RNAi-mediated silencing of prohormone convertase (PC) 5/6 expression leads to impairment in processing of cocaine- and amphetamine-regulated transcript (CART) precursor

Jeffrey STEIN*, Rohan SHAH*, Donald F. STEINER*† and Arunangsu DEY*¹

*Department of Biochemistry and Molecular Biology, University of Chicago, 5841 South Maryland Avenue, MC-1027, Chicago, IL 60637, U.S.A., and †Howard Hughes Medical Institute, University of Chicago, 5841 South Maryland Avenue, MC-1028, Chicago, IL 60637, U.S.A.

Understanding the functions of the widely expressed PCs (prohormone/proprotein convertases), including PC5/6, furin and PACE4 (paired basic amino acid cleaving enzyme 4), in animal models is difficult since individual knockouts of these PCs in mice exhibit early embryonic lethality. To investigate the roles of PC5/6 in processing pro-CART (pro-cocaine- and amphetamine-regulated transcript), an important anorexigenic peptide precursor, we have generated GH3 cells silenced for PC5/6 expression by RNAi (RNA interference). We show, following transient knockdown of PC5/6 in these neuroendocrine cells, that generation of the two bioactive forms, CART I (amino acids 42–89/55–102) and CART II (amino acids 49–89/62–102), from pro-CART is impaired due to a lack particularly of the A isoform of PC5/6. The results indicate that PC5/6A shares specificities primarily

with PC2 (PC5/6A < PC2) in cleaving the pairs of basic residues, KR (40, 41↓/53, 54↓) and KK (47, 48↓/60, 61↓), within the pro-CART isoforms [see Dey, Zhu, Carroll, Turck, Stein and Steiner (2003) *J. Biol. Chem.* **278**, 15007–15014]. We do not find any significant role of PC5/6A in processing the RXXR (29–32↓) site for production of intermediate CART (amino acids 33–102) from long pro-CART. The findings taken altogether indicate that PC5/6 participates in normal processing of pro-CART.

Key words: cocaine- and amphetamine-regulated transcript (CART), paired basic amino acid cleaving enzyme (PACE), prohormone/proprotein convertase 5/6 (PC5/6), protein precursor processing, RNA interference (RNAi), short hairpin RNA (shRNA).

INTRODUCTION

PCs (prohormone/proprotein convertases) are a group of calcium-dependent serine endoproteases that are related to yeast kexin and bacterial subtilisin [1]. In mammals, seven PC members have been identified so far and these include PC2, PC1/3, furin, PACE4 (paired basic amino acid cleaving enzyme 4), PC5/6, PC7/8 and PC4 [1–3]. While four of the PCs, furin, PACE4, PC5/6 and PC7/8, are widely distributed, PC2 and PC1/3 are primarily expressed in neuroendocrine/endocrine tissues and PC4 is expressed only in germ cells [1–4]. PCs post-translationally and endoproteolytically process numerous inactive protein precursors to generate smaller peptides and the biological substrates for PCs include various prohormones, proneuropeptides, growth factors and growth factor receptors, adhesion molecules, matrix metalloproteinases, viral coat proteins and bacterial toxins [1–3,5,6]. PCs in general cleave at the C-terminus of a pair of basic (KR↓/RR↓, more rarely at KK↓/RK↓) or a single basic (K↓/R↓) site without or with upstream basic residues (R or K) at the P4, P6 or P8 positions [1–3,7,8]. However, other amino acids present at or near a cleavage site are likely to confer specificity for PC-mediated protein precursor processing [9].

Unlike PC2 and PC1/3 for which null and viable animal models are available [10,11], investigating physiological function of the widely distributed PCs in animal models is difficult, since furin or PACE4-null mice die early in embryonic development [12–15]. A recent study on homozygous deletion of PC5/6 catalytic domain shows early embryonic lethality in mice [16]. PC5/6 has two isoforms, A (soluble) (915 amino acids) and B (membrane-bound)

(1877 amino acids), of which A is sorted to both the regulated and constitutive secretory pathways, while the C-terminally extended B isoform is localized in the Golgi [17–19]. PC5/6A having a shorter C-terminal CRD (cysteine-rich domain) is shown to interact with TIMPs (tissue inhibitors of metalloproteinases), indicating a potential role of this convertase to cleave protein precursors that are localized at the cell surface [18]. Our previous studies and those of others have shown that PC5/6A shares specificities with PC2 and/or PC1/3, the two neuroendocrine-specific convertases, in cleaving several important hormone and neuropeptide precursors including proglucagon, pro-GHRH (pro-growth hormone-releasing hormone), pro-NT/NN (pro-neurotensin/neuromedin) and pro-CCK (pro-cholecystokinin) [20–25].

