Biological Processing of the CART Precursors by Prohormone Convertases, PC2 and PC1/3

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Running Title: Biological processing of proCART by PCs
Abbreviations: CART, cocaine and amphetamine regulated transcript; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; POMC, proopiomelanocortin; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate buffered saline; BSA, bovine serum albumin; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PTH, phenylthiohydantoin; icv, intracerebroventricular; NPY, neuropeptide Y; NMR, nuclear magnetic resonance.
ABSTRACT:

Cocaine and amphetamine regulated transcript (CART), a neuroendocrine 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 proCART molecules of either 102 or 89 aa. In the present study, we have shown that proCART 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 proCART 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 proCART 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 HPLC and mass spectrometry. We have shown that PC2 is more efficient in generating bioactive CART I compared to PC1/3, while 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 proCART respectively. To compare in vitro and in vivo processing of proCART, 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 while 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*. 
INTRODUCTION:

CART or cocaine and amphetamine regulated transcript 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, in 1981, had identified a CART peptide fragment from ovine hypothalamus but its functional significance was not explored further (3). Characterization studies have demonstrated that CART protein, first shown to be induced 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 proCART, generated due to alternate splicing, is composed of 102 aa and has an extra 13 aa stretch located within the protein coding region (1) while the short proCART consists of 89 aa following an N-terminal hydrophobic signal sequence of 27 aa. Both the 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 (icv) injections of bioactive CART fragments cause a significant block of normal and starvation induced feeding as well as totally inhibiting neuropeptide Y (NPY) augmented feeding responses. They have also shown that following icv injection of anti-CART antiserum, 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 icv 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/ fa) 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 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 cleave several precursor proteins, including proinsulin, POMC, proglucagon, proenkephalin, prodynorphin, prosomatostatin and proGHRH (Dey et al, unpublished results) 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 P4 and/or P6 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 proCART peptide is shown to have 98% and 96% homology with the rat and human sequences,
respectively. However, homozygous inactivation 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, adrenalectomized 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 proCART processing to generate the bioactive forms.

In the current study, we have examined the roles of PC2 and PC1/3 in processing proCART. 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 vs. PC1/3 in generating the CART peptides. Also, we have observed defects in hypothalamic processing of proCART 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 proCART 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 proCART) were raised in both rabbits and guinea pigs (Covance, Richmond, CA). Significant titres 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 GIBCO-BRL. Effectene, the transfection reagent, and the Endo Free Plasmid Kit were from Qiagen. All the radioactive amino acids were from Amersham. Monoclonal anti Flag antibody was from Sigma.

Cell Culture, Metabolic Labeling, Immunoprecipitation, and Western Blot:

