Chromogranin A Processing and Secretion

Specific Role of Endogenous and Exogenous Prohormone Convertases in the Regulated Secretory Pathway

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Abstract

Chromogranins A and B and secretogranin II are a family of acidic proteins found in neuroendocrine secretory vesicles; these proteins contain multiple potential cleavage sites for proteolytic processing by the mammalian subtilisin-like serine endoproteases PC1 and PC2 (prohormone convertases 1 and 2), and furin. We explored the role of these endoproteases in chromogranin processing in AtT-20 mouse pituitary corticotropes. Expression of inducible antisense PC1 mRNA virtually abolished PC1 immunoreactivity on immunoblots. Chromogranin A immunoblots revealed chromogranin A processing, from both the NH_2 and COOH termini, in both wild-type AtT-20 and AtT-20 antisense PC1 cells. After antisense PC1 induction, an $\sim$ 66-kD chromogranin A NH_2-terminal fragment as well as the parent chromogranin A molecule accumulated, while an $\sim$ 50 kD NH_2-terminal and an $\sim$ 30 kD COOH-terminal fragment declined in abundance. Chromogranin B and secretogranin II immunoblots showed no change after PC1 reduction. [^{35}S]Methionine/cysteine pulse–chase metabolic labeling in AtT-20 antisense PC1 and antisense furin cells revealed reciprocal changes in secreted chromogranin A COOH-terminal fragments (increased $\sim$ 82 kD and decreased $\sim$ 74 kD forms, as compared with wild-type AtT-20 cells) indicating decreased cleavage, while AtT-20 cells overexpressing PC2 showed increased processing to and secretion of $\sim$ 71 and $\sim$ 27 kD NH_2-terminal chromogranin A fragments. Antisense PC1 specifically abolished regulated secretion of both chromogranin A and β-endorphin in response to the usual secretagogue, corticotropin-releasing hormone. Moreover, immunocytochemistry demonstrated a relative decrease of chromogranin A in processes (where regulated secretory vesicles accumulate) of AtT-20 cells overexpressing either PC1 or PC2. These results demonstrate that chromogranin A is a substrate for the endogenous endoproteases PC1 and furin in vivo, and that such processing influences its trafficking into the regulated secretory pathway; furthermore, lack of change in chromogranin B and secretogranin II cleavage after diminution of PC1 suggests that the action of PC1 on chromogranin A may be specific within the chromogranin/secretogranin protein family. (*J. Clin. Invest.* 1996. 98:148–156.) Key words: proopiomelanocortin • prohormone convertase • corticotrope • AtT-20

Introduction

The chromogranins/secretogranins are a family of acidic secretory proteins found in virtually all neuroendocrine tissues, where they are co-stored with amine and peptide neurotransmitters and peptide hormones in secretory vesicles (for reviews see references 1 and 2). The precursors of bioactive peptides contain multiple sites of paired basic residues (3) which are potential sites for proteolytic processing to biologically active peptides. Chromogranins A and B and secretogranin II undergo proteolytic processing during their routing to and storage in secretory vesicles (4–6). Their biologically active proteolytic fragments include pancreastatin (7–9), β-granin (10), parastatin (11), and vasostatin (12), in the case of chromogranin A, and secretoneurin (13), in the case of secretogranin II.

A new family of mammalian subtilisin- or *Kex2*-like enzymes that cleave at paired basic amino acid residues has been characterized (14–17). Two of these prohormone convertases (PC),[1] PC1 (also known as PC3) and PC2, are found almost exclusively in neuroendocrine tissues (14–18) and process prohormones destined for the regulated-secretory pathway, while furin is found in all tissues. By expressing antisense PC1 mRNA in AtT-20, a mouse cell line that contains high amounts of endogenous PC1, Bloomquist et al. (18) demonstrated the role of PC1 in the initial steps of endoproteolytic processing of the proopiomelanocortin prohormone to its biologically active peptide fragments, while PC2 acts later in the secretory pathway, mediating cleavages seen in the intermediate pituitary (14, 15, 17, 19).

Using region-specific antibodies to the NH_2 and COOH termini of the chromogranins, we explored the role of PC1, PC2, and furin in chromogranin/secretogranin processing in the mouse pituitary corticotrope line AtT-20. We examined AtT-20 cells which inducibly express antisense mRNA to PC1, to specifically lower PC1 protein, thereby disrupting proopiomelanocortin processing (18), and also cells expressing antisense furin (aFur) mRNA. In addition, AtT-20 cells overexpressing recombinant PC1 or PC2 (19) were used to determine the roles of both prohormone convertases in chromogranin A processing and intracellular trafficking.

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1. *Abbreviations used in this paper:* aFur, antisense furin; CRH, corticotropin-releasing hormone; ELH, egg-laying hormone; PC, prohormone convertase.

Methods

Chemicals and reagents. DME/F12 (50:50) medium, glutamine, penicillin/streptomycin, Geneticin (G-418), fetal bovine serum, and horse serum were obtained from GIBCO-BRL (Bethesda, MD). NuSerum was obtained from Collaborative Research Inc. (Waltham, MA). Lima bean trypsin inhibitor, bacitracin, insulin, transferrin, and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO). Protein A–Sepharose CL-4B was obtained from Pharmacia Biotech Inc. (Piscataway, NJ). $CdCl_2$ was purchased from Fisher Scientific (Fairlawn, NJ). Human corticotropin-releasing hormone (CRH) was obtained from Peninsula Laboratories, Inc. (Belmont, CA). Precast 10% SDS-PAGE gels and nitrocellulose and polyvinylidenedifluoride papers were from Schleicher & Schuell, Inc. (Keene, NH). DTT was purchased from Calbiochem-Novabiochem (La Jolla, CA). Vectastain reagents for color development of immunoblots were obtained from Vector Laboratories (Burlingame, CA). Protein was assayed by the Coomassie blue dye–binding method (Bio-Rad Laboratories, Richmond, CA).

Cell culture and secretory vesicle preparation. AtT-20/D16v mouse pituitary corticotropes were maintained as described in Bloomquist et al. (18). Some AtT-20 cell lines were also obtained from the laboratory of Michael G. Rosenfeld (University of California, San Diego). The A5-5 cell line is an AtT-20 cell line stably expressing antisense PC1 mRNA, under the control of a Cd^{2+}-inducible metallothionein promoter (18). Cells were grown to $\sim$ 80% confluency in DME/F12 (50:50) medium supplemented with 10% fetal bovine serum, 10% horse serum, and 10% NuSerum. For A5-5 cells, G-418 (0.5 mg/ml) was added at all times. Cells were washed twice with serum-free medium containing 0.1 mg/ml lima bean trypsin inhibitor, 0.1 mg/ml bacitracin, 2.5 μg/ml insulin, and 0.1 μg/ml transferrin. The cells were treated for 12 h with 10 μM cadmium chloride in the above medium, without serum, to induce expression of the PC1 antisense transcript through activation of the mouse metallothionein-1 promoter (18). For chromogranin immunoblots, the cells were then detached with a rubber policeman, transferred to 15-ml conical tubes, rinsed twice with phosphate-buffered saline, and lysed with 1 ml 1% SDS in 5 mM Na-Hepes, pH 7.0. Lysates in microfuge tubes were vortexed, boiled for 10 min, and centrifuged to remove debris. Supernatants were assayed for protein by the Coomassie blue dye–binding method (20).