CART (cocaine- and amphetamine-regulated transcript) is an important anorexigenic peptide and is expressed in the hypothalamus [26,27]. It has recently been implicated in the regulation of secretion of islet hormones, including insulin [28]. CART was first cloned in the rat [29] and later in human [30]. The mouse *Cart* gene was then identified [31] based on its sequence similarity to the rat and human cDNAs. While both rats and mice express two pro-CART isoforms, long (102 amino acids) and short (89 amino acids), produced from a single-copy *Cart* gene, only the short isoform is found in humans [30] (Figure 1A). The long isoform has an extra 13-amino-acid stretch (residues 27–39) (Figure 1A) and is generated by alternative splicing [29]. Both isoforms have a common N-terminal signal peptide of 27 amino acids [29,30], which is deleted following their entry into the secretory pathway like most other neuroendocrine precursors [32]. In our earlier

Abbreviations used: CART, cocaine- and amphetamine-regulated transcript; IP, immunoprecipitation; PACE, paired basic amino acid cleaving enzyme; PC, prohormone/proprotein convertase; pro-GHRH, pro-growth hormone-releasing hormone; pro-NT/NN, pro-neurotensin/neuromedin; RNAi, RNA interference; RT, reverse transcriptase; shRNA, short hairpin RNA; UTR, untranslated region; WB, Western blotting.

¹ To whom correspondence should be addressed (email adey@bsd.uchicago.edu).

Figure 1 Schematic representation of the pro-CART (A) and PC5/6 (B) isoforms

(A) Schematic structures of the pro-CART isoforms. Each of the short (1–89) and long (1–102) pro-CART isoforms represented by a solid box has a common signal peptide of 27 residues (empty box). Each upper-case letter represents a single amino acid (aa), e.g. K for Lys and R for Arg. All the PC cleavage sites (single/pairs of basic) within the pro-CART isoforms are indicated by arrows. The triangle represents a stretch of extra 13 amino acids present within the long pro-CART. (B) Schematic representation of the PC5/6 isoforms and various shRNA target sequences for PC5/6 silencing. (a) The two PC5/6 isoforms, A and B and their different coding regions were selected for several shRNA syntheses. The letters D, H, N and S shown on the two isoforms represent aspartic acid, histidine, asparagine and serine residues respectively. The common peptide domains of the two isoforms are, from the N- to C-terminus, signal peptide, prodomain, catalytic domain, P domain and Cys-rich domain, while the cytoplasmic and transmembrane domains belong only to the B isoform. The B isoform also has a much larger cysteine-rich domain. The circles represent potential glycosylation sites. (b) Sense strand sequences, 19 nt in length, for shRNAs.

studies on PC-mediated processing of both the long and short pro-CART isoforms, we have shown that PC2 is the major convertase for production of the two bioactive peptides, I (amino acids 42–89/55–102) and II (amino acids 49–89/62–102), whereas PC1/3 is primarily required to generate the intermediate forms (amino acids 33–102 and 10–89) [33].

In the present study, we have developed PC5/6-silenced GH3 cells by RNAi (RNA interference) in order to examine the roles of PC5/6 on pro-CART processing.

EXPERIMENTAL

Materials

Both the long (102 amino acids) and short (89 amino acids) rat pro-CART-expressing constructs, CART antibodies including the monoclonal antibody against the CART peptide (amino acids 41–89) and the recombinant CART peptides were gifts from Dr L. Thim, Dr S. Hastrup and Dr J. T. Clausen (Novo Nordisk, Bagsværd, Denmark). The rabbit antisera against human PC5/6

peptide (N-terminal) were gifts from Dr Robert Day (University of Sherbrooke, Sherbrooke, Canada). The polyclonal antibodies against PC2 and PC1/3 were raised in our laboratory. The rabbit antisera against 7B2 peptide were gifts from Dr Iris Lindberg (Louisiana State University Health Science Center, New Orleans, U.S.A.). RNAi-Ready pSIREN-Shuttle vector was purchased from BD Biosciences Clontech (Palo Alto, CA, U.S.A.). The mammalian expression vector, pcDNA3.1, the transfection reagent, Lipofectamine™ 2000, the Superscript first-strand synthesis system for RT (reverse transcriptase)–PCR and all the cell-culture media and reagents were bought from Invitrogen (Carlsbad, CA, U.S.A.). The transfection reagent, Effectene, and the Endo-free plasmid purification kit and Total RNeasy kit were bought from Qiagen (Valencia, CA, U.S.A.).