αTC1-6, βTC-3 and AtT20 cells were cultured in DMEM (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 PBS (37°C) and incubated in a deficient medium (lacking amino acids used for labeling) without serum (+0.15% BSA + high glucose) for 1 h. Labeling was performed using various mixtures of tritiated amino acids alone or with 35S-Met (100-200µCi of each / ml of deficient medium) for 30 min. and following washing with PBS, cells were chased for different time periods in a complete medium without serum (+0.15% BSA + high glucose) and also in the presence of a 10x excess of the cold amino acids. Cells (both pulsed and chased) following PBS wash and chased media, were stored at –80°C for immunoprecipitation (IP). For IP, cells were scraped from plates into ice cold IP buffer.
(Tris.HCl-50mM, pH 7.5, NaCl-150mM, EDTA-1mM, Tween 20-0.1%, Na-deoxycholate-0.05% and glycerol-10% with freshly added protease inhibitor cocktail containing PMSF-0.2mM, leupeptin-2.5µg/ml, pepstatinA-2µM, aprotinin-1µg/ml, β-glycerophosphate-10mM, NaF-1mM and Na-orthovanadate-0.1mM), followed by brief sonication (10sec., thrice) on ice, and the whole cell lysates were microcentrifuged at 12,000g for 10 min. at 4°C. Bovine serum albumin (final conc. 1 mg/ml) was added to the clear 12,000g supernatants; chase media samples received the above protease inhibitor cocktail. Equal aliquots were incubated overnight (O/N) with the required antibody at 4°C on a tube rocker. Next day, following addition of either protein G or protein A-agarose (Boehringer Mannheim), incubation was continued for O/N at 4°C. Beads bound with the Ab-Ag complexes were subjected to washing, twice each with the ice cold IP buffer followed by a high salt (Tris.HCl-50mM, pH7.5, NaCl-500mM, Tween20-0.1% and Na-deoxycholate-0.05%) and low salt buffer (Tris.HCl-50mM, pH7.5, Tween20-0.1% and Na-deoxycholate-0.05%). In case of non-reducing gels, Ab-Ag complexes, bound to beads, were first extracted in 100mM glycine (pH2.7) and following adjustment of pH by 1N NaOH, were boiled in Laemmli sample buffer (without βME) and resolved in Tricine-SDS-PAGE (47). For reducing gels, beads, following IP washes, were directly boiled in Laemmli sample buffer (+βME). Resolved peptides were fixed in a mixture of water [65]: isopropanol [25]: 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 out aseptically from control and knock out mice, each pair combined and quickly homogenized in the cold IP buffer (with freshly added protease inhibitor cocktail) 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 10 min. spin at maximum speed in a micro centrifuge. Following Bio Rad protein assay, equal amounts of hypothalamic extracts were boiled in Laemmli sample buffer without or with βME and resolved in gradient Tricine-SDS-PAGE. The resolved peptides, after being transferred onto Hybond P membrane (Amersham Pharmacia), were incubated either with mouse monoclonal anti CART (41-89) or purified rabbit anti CART (42-55) antibody at 1µg/ml or 1:1000 dilution for O/N at 4°C followed by HRP conjugated anti mouse or anti rabbit IgG (Amersham Pharmacia) at 1:3000 dilution for 1h at RT. Finally, immunoreactive bands were visualized by ECL Plus (Amersham Pharmacia). 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 0.5-1.0 pmole/lane.

Microsequencing:

Radioactively labeled (tritiated) peptides were transferred from Tricine-SDS-PAGE onto PVDF membranes (Bio-Rad) and the band areas on PVDF membranes corresponding to the bands on autoradiograms were cut out carefully. These were subjected to automated Edman degradation using a PE/ABD protein sequencer, model 492, and a blot sequencing cartridge (PE/ABD, Foster City, CA). Following each sequencing cycle, the PTH amino acids were collected and counted in a scintillation counter.

Electroelution, HPLC, and Mass Spectrometry:

Following transient transfection of proCART 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 the CART I fragment 55-102 with reference to the authentic as well as pre-stained 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 microcon filter devices (Millipore) 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 x 250 mm) equilibrated with 0.1% (vol./vol.) trifluoroacetic acid in 5% acetonitrile. The concentration of the acetonitrile in the eluting solvent was increased to 65% (vol./vol.) over 40 min period and the absorbency 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 pump speed was 5µl/min for this instrument. Calibration was performed using the external standards and the accuracy of the mass determination was within 2 mass units.

RESULTS:

Processing of proCART in Transfected Cell Lines:

We have studied the processing of both long (102aa) and short (89aa) proCART 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 proCART 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, while AtT20 expresses PC1/3 at high levels with very low or undetectable levels of endogenous PC2 (33, 48) as we also confirmed by northern and western blot analyses (data not shown). In order to examine the processing of proCART by these two prohormone convertases, both long and short proCART isoforms were transiently transfected into the above cell lines using Effectene from Qiagen (see
Methods). Cells were metabolically labeled (see Materials and Methods) and 12,000g clear supernatants were subjected to IP using either the monoclonal anti-CART antibody (generated against recombinant CART 41-89 and characterized by Thim et al as described in ref. 11) or the rabbit anti-CART antibody that we generated (see Methods). Immunoprecipitates, either extracting in 100mM glycine (pH2.7) followed by boiling in Laemmli sample buffer without βME or directly boiling in Laemmli sample buffer with βME, were resolved in Tricine-SDS-PAGE (47) followed by fixation and fluorography. We first examined the processing of long proCART in αTC1-6 cells where, following pulse (30-min) and chase (45-min and 3h), 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 proCART, were the bioactive forms I (55-102) and II (62-102) (lanes 2-6) corresponding to the authentic iodinated markers in lane 1, while a novel intermediate, CART 33-102, arising from a cleavage at arginine 32 that lies in the inserted region of long proCART, was produced in very low amounts (lanes 5 and 6). Densitometric scanning of Fig.2A shows that following the 30-min pulse (lane 2), about 20% of total proCART 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) proCART had dropped to 23% of total and CART II had risen significantly to 55%, while CART I was 22%. After the 3-hr chase (lane 4), CART II comprised about 75% of total product radioactivity (5 times more than the level of CART I), indicating PC2’s robust activity 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 proCART as well. As expected, the amount of CART II in the media samples increased by two-fold during the longer chase interval, consistent with a slow basal secretory
When long proCART processing in AtT20 cells was analyzed under similar conditions (Fig. 2B), we observed the generation mainly of intermediate CART 33-102 (lanes 2-6), while bioactive CART I was present at much reduced levels (lanes 4 and 6) and no CART II was seen. Densitometric scanning data demonstrated that in AtT20 cells, after a 30-min pulse (lane 2), approximately 20% of the total pool of long proCART was processed into its intermediate 33-102 which continued to rise in cells during the 3-hr chase periods to about 70% of total product, while CART I, initially found at negligible levels (lanes 2 and 3), amounted to almost 1/3rd 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 approximately 3:1 (lane 6) along with considerable amounts of unprocessed long proCART (approximately 40% of total initial proCART), indicating PC1/3’s much lower efficiency compared to PC2 in processing long proCART and intermediate CART.