Some experiments were done with AtT-20 cell lines (19) stably overexpressing prohormone convertase 1 (sPC1) or prohormone convertase 2 (sPC2), or with cells stably expressing aFur mRNA (21).

Bovine pituitary vesicle soluble core proteins (lysates) were prepared as described previously (4). Mouse adrenal glands were crushed and homogenized in a hypotonic solution of 0.1% SDS in 5 mM Na-Hepes, pH 7.0. Homogenates were vigorously vortexed, boiled for 5 min, and centrifuged. Supernatants were assayed for protein and frozen at −70°C.

Antisera. Region-specific polyclonal rabbit antisera to chromogranins A and B were developed and characterized as described previously (4, 5, 22). The antibodies are directed against the chromogranin A NH_2 terminus (human/bovine chromogranin $A_{1–16}$-[tyr_{17}]), chromogranin A COOH terminus ([tyr_0]-bovine chromogranin $A_{417\,431}$), chromogranin A whole molecule 137 (bovine chromogranin $A_{163–181}$); chromogranin B NH_2 terminus (human chromogranin $B_{1–15}$-[tyr_{16}]), and chromogranin B COOH terminus ([tyr_0]-human chromogranin $B_{644–657}$). For these synthetic peptides, amino acid numbering begins in the mature proteins, after NH_2-terminal signal peptide removal.

To generate region-specific rabbit polyclonal antibodies directed against secretogranin II and the midmolecule portion of chromogranin A, peptides were solid-phase–synthesized (23), and the peptide haptens were coupled to a keyhole limpet hemocyanin carrier as described previously (5). Human secretogranin II peptides corresponding to the NH_2 terminus (human secretogranin $II_{1–11}$-[tyr_{12}]) and COOH terminus ([tyr_0]-human secretogranin $II_{576–587}$) were used. A chromogranin A midmolecule synthetic peptide corresponded to rat chromogranin $A_{131–150}$ (24). Anti PC1 rabbit antisera JH888 and JH887 were raised against a synthetic peptide corresponding to amino acid residues 359–373 of rat PC1 (25).

SDS-PAGE and immunoblotting. Protein samples were mixed with sample buffer containing 1% SDS, 20% glycerol, 0.005% bromophenol blue, and 5 mM Na-Hepes, pH 7.0. The samples were heated for 5 min at 100°C, followed by loading onto SDS-PAGE 10% slab gels (Schleicher & Schuell). Immunoblotting was performed using an avidin–biotin complex bridge (Vectastain; Vector Laboratories), after electrophoretic transfer to nitrocellulose or polyvinylidenedifluoride sheets (Schleicher & Schuell). Rabbit antisera to chromogranins A and B and secretogranin II were used at titers of 1:100 to 1:1,000 (vol/vol) at 4°C, overnight. Visualization of the antigen/antibody complex was carried out using peroxidase-coupled goat anti–rabbit IgG and the chromogenic substrate 4-chloro-1-naphthol. Rabbit antisera to PC1 were used at a titer of 1:4,000 (vol/vol). Total proteins were visualized by amido black stain (26). Reflectance densitometry of unprocessed (parent) immunoblot bands was accomplished with the program Scan Analysis (Biosoft, Ferguson, MO) for the Macintosh microcomputer.

Pulse–chase biosynthetic labeling experiments. Biosynthetic labeling experiments on AtT-20 cells expressing different prohormone convertase profiles were carried out as described previously (19, 27). Briefly, cells were first incubated in methionine- and cysteine-depleted serum-free medium for 5 min, then incubated with medium containing [^{35}S]methionine/cysteine (1,000 Ci/mmol, in vitro cell labeling mix; Amersham Corp., Arlington Heights, IL) for 30 min with or without subsequent chase-incubations in nonradioactive complete media. Media were collected and cells were extracted with 50 mM sodium phosphate, pH 7.4, containing 1% SDS, 50 mM β-mercaptoethanol, 2 mM EDTA, and protease inhibitors, and preheated to 95°C (boiling SDS buffer) (27). After absorbing SDS with NP-40, samples were incubated with the primary antibodies at 4°C overnight in the presence of protease inhibitors, followed by incubation with protein A–Sepharose beads (19). Immunoprecipitated samples were dissolved in SDS gel buffer, boiled, and electrophoresed on 10% SDS-PAGE (26), followed by fluorography.

Immunocytochemical (immunofluorescence) studies. Immunostaining was performed on nontransfected (wild-type) and various stably transfected AtT-20 cell lines, as described (27).

Regulated secretion studies. 1×10^5 cells (wild-type AtT-20 or A5-5 cells) were grown to $\sim$ 60% confluency. The cells were treated with $CdCl_2$ (10 μM) and/or dexamethasone (1 μM) in serum-free medium for $\sim$ 12 h, followed by several washes with serum-free medium. DME/F12 medium supplemented with serum (as described above) was added to the cells for 1 h, followed by several rinses in serum-free media. Human CRH (100 nM) was added to the cells for 3 h. After the 3-h secretion period (28), the medium was removed and analyzed for β-endorphin and chromogranin A immunoreactivities. β-Endorphin was measured by a solid-phase immunoradiometric assay (Nichols Institute, San Juan Capistrano, CA), in which the β-endorphin precursor β-lipotropin also cross-reacts by 16%. Chromogranin A was quantified by a previously described radioimmunoassay (29); this assay, based on the synthetic chromogranin A NH_2 terminus, recognizes intact chromogranin A and its NH_2 terminus with parallel molar equivalency; it does not recognize the isolated COOH terminus of chromogranin A (29). The cells were lysed with 300 μl 10 mM Na-Hepes buffer, pH 7.0, boiled for 10 min, and the lysates were kept at −70°C before assay.

Statistics. Results are reported as the mean value±1 SEM. Groups were compared by ANOVA or paired *t* test, as appropriate. The level for significance chosen was $P < 0.05$.

Results

PC1 antisense mRNA. Suppression of PC1 expression by Cd^{2+} induction of PC1 antisense mRNA in A5-5 cells was con-

firmed by PC1 immunoblots. An ~ 66-kD PC1-immunoreactive band was detected in normal anterior pituitary hormone storage vesicles, as well as in wild-type AtT-20 and A5-5 cells (Fig. 1). Fig. 1 shows virtual abolition of PC1 protein expression (~ 66-kD band) in the A5-5 cells expressing the antisense PC1 mRNA. By contrast, Cd^{2+} had no effect on PC1 expression by wild-type AtT-20 cells, confirming the specificity of the Cd^{2+} effect through antisense PC1 mRNA.

