in hypothalamic extracts of rats, confirming the notion that full-length CART transcripts encode precursor proteins, which undergo processing to exert their physiological function(s).

Following the discovery of Kex2, a subtilisin-like serine endoprotease in yeast (12, 13), several mammalian homologs of Kex2 have been identified and shown to be involved in the processing of precursor proteins (14–23). Proteolytic processing of precursor peptides is implicated in regulation of several physiological processes in yeasts as well as many others in higher vertebrates including mammals. These include neural/endocrine regulation, gene expression, embryogenesis, cell cycle control, apoptosis, and intracellular protein targeting (see reviews in Refs. 13, 14, 17, 19–21). Among the seven prohormone convertases identified so far, PC2 (SPC2) and PC1/3 (SPC3) (24–27) are abundantly expressed in neuroendocrine tissues including brain (25, 27–29) and have been shown to prohormone convertases; Tricine, N-[2-hydroxy-1,1-bis(hydroxy-methyl)ethyl]glycine.
cleave several precursor proteins, including proinsulin, proopiomeselanocortin, proglucagon, proenkephalin, prodynorphin, prosomatostatin, and pro-GHRH\(^{19}\) generating their mature bioactive forms for their diverse biological functions (30–41). PC2 and PC1/3 cleave precursors at dibasic residue sites, usually KR or RR, but also are positively influenced by upstream basic residues at the P\(_2\) and/or P\(_3\) positions, as well as by other more subtle features in or near these sites (14, 19, 42, 43).

Analysis of the CART precursor sequences (both long and short) reveals the presence of several conserved mono- and dibasic potential processing sites consistent with the likelihood that these are substrates of the prohormone convertases PC2 and/or PC1/3, both of which are expressed at high levels in the arcuate nucleus (29). Recently, the mouse Cart gene has been identified (9) based on the sequence similarities of rat and human cDNAs (1, 2) and the mouse pro-CART peptide is shown to have 98 and 96% homology with the rat and human sequences, respectively. However, homozygous activation of PC2 (exon 3 deletion) in mice shows no apparent effect on feeding/appetite and obesity (38). Also, mice null for 7B2, a neuroendocrine protein required for the activation of PC2, do not show any severe abnormality in feeding behavior or obesity (44). Recently, adenrelecanotized male mice lacking 7B2 have been shown to be significantly obese at around 12 weeks of age (45). Compound heterozygosity for inactivating mutations in the PC1/3 gene in man has been shown to be associated with severe obesity beginning early in life (46). On the contrary, PC1/3 null mice, recently created in our laboratory, exhibit growth retardation without obesity (41). However, the levels of the bioactive forms of CART in the arcuate nucleus of the hypothalamus have not yet been analyzed in any of these convertase-deficient models. The co-localization of PC2 and PC1/3 along with CART in hypothalamic nuclei thus strongly suggests that these are physiologically important sites for pro-CART processing to generate the bioactive forms.

In the current study, we have examined the roles of PC2 and PC1/3 in processing pro-CART. Our findings indicate that both convertases are required for efficient processing of the bioactive forms of CART, but there are significant differences in the ability of PC2 versus PC1/3 in generating the CART peptides. Also, we have observed defects in hypothalamic processing of pro-CART in PC2 and PC1/3 null mice in vivo that confirm our in vitro findings.

MATERIALS AND METHODS

Sources—Both the short (89 aa) and long (102 aa) rat pro-CART expression plasmids (pSX631 and pSX632), the monoclonal (CA6–1F4A1B1C1D4) and polyclonal (CA4) antibodies against CART as well as the purified CART peptides (10–89, 41–89, and 48–89) were kind gifts from Dr. Lars Thim and Dr. Sven Hastrup of Novo Nordisk. Polyclonal antisera against the N-terminal 14 residues of CART I (residues 42–55 of short pro-CART) were raised in both rabbits and guinea pigs (Covance, Richmond, CA). Significant titers of CART antibody were detected in the 3rd bleed sera, and IgG fractions were purified by means of Affi-Gel protein A-agarose (Bio-Rad). All the cell culture media (complete and deficient) and reagents were purchased from Invitrogen. Effectene, the transfection reagent, and the Endo Free Plasmid Kit were from Qiagen. All the radioactive amino acids were from Amersham Biosciences. Monoclonal anti-FLAG antibody was from Sigma.