Cell culture, metabolic labelling, IP (immunoprecipitation) and WB (Western blotting)

GH3 cells were cultured in F10 medium containing 10% (v/v) foetal bovine serum. Cells were dissociated in an enzyme-free

Hanks-based buffer (cell dissociation buffer) for propagation. Metabolic labelling using [35 S]Met (150 μ Ci/ml), IP and WB of pro-CART and its various processed forms were performed as described previously [33]. The band intensities of various CART peptides following separation in gels were determined by densitometric scanning using 1D Image Analysis software (Kodak, Rochester, NY, U.S.A.). For WB of PC5/6A, equal numbers of cells were boiled directly in 1 $\times$ Laemmli sample buffer (200 μ l) containing 5% (v/v) 2-mercaptoethanol for 10 min, and following a brief centrifugation in a microcentrifuge at 12000 *g*, equal volumes (40 μ l) of whole cell lysates were resolved by SDS/8% PAGE and peptides were transferred on to Hybond-P membrane (Amersham Biosciences, Little Chalfont, Bucks., U.K.). Peptides were incubated with the rabbit polyclonal antibody against PC5/6 (Alexis Biochemicals, San Diego, CA, U.S.A.) and the immunoreactive bands were detected by ECL[®] (enhanced chemiluminescence).

Synthesis of shRNA (short hairpin RNA) and transient transfection

Different segments of the coding region of mouse PC5/6 cDNA (GenBank[®] accession number L14932), excluding the 5'- and 3'-UTRs (untranslated regions), were selected to design several shRNA target sequences corresponding to the motif, AA (N19) NN, where N is any nucleotide [34–36] (Figure 1B, panel b). Each target sequence [annealed complementary oligonucleotides with an orientation of (5')-sense-hairpin loop-antisense-(3') sequences] was cloned downstream of a Pol III promoter (U6) [37–39] in a plasmid expression vector (see the Materials subsection). We ligated annealed oligonucleotides into the pSIREN-Shuttle vector following the manufacturer's instructions and finally transformed into DH-5 α competent cells (Invitrogen) for large-scale DNA purification. Several different shRNA expression constructs, including experimental (Figure 1B) and control (luciferase) (supplied by BD Biosciences-Clontech), were generated. Each shRNA was transiently transfected in GH3 and GH3-CART cells overexpressing short pro-CART (89 amino acids). To generate GH3-CART cells stably overexpressing short pro-CART (89 amino acids), we subcloned the small pro-CART cDNA in a mammalian expression vector, pcDNA3.1/Zeo (Invitrogen), and following transient transfection of the recombinant plasmid in GH3 cells, we grew several individual clones under the selection of zeocin and the clone expressing maximum levels of CART peptides was selected for further studies.

RT-PCR

Total RNA for each sample was extracted from equal numbers of cells grown in a 60 mm dish using the RNeasy mini kit from Qiagen. The first strand cDNA was synthesized from 1 μ g of total RNA for all the genes including PC5/6, furin, PACE4 and β -actin using the Superscript first-strand RT-PCR kit. Equal amounts of first strand cDNA were amplified by PCR using specific forward and reverse primers for each gene. We used 30–35 PCR cycles for amplification of all the genes except for β -actin where 25 cycles were employed.

RESULTS

To investigate the roles of PC5/6 in pro-CART processing, we generated transient PC5/6-null GH3 cells using plasmid vector-mediated shRNAs (see the Experimental section for details). We employed GH3 cells because of higher levels of PC5/6 and low amounts of both PC2 and PC1/3 mRNA expression (see

Figures 2A and 2B) [40]. We examined the protein levels for both PC2 and PC1/3 in GH3 cells by immunoblotting and found low levels (results not shown) as shown previously [41]. The GH3 somatolactotroph cells expressing growth hormone were derived from rat pituitary anterior lobe [42]. The rat anterior pituitary lobe has considerable levels of PC5/6 mRNA [43]. To investigate the effects of PC5/6 silencing on pro-CART processing, we generated seven experimental shRNA expression plasmids (see the Experimental section and Figure 1B). We show that three are particularly effective following individual transfection and cause silencing of PC5/6 gene expression in both GH3 and GH3-CART cells expressing small pro-CART, as measured by RT-PCR, and the constructs are 584-shRNA (III), 1851-shRNA (IV) and 2542-shRNA (VII) (Figures 2C, panel a, and 2D, panel a). We examined the levels of β -actin and other PC genes, including furin and PACE4, by RT-PCR and found no significant changes in their levels of expression (Figures 2C, panels b–d, and 2D, panels b–d). We found comparable levels of both PC2 and PC1/3 following shRNA transfection as shown in non-transfected parental GH3 cells (see Figure 2B). The results altogether indicate specificity of RNAi in silencing PC5/6 gene expression.