We next examined, under similar non-reducing conditions, the processing of short proCART in αTC1-6 cells. As shown in Fig. 3A, following the pulse (30-min, lane 2) and chase (45-min and 3h, lanes 3 and 4), the short CART precursor also generated both bioactive forms, CART I (42-89) and CART II (49-89) corresponding to the two authentic iodinated CART markers (lane 1). As also seen in Fig. 2A, the bioactive CART II level was negligible after the 30-min pulse (lane 2), but rose considerably to a ratio of ~2:1 relative to CART I during the chase period, especially at 3-h (lane 4), indicating again the absolute requirement for PC2 for the processing of CART II. During the 3h chase period, short proCART was almost quantitatively converted to products recovered in cells and/or media (not shown). When short proCART was pulse-labeled and chased in AtT20 cells (Fig. 3B), we found an intermediate band (lanes 2 – 4), corresponding
to the authentic iodinated CART 10-89 marker (lane 1). Densitometric scanning showed that after the 30-min pulse (lane 2), the amounts of short proCART vs intermediate CART were 3:1 while this ratio was almost reversed after the 45-min chase (lane 3). During the 3h chase period (lane 4), most of the short proCART was processed into intermediate 10-89, indicating a major role for PC1/3 in generating this intermediate as we demonstrated earlier in the case of the other intermediate, 33-102, that arises from long proCART (see Fig.2B). Also, low levels of bioactive CART I (42-89) were seen during the chase (Fig.3B, lanes 3 and 4) but without any detectable levels of bioactive form II (49-89). We checked the amounts of bioactive CART I secreted into the chase medium of AtT20 cells and found it comparable to the low levels in the cell extracts (data not shown), indicating that PC2, lacking in AtT20 cells, is required for efficient processing of both short proCART and the intermediate CART 10-89. Thus, PC2 plays a more important role in generating both the bioactive CART forms and especially the shorter CART II. We confirmed the generation of the two bioactive peptides, I and II, as well as intermediate CART 10-89 in similar experiments by expressing a chimeric short proCART tagged with a Flag epitope (8aa 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 proCART in greater detail, using rabbit antiserum (CART 42-55) and reducing conditions (see 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 proCART, varied between 20 to 30% of total proCART 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 proCART, in turn was present in all three cell lines, with almost 3-fold more formed in both AtT20 and ßTC-3 cells (lanes 3 and 5) implying that both PC1/3 and PC2 (PC1/3>>PC2) are able to generate this intermediate. The antiserum 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 ßTC-3 and αTC1-6 cells due to the presence of high levels of PC2 compared to the AtT20 cells expressing only PC1/3.