Cell Culture, Metabolic Labeling, Immunoprecipitation, and Western Blot—At 1–6, βTC-3, and AT20 cells were cultured in Dulbecco’s modified Eagle’s medium (high glucose) with 10% FBS. Following transient transfection (according to Qiagen’s protocol) and before labeling, cells in 6-cm dishes (almost 80% confluency) were washed twice with phosphate-buffered saline (37 °C) and incubated in a deficient medium (lacking amino acids used for labeling) without serum (+0.15% bovine serum albumin + high glucose) for 1 h. Labeling was performed using various mixtures of tritiated amino acids alone or with [\(^{35}\)S]Met (100–200 μCi of each/ml of deficient medium) for 30 min and following washing with phosphate-buffered saline, cells were chased for different time periods in a complete medium without serum (+0.15% bovine serum albumin + high glucose) and also in the presence of 10× excess cold amino acids. Cells (both pulsed and chased), following the phosphate-buffered saline wash and chased media, were harvested (48 °C for immunoprecipitation (IP). For IP, cells were scraped from plates into ice-cold IP buffer (Tris-HCl, 50 mM, pH 7.5; NaCl, 150 mM; EDTA, 1 mM; Tween 20, 0.1%; Na-deoxycholate, 0.05%; and glycerol, 10%, with freshly added protease inhibitor mixture containing phenylmethylsulfonyl fluoride, 0.2 mM; leupeptin, 2.5 μg/ml; pepstatin A, 2 μM; aprotinin, 1 μg/ml; β-glycerophosphate, 10 mM; NaF, 1 mM; and Na-orthovanadate, 0.1 mM), followed by brief sonication (10 s, three times) on ice, and the whole cell lysates were microcentrifuged at 12,000 × g for 10 min at 4 °C. Bovine serum albumin (final concentration 1 mg/ml) was added to the clear 12,000 × g supernatants; chase media samples received the above protease inhibitor mixture. Equal aliquots were incubated overnight with the required antibody on a tumbler. The next day, following addition of either protein G- or protein A-agarose (Roche Molecular Biochemicals), incubation was continued overnight at 4 °C. Beads bound with the antibody-antigen complexes were subjected to washing, twice each with ice-cold IP buffer followed by a high salt (Tris-HCl, 50 mM, pH 7.5; NaCl, 500 mM; Tween 20, 0.1%; and Na-deoxycholate, 0.05%) and low salt buffer (Tris-HCl, 50 mM, pH 7.5; Tween 20, 0.1%; and Na-deoxycholate, 0.05%). In the case of non-reducing gels, antibody-antigen complexes, bound to beads, were first extracted in 100 μl glycine (pH 2.7) and following adjustment of pH by 1 N NaOH, were boiled in Laemmli sample buffer (without β-mercaptoethanol) and resolved in Tricine/SDS-PAGE (47). For reducing gels, after incubation, the beads released from the beads, following IP washes, were directly loaded in Laemmli sample buffer (+ β-mercaptoethanol). Resolved peptides were fixed in a mixture of water (25), isopropyl alcohol (65), acetic acid (10) for 30 min, treated (Amplify, Amersham) for another 30 min, and finally, the dried gels were exposed to Hyperfilm MP (Amersham Pharmacia Biotech) at 80 °C. For Western blot analysis, intact hypothalami (2 of each) were dissected aseptically from control and knockout mice, each pair combined and quickly homogenized in the cold IP buffer (with freshly added protease inhibitor mixture) as mentioned above using a Dounce glass homogenizer (25–30 strokes on ice). The homogenized materials were incubated on ice for 40 min and clear supernatants were collected following a 10-min spin at maximum speed in a microcentrifuge. Following the Bio-Rad protein assay, equal amounts of hypothalamic extracts were boiled in Laemmli sample buffer without with β-mercaptoethanol and resolved in gradient Tricine/SDS-PAGE. The resolved peptides, after being transferred onto Hybond P membrane (Amersham Biosciences), were incubated either with mouse monoclonal anti-CART (41–89) or purified rabbit anti-CART-(42–45) antibody at 1 μg/ml or 1 μg/ml dilution overnight at 4 °C followed by horseshadish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences) at 1:3000 dilution for 1 h at room temperature. Finally, immunoreactive bands were visualized by ECL Plus (Amersham Biosciences). Purified rat CART peptides (CART-(10–89), CART-(41–89), and CART-(48–89)) used as standards were recovered and detected with equal efficiency regardless of their size when applied in the range of 0.5–1 pmol/lane.

