Several immortalized cell lines serve as models for procholecystokinin (pro-CCK) processing. Rin5F cells, derived from a rat insulinoma, and STC-1 cells, derived from a murine intestinal tumor, process pro-CCK mainly to amidated CCK 8. Both also make significant quantities of amidated CCK 22, a slightly larger form found in the gut. Many modifications are necessary during pro-CCK processing including cleavages performed by endoproteases, the identities of which are unknown. A candidate endoprotease is prohormone convertase 1 (PC1) also known as PC3, a Ca\textsuperscript{2+}-dependent serine endoprotease of the subtilisin family.

Constitutive expression of antisense PC1 message in stably transfected Rin5F cells resulted in a significant reduction of the cellular content of CCK 8 as measured by radioimmunoassay. Several affected cell lines displayed about 80% reduction in CCK content in early passages after transfection. Expression of antisense PC1 message in these cell lines resulted in a selective depletion of CCK 8 and a comparative sparing of CCK 22. The induction of antisense PC1 message within a single subclone of Rin5F cells using the Lac Switch system also resulted in a significant inhibition of CCK content. Expression of antisense PC1 message in a stably transfected STC-1 cell line also resulted in a decrease in CCK content and in PC1 protein expression, and the specific depletion of CCK 8 with comparative sparing of CCK 22. These observations support the hypothesis that PC1 is necessary for pro-CCK processing in Rin5F and STC-1 cells and suggests a role for PC1 endoprotease in the biosynthesis of CCK 8 \textit{in vivo}.

Cholecystokinin (CCK)\textsuperscript{1} is a peptide found in both the digestive tract and the brain. It is released from the intestine following the ingestion of food and causes the contraction of the gall bladder and the release of digestive enzymes from the pancreas (1, 2). Like other gut peptides, CCK also serves as a neurotransmitter, and next to neuropeptide Y (3), is the most abundant and is widely distributed in most areas of the brain (4–6). Its role in the nervous system is less clear but appears to serve as a neurotransmitter (7, 8) or a neuromodulator (9–12). The predominant form of CCK in the brain is the eight-amino acid CCK 8, whereas the larger forms, such as CCK 58, CCK 33, and CCK 22, predominate in the gut. Various forms of CCK found either in the brain or the gut appear to be the result of differential processing of the same pro-CCK precursor. The processing of pro-CCK in the gut is complex and species-dependent.

Modifications on pro-CCK during processing include sulfation of three carboxyl-terminal tyrosines, cleavage at dibasic or monobasic sites, the possible action of amino- or carboxypeptidases, and amidation of the carboxyl-terminal to release bioactive forms of CCK. Among these modifications, cleavages produced by endoproteases are the keys to differential processing. The identity of these endoproteases in pro-CCK processing are, for the most part, unknown. Recently, the isolation of CCK 8-generating enzyme from rat brain synaptosomes and its ability to cleave CCK 33 to form CCK 8 have identified at least one endoprotease that may be involved in CCK 8 biosynthesis (13).

The cloning of prohormone convertase 1 (PC1) (14, 15), also known as PC3, a member of the subtilisin family of enzymes, has identified another candidate. PC1 is a Ca\textsuperscript{2+}-dependent serine endoprotease known to cleave propeptides at dibasic residues (16, 17), and there is increasing evidence that it can also cleave at mono-arginyl sites as well (18, 19). Evidence to support its role in pro-CCK processing includes the following.

1) PC1 and CCK are both found in neuroendocrine cells (20) and share a similar tissue distribution, and both PC1 and CCK are found within the regulated secretory pathway. 2) PC1 is expressed in a number of endocrine cell lines that express CCK mRNA and correctly process it to carboxamidated products. These include Rin5F cells derived from a rat insulinoma (21), STC-1 cells derived from a murine intestinal tumor, WE cells derived from a mouse medullary thyroid tumor, and AtT20 cells derived from mouse pituitary cells (22). PC2 is also expressed by all of these lines, except At-T20 cells (22). 3) Pro-CCK contains several dibasic and mono-arginyl residues that are potential sites of cleavage for PC1. 4) PC1 has recently been shown to cleave a number of propeptides including pro-opiomelanocortin (POMC) (23, 24), proinsulin (25), proenkephalin (26), and prosomatostatin (27).