Our findings on the cleavage of long (1-102) and short (1-89) proCART 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 proCART isoforms, following enzymatic processing at two dibasic sites, (K53/40, R54/41) and (K60/47, K61/48) respectively. PC1/3 alone is unable to cleave the dibasic site, KK (60/47, 61/48), while the cleavage of the other dibasic site, KR (53/40, 54/41) by PC1/3 is done less efficiently compared to the more complete enzymatic processing by PC2. The diagram also shows that PC1/3 is crucial in generating intermediate CART 10-89 from short proCART by cleaving at single basic residue R9 while both PC1/3 and PC2 (PC1/3>>PC2) are able to produce the other intermediate 33-102 from long proCART 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 PVDF membranes (Bio-Rad). First, the CART peptides, transiently expressed in all the three cell lines, AtT20, ßTC-3 and αTC1-6, were metabolically labeled with two tritiated amino acids, 3H-isoleucine (I) and 3H-tyrosine (Y). 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 (55-102/42-89 and 62-102/49-89), significant counts (CPM) were recovered in cycle numbers 1 and 3 as well as in 4 and 8 due to incorporation of the labeled amino acids at positions, 55 / 42, 57 / 44, 58 / 45 and 62 / 49 in the long / short proCART peptide sequence, as shown in Fig.5B. For the intermediate CART peptide, 33-102, significant counts (CPM) were recovered only in cycle number 8 due to isoleucine incorporation at aa40, while the unprocessed proCART molecule did not reveal any significant incorporated count (CPM) up to 15 cycles. These data support the identification of both the bioactive forms, I and II, generated from both short and long proCART as well as one of the intermediates, 33-102, as demonstrated in Fig. 2A+B and 3A+B.

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 in Methods. The HPLC chromatogram showed a single major peak at 25 min (Fig.6A) and the peptides eluted at that time point were analyzed by the 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 proCART:**

To confirm our *in vitro* findings on proCART 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 proCART processing in three prohormone convertase null mice, i.e., PC2 (38), 7B2(44) and PC1/3 (41). We first
examined the ratio of short vs. long proCART mRNA by RT PCR in hypothalamic tissues of wild type (WT) and knock out (KO) mice and found it to be approximately 2:1 (data not shown). Since, 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 proCART processing defects. For qualitative quantitation of the various processed forms, we analyzed equal amounts of extracts from animals 12-16 weeks of age under non-reducing gel conditions (see Methods), which allowed clear identification of both bioactive CART I and II, as demonstrated in the foregoing transient transfection studies. Fig.7A shows that in WT control animals, both bioactive forms I and II were present in considerable amounts and the level of bioactive CART I was higher than that of CART II (lane 4). Both short and long proCART levels were undetectable while 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 for cleaving the dibasic KK site to generate bioactive CART II. Also, the barely detectable level of CART I implies PC1/3’s only partial ability to cleave the dibasic KR site, as was also demonstrated _in vitro_. However, as a result of active PC1/3 in PC2 null mice, we found considerable levels of the intermediate CART 10-89 and also a low accumulation of unprocessed short proCART, while long proCART was fully processed into its only recognizable intermediate, 33-102, as shown in Fig.7A, lane 3. Likewise, in 7B2 KO animals, compared to their WT littermates, similar defects in proCART processing were found in hypothalamic extracts (Fig.7B, lane 3 vs. 4), indicating that PC2
enzymatic activity, conferred by 7B2, is crucial for generating the two bioactive CART forms as shown \textit{in vitro}.

In contrast, as demonstrated in Fig. 7C, no apparent processing defects were seen in generating the two bioactive forms I and II in PC1/3 KO hypothalamic extracts compared to their control littermates (lane 4 vs. lanes 5 and 6) indicating PC2’s high efficiency in processing these two forms. Also, in PC1/3 KO animals, significant amounts of long proCART accumulated and correspondingly, intermediate CART 33-102 was undetectable (Fig. 7C, lane 4) implying that PC1/3 is mostly responsible for cleavage at the RQLR site of long proCART as demonstrated before in our transfection studies. Also, in control animals (WT and PC1/3 heterozygotes), we found considerable levels of intermediate CART 10-89 (lanes 5 and 6) along with some unprocessed short proCART, forming a doublet with the 10-89 intermediate, only in heterozygotes (lane 5), reinforcing the idea that PC1/3 is the major enzyme cleaving at the single basic residue, R9 (see Fig. 5A). Note that we detected intermediate CART 33-102 at very low levels, in PC1/3 control animals (lanes 5 and 6), although it was not evident in PC2 and 7B2 WT littermates (Fig. 7A and B, lane 4), possibly due to differences among the various KO strains.