Microsequencing—Radiolabeled (tritiated) peptides were transferred from Tricine/SDS-PAGE onto polyvinylidene difluoride membranes (Bio-Rad) and the bands areas on polyvinylidene difluoride
membranes corresponding to the bands on autoradiograms were cut out carefully. These were subjected to automated Edman degradation using a PerkinElmer/Applied Biosystems protein sequencer, model 492, and a blot sequencing cartridge (PerkinElmer/Applied Biosystems, Foster City, CA). Following each sequencing cycle, the phenylthiohydantoin-derivatives were collected and counted in a scintillation counter.

Electroelution, HPLC, and Mass Spectrometry—Following transient transfection of pro-CART expression plasmid into βTC-3 cells, cell lysates containing pro- and processed forms of CART were immunoprecipitated using monoclonal anti-CART antibody and the immunoprecipitates were resolved in Tricine/SDS-PAGE. The gel portions having only CART I fragment 55–102 with reference to the authentic as well as preformed molecular weight markers were carefully cut out and the peptide band was electroeluted using the Bio-Rad Electro-Eluter (model 422) following the company’s protocol. Salt was removed from the eluate by diluting in water and spinning through microfiter devices (Milipore) several times. Finally, the eluate was dried in a vacuum centrifuge (Speed Vac) and dissolved in a solution of 25% methanol, 1% acetic acid, and 74% water. This solution was injected onto a Vydac reversed-phase C4 HPLC column (4.6 × 250 mm) equilibrated with 0.1% (v/v) trifluoroacetic acid in 5% acetonitrile. The concentration of the acetonitrile in the eluting solvent was increased to 65% (v/v) over a 40-min period and the absorbance was measured at 214 nm. Peptide materials corresponding to peaks were collected for analysis by electrospray ionization mass spectrometry (PE-SCIEX, model API 150 EX). The syringe corresponds to peaks were collected for analysis by electrospray ionization mass spectrometry (PE-SCIEX, model API 150 EX). The syringe peak corresponding to peaks were collected for analysis by electrospray ionization mass spectrometry (PE-SCIEX, model API 150 EX). The syringe peak corresponding to peaks were collected for analysis by electrospray ionization mass spectrometry (PE-SCIEX, model API 150 EX). The syringe peak corresponding to peaks were collected for analysis by electrospray ionization mass spectrometry (PE-SCIEX, model API 150 EX).