To examine PC5/6 protein levels following RNAi-mediated silencing, we performed WB using GH3 or GH3-CART cells stably overexpressing short pro-CART (89 amino acids). We show that construct III is particularly effective in silencing PC5/6 expression relative to the other shRNA constructs examined (Figure 2E). We found comparable levels of tubulin for all the samples, indicating equal loading of whole cell lysates (results not shown). In our immunoblot studies using the polyclonal antibody against PC5/6 (see the Experimental section), we detected primarily the PC5/6A isoform as shown previously [17,22]. It is likely that these neuroendocrine cells have very low endogenous levels of the PC5/6B isoform which are below the detection limits of this antibody.

Next, we examined the effects of transient silencing of PC5/6 expression in processing both the rat pro-CART isoforms, long (102 amino acids) and short (89 amino acids). PC5/6A cleaves neuroendocrine precursors at pairs of basic or single basic sites without or with upstream basic residues [20–24]. PC5/6 is expressed in the hypothalamus [43] and its A isoform is often co-expressed with PC2 and/or PC1/3 in various endocrine/neuroendocrine tissues, including the hypothalamus [17,23,25]. To analyse whether PC5/6A has any significant ability to cleave the pro-CART isoforms, we transiently co-transfected GH3 cells with long pro-CART expression vector and either luciferase-shRNA (negative control) or 584-shRNA (III) expression plasmid (Figure 3A). Also, we transiently transfected GH3-CART cells overexpressing small pro-CART (see the Experimental section) with luciferase-shRNA or 584-shRNA (Figure 4A). The densitometric scanning data show that the levels of CART I (amino acids 55–102) and CART II (amino acids 62–102) generated from long pro-CART in GH3 cells are inhibited 2.3- and 1.9-fold respectively in the presence of the 584-shRNA relative to the luciferase control (Figure 3B). Similarly the scanning data of short pro-CART processing exhibit 2-fold lower production of CART I (amino acids 42–89) in GH3-CART cells by the 584-shRNA relative to the control (Figure 4B). The findings taken together suggest significant yet moderate impairment of bioactive CART production from the two pro-CART isoforms due to silencing of PC5/6A expression and indicate functional redundancy primarily between PC5/6A and PC2 (PC2 > PC5/6A) in cleaving the bioactive CART fragments, I and II (Figure 5). The results also demonstrate lack of any major defects in processing of the intermediate CART (amino acids 33–102) from long pro-CART in the presence of the 584-shRNA (Figure 3),

Figure 2 Assessment of the levels of various PCs (A and B) and shRNA-mediated PC5/6 silencing (C–E)

(A and B) RT–PCR analysis of PC5/6 as well as PC2 and PC1/3 levels. Total RNA following extraction from parental GH3 cells was subjected to RT–PCR assays (see the Experimental section). In (A), genes including β -actin (249 bp) (6 μ l) and PC5/6 (363 bp) (8 μ l) were PCR-amplified for 30 cycles. In (B), each (6 μ l) of PC2 (380 bp), PC1/3 (748 bp) and β -actin (249 bp) was PCR-amplified for 25 cycles. Lane 1 in both (A) and (B) shows the locations of the 1 kb DNA fragments. Lanes 3 and 4 in (B) represent the positive controls for PC2 and PC1/3 levels respectively using similar RT–PCR conditions as employed for GH3 cells. (C and D) Silencing of PC5/6 gene expression by RNAi. For transient silencing of PC5/6 gene expression, equal numbers of cells (1 million/60 mm dish) were plated and transfected with each of the shRNA expression plasmids (1 μ g), control (luciferase) or experimental, as described in the Experimental section. Total RNA was extracted from each sample and first strand cDNAs were amplified as described in the Experimental section to examine the levels of PC5/6 (363 bp), β -actin (540 bp), furin (298 bp) and PACE4 (157 bp) genes. Lanes 2–7 in (C and D) represent the following shRNAs: luciferase, I, II, III, IV and V respectively and lane 8 in (C) represents VII. We found the construct VI ineffective in silencing PC5/6 expression and is thus not included here. We have used different forward and reverse primers for β -actin, generating a larger fragment compared with (A and B). (E) Analysis of PC5/6 protein levels following RNAi-mediated silencing. Equal numbers of cells were transiently transfected with different individual shRNA in various amounts as shown and cells were lysed and whole cell lysates were immunoblotted as described in the Experimental section. The major immunoreactive PC5/6A band of 117 kDa [17], detected by ECL[®], is present significantly in lane 2 (luciferase control), lanes 3 and 4 (1851-shRNA) and lane 6 (241-shRNA) relative to lane 5 (1851-shRNA) and lanes 7–9 (584-shRNA). Lane 1 represents the locations of the pre-stained high-molecular-mass marker.