**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 \textit{in vitro} and \textit{in vivo}. We have shown that PC1/3 is much more potent than PC2 in generating the intermediate form, 33–102, from long proCART and is exclusively responsible for generating the intermediate, 10-89, from the short precursor (see Fig. 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 Fig. 2, 3 and 7). Accordingly, in $\alpha$TC1-6
cells, expressing only PC2, we reproducibly observed production mainly of both the bioactive CART fragments, I and II, from both short or long proCART, while 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, while 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 proCART 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 due to more subtle structural features within or near this site. Similarly, a dibasic KK site is located at positions 47,48 in short proCART (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 RXXR sites (see reviews 14, 43 and 49), one of which is present in the long proCART, between residues 29–32 (RQLR), a part of the 13 aa stretch that is absent in short proCART due to 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 proCART, 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. ProCART was efficiently expressed and secreted, but no processing was seen in either cell line (data not shown). The CART 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 proCART isoform) and *in vivo* (using hypothalamic extracts) studies (see Fig. 3, 4 and 7) and have shown that this 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 P2, P4, or P6 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 proCART itself, isolated from different cell lines, was unsuccessful, probably due to 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 proCART 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 proCART indicates either that processing is incomplete in these cell lines or that some proportion of the precursor may be secreted via constitutive pathways since 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 hypothalamus 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 icv injections of CART 55-102 not only inhibit feeding, but also reduce NPY-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 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 proCART processing in hypothalamic extracts from PC2, 7B2 and PC1/3 null mice compared to 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 proCART processing in any single strain of knock-out mice we have studied, lack of either PC2 or 7B2 (a peptide required for proPC2 activation) causes significantly greater deficiencies of both the bioactive forms of CART compared to the PC1/3 null condition (see Fig.7A+B+C), confirming PC2's much greater efficiency in producing the bioactive forms, I and II. Undetectable levels of short proCART and higher levels of unprocessed long proCART in PC1/3 null mice compared to 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 proCART 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 proCART 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.
ACKNOWLEDGEMENT: We thank Dr. L. Thim, S. Hastrup and J. T. Clausen of Novo Nordisk, Denmark for providing the CART expression plasmids, antibodies, and purified peptides. Paul Gardner is thanked for making oligonucleotides and Christina Norrbom for her technical assistance. We also wish to thank Rosie Ricks for expert secretarial assistance. This work has been supported by NIH grants DK13914 and DK20595 and by the Howard Hughes Medical Institute.
REFERENCES:


FIGURE LEGENDS:

FIG. 1: Schematic structures of the long and short proCART isoforms and derived proteolytic processing products discussed in this report. The inserted sequence in the long isoform due to alternative splicing (residues 27-39) is indicated by the thin line.

FIG. 2: Processing of long proCART by endogenous PC2 or PC1/3. Cells, αTC1-6 (panel A) and AtT20 (panel B), were transiently transfected with 1µg of long proCART expression plasmid and pulsed for 30min with either 3H-isoleucine and 3H-tyrosine or 35S-met followed by chase for 45min and 3h. Both the cell extracts and media were IP with monoclonal anti CART antibody and the immunoprecipitated peptides were resolved, under non-reducing conditions (see Methods), in Tricine-SDS-PAGE, followed by fixation and fluorography. In both panels A and B, lane 2 represents 30-min pulse while lanes 3, 4 and 5, 6 indicate 45min and 3h chased cell extracts (CE) and media (MED) respectively. Lane 1 shows the locations of the three iodinated authentic peptides (top - int. CART 10-89, middle - CART I and bottom - CART II) as well as the pre-stained protein molecular weight markers.

FIG. 3: Processing of short proCART by endogenous PC2 or PC1/3. Cells, αTC1-6 (panel A) and AtT20 (panel B) following transient transfection of 1µg short proCART expression plasmid, were metabolically labeled with either 3H-isoleucine and 3H-tyrosine or 35S-met for 30 min. Cell lysates were IP with monoclonal anti-CART antibody and the immunoprecipitates, under non-reducing conditions, were analyzed in Tricine-SDS-PAGE. In both panels A and B, lane 2 represents 30-min pulse and lanes 3 and 4 indicate 45-min and 3h chase of cell extracts,
respectively. Lane 1 shows the locations of the three iodinated authentic peptides as described in Fig.2 legend and pre-stained protein markers.