Immunoblot analyses of effects of PC1 antisense mRNA on chromogranin A processing. Fig. 2 diagrams the primary structure of mouse chromogranin A (29) and the regions (epitopes) against which antibodies are directed. The mature 445–amino acid protein contains eight sets of paired basic residues that are potential sites for proteolytic cleavage by prohormone convertases, as well as nine methionine and two cysteine residues for [^{35}S]methionine/cysteine metabolic labeling (though none near the COOH terminus).

Fig. 3 depicts the chromogranin A immunoblotting patterns of mouse adrenal homogenate, bovine anterior pituitary secretory vesicles, and mouse pituitary corticotropes (AtT-20 and A5-5 cell lysates). The blots demonstrate extensive processing of chromogranin A, from both the NH_2 and COOH termini, in normal pituitary vesicles as well as both wild-type AtT-20 and A5-5 cells, with especially prominent accumulation of low molecular mass proteins retaining the COOH-terminal epitope. The NH_2-terminal antiserum initially recognized major bands at ~ 80, ~ 70, and ~ 50 kD, while the COOH-terminal antiserum initially recognized a major band at ~ 70 kD and lower molecular mass bands at ~ 32.5, ~ 30.5, and ~ 27 kD.

Exposure of wild-type AtT-20 cells to Cd^{2+} did not affect the apparent processing pattern of chromogranin A, as gauged by both NH_2- and COOH-terminal antisera. By contrast, the specific decrease of PC1 in Cd^{2+}-treated A5-5 cells produced several changes (Fig. 3): accumulation of an ~ 66-kD COOH-terminal immunoreactive chromogranin A fragment (Fig. 3 *B, open triangle*) as well as the parent chromogranin A molecule (Fig. 3, *A* and *B, arrows*), with concomitant diminution of both an ~ 50-kD NH_2-terminal chromogranin A fragment (Fig. 3 *A, star*) and an ~ 30-kD COOH-terminal chromogranin A fragment (Fig. 3 *B, closed triangle*). By densitometric scanning, the unprocessed chromogranin A band increased (accumulated) by a mean of 3.01-fold (Fig. 3) after Cd^{2+} induction of PC1 antisense mRNA in A5-5 cells.

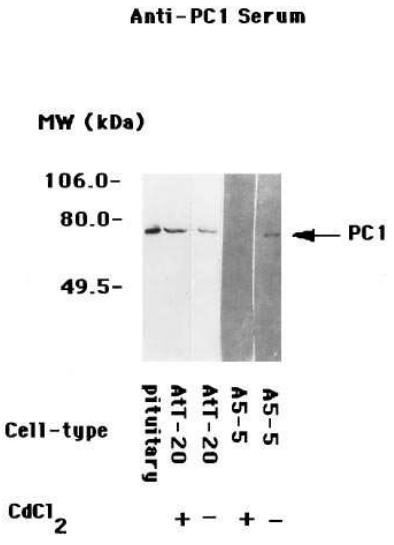

Figure 1. Reduction of PC1 expression after induction of antisense PC1 mRNA in A5-5 cells. 100 μg of cellular protein from wild-type AtT-20 corticotropes, or the antisense PC1 mRNA Cd^{2+}-inducible AtT-20 derivative A5-5 cells, was immunoblotted with a rabbit antiserum (JH888) directed against rat prohormone convertase (PC1). The arrow indicates the ~ 66-kD PC1 immunoreactive band. 10 μM $CdCl_2$ is the inducer of PC1 antisense mRNA expression in A5-5 cells (see Methods). *pituitary*, bovine anterior pituitary hormone secretory vesicle soluble core proteins (50 μg). Numbers at the left are molecular masses, in kD, of size standards.

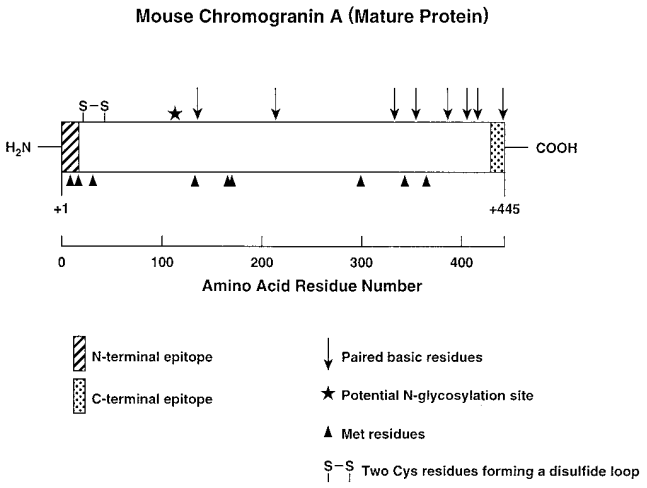

Figure 2. Domains in the primary structure of the mouse chromogranin A mature protein (31). Numbered amino acid residues (+1 to +445) are those present in the mature protein after NH_2-terminal signal peptide cleavage. The NH_2- and COOH-terminal epitopes refer to the synthetic peptides against which antisera were developed (see Methods). Cysteine and methionine residues are subject to metabolic labeling during [^{35}S]cys/met pulse–chase labeling.

The chromogranin A processing pattern and its disruption by antisense PC1 expression (Fig. 3) were observed consistently in replicated immunoblot experiments.

Chromogranin B and secretogranin II processing: no effect of PC1 antisense on region-specific immunoblots. Both normal anterior pituitary hormone storage vesicles and mouse pituitary corticotropes (wild-type AtT-20 and A5-5) contained immunoreactive chromogranin B (Fig. 4) and secretogranin II (Fig. 5), and both chromogranin B and secretogranin II showed evidence of extensive NH_2- and COOH-terminal processing in each cell type. For chromogranin B in AtT-20 cells, a major ~ 90-kD protein was recognized by both the NH_2- and COOH-terminal antisera, as well as lower molecular mass forms between ~ 60 and ~ 70 kD. Only the NH_2-terminal antiserum recognized a low molecular mass band at ~ 30 kD. For secretogranin II, a mixture of NH_2- and COOH-terminal proteins was found: in AtT-20 cells, the COOH-terminal antiserum recognized ~ 80-kD and ~ 65-kD forms, while the NH_2-terminal antiserum recognized a major band at ~ 65 kD; these findings suggest substantial secretogranin II processing, especially towards the COOH terminus. AtT-20 and A5-5 corticotropes did not differ in apparent processing pattern of either chromogranin B or secretogranin II.