suggesting that PC5/6A preferentially cleaves the pairs of basic sites, KR↓/KK↓, of the CART precursor isoforms (Figures 3–5). CART II could not be detected in GH3-CART cells possibly due to inefficient cleavage by the rather low endogenous levels of PC2 in these cells as compared with the α TC1-6 cells that were employed for CART II detection in the initial studies [33]. The findings help to explain our previous *in vivo* results showing that while intermediate CART production in PC1/3-null hypothalamus is severely affected, production of bioactive CART fragments remains comparable with that in normal littermates [33]. Thus the low levels of both forms of bioactive CART (CART I > CART II) found in both PC2 and 7B2-null animals indicate overlapping specificities of PC2 more with PC5/6A than PC1/3 in processing the pairs of basic sites, KR↓ and KK↓, of pro-CART (Figure 5).

DISCUSSION

In the present study, we have analysed processing defects of both the rat pro-CART isoforms, long (102 amino acids) and short

(89 amino acids), using RNAi-mediated PC5/6-null GH3 cells. We show that PC5/6A is functionally redundant more with PC2 (PC2 > PC5/6A) than PC1/3 in cleaving the pairs of basic sites, KR (53, 54/40, 41)↓ and KK (60, 61/47, 48)↓, of both pro-CART isoforms and thus production of the bioactive fragments, I and II, is significantly but moderately affected. Our results also demonstrate unimpaired generation of intermediate CART (amino acids 33–102) indicating that PC5/6A is unable to process the RXXR site, RQLR (29–32↓), within the long pro-CART. GH3 cells have much lower levels of both PC2 and PC1/3 compared with other widely used endocrine/neuroendocrine cell types such as β -TC3 (both PC2 and PC1/3 are high; see Figure 2B), α TC1-6 (PC2 is high) and AtT20 (PC1/3 is high). Silencing of PC5/6 gene expression in the context of much lower endogenous levels of both PC2 and PC1/3 thus facilitates better analysis of the effects of PC5/6A in processing pro-CART. The results correspond to the previous findings that PC5/6A shares specificities with PC2 in processing KR residues present in proglucagon and pro-NT/NN [20,22,23]. PC5/6A is co-expressed with PC2 in several hypothalamic nuclei, particularly in the arcuate nucleus [23],

Figure 3 Silencing of PC5/6A causes inhibition of CART I and II production from long pro-CART

(A) Cells were transiently co-transfected with long pro-CART (102 amino acids) expression plasmid (1 μ g) and either luciferase or 584-shRNA (III) expression construct as shown and were metabolically labelled for 45 min following our previous experimental methods as mentioned in the Experimental section. Total cell extracts (CE) were prepared and equal amounts were subjected to IP using the mouse monoclonal anti-CART antibody and resolved by Tricine-SDS/PAGE as described above (see the Experimental section). Lane 1 shows the locations of pre-stained low-molecular-mass protein markers and iodinated authentic CART I and II peptides. (B) Quantification by densitometric scanning (see the Experimental section) for long pro-CART, intermediate CART (Int. CART), CART I and CART II. Statistical analysis: all values are expressed as the means $\pm$ S.E.M. ($n=3$). * $P < 0.05$.

which is a major site of abundant CART expression [26,44], indicating *in vivo* overlapping specificities between these two convertases in processing pro-CART. In our previous studies on mouse pro-GHRH processing, we demonstrated much lower but detectable ability of PC5/6A (PC5/6A $<$ PC1/3 $\ll$ furin) to cleave the N-terminal RXXR site, RMQR $\downarrow$ [21]. In contrast, for a similar cleavage site, RQLR $\downarrow$, located within the long pro-CART isoform, silencing of PC5/6A expression fails to cause any appreciable processing defects, suggesting likely effects of other residues at or near a cleavage site in influencing specificity of a convertase [7,9]. In this context, quantitative studies on the co-expression of PC2 and PC5/6A in the CART-expressing cells in the hypothalamus will be of importance. In both of our previous and present studies on pro-CART processing, we were unable to detect any significant cleavage at the other pairs of basic sites, KK (48, 49 $\downarrow$ /35, 36 $\downarrow$) and RK (77, 78 $\downarrow$ /64, 65 $\downarrow$). It is worth noting that a large aliphatic residue, e.g. leucine, present at the P1' position of the site KK (48, 49 $\downarrow$ /35, 36 $\downarrow$) may render significant steric hindrance to a convertase from cleaving this or