RESULTS

Processing of Pro-CART in Transfected Cell Lines—We have studied the processing of both long (102 aa) and short (89 aa) pro-CART by PC2 and PC1/3 in several neuroendocrine cell lines. We employed three PC1/3 and/or PC2-expressing murine neuroendocrine cell lines, αTC1–6 and βTC-3, derived from mouse pancreatic islets and AtT20, derived from rat anterior pituitary corticotrophs, for most of our studies of pro-CART processing. While αTC1–6 is known to have high levels of PC2 and undetectable levels of PC1/3, βTC-3 cells express substantial amounts of both convertases, whereas AtT20 expresses PC1/3 at high levels with very low or undetectable levels of endogenous PC2 (33, 45) as we also confirmed by Northern and Western blot analyses (data not shown). To examine the processing of pro-CART by these two prohormone convertases, both long and short pro-CART isoforms were transiently transfection into the above cell lines using Effectene from Qiagen (see “Materials and Methods”). Cells were metabolically labeled (see “Materials and Methods”) and 12,000 g supernatants as well as chased media were subjected to IP using either the monoclonal anti-CART antibody (generated against recombinant CART-(41–89) and characterized by Thin et al. (as described in Ref. 11) or the rabbit anti-CART antibody that we generated (see “Materials and Methods”). Immunoprecipitates, either extracted in 100 mM glycine (pH 2.7) followed by boiling in Laemmli sample buffer without β-mercaptoethanol or directly boiling in Laemmli sample buffer with β-mercaptoethanol, were resolved in Tricine/SDS-PAGE (47) followed by fixation and fluorography. We first examined the processing of long pro-CART in αTC1–6 cells where, following pulse (30 min) and chase (45 min and 3 h), long CART precursor cleavage was analyzed under non-reducing conditions in both the cell extracts and media as shown in Fig. 2A. These results demonstrated that the two major peptides, generated from long pro-CART, were the bioactive forms I (55–102) and II (62–102) (lanes 2–6) corresponding to the authentic iodinated markers in lane I, whereas a novel intermediate, CART-(33–102), arising from a cleavage at arginine 32 that lies in the inserted region of long pro-CART, was produced in very low amounts (lanes 5 and 6). Denatometric scanning of Fig. 2A shows that following the 30-min pulse (lane 2), about 20% of total pro-CART radioactivity was present as CART I in cells while CART II was negligible (about 4%). After the 45-min chase in the cells (lane 3) pro-CART had dropped to 23% of total and CART II had risen significantly to 55%, whereas CART I was 22%. After the 3-h chase (lane 4), CART II comprised about 75% of the total product radioactivity (5 times more than the level of CART I), indicating the robust activity of PC2 in secretory vesicles. Approximately 30% of CART radioactivity appeared in the chase media (lanes 5 and 6) in ratios of CART II/CART I similar to those seen in the cells and with small amounts of pro-CART as well. As expected, the amount of CART II in the media samples increased by 2-fold during the longer chase interval, consistent with a slow basal secretory rate.

When long pro-CART processing in AtT20 cells was analyzed under similar conditions (Fig. 2B), we observed the generation mainly of intermediate CART-(33–102) (lanes 2–6), whereas bioactive CART I was present at much reduced levels (lanes 4 and 6) and no CART II was seen. Denatometric scanning data demonstrated that in AtT20 cells, after a 30-min pulse (lane 2), ~20% of the total pool of long pro-CART was processed into its intermediate 33–102 that continued to rise in cells during the 3-h chase periods to about 70% of total product, whereas CART I, initially found at negligible levels (lanes 2 and 3), amounted to almost 1/5 of the levels of intermediate CART-(33–102) (lane 4). Both of these processed peptides, intermediate 33–102 and CART I, were secreted into chase media at a similar ratio of ~3:1 (lane 6) along with considerable amounts of unprocessed long pro-CART (~40% of total initial pro-CART), indicating much lower efficiency of PC1/3 compared with PC2 in processing long pro-CART and intermediate CART.

We next examined, under similar non-reducing conditions, the processing of short pro-CART in αTC1–6 cells. As shown in Fig. 3A, following the pulse (30 min, lane 2) and chase (45 min...
epitope (8 aa residues) at its C terminus and using monoclonal anti-FLAG antibody for IP (data not shown).

Next, we examined the early events in the processing of both long and short pro-CART in greater detail, using rabbit anti-serum (CART-(42–55)) and reducing conditions (see “Materials and Methods”), in three cell types, AtT20, βTC-3, and αTC1–6 as shown in Fig. 4. These conditions gave improved resolution of intermediate products. We again demonstrated using 30-min pulsed cell extracts that in both AtT20 and βTC-3 cells, the amounts of intermediate CART-(10–89), generated from short pro-CART, varied between 20 and 30% of total pro-CART levels (lanes 2 and 4) and in the αTC1–6 cells, this intermediate form was completely lacking (lane 6), indicating the exclusive importance of PC1/3 in generating it. The other intermediate form, 33–102, processed only from long pro-CART, in turn was present in all three cell lines, with almost 3-fold more formed in both AtT20 and βTC-3 cells (lanes 3 and 5) implying that both PC1/3 and PC2 (PC1/3 ≫ PC2) are able to generate this intermediate. The antisera used in this experiment was able to detect only bioactive CART I-(55–102/42–89), which, as before, was most efficiently generated in both βTC-3 and αTC1–6 cells because of the presence of high levels of PC2 compared with the AtT20 cells expressing only PC1/3.