Antisense PC1 expression induced by Cd^{2+} in A5-5 cells did not affect the distribution of processed (lower molecular mass) forms of either chromogranin B (Fig. 4) or secretogranin II (Fig. 5). There were only marginal increases in the unprocessed (parent) chromogranin B (mean, 1.37-fold; Fig. 4) or secretogranin II (mean, 1.30-fold) bands after Cd^{2+} in A5-5 cells. As a control for specificity of Cd^{2+} effects, chromogranin

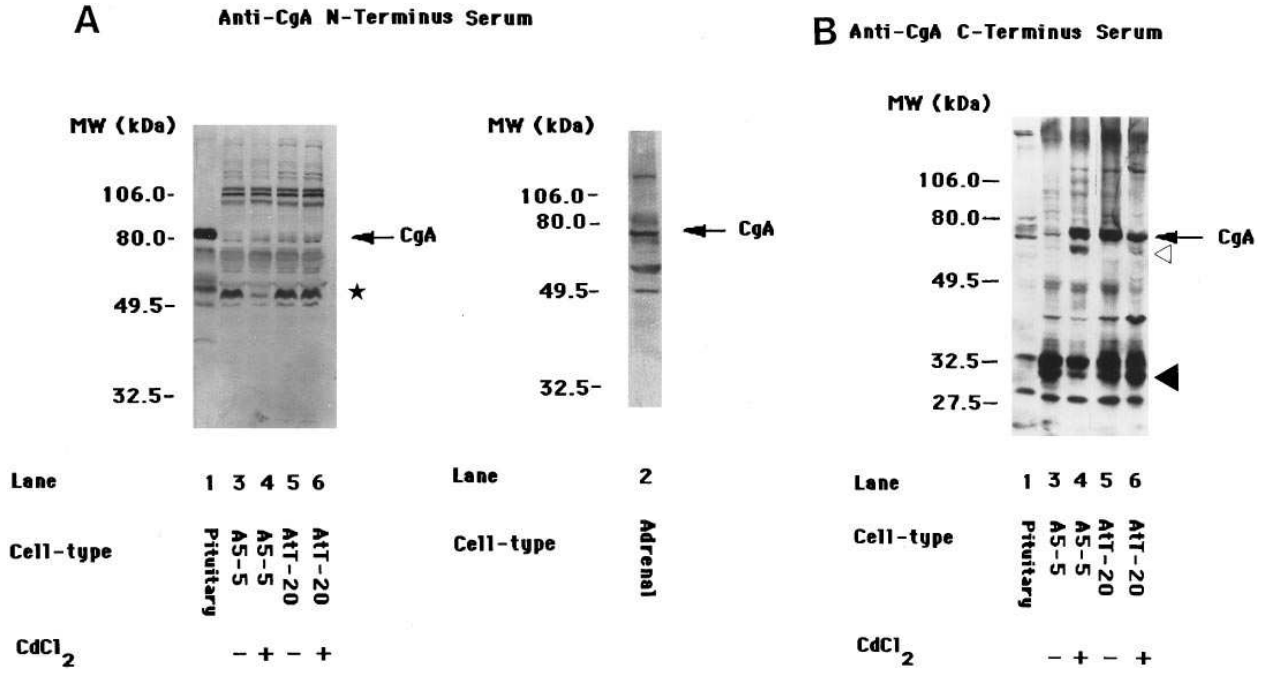

Figure 3. Effect of PC1 ablation on chromogranin A processing: region-specific immunoblots. Mouse adrenal homogenate (*Adrenal*, 100 μg protein), bovine anterior pituitary hormone secretory vesicle soluble core proteins (*Pituitary*, 50 μg), A5-5 or wild-type AtT-20 cell lysates (100 μg) were immunoblotted with anti–chromogranin A (*CgA*) antisera directed against the NH_2 or COOH terminus. (*A*) Immunoblot using antiserum against the chromogranin A NH_2 terminus. (*B*) Immunoblot using antiserum against the chromogranin A COOH terminus. In this SDS-PAGE system, intact (unprocessed) chromogranin A migrates at ~ 80 kD (*arrow*) (see Results); higher molecular mass (~ 106 kD) chromogranin A–immunoreactive bands are consistent with the chromogranin A–core proteoglycan (4). *Open triangle*, ~ 66-kD COOH-terminal chromogranin A fragment which accumulates after antisense PC1 mRNA induction; *star*, ~ 50-kD NH_2-terminal chromogranin A fragment which is diminished after antisense PC1 mRNA induction; *closed triangle*, ~ 30-kD COOH-terminal chromogranin A fragment which is diminished after antisense PC1 mRNA induction by 10 μM $CdCl_2$. Numbers at the left are molecular masses, in kD, of size standards. To confirm that electrophoretic protein loads from wild-type AtT-20 and A5-5 cells were comparable, cell lysates were blotted and stained with amido black (data not shown). As a negative control for immunoblots, cell lysates were blotted and treated with preimmune rabbit serum (at 1:100 dilution, similar to the anti–chromogranin/secretogranin dilutions); no immunoreactive bands were seen with preimmune sera (data not shown).

B (Fig. 4, lanes *5* and *6*) and secretogranin II (Fig. 5, lanes *5* and *6*) fragment distributions did not change in wild-type AtT-20 cells after Cd^{2+}.

Changes in chromogranin A processing in AtT-20 cells under- or overexpressing PC1, furin, or PC2: metabolic labeling studies. In another approach to investigate the role of PC1 and possible involvement of other prohormone convertases in chromogranin A processing, metabolic labeling experiments were conducted on AtT-20 cells with different PC profiles.

Within wild-type AtT-20 cells during the pulse-labeling period, an ~ 94-kD intact chromogranin A protein was recognized by both NH_2-terminal (Fig. 6 *B*) and COOH-terminal (Fig. 6 *A*) antisera; the NH_2-terminal antiserum also detected an ~ 83-kD fragment, while the COOH-terminal antiserum found an additional ~ 82-kD target. During the subsequent first half-hour of chase (nonradioactive) incubation, as examined by a COOH-terminal antiserum (Fig. 6 *A*), the cellular ~ 94-kD and ~ 82-kD forms each declined substantially, while an ~ 74-kD protein appeared in the medium. After a 4-h chase, no cellular chromogranin A could be detected by the COOH-terminal antiserum, while both ~ 82-kD and ~ 74-kD forms were seen in the medium, with the ~ 74-kD form predominant. The NH_2-terminal antiserum (Fig. 6 *B*) recognized the intracellular ~ 94-kD form through the entire 4-h chase period in cell extracts, as well as an ~ 83-kD intracellular form at up to 0.5 h of chase, but only the ~ 83-kD form appeared in the secretion medium.

In both antisense PC1 (aPC1) cells and aFur AtT-20 cells, the COOH-terminal chromogranin A antiserum (Fig. 6 *A*) demonstrated reciprocal changes in two secreted forms: an increase in the ~ 82-kD form (Fig. 6 *A*, *asterisk*) with a decrease of the ~ 74-kD form in chase media, as compared with wild-type AtT-20 cells. There was also a decrease in the ~ 83-kD chromogranin A NH_2-terminal form secreted into chase medium after 4 h by aPC1 cells, as compared with wild-type AtT-20 cells. Within aPC1 (though not aFur) cells, there was also a relative intracellular accumulation of the ~ 83-kD NH_2-terminal chromogranin A fragment at the 0.5-h chase time point, as compared with wild-type AtT-20 cells (Fig. 6 *B*).

The NH_2-terminal chromogranin A antiserum detected secretion of two additional forms at ~ 71 and ~ 27 kD (Fig. 6 *B*, *asterisks*) in chase medium from AtT-20 cells overexpressing PC2 (sPC2).