Figure 4 Impairment in CART I generation from short pro-CART due to PC5/6A silencing

(A) Cells overexpressing short pro-CART (89 amino acids) (see the Experimental section) were transiently transfected with either luciferase or 584-shRNA (III) expression plasmid as shown. Equal amounts of total cell extracts (CE; 200 μ g of total protein/sample) were subjected to IP with the monoclonal anti-CART antibody and following separation by Tricine-SDS/PAGE, were immunoblotted with the same antibody as described above (see the Experimental section). The locations of pre-stained low-molecular-mass protein markers are shown on the left and lane 4 represents the positions of 125 I-CART I and II peptides. (B) Quantification by densitometric scanning (see the Experimental section) for pro-CART and CART I. Statistical analysis: all values are expressed as the means $\pm$ S.E.M. ($n=3$). * $P < 0.05$.

Figure 5 Schematic representation of functional redundancy between PC5/6A and PC2 in processing of pro-CART

PC5/6A shares specificities with PC2 in cleaving the pairs of basic sites, KR (Lys, Arg) and KK (Lys, Lys), within the two pro-CART isoforms and generates both the bioactive fragments, CART I and II. The processing scheme is a modified version of our previous studies [33] based on the present findings.

similar sites, as suggested by Cameron et al. [9]. Also, *in vivo* cleavage at the RK (77, 78/64, 65) site possibly will not favour release of processed peptides such as 55–76/42–63 and 62–76/49–63 because these fragments will very likely remain anchored by the three disulfide linkages formed between cysteine residues that are located C-terminally in the pro-CART isoforms [45]. In our studies on pro-CART processing, we have failed to detect a smaller CART fragment, 33–52, following processing at the sites RXXR (29–32↓) and KR (53, 54↓) within the long pro-CART isoform, most likely due to very low levels of production of the fragment. The findings altogether are consistent with our previous *in vivo* results on pro-CART processing examining hypothalamic extracts from PC1/3, PC2 and 7B2 (an essential PC2-interacting protein)-null animals and help to explain the severe lack of the intermediate CART fragments in PC1/3-null mice compared with their normal littermates [33]. Also these results are consistent with the production of low levels of bioactive CART forms (CART I > CART II) in PC2 and 7B2-null mice relative to their normal controls.

We thank Margaret Milewski for her assistance in cell culture and Paul Gardner for synthesizing oligonucleotides. We also thank Dr George Greeley (University of Texas Medical Branch, Galveston, TX, U.S.A.) for the gifts of PC5/6 antisera. This work was supported by NIH (National Institutes of Health) grants DK13914 and DK20595, and by the Howard Hughes Medical Institute.