Our findings on the cleavage of long (1–102) and short (1–89) pro-CART by PC1/3 and PC2 are summarized in Fig. 5A. The schematic diagram indicates that in the presence of PC2 alone, both the bioactive CART peptides, i.e. 55–102/42–89 (I) and 62–102/49–89 (II), are generated from both the pro-CART isoforms, following enzymatic processing at two dibasic sites, (Lys-53/40, Arg-54/41) and (Lys-60/47, Lys-61/48), respectively. PC1/3 alone is unable to cleave the dibasic site, KK (60/47), whereas the cleavage of the other dibasic site, KR (53/40, 54/41) by PC1/3 is done less efficiently compared with the more complete enzymatic processing by PC2. The diagram also shows that PC1/3 is crucial in generating intermediate CART-(10–89) from short pro-CART by cleaving at the single basic residue Arg-9, whereas both PC1/3 and PC2 (PC1/3 ≫ PC2) are able to produce the other intermediate 33–102 from long pro-CART at the site RQLR.

Characterization of Processed Peptides—To confirm the identity of these processed peptides, microsequencing (see “Materials and Methods”) was performed on individual, labeled peptide bands following transfer onto polyvinylidene difluoride membranes (Bio-Rad). First, the CART peptides, transiently expressed in all the three cell lines, AtT20, βTC-3, and αTC1–6, were metabolically labeled with two tritiated amino acids, [3H]isoleucine and [3H]tyrosine. Each doubly labeled peptide, derived from each cell line, was then subjected to microsequencing up to 15 cycles. In case of the bioactive CART peptides...
Materials and Methods

We also confirmed the identity of CART I-(55–102/42–89) by HPLC and mass spectrometry after its elution from the Tricine/SDS-PAGE following immunoprecipitation of the transiently expressed CART as described under "Materials and Methods." The HPLC chromatogram showed a major peak at 25 min (Fig. 6A) and the peptides eluted at that time point were analyzed by electrospray ionization mass spectrometry. The mass of the bioactive CART I, deduced by mass spectrometry, was found to be 5260.4 (Fig. 6B), which was in good agreement with the molecular mass of CART I-(42–89), i.e. 5259.3, as calculated from the published amino acid sequence.

In Vivo Processing of Pro-CART—To confirm our in vitro findings on pro-CART processing, whether both PC2 and PC1/3 are required and also, whether both of the prohormone convertases have varying degrees of efficiencies in generating both the bioactive forms (I and II) as well as the two intermediate peptides as described above, we analyzed the possible defects, if any, in pro-CART processing in three prohormone convertase null mice, i.e. PC2 (38), 7B2 (44), and PC1/3 (41). We first examined the ratio of short versus long pro-CART mRNA by reverse transcriptase-PCR in hypothalamic tissues of wild type (WT) and knockout (KO) mice. Because, both of the bioactive forms of CART (I and II) were shown to be present in rat hypothalamus (11), we compared whole hypothalamic extracts prepared from WT and KO mice by Western blotting to detect pro-CART processing defects. For qualitative quantitation of the various processed forms, we analyzed equal amounts of extracts from animals 8–12 weeks of age under non-reducing gel conditions (see "Materials and Methods").
pro-CART levels were undetectable whereas the level of intermediate CART-(10–89) was low, indicating very efficient processing of both precursor forms (Fig. 7A, lane 4).