The development of specific inhibitors of PC1 that are not toxic to cells in culture has lagged behind the discovery of PC1. To evaluate the importance of PC1 in the processing of pro-CCK in specific cells, an antisense strategy was adopted. The use of stable expression of PC1 antisense message to inhibit an PC1 expression was first used by Bloomquist et al. (28) to support a role for PC1 in POMC processing in AtT20 cells. Subsequently, it has been used to demonstrate the role of PC2 in proenkephalin (29), POMC (30), and proglucagon processing (31). In this study, we have inhibited endogenous PC1 expression by stable expression of PC1 antisense mRNA in Rin5F (32) and STC-1 cells (22), which express CCK mRNA and process pro-CCK to CCK 8 and CCK 22. Here we present evidence that

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\textsuperscript{1} The abbreviations used are: CCK, cholecystokinin; PC, prohormone convertase; POMC, pro-opiomelanocortin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; RSV, Rous sarcoma virus; RIA, radioimmunoassay; TBS, Tris-buffered saline; kb, kilobase(s); CMV, cytomegalovirus; MOPS, 3-(N-morpholino)propanesulfonic acid.

\textsuperscript{¶} This paper is available on line at http://www-jbc.stanford.edu/jbc/
the expression of PC1 antisense mRNA inhibits CCK 8 formation within these cells.

MATERIALS AND METHODS

Construction of Antisense Expression Plasmids—Constitutively expressing antisense PC1 plasmid pCMV5/anti-PC1 was constructed using the first 491 bases of the PC1 cDNA insert from prPC1.491EX, kindly provided by Dr. Richard Mains at Johns Hopkins University. This PC1 fragment was ligated into the pCMV5 mammalian expression vector (33) using a ratio of 8:1 insert to vector (34) in the antisense orientation in the HindIII and XbaI sites so that its expression was driven by the cytomegalovirus promoter. The orientation of the PC1 cDNA insert was verified by restriction digestion and polymerase chain reaction.

Constitutively expressing antisense PC2 plasmid pCMV5/anti-PC2 was constructed using the first 480 bases of the PC2 cDNA contained within the prPC2 490EX plasmid also provided by Dr. Richard Mains. This PC2 fragment was inserted into pCMV5 at the KpnI and XbaI sites in the antisense orientation. The orientation of the insert was confirmed by endonuclease digestion.

The Lac Switch inducible expression system (Stratagene) was employed for expressing antisense PC1 sequences in STC-1 cells. This system employs two plasmids: the first plasmid, p3SS, expresses the Lac repressor protein that blocks expression from a second expression plasmid, containing the sequence of interest. The expression plasmid can be de-repressed by the addition of 1–10 mM isopropyl-1-thio-D-β-galactopyranoside (IPTG). IPTG binds to the Lac repressor protein and causes a conformational change to decrease its affinity to Lac operator sequences located between the Rous sarcoma virus (RSV-long terminal repeat) promoter and the sequence of interest within the expression plasmid. The first 491 bases of the PC1 cDNA was cloned into the expression plasmid, POPRSVICAT, in the antisense orientation. Orientation of the PC1 insert was verified by restriction digestion analysis, polymerase chain reaction, and DNA sequencing.

Maintenance and Transfection of Tissue Culture Cells—Rin5F cells and STC-1 cells were maintained in Dulbecco’s modified Eagle’s minimal medium containing 20% newborn calf serum, 10% horse serum, and 1% penicillin and streptomycin or 1% gentamicin in a humidified 37 °C incubator at 5% CO2. Cells were grown to 80% confluency by trypsinization. STC-1 cells were a gift of Dr. Douglas Hanahan at the Hormone Research Institute of the University of California, San Francisco.

Expression plasmids were transfected into Rin5F and STC-1 cells by electroporation at 2000V/800 microfarads using the Bio-Rad Gene Pulser electroporator. Cells were grown to 80% confluency by trypsinization. STC-1 cells were a gift of Dr. Douglas Hanahan at the Hormone Research Institute of the University of California, San Francisco.

Expression plasmids were transferred to Rin5F and STC-1 cells by electroporation at 2000V/800 microfarads using the Bio-Rad Gene Pulser electroporator. Cells were grown to 80% confluency, trypsinized, pelleted, and resuspended to 5 × 10^6 cells/0.8 ml of growth medium, along with 5–50 μg of plasmid DNA, in a Bio-Rad 0.6 cm electroporation cuvette. The cuvette was pulsed on ice for 10 min prior to electroporation and again for 10 min after electroporation. The cells were allowed to recover for 48 h in normal growth medium and then diluted into 96-well plates containing the appropriate antibiotic for selection. Single colonies were isolated and established as stable cell lines.