REFERENCES

- Zhou, A., Webb, G., Zhu, X. and Steiner, D. F. (1999) Proteolytic processing in the secretory pathway. *J. Biol. Chem.* **274**, 20745–20748
- Seidah, N. G. and Chretien, M. (1999) Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. *Brain Res.* **848**, 45–62
- Thomas, G. (2002) Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat. Rev. Mol. Cell Biol.* **3**, 753–766
- Mbikay, M., Tadros, H., Ishida, N., Lerner, C. P., De Lamirande, E., Chen, A., El-Alfy, M., Clermont, Y., Seidah, N. G., Chretien, M. et al. (1997) Impaired fertility in mice deficient for the testicular germ-cell protease PC4. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6842–6846
- Taylor, N. A., Van de Ven, W. J. M. and Creemers, J. W. M. (2003) Curbing activation: proprotein convertases in homeostasis and pathology. *FASEB J.* **17**, 1215–1227
- Khatib, A. M., Siegfried, G., Chretien, M., Metrakos, P. and Seidah, N. G. (2002) Proprotein convertases in tumor progression and malignancy. Novel targets in cancer therapy. *Am. J. Pathol.* **160**, 1921–1935
- Rockwell, N. C. and Thorner, J. W. (2004) The kindest cuts of all: crystal structures of Kex2 and furin reveal secrets of precursor processing. *Trends Biochem. Sci.* **29**, 80–87
- Henrich, S., Cameron, A., Bourenkov, G. P., Kiefersauer, R., Huber, R., Lindberg, I., Bode, W. and Than, M. E. (2003) The crystal structure of the proprotein processing proteinase furin explains its stringent specificity. *Nat. Struct. Mol. Biol.* **10**, 520–526
- Cameron, A., Apletalina, E. V. and Lindberg, I. (2002) The enzymology of PC1 and PC2. In *The Enzymes* (Dalbey, R. E. and Sigman, D. S., eds.), pp. 291–328, Academic Press, New York
- Furuta, M., Yano, H., Zhou, A., Rouille, Y., Holst, J. J., Carroll, R., Ravazzola, M., Orci, L., Furuta, H. and Steiner, D. F. (1997) Defective prohormone processing and altered pancreatic islet morphology in mice lacking active SPC2. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6646–6651
- Zhu, X., Zhou, A., Dey, A., Norrbom, C., Carroll, R., Zhang, C., Laurent, V., Lindberg, I., Ugleholdt, R., Holst, J. J. and Steiner, D. F. (2002) Disruption of PC1/3 expression in mice causes dwarfism and multiple neuroendocrine peptide processing defects. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 10293–10298
- Roebroek, A. J., Umans, L., Pauli, I. G., Robertson, E. J., van Leuven, F., Van de Ven, W. J. and Constam, D. B. (1998) Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase furin. *Development* **125**, 4863–4876
- Constam, D. B. and Robertson, E. J. (2000) SPC4/PACE4 regulates a TGF- β signaling network during axis formation. *Genes Dev.* **14**, 1146–1155
- Constam, D. B. and Robertson, E. J. (2000) Tissue-specific requirements for the preprotein convertase furin/SPC1 during embryonic turning and heart looping. *Development* **127**, 245–254
- Beck, S., Le Good, J. A., Guzman, M., Ben Haim, N., Roy, K., Beermann, F. and Constam, D. B. (2002) Extraembryonic proteases regulate Nodal signalling during gastrulation. *Nat. Cell Biol.* **4**, 981–985
- Essalmani, R., Hamelin, J., Marcinkiewicz, J., Chamberland, A., Mbikay, M., Chretien, M., Seidah, N. G. and Prat, A. (2006) Deletion of the gene encoding proprotein convertase 5/6 causes early embryonic lethality in the mouse. *Mol. Cell. Biol.* **26**, 354–361
- De Bie, I., Marcinkiewicz, M., Malide, D., Lazure, C., Nakayama, K., Bendayan, M. and Seidah, N. G. (1996) The isoforms of proprotein convertase PC5 are sorted to different subcellular compartments. *J. Cell Biol.* **135**, 1261–1275
- Nour, N., Mayer, G., Mort, J. S., Salvat, A., Mbikay, M., Morrison, C. J., Overall, C. M. and Seidah, N. G. (2005) The cysteine-rich domain of the secreted proprotein convertases PC5A and PACE4 functions as a cell surface anchor and interacts with tissue inhibitors of metalloproteinases. *Mol. Biol. Cell* **16**, 5215–5226
- Xiang, Y., Molloy, S. S., Thomas, L. and Thomas, G. (2000) The PC6B cytoplasmic domain contains two acidic clusters that direct sorting to distinct *trans*-Golgi network/endosomal compartments. *Mol. Biol. Cell* **11**, 1257–1273
- Dey, A., Lipkind, G. M., Rouille, Y., Norrbom, C., Stein, J., Zhang, C., Carroll, R. and Steiner, D. F. (2005) Significance of prohormone convertase 2, PC2, mediated initial cleavage at the proglucagon interdomain site, Lys70-Arg71, to generate glucagon. *Endocrinology* **146**, 713–727
- Dey, A., Norrbom, C., Xhu, X., Stein, J., Zhang, C., Ueda, K. and Steiner, D. F. (2004) Furin and prohormone convertase 1/3 are major convertases in the processing of mouse pro-growth hormone-releasing hormone. *Endocrinology* **145**, 1961–1971
- Barbero, P., Rovere, C., De Bie, I., Seidah, N. G., Beaudet, A. and Kitabgi, P. (1998) PC5-A-mediated processing of pro-neurotensin in early compartments of the regulated secretory pathway of PC5-transfected PC12 cells. *J. Biol. Chem.* **273**, 25339–25346
- Villeneuve, P., Lafortune, L., Seidah, N. G., Kitabgi, P. and Beaudet, A. (2000) Immunohistochemical evidence for the involvement of protein convertases 5A and 2 in the processing pro-neurotensin in rat brain. *J. Comp. Neurol.* **424**, 461–475
- Cain, B. M., Vishnuvardhan, D. and Beinfeld, M. C. (2001) Neuronal cell lines expressing PC5, but not PC1 or PC2, process pro-CCK into glycine-extended CCK 12 and 22. *Peptides* **22**, 1271–1277
- Cain, B. M., Connolly, K., Blum, A. C., Vishnuvardhan, D., Marchand, J. E. and Beinfeld, M. C. (2003) Distribution and colocalization of cholecystokinin with the prohormone convertase enzymes PC1, PC2, and PC5 in rat brain. *J. Comp. Neurol.* **467**, 307–325
- Kristensen, P., Judge, M. E., Thim, L., Ribel, U., Christjansen, K. N., Wulff, B. S., Clausen, J. T., Jensen, P. B., Madsen, O. D., Vrang, N. et al. (1998) Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* **393**, 72–76
- Hunter, R. G., Philpot, K., Vicentic, A., Dominguez, G., Hubert, G. W. and Kuhar, M. J. (2004) CART in feeding and obesity. *Trends Endocrinol. Metab.* **15**, 454–459
- Wierup, N., Bjorkqvist, M., Kuhar, M. J., Mulder, H. and Sundler, F. (2006) CART regulates islet hormone secretion and is expressed in the β -cells of type 2 diabetic rats. *Diabetes* **55**, 305–311
- Douglass, J., McKinzie, A. A. and Couceyro, P. (1995) PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. *J. Neurosci.* **15**, 2471–2481
- Douglass, J. and Daoud, S. (1996) Characterization of the human cDNA and genomic DNA encoding CART: a cocaine- and amphetamine-regulated transcript. *Gene* **169**, 241–245
- Adams, L. D., Gong, W., Vechia, S. D., Hunter, R. G. and Kuhar, M. J. (1999) CART: from gene to function. *Brain Res.* **848**, 137–140
- Blobel, G. (2000) Protein targeting. *Biosci. Rep.* **20**, 303–344
- Dey, A., Zhu, X., Carroll, R., Turck, C. W., Stein, J. and Steiner, D. F. (2003) Biological processing of the cocaine and amphetamine-regulated transcript precursors by prohormone convertases, PC2 and PC1/3. *J. Biol. Chem.* **278**, 15007–15014
- Dykxhoorn, D. M., Novina, C. D. and Sharp, P. A. (2003) Killing the messenger: short RNAs that silence gene expression. *Nat. Rev. Mol. Cell Biol.* **4**, 457–467
- Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498
- Harborth, J., Elbashir, S. M., Vandenburgh, K., Manniga, H., Scaringe, S. A., Weber, K. and Tuschl, T. (2003) Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. *Antisense Nucleic Acid Drug Dev.* **13**, 83–105
- Mcmanus, M. T. and Sharp, P. A. (2002) Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.* **3**, 737–747
- Brummelkamp, T. R., Bernards, R. and Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550–553
- Paddison, P. J., Caudy, A. A. and Hannon, G. J. (2002) Stable suppression of gene expression by RNAi in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 1443–1448