PC1 involvement in regulated peptide secretion. Fig. 7 shows the effect of antisense PC1 expression on CRH-stimulated secretion of β-endorphin (Fig. 7 *A*) and chromogranin A (Fig. 7 *B*).

CRH (100 nM) stimulated secretion of both β-endorphin (almost doubling basal secretion; Fig. 7 *A*) and chromogranin A (more than doubling basal secretion; Fig. 7 *B*) in wild-type AtT-20 cells, as well as in noninduced A5-5 cells. CRH also

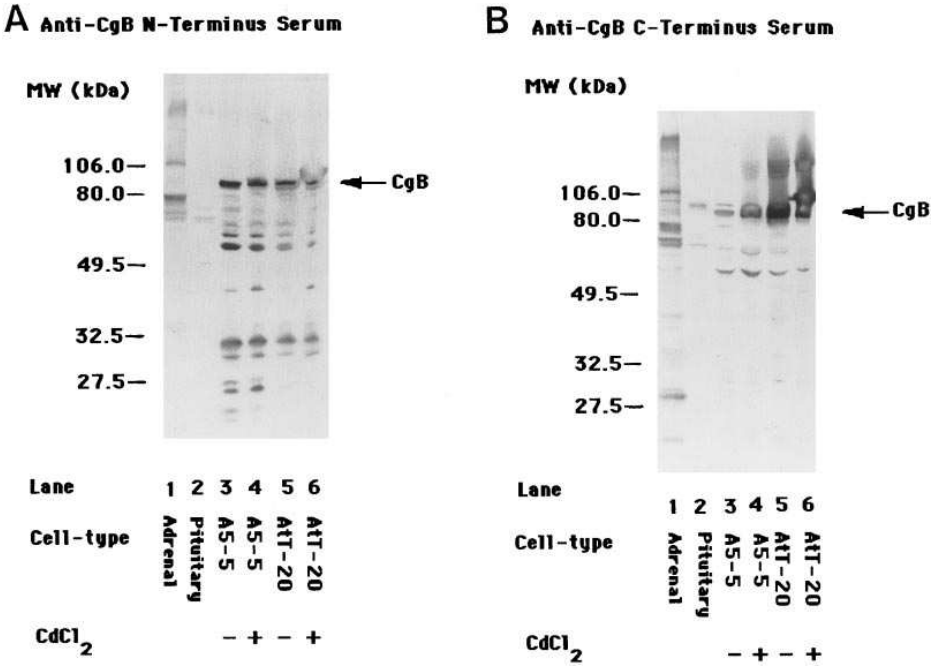

Figure 4. Lack of effect of antisense PC1 on chromogranin B processing: region-specific immunoblots. Mouse adrenal homogenate (*Adrenal*, 100 μg protein), bovine anterior pituitary hormone secretory vesicle soluble core proteins (*Pituitary*, 50 μg), A5-5 or AtT-20 cell lysates (100 μg) were immunoblotted with anti–chromogranin B (*CgB*) antisera directed against the NH_2 or COOH terminus. (*A*) Immunoblot using antiserum against the NH_2 terminus of chromogranin B. (*B*) Immunoblot using antiserum against the COOH terminus of chromogranin B. *Arrow*, parent (unprocessed) chromogranin B. Numbers at the left are molecular masses, in kD, of size standards.

stimulated β-endorphin and chromogranin A secretion in wild-type AtT-20 cells in the presence of $CdCl_2$, to the same extent as in the absence of $CdCl_2$. In striking contrast, β-endorphin (Fig. 7 *A*) and chromogranin A (Fig. 7 *B*) secretion in antisense PC1 expressing A5-5 cells were not stimulated by CRH (both $P > 0.05$).

As expected from the known positive regulation of chromogranin A by glucocorticoids (28, 30), pretreatment with dexamethasone increased basal secretion of chromogranin A from wild-type AtT-20 cells by ~ 160%, and from A5-5 cells by ~ 300%. In contrast, dexamethasone diminished basal β-endorphin secretion from wild-type AtT-20 cells by ~ 70%, though not from A5-5 cells. In the presence of $CdCl_2$, dexamethasone did not have any effect on basal chromogranin A and β-endorphin levels (data not shown).

Addition of dexamethasone did not affect CRH stimulation of β-endorphin or chromogranin A secretion from AtT-20 or A5-5 cells (Fig. 7, *A* and *B*).

Immunocytochemical localization of chromogranin A in AtT-20 cells. Fig. 8 depicts the cellular localization of chromogranin A in AtT-20 cells. When probed with antisera against chromogranin A, a typical distribution pattern within the regulated secretory pathway was observed in wild-type AtT-20 (Fig. 8 *a*: antiserum against intact [full-length] chromogranin A; Fig. 8 *d*: antiserum against the NH_2 terminus of chromogranin A). In AtT-20 cells overexpressing exogenous

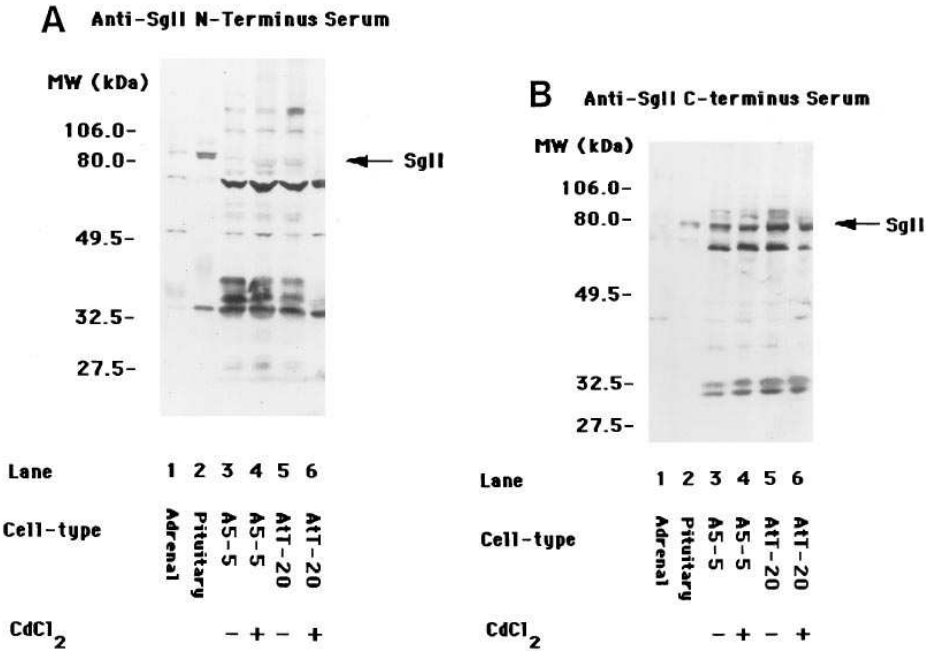

Figure 5. Lack of effect of antisense PC1 on secretogranin II processing: region-specific immunoblots. Mouse adrenal homogenate (*Adrenal*, 100 μg protein), bovine anterior pituitary hormone secretory vesicle soluble core proteins (*Pituitary*, 50 μg), A5-5 or AtT-20 cell lysates (100 μg) were immunoblotted with anti–secretogranin II antisera directed against the NH_2 or COOH terminus. (*A*) Immunoblot using antiserum against the NH_2 terminus of secretogranin II. (*B*) Immunoblot using antiserum against the COOH terminus of secretogranin II. *SgII*, secretogranin II. *Arrow*, parent (unprocessed) secretogranin II. Numbers at the left are molecular masses, in kD, of size standards.