Constitutively expressing plasmids pCMV5/anti-PC1 and pCMV5/ anti-PC2 were co-transfected with pMNeo, which confers resistance to the antibiotic G418, in a molar ratio of 3:1:5, respectively. The two plasmids of the inducible expression system were transfected sequentially. Plasmid p3SS was initially transfected into STC-1 cells and selected with hygromycin for successful plasmid incorporation. Production of Lac repressor protein was verified by histochemistry using an antiseraum purchased from Stratagene. A positive stromal was then selected for subsequent transfection of the second plasmid, expressing antisense PC1 sequences, and screened with a combination of hygromycin and G418.

Northern Blot Analysis—Total RNA was isolated from tissue culture cells using guanidine isothiocyanate (36), and 20–40 μg were used for analysis. Polyadenylated mRNA was isolated using the Riboprobe isolation kit from Collaborative Biosciences and Products, and 5 μg were used for analysis. Northern analysis was performed as described elsewhere (37) with slight modifications. Briefly, RNA was fractionated by electrophoresis through a 2.2 M formaldehyde, 1% agarose gel in 1 × MOPS buffer, transferred overnight to Magna nylon filter (Micron Separations Inc.) in 10 × sodium chloride/sodium citrate transfer buffer (SSC), cross-linked to the filter by UV illumination, and placed with 10 ml of hybridization solution in a glass bottle set in a hybridization oven at 42 °C. A small amount of ethidium bromide was included in the sample buffer so that the uniformity of RNA loading and transfer could be verified by ethidium bromide staining and photography of agarose gel and the blot after transfer. Prehybridization solution and hybridization solution consisted of 50% formamide, 5 × SSC, 5 × Denhardt’s, 0.5% sodium dodecyl sulfate (SDS), and 100 μg of denatured salmon sperm DNA/ml.

The hybridization probe was produced using the same portions of the PC1 or PC2 cDNA described in the antisense experiments as templates for random primed labeling using the RPL kit from New England Biolabs. These double-stranded probes allowed detection of both the sense strand antisense RNA. A labeled DNA was separated from unincorporated nucleotides through a G-50 column, denatured along with 300 μg of salmon sperm DNA by heating in a boiling water bath, and placed into the hybridization cylinder at a specific activity of 1 × 10^9 cpm/ml. Filters were washed within the hybridization cylinders twice at room temperature with 2 × SSC, 0.1% SDS for 10 min; twice at room temperature with 0.2 × SSC, 0.1% SDS for 10 min; and twice at 42 °C with 0.1 × SSC, 0.1% SDS for 15 min. Autoradiography was performed with an intensifying screen at −80 °C for several hours to days as appropriate.

Western Blot Analysis—Polyclonal antibodies recognizing PC1 and PC2 proteins were generously provided by Iris Lindberg at the Louisiana State Medical Center (38, 39). Western analysis was performed with the following modifications to the protocol described in the protocolary. Tissue culture cells were grown to near confluency and harvested for protein analysis by scraping from dishes with 1 ml of phosphate-buffered saline, after which an aliquot was taken for total protein measurement by the Lowry assay (40). After centrifugation, cells were extracted by resuspending in 1 × SDS protein loading buffer (25 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromphenol blue), sonication, and boiling for 5 min. Protein samples were fractionated by electrophoresis at 100 V for 2 h through a 10% polyacrylamide gel in protein electrophoresis buffer (0.1% SDS, 0.25 mM Tris, 192 mM glycine) using a protein minigel apparatus (Bio-Rad). The fractionated proteins were electroblotted onto nitrocellulose in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 4 °C overnight at 0.25 mA/gel. The membranes were then incubated with 1:5000, respectively. Visualization by alkaline phosphatase was performed according to manufacturer’s protocol. Visualization by enzyme chemiluminescence using the horseradish peroxidase enzyme was performed according to manufacturer’s protocol from the ECL Western detection kit from Amersham Life Science.

Quantitation of Western protein levels were performed by densitometry using the Image Quant software analysis program.