**FIG. 4:** Early events in the processing of long and short proCART by endogenous PC2 and/or PC1/3 under reducing gel conditions. AtT20, βTC-3 and αTC1-6 cells, following transient transfection and metabolic pulse labeling (30 min), were IP with rabbit anti-CART (42-55) antibody (see Methods for details) and the immunoprecipitated peptides were resolved in Tricine-SDS-PAGE. Lanes 2, 4, 6 and lanes 3, 5 and 7 represent short and long proCART transfection, respectively. Lane 1 shows the locations of the three iodinated authentic peptides (top - int. CART 10-89, middle - CART I and bottom - glucagon) and pre-stained protein molecular weight markers.

**FIG. 5:** Schematic representation of the enzymatic processing of both the long (purple) and short (green) proCART isoforms by PC2 and/or PC1/3 (A) and amino acid sequence of mouse proCART isoforms (B). A. The processing scheme is based on the analysis of radiomicrosequencing data derived from each of 3H-labeled peptides transferred onto PVDF membranes following transient transfection in αTC1-6, AtT20 and βTC-3 cells (see Methods for details). The inserted sequence in long proCART is denoted by the triangle (residues 27-39). The letters K and R represent the two basic amino acids, lysine and arginine, respectively, in the CART peptide sequence. B. In both long and short proCART isoforms, the 3H-isoleucine and 3H-tyrosine labeled amino acids, identified by microsequencing following enzymatic processing, were marked with asterisks.
FIG. 6: HPLC and Mass spectroscopic analysis of the bioactive CART I, 55-102/42-89. A. shows the reversed phase HPLC on a Vydac C4 column of the electroeluted bioactive CART, derived from neuroendocrine cells transiently transfected with the proCART expression plasmids (see Methods for details). B. shows the mass spectrum of the bioactive CART peptide, eluted in the HPLC peak at 25 min as shown in A. The single mass spectrum peak corresponds to CART 55-102/42-89 (theoretical mass = 5.2592 Kd).

FIG. 7: Western blot of hypothalamic extracts. Equal amounts of hypothalamic extracts from control and knockout animals, 150 µg of total protein for each of PC2, 7B2 and PC1/3, were resolved, under non-reducing conditions, in Tricine-SDS-PAGE and following membrane transfer and incubation with monoclonal anti-CART antibody, immunoreactive bands were reproducibly detected by ECL Plus (see Methods for detail). In panels A and B, lanes 3 and 4 represent PC2 and 7B2 knockout and WT animals, respectively while in panel C, lanes 4, 5 and 6 indicate PC1/3 knockout, heterozygous and WT respectively. In panel A, lane 1 shows the location of the authentic CART II peptide and lane 2 represents the locations of the other two authentic peptides, int. CART 10-89 (top) and CART I (bottom) while in panel B, lane 1 indicates both the authentic CART I (top) and II (bottom) and lane 2 represents the authentic int. CART 10-89. In panel C, lanes 1, 2 and 3 show the authentic CART II, int. CART 10-89 and CART I locations respectively. The pre-stained protein molecular weight markers are shown on left of each of panels A, B and C.
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ProCART (1-102)
Int. CART (33-102)
CART I
CART II

αTC1-6 Cells
5B.

Long: QEDAELOPRALDIYSAVDDASHEKELPRRQLRAPGAMLQI
Short: QEDAELOPRALDIYSAVDDASHEKEL

Long: EALQEVLKKLKLKSKRIPIYEKKYGQVPMCDAGEQCAVRKGA
Short: EALQEVLKKLKLKSKRIPIYEKKYGQVPMCDAGEQCAVRKGA

Long: RIGKLCDCPRGTSNFLLKCL
Short: RIGKLCDCPRGTSNFLLKCL
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**7B2 Hypothalamic Extracts**

- ProCART (1-89)
- Int. CART (10-89)
- Int. CART (33-102)
- CART I
- CART II
Addition and Correction


Biological processing of the cocaine and amphetamine-regulated transcript precursors by prohormone convertases, PC2 and PC1/3.

Arunangsu Dey, Xiaorong Zhu, Raymond Carroll, Christopher W. Tureck, Jeffrey Stein, and Donald F. Steiner

Dr. Zhu's name was misspelled.

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Biological processing of the CART precursor by prohormone convertases PC2 and PC1/PC3
Arunangsu Dey, Xiaorong Zhu, Raymond Carroll, Christopher W. Turck, Jeffrey Stein and Donald F. Steiner

J. Biol. Chem. published online February 12, 2003

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