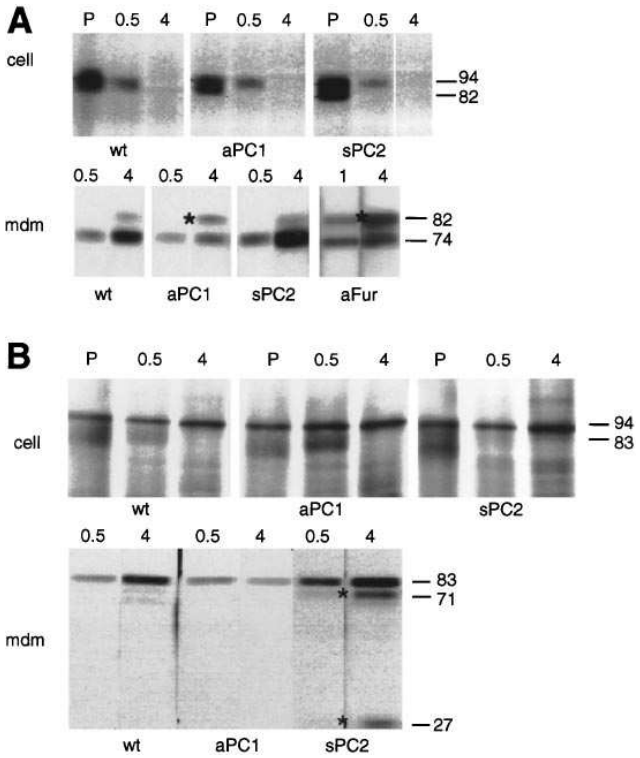

Figure 6. Biosynthetic processing of metabolically labeled chromogranin A in AtT-20 cells. AtT-20 cells (wild-type [*wt*]; antisense PC1 [A5-5; *aPC1*]; overexpressing PC2 [*sPC2*]; or aFur) were pulse-labeled (*P*) with [^{35}S]methionine/cysteine for 30 min, then chase-incubated in nonradioactive medium for 0.5 h (or 1 h for aFur cells) or 4 h. Cell extracts and collected media equivalent to 5×10^6 cpm TCA-precipitable protein were immunoprecipitated with appropriate antisera and analyzed as described in Methods. (*A*) Immunoprecipitation with anti–chromogranin A COOH-terminal antiserum (×7, prolonged exposure time for autoradiograph of media samples). (*B*) Immunoprecipitation with anti–chromogranin A NH_2-terminal antiserum (×5, prolonged exposure time for autoradiograph of media samples). Identical results were obtained in another independent experiment. *cell*, cell lysates; *mdm*, medium. Numbers at the top of each panel are hours of chase. Numbers at the right are molecular masses, in kD, of size standards. The most significant changes in chromogranin A processing are marked by asterisks. In this SDS-PAGE system, intact (unprocessed) chromogranin A migrates at ~ 94 kD (see Results).

PC1 (Fig. 8 *b*) or PC2 (Fig. 8 *c*), there were dramatic decreases of chromogranin A staining in the cell processes, where regulated secretory vesicles reside, as compared with wild-type cells. In cells underexpressing PC1, there was a small increase in chromogranin A staining in the processes (not shown).

Fig. 8, *e* and *f*, demonstrates PC1 immunostaining in wild-type (Fig. 8 *e*) and antisense PC1 (Fig. 8 *f*) cells, with the expected diminution of PC1 expression in antisense PC1 cells.

Discussion

The primary structure of chromogranin A (Fig. 2), a precursor of biologically active peptides (1–12), contains multiple pairs of basic amino acids which are potential sites for cleavage by the endoproteases PC1 and PC2.

We found that pituitary corticotrope chromogranin A can be cleaved by PC1 (Figs. 3 and 6), PC2 (Fig. 6), and furin (Fig. 6) in vivo. Furthermore, the PC1 antisense studies (Figs. 3 and 6) demonstrate that endogenous PC1 is a chromogranin A-processing enzyme in pituitary corticotropes, since: (*a*) on chromogranin A region-specific immunoblots (Fig. 3), an NH_2-terminal fragment of ~ 50 kD and a COOH-terminal fragment of ~ 30 kD seem to result from PC1 proteolytic action on intact chromogranin A (~ 80 kD) and an ~ 66-kD COOH-terminal fragment; and (*b*) in pulse–chase immunoprecipitations (Fig. 6), conversion of secreted COOH-terminal fragments of chromogranin A from ~ 82 to ~ 74 kD (Fig. 6 *A*) and generation of a secreted NH_2-terminal ~ 83-kD fragment (Fig. 6 *B*) also seem to be mediated by PC1. Similarly, the furin antisense studies (Fig. 6 *A*) also implicate endogenous furin in cleavage of secreted chromogranin A COOH-terminal fragments from ~ 82 to ~ 74 kD.

Two points idiosyncratic to chromogranin A influence interpretation of our immunoblotting (Fig. 3) and metabolic labeling (Fig. 6) results. First, because there are no methionine or cysteine residues near the COOH terminus of mouse (31) chromogranin A (Fig. 2), small COOH-terminal fragments of chromogranin A (such as those visualized on immunoblots, Fig. 3 *B*) cannot be detected on fluorography after [^{35}S]methionine/cysteine pulse-labeling and immunoprecipitation (Fig. 6 *A*). Second, the mobility of chromogranin A on SDS-PAGE is highly anomalous: though its molecular mass (deduced from cDNA sequences [1–3, 24, 31]) is ~ 48–50 kD, its acidic amino acid composition (1, 3, 9, 24) may inhibit SDS binding, resulting in diminished relative SDS-PAGE mobility and consequently increased apparent (standard-interpolated) molecular mass (3, 4); in the two SDS-PAGE systems used in these experiments, the apparent molecular mass of intact (unprocessed) chromogranin A ranged from ~ 80 kD (Fig. 3) to ~ 94 kD (Fig. 6).

While our results document a role for endogenous PC1 in processing of chromogranin A in vivo, by contrast Arden et al. (32) reported that PC2 (though not PC1) cleaved chromogranin A in vitro; however, it may be difficult to ensure optimal in vitro cleavage conditions for PC1. Furthermore, we cannot exclude the possibility that PC1's function may be to proteolyze and thereby activate another prohormone processing enzyme, which then affects the cleavage of chromogranin A.