Radioimmunoassay—The radioimmunoassay (RIA) to measure CCK 8 from cell extracts was performed as described previously (4). The rabbit polyclonal CCK antibody (R5) is specific for the amidated form of CCK 8. As tracer, the RIA utilized gastrin 17 produced by iodination using the chloramine-T method (41).

Chromatography—Cells from several plates were extracted with 0.1 N HCl, pooled, and concentrated by vacuum centrifugation. Cell extracts were separated by Sephadex G50 chromatography in a 35 × 1 cm column run at 4 °C in 50 mM Tris, 100 mM NaCl, pH 7.8, containing 0.1% BSA and 0.05% sodium azide. Fractions of 1.0 ml were collected and aliquots removed for the CCK RIA.

RESULTS

Constitutive Expression of Antisense PC1 in Rin5F Cells—Rin5F cells were engineered to express the first 491 bases of the rat PC1 cDNA in the antisense orientation from the cyto-megalovirus promoter, or the pCMV5 vector with no insert, to serve as a control. Positive transfectants were screened after dilution and replating using Genetec (G418) and established
as stable cell lines. When CCK levels of anti-PC1-transfected subclones were measured by radioimmunoassay, they displayed a wide range of CCK content. Some were similar to both the parental Rin5F and Rin5F cells transfected with pCMV5 while the most severely affected clones R1E8 and R1E9 showed about an 80% reduction as compared with control (Fig. 1). Northern analysis revealed that the dramatic decrease in CCK 8 was not a result of lower levels of CCK mRNA expression in these cells because they expressed similar amounts of CCK relative to both parental Rin5F cells and Rin5F cells transfected with pCMV5 plasmid (data not shown).

To examine the effect of expression of antisense PC1 message on PC1 sense message in one of these cells lines (R1E8), the expression of both sense and antisense PC1 messages were measured by Northern analysis. Because the endogenous PC1 messages and the antisense PC1 message are of different sizes, they could both be visualized after hybridization (Fig. 2). Rin5F cells, RCC9 cells, transfected with the pCMV5 vector alone, and R1E8 cells all expressed endogenous alternatively spliced sense PC1 transcripts of approximately 2.6, 4.0, and 5.0 kb. In R1E8 cells, in addition to larger amounts of these three transcripts, we also observed a large amount, at least in equal concentration to the endogenous messages, of antisense PC1 message of approximately 0.6 kb. Furthermore, we observed several other previously unreported alternatively spliced forms of endogenous PC1 message, the formation of which are presumably due to the interference of antisense in the processing of the primary mRNA transcript. Others have reported the inhibition of primary transcript processing (42) and the accumulation of target message (43) during successful antisense inhibition. The observations on the effect of PC1 message in R1E8 cells support these as possible mechanisms of antisense action.

In Fig. 3, the PC1 and PC2 protein levels in anti-PC1 Rin5F subclones are compared with control cell lines. Because these blots were stripped and reprobed, it is possible to directly compare the ratio of PC1 to PC2. Two major forms of PC1 protein identified by Western analysis in Rin5F cells were 87 and 66 kDa in size. The antisense PC1 cells (R1E8, R1E9, and R1E11) that had reduced amounts of CCK 8, also had decreased levels of PC1 protein both absolutely and relative to PC2. Both the 87- and 66-kDa proteins were reduced equally and confirmed that the expression of PC1 antisense resulted in a dramatic inhibition of PC1 protein as compared with Rin5F and RCC9 cells. In these cells, although the PC1 level was reduced, it was not completely eliminated. After as long as 1 year in culture, PC1 levels were still very low in the R1E8 and R1E9 cells (data not shown).

Sephadex Chromatography of CCK Peptides in Rin5F Wild Type and PC1 Antisense-expressing Cell Lines—Sephadex G50 chromatography of extracts of Rin5F, R1E8, and R1E9 shows that expression of PC1 antisense message, which causes a decrease in PC1 protein, produces a substantial depletion of CCK 8 relative to CCK 22. (Fig. 4). If the areas under the peak of CCK 8 is compared with CCK 22, the wild type is 0.9, while the antisense lines are from 0.1 to 0.2. After as long as 1 year in culture, the ability of R1E8 and R1E9 to produce CCK 8 did not recover (data not shown). The levels of total CCK did increase with time, suggesting a possible up-regulation of CCK expression or processing to CCK 22.