In contrast, in PC2 KO animals, generation of both bioactive forms was severely hampered, with only a barely detectable level of bioactive CART I and no detectable levels of CART II (Fig. 7A, lane 3), confirming the exclusive importance of PC2 in generating the intermediate, CART-(10–89), from the short precursor (see Figs. 2, 3, 4, and 7). In contrast, PC2 is much more efficient than PC1/3 in generating bioactive form I and the smaller bioactive form II is produced exclusively by PC2 (see Figs. 2, 3, and 7). Accordingly, in αTC1–6 cells, expressing only PC2, we reproducibly observed production mainly of the bioactive CART fragments, I and II, from both short or long pro-CART, whereas in AtT20 cells, expressing PC1/3, we reproducibly demonstrated increased production of both intermediates, 10–89 and 33–102, from long pro-CART and is exclusively responsible for generating the intermediate, 10–89, from the short precursor (see Figs. 2, 3, 4, and 7). In contrast, PC2 is much more efficient than PC1/3 in processing both precursor forms, long (1–102) and short (1–89) both in vitro and in vivo. We have shown that PC1/3 is much more potent than PC2 in generating the intermediate form, 33–102, from long pro-CART and is exclusively responsible for generating the intermediate, 10–89, from the short precursor (see Figs. 2, 3, 4, and 7). In contrast, PC2 is much more efficient than PC1/3 in generating bioactive form I and the smaller bioactive form II is produced exclusively by PC2 (see Figs. 2, 3, and 7). Accordingly, in αTC1–6 cells, expressing only PC2, we reproducibly observed production mainly of both the bioactive CART fragments, I and II, from both short or long pro-CART, whereas in AtT20 cells, expressing PC1/3, we reproducibly demonstrated increased production of both intermediates, 10–89 and 33–102, from the short and long precursors, respectively, and only bioactive CART I. These results are consistent with the findings of Thim et al. (11) that CART I could be isolated from the PC1/3-rich rat anterior pituitary, whereas only CART II was found in the pituitary intermediate lobe, which contains high levels of PC2 in addition to PC1/3 (32).

PC2 and PC1/3 have preferences in their ability to cleave after dibasic residue sites, primarily KR (Lys-Arg) or RR (Arg-Arg) (14). In both the short and long pro-CART isoforms, a typical dibasic KR processing site is located at residues 40, 41 and 53, 54, respectively, resulting in the generation of bioactive CART I. The preferential cleavage after this site by PC2 is likely because of more subtle structural features within or near this site. Similarly, a dibasic KK site is located at positions 47, 48 in short pro-CART (corresponding to 60, 61 in the long isoform), leading to production of bioactive CART II by PC2, which is known to preferentially cleave after KK (Lys-Lys) or KR (Arg-Lys) sites (14 and 43). PC1/3, like furin, is known to cleave after RXRR sites (see reviews in Refs. 14, 43, and 49), one of which is present in the long pro-CART, between residues 29 and 32 (RQLR), a part of the 13-aa stretch that is absent in

**DISCUSSION**

In the present study, we have demonstrated the importance of the prohormone convertases, PC2 and PC1/3, for the physiological processing of both the CART precursors, long (1–102) and short (1–89) both in vitro and in vivo. We have shown that PC1/3 is much more potent than PC2 in generating the intermediate form, 33–102, from long pro-CART and is exclusively responsible for generating the intermediate, 10–89, from the short precursor (see Figs. 2, 3, 4, and 7). In contrast, PC2 is much more efficient than PC1/3 in generating bioactive form I and the smaller bioactive form II is produced exclusively by PC2 (see Figs. 2, 3, and 7). Accordingly, in αTC1–6 cells, expressing only PC2, we reproducibly observed production mainly of both the bioactive CART fragments, I and II, from both short or long pro-CART, whereas in AtT20 cells, expressing PC1/3, we reproducibly demonstrated increased production of both intermediates, 10–89 and 33–102, from the short and long precursors, respectively, and only bioactive CART I. These results are consistent with the findings of Thim et al. (11) that CART I could be isolated from the PC1/3-rich rat anterior pituitary, whereas only CART II was found in the pituitary intermediate lobe, which contains high levels of PC2 in addition to PC1/3 (32).
short pro-CART because of alternative splicing. We have demonstrated that PC1/3 is much more potent in processing after this RQLR site than PC2. To check whether furin could generate the intermediate form, 33–102, through processing at this RQLR motif in long pro-CART, NIH-3T3 and COS-7 cells expressing considerable amounts of furin but undetectable levels of PC2 and PC1/3 (48) were studied by transient transfection. Pro-CART was efficiently expressed and secreted, but no processing was seen in either cell line (data not shown). The CART precursor was efficiently expressed and secreted, but no processing of the intermediate, 10–89, was first identified in rat adrenal glands (11). However, we have detected it in both of our in vitro (using the short pro-CART isoform) and in vivo (using hypothalamic extracts) studies (see Figs. 3, 4, and 7) and have shown that the intermediate is produced exclusively by PC1/3 via cleavage at a monobasic site, Arg-9. Similarly, proglucagon is processed by PC1/3 at a single arginine at position 77 (14, 33), lacking upstream basic amino acids at the important amino acids at the important positions.