-
- 40 Posner, S. F., Vaslet, C. A., Jurofcik, M., Lee, A., Seidah, N. G. and Nillni, E. (2004) Stepwise posttranslational processing of progrowth hormone-releasing hormone polypeptide by furin and PC1. *Endocrine* **23**, 199–213
- 41 Marzban, L., Trigo-Gonzalez, G., Xhu, X., Rhodes, C. J., Halban, P. A., Steiner, D. F. and Verchere, C. B. (2004) Role of β -cell prohormone convertase (PC) 1/3 in processing of pro-islet amyloid polypeptide. *Diabetes* **53**, 141–148
- 42 Tashjian, A. H., Yasumura, Y., Levine, L., Sato, G. H. and Parker, M. L. (1968) Establishment of clonal strains of rat pituitary tumor cells that secrete growth hormone. *Endocrinology* **82**, 342–352
- 43 Lusson, J., Vieau, D., Hamelin, J., Day, R., Chretien, M. and Seidah, N. G. (1993) cDNA structure of the mouse and rat subtilisin /kexin-like PC5: a candidate proprotein convertase expressed in endocrine and neuroendocrine cells. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6691–6695
- 44 Thim, L., Kristensen, P., Nielsen, P. F., Wulff, B. S. and Clausen, J. T. (1999) Tissue-specific processing of cocaine- and amphetamine-regulated transcript peptides in the rat. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 2722–2727
- 45 Ludvigsen, S., Thim, L., Blom, A. M. and Wulff, B. S. (2001) Solution structure of the satiety factor, CART, reveals new functionality of a well-known fold. *Biochemistry* **40**, 9082–9088
-

Received 6 April 2006/12 June 2006; accepted 27 June 2006

Published as BJ Immediate Publication 27 June 2006, doi:10.1042/BJ20060506