Inducible Expression of Antisense PC1 in Rin5F Cells—To further rule out the possibility that constitutively expressing antisense PC1 Rin5F subclones were selected that were already low in CCK 8 content, we attempted to further correlate the expression of antisense with inhibition of CCK 8 formation in a single subclone using the Lac Switch mammalian expres-
tion system. This expression system displayed tight basal control of antisense PC1 message before induction (Fig. 5). Three major transcripts of PC1 of 2.6, 4.0, and 5.0 kb were observed as seen previously. After induction with 10 mM IPTG, a 0.6-kb antisense message was also observed, which was driven by the RSV-long terminal repeat promoter. However, it was apparent that induction of the expression of antisense PC1 from the RSV promoter in these cells was not comparable to the amount of expression observed from the constitutive CMV promoter in R1E8 or the other antisense cell lines (Fig. 2) but did result in a 20% reduction of CCK 8 levels (Fig. 6). Northern analysis also revealed that CCK message did not decrease after the introduction of IPTG and verified that the 20% reduction of CCK 8 in RLS5E9 cell content was not due to the decrease in CCK expression (data not shown).

Nevertheless, this observation that the expression of antisense PC1 message correlates with the inhibition of CCK 8 content further supports the connection between antisense PC1 expression and levels of immunoreactive CCK 8.

Constitutive Expression of Antisense PC1 in STC-1 Cells—Using a similar strategy as described for Rin5F cells, STC-1 cells expressing anti-PC1 and anti-PC2 mRNA were generated. Like the Rin5F cells, they also had greatly reduced levels of CCK by RIA. The most severely affected (S1F2) having about 26% of parental levels of CCK was selected for further analysis in comparison with parental STC-1 cells and cells expressing anti-PC2 mRNA (S2H4) and control STC-1 cells (SCC9) transfected with pCMV5 without any insert.

Measurement of sense and antisense PC1 messages by Northern analysis revealed that the two predominant endogenous PC1 transcripts in STC-1 cells are the 2.6- and 4.0-kb messages (Fig. 7A). These transcripts were observed in STC-1 cells, control SCD9 cells, and anti-PC2 S2H4 cells. In S1F2 cells, however, antisense PC1 message was not observed and only very low levels of sense PC1 messages were present. This was assumed to be due to the degradation of the sense and antisense RNA duplex.

Northern analysis on the same cell lines using PC2 as a probe revealed that all four cell lines expressed similar levels of a single endogenous PC2 transcript of 2.2 kb (Fig. 7B). In addition to the endogenous PC2 message, S2H4 cells expressed a large amount of antisense PC2 message, which was measured by densitometry to be about 15-fold greater than the sense message. Although the expression of antisense PC1 message resulted in almost complete disappearance of the endogenous PC1 message in S1F2 cells, it appeared to have little effect on the expression of PC2 message. Similarly, the expression of a large amount of antisense PC2 message in S2H4 cells appeared to have little effect on the expression of PC1 message. This suggests that the effect of antisense is largely specific on the nucleic acid level to its targets in STC-1 cells.
Protein measurements on these cell lines by Western blot analysis confirmed the inhibition of PC1 protein expression (Fig. 8A). The two predominant forms of PC1 in STC-1 cells were 87 and 66 kDa, similar to the forms seen in Rin5F cells. Both forms were found to be greatly reduced in S1F2 cells. Quantitation by densitometry showed approximately an 80% inhibition as compared with STC-1, SCD9, and S2H4. The observation that neither the 75-kDa nor the 66-kDa PC2 protein levels in S1F2 cells were decreased demonstrates that the antisense inhibition in these cells is specific (Fig. 8B). Interestingly, although the endogenous PC2 message was not altered after the expression of antisense PC2 in S2H4 cells, we also observed a reduction in PC2 protein level of approximately 50% in S2H4 cells.

STC-1 cells produce a CCK 8 immunoreactive peptide, which co-elutes with CCK 8 on high performance liquid chromatography in STC-1 cells (22). Further analysis using Sephadex G-50 chromatography revealed that there are also significant amounts of a larger amidated form that co-elutes with a porcine CCK 22 standard produced by endoproteolysis of porcine CCK 33 with endo-Lys C (Fig. 9A). Further chemical characterization of this peptide has not been possible as a synthetic standard corresponding to rat CCK 22 is not available for high performance liquid chromatography analysis.
of extracts of anti-PC1 S1F2 cells demonstrated a specific ablation of CCK 8 relative to CCK 22 (Fig. 9B). Similar analysis of extracts of control SCD9 cells did not reveal depletion of either CCK 22 or CCK 8 (data not shown).