In this study, the identities of both the bioactive forms of CART, I and II, and intermediate CART (33–102) were confirmed by microsequencing. Microsequencing of pro-CART itself, isolated from different cell lines, was unsuccessful, probably because of the presence of an N-terminal glutamine that may undergo cyclization, preventing reaction with the Edman reagent (50, 51), as noted also by Thim et al. (11). The identity of bioactive form I, which has been used for many of the biological studies in vivo, was also confirmed by HPLC and mass spectrometry. Efficient secretion of all the processed forms of pro-CART was observed in our studies, indicating that following processing, these peptide fragments are retained in secretory granules and then secreted into the medium. The secretion of appreciable amounts of pro-CART indicates either that processing is incomplete in these cell lines or that some proportion of the precursor may be secreted via constitutive pathways because elevated constitutive secretion is a common property of transformed neuroendocrine cell lines.

Both bioactive CART I and II have been shown to be the most abundant forms in rat hypothalamus (11), and have the highest potency in anorexigenesis (5, 9, 10). Two recent studies in rats (52, 53) have indicated that bioactive CART potentiates anorexia either through inhibition of dopaminergic signaling in hypothalamic centers or directly via elevation of CART levels in hypophyseal neurons with aging. CART has been found in some islet tumors, including those arising from the somatostatin-producing D cells (54) and in this context, a group has demonstrated (55) that CART (55–102) inhibits insulin secretion significantly in perfused pancreatic islets. Recently, Volkoff et al. (56) have shown in goldfish that intracerebroventricular injections of CART (55–102) not only inhibit feeding, but also reduce neuropeptide Y-augmented food intake, indicating that the action of CART on feeding is conserved in evolution. CART also induces c-fos, an immediate early gene, in hypothalamus (57). The putative CART promoter (5′-flanking region) has been recently sequenced and shown to have a polymorphic site(s) associated with human obesity (58). Recently, a CART missense mutation of G729C in exon 2 causing substitution of Leu-34 to Phe was detected in a 10-year-old boy who had been obese since 2 years of age (59). In the same report, this autosomal dominant missense mutation was shown to cosegregate in three generations along with the phenotypes of severe obesity and type 2 diabetes. Also, CART has been identified in prenatal rat brain at embryonic day 11 indicating its possible role in brain development (60). All these studies establish the biological significance of CART as an important neuropeptide that acts downstream of leptin in the satiety signaling pathway (5).

Our studies of altered pro-CART processing in hypothalamic extracts from PC2, 7B2, and PC1/3 null mice compared with their controls are consistent with our conclusions from the transfection studies in neuroendocrine cell lines. PC1/3 and PC2 work in concert to generate normal levels of bioactive forms from both the precursor isoforms. Although we have not seen a complete block of pro-CART processing in any single strain of knockout mice we have studied, lack of either PC2 or 7B2 (a peptide required for pro-PC2 activation) causes significantly greater deficiencies of both the bioactive forms of CART compared with the PC1/3 null condition (see Fig. 7, A–C), confirming the much greater efficiency of PC2 in producing the bioactive forms, I and II. Undetectable levels of short pro-CART and higher levels of unprocessed long pro-CART in PC1/3 null mice compared with their control littermates as well as much enhanced accumulation of both the intermediate CART, 10–89 and 33–102, along with some levels of unprocessed short pro-CART in both PC2 and 7B2 null relative to their wild type littermates reflect varying degrees of processing of pro- and intermediate CART by PC2 and PC1/3.

Altogether, the present studies indicate that a complex but highly coordinated interplay among neuroendocrine processing enzymes ensures the correct, physiological processing of pro-CART in critical hypothalamic regions. Recently, the three-dimensional structure of the C-terminal region of human bioactive CART residues, 48–89, has been determined by NMR spectroscopy and shown to consist of a globular disulfide knot structure that has a large hydrophobic surface for potential receptor interactions (61). Identification of the CART receptor would greatly facilitate studies on the physiology and neurobiology of this potentially important neuroendocrine peptide and would help to clarify the signaling potential, if any, of the intermediate forms and of the precursor itself.

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Dr. Zhu’s name was misspelled.