Specificity of PC1 cleavage of chromogranin A in corticotropes was evidenced by lack of effect of PC1 antisense on cleavage of chromogranin B (Fig. 4) or secretogranin II (Fig. 5). Since chromogranin B and secretogranin II also contain multiple sites of paired basic residues (1, 2, 5, 6, 33), this specificity suggests that the precise flanking amino acid sequences (or structural context) around putative dibasic cleavage sites may influence PC1 cleavage specificity. Similarly, PC1 cleaves proenkephalin but not proopiomelanocortin at lysine-lysine residue pairs in transfected cells, and only PC2 seems capable of cleavage at the lysine-lysine pair in proopiomelanocortin (34, 35). By contrast, Hoflehner et al. (36) showed that vaccinia virus–overexpressed PC1 cleaved secretogranin II, and Egger et al. (37) found that secretogranin II processing in adrenal medulla and sympathetic axons correlated with content of soluble PC1 and PC2; however, neither of these observations necessarily pertains to the effects of endogenous PC1 in corticotropes.

Although the chromogranins/secretogranins share common properties (for example, each has an acidic amino acid

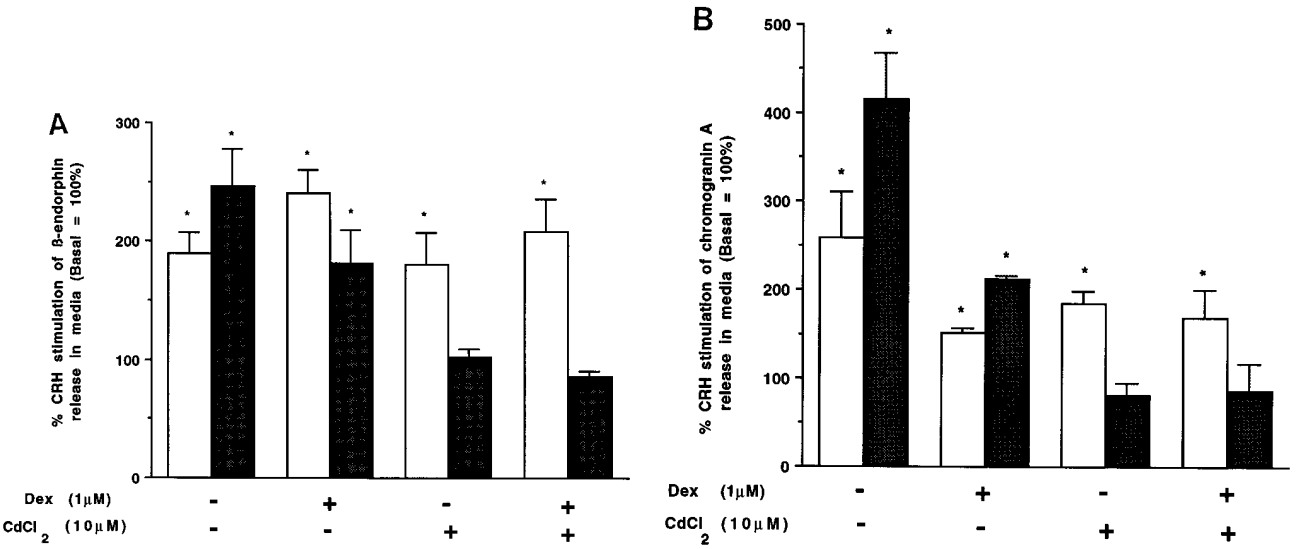

Figure 7. PC1 and the regulated secretory pathway. The effect of antisense PC1 on CRH (100 nM)-stimulated secretion of β-endorphin and chromogranin A from pituitary corticotropes. Secretion in response to 3 h of CRH was studied in the presence or absence of 12-h pretreatment with $CdCl_2$ (10 μM) and/or dexamethasone (1 μM), in wild-type AtT-20 (*open bars*) and A5-5 cells (*filled bars*). Results are the mean±SEM for β-endorphin or chromogranin A secretion compared with controls from $n = 3$–4 experiments per condition. Pretreatment with the glucocorticoid dexamethasone (1 μM, 12 h) in wild-type AtT-20 cells increased basal secretion of chromogranin A (from 3.2±0.4 to 5.2±0.6 fmol chromogranin A/μg protein, $P = 0.03$), while diminishing the basal secretion of β-endorphin (from 52±5 to 14±3 fmol β-endorphin/μg protein, $P = 0.009$). (*A*) CRH-stimulated β-endorphin secretion (increased by 180±20% in wild-type AtT-20 cells, $P = 0.01$; by 250±30% in noninduced A5-5 cells, $P = 1 \times 10^{-6}$). (*B*) CRH-stimulated chromogranin A secretion (increased by 260±50% in wild-type AtT-20 cells, $P = 0.02$; by 410±80% in noninduced A5-5 cells, $P = 0.003$). Basal secretion (without CRH) is defined as 100%. *Dex*, dexamethasone (1 μM). CRH, 100 nM. $^*P < 0.05$ (versus basal [without CRH]).

composition, contains multiple sets of paired basic residues, and binds calcium with moderate affinity [33, 38, 39]), sequence homology among them is confined to moderately homologous NH_2 and COOH termini between chromogranins A and B (1, 31), and a short (10 amino acid) region of partial homology towards the COOH terminus, among chromogranins A and B and secretogranin II (1).

Processing of chromogranin A is tissue- and cell type–specific, varying at different neuroendocrine sites (2, 4, 5, 40). Metz-Boutigue et al. (41) determined at least 12 cleavage sites for chromogranin A in bovine adrenal medulla, wherein the protein is cleaved both intra- and extracellularly; 8 of these 12 cleavage sites are at paired basic residues. Mouse chromogranin A also contains eight paired basic sites (31), potential proteolytic recognition points for PC1 and PC2.

The data presented (Figs. 3–5) indicate that processing of chromogranins/secretogranins in mouse corticotropes proceeds from both the NH_2- and COOH-terminal ends, as initially demonstrated for chromogranin A by Wohlfarter et al. (42) in the bovine adrenal medulla, and for chromogranins A and B in a variety of neuroendocrine sites (2, 4, 5). Accumulation of chromogranin A NH_2-terminal fragments of ~ 50–70 kD (Fig. 3 *A*), along with COOH-terminal fragments of ~ 28–33 kD (Fig. 3 *B*), suggests predominant chromogranin A cleavage towards the COOH terminus. For chromogranin B (Fig. 4), more processing was seen from the NH_2-terminal end, while for secretogranin II (Fig. 5) both NH_2- and COOH-terminal cleavage products were prevalent.

Is there a necessary role for endoproteolytic cleavage by prohormone convertases during intracellular trafficking of peptides to storage vesicles of the regulated secretory pathway? The ability of CRH to stimulate secretion of both β-endorphin (Fig. 7 *A*) and chromogranin A (Fig. 7 *B*) in wild-type AtT-20 cells and noninduced (i.e., PC1-expressing) A5-5 cells, but not in antisense PC1 (i.e., PC1-deficient) A5-5 cells, suggests that additional changes have occurred in antisense PC1 cells in the regulated secretory pathway. Jung et al. (43, 44) demonstrated that in AtT-20 cells transfected with the Aplysia egg-laying hormone (ELH) prohormone, mutation of ELH prohormone cleavage sites reroutes the trafficking of some ELH fragments to constitutive-like (instead of regulated) secretory vesicles. While Jung et al. (43, 44) studied cleavage-dependent trafficking of exogenous (transfected ELH) neuropeptides, our results document cleavage-dependent trafficking of endogenous (chromogranin A, β-endorphin) neuropeptides. By contrast, expression of PCs may not be entirely sufficient to ensure regulated secretion, since the AtT-20/myeloma fusion cell line HYA.15.6.T.3 (45), which expresses both PC1 and PC2, lacks dense core vesicles and a regulated pathway of secretion; in the HYA.15.6.T.3 line, regulated secretion of proopiomelanocortin can be restored by cyclic AMP treatment (45). Likewise, in at least some cell types, cleavage of chromogranin A may not be entirely necessary for its entry into the regulated secretory pathway; for example, chromogranin A is only partially processed in chromaffin vesicles, and intact chromogranin A is released from chromaffin cells in response to physiologic (nicotinic cholinergic) secretory stimulation (2, 4, 41).

Finally, disruption of the usual patterns of chromogranin A localization in the secretory pathway on immunocytochemistry of AtT-20 cells under- or overexpressing prohormone convertases (Fig. 8) is also consistent with an influence of both prohormone convertases, and their cleavage of the substrate chromogranin A, on its intracellular trafficking. Overexpression of

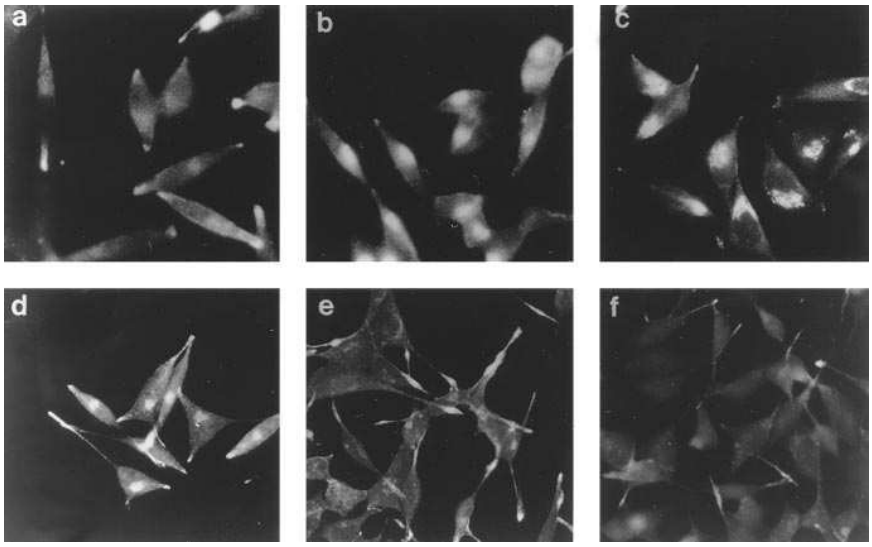

Figure 8. Immunofluorescence studies of chromogranin A localization and PC1 expression in AtT-20 cells. Immunostaining of AtT-20 cells with various PC profiles was performed as described in Methods. (*a–c*) Immunostaining of chromogranin A using an antiserum to intact (full-length) chromogranin A, in wild-type AtT-20 cells, AtT-20 sPC1 cells (overexpressing PC1), or AtT-20 sPC2 cells (overexpressing PC2), respectively. (*d*) Immunostaining of wild-type AtT-20 cells using a chromogranin A NH_2-terminal antiserum. (*e* and *f*) Immunostaining of PC1 in wild-type AtT-20 cells (*e*) or antisense PC1 (A5-5) cells (*f*), using PC1 antiserum JH887. Both wild-type and antisense PC1 cells were treated with 10 μM $CdCl_2$ overnight in complete serum-free medium before immunostaining.

either PC1 or PC2 seemed to emphasize the immunostaining of chromogranin A in the *trans*-Golgi region, seemingly with depletion of chromogranin A in peripheral secretory vesicles, while underexpression of PC1 resulted in apparent emptying of the *trans*-Golgi region of chromogranin A, with accumulation in peripheral vesicular sites. Other determinants of correct trafficking of chromogranins into the regulated secretory pathway include an intact intramolecular disulfide loop in chromogranin B (46) and as yet undefined elements in the NH_2 terminus of chromogranin A which can divert even ordinarily nonsecreted proteins into the regulated pathway (47). Thus, chromogranin A may contain a dominant targeting signal for the regulated pathway (47), and chromogranin A also may assist in trafficking (perhaps by aggregation) of other secreted peptides into the regulated pathway (1, 2, 48, 49); if the entry of chromogranin A into that pathway is impaired by disruption of its proteolysis (Fig. 7 *B*), then perhaps failure of entry of β-endorphin into the pathway (Fig. 7 *A*) may in part be secondary or attributable to disruption of trafficking of chromogranin A (Fig. 7 *B*).

We used Cd^{2+} to induce a metallothionein promoter controlling PC1 antisense mRNA (18), thereby decreasing PC1 expression in these experiments (Fig. 1). Although Cd^{2+} may be cytotoxic at high dosage (50, 51), in these experiments Cd^{2+} did not impair two crucial functions of wild-type AtT-20 cells: prohormone processing (Figs. 3–6) and regulated secretion (Fig. 7). Thus, the effects of Cd^{2+} on A5-5 cell functions seem to be specifically mediated by the induction of PC1 antisense mRNA expression.

As previously documented (28, 30), dexamethasone had opposite effects on basal secretion of chromogranin A and β-endorphin, increasing chromogranin A release while diminishing β-endorphin secretion. Glucocorticoids exert both direct and indirect effects to diminish proopiomelanocortin fragment release in corticotropes, where the indirect effects may require the action of an intervening gene (52). Wand et al. (28) found that chromogranin A (or its fragments) suppressed proopiomelanocortin release; since chromogranin A is augmented by glucocorticoid (28, 30). Wand et al. (28) proposed that chromogranin A might, in part, mediate the effects of glucocorticoid to inhibit proopiomelanocortin secretion. In the present experiments, we observed that disruption of chromogranin A processing (by PC1 ablation in A5-5 cells) also disrupted regulated secretion of β-endorphin (Fig. 7 *A*), consistent with the notion that chromogranin A proteolytic fragments modulate proopiomelanocortin fragment release (28). However, it should be noted that not all observers find that chromogranin A inhibits proopiomelanocortin release (53).

These results demonstrate that chromogranin A is a substrate for the endogenous endoproteases PC1 and furin in vivo; furthermore, lack of change in chromogranin B and secretogranin II cleavage after ablation of PC1 suggest that the action of PC1 on chromogranin A is specific within the chromogranin/secretogranin protein family. Finally, disruption of prohormone proteolytic processing results in interference with entry and traffic of chromogranin A and proopiomelanocortin into the regulated secretory pathway.

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