DISCUSSION

The complexity of the differential processing of pro-CCK to various bioactive products is reflected by the large number of amidated forms that have been isolated from different tissues and species including CCK 8 (44, 45), CCK 22 (46), CCK 33 (47), CCK 58 (48), and CCK 83 (48). Several different processing pathways have been proposed based on these CCK intermediates (49, 50), which suggests a regulatory mechanism for the fate of pro-CCK during processing. The most likely mechanism underlying differential processing is by the control of the activity of different endoproteases. In an attempt to identify endoproteases that are involved in pro-CCK processing, we have correlated the inhibition of PC1 expression in both STC-1 and Rin5F cells with specific alteration of CCK 8 content using antisense inhibition.

The stable expression of anti-PC1 mRNA results in a long-lasting inhibition of PC1 protein expression with no alteration of CCK mRNA or PC2 mRNA or protein levels. This is accompanied by a long-lasting inhibition of CCK 8 production. A small but significant decrease in CCK levels was also observed as a result of transient expression of anti-PC1 mRNA using the Lac Switch system. In these experiments, the decrease in CCK content was roughly proportion to expression of anti-PC1 mRNA.

The observation from these studies that the inhibition of PC1 correlates with the inhibition of CCK 8 cell content suggests that PC1 is necessary for CCK 8 biosynthesis. In support of this proposal is the observation that stable expression of the rat CCK cDNA in At-T20 cells, which express PC1 but not PC2, results in the synthesis and regulated secretion of large amounts of amidated CCK 8.2

The comparative sparing of CCK 22 suggests that PC1 is necessary for the formation of CCK 8 but not for CCK 22. Furthermore, the depletion of CCK 8 selectively demonstrates that the effect of antisense is specific for CCK 8 and is not the result of decreased production of pro-CCK.

Recent studies3 have shown that inhibition of expression of PC2 in STC-1 and Rin5F cell lines results in the opposite phenotype (depletion of CCK 22 relative to CCK 8). These results, taken together with the observation that both CCK 22 and CCK 8 are secreted by wild type STC-1 and Rin5F,4 suggest that CCK 8 and CCK 22 are both end products of pro-CCK processing which do not readily interconvert and that they are produced by independent processing pathways of pro-CCK. These results are consistent with the hypothesis that the differences in processing of pro-CCK observed in brain and gut are related to tissue differences in expression or activity of PC1 and PC2.

FIG. 10. Model of pro-CCK processing. The rat prepro-CCK sequence is shown with the major forms indicated above the prohormone. The major cleavage sites are indicated by the single-letter amino acid abbreviation form. The proposed branched pathway that would generate CCK 22 and CCK 8 independently is indicated below.

2 M. C. Beinfeld, unpublished observation.
3 J. Yoon and M. C. Beinfeld, manuscript in preparation.
4 J. Yoon and M. C. Beinfeld, unpublished observation.
The site where PC1 is acting on the pro-CCK sequence is still under investigation. PC1 is probably cleaving at an Arg-Asp site to release carboxyl-terminal extended CCK 8 from pro-CCK (Fig. 10), although it may also be cleaving further upstream in pro-CCK. It is also possible that PC1 may be acting indirectly to regulate the activity of another enzyme that acts directly on pro-CCK. Purified CCK 8-generating enzyme and purified recombinant YAP3 and PC 2 are also able to cleave amidated, sulfated porcine CCK 33 at this same site to generate amidated CCK 8 (13, 55, 56), so that there are a number of possible endoproteases which could potentially process pro-CCK in different tissues.

Because specific, non-cytotoxic inhibitors of PC1 are not available, we inhibited PC 1 expression by an antisense strategy. Studies using antisense inhibition have been criticized because of possible nonspecific cellular effects complicating the interpretation of the results. In light of these concerns, we performed several controls to ensure that the effects of antisense expression on the processing of pro-CCK were specific. The two most important controls performed were to measure the effects of antisense expression on target mRNA and target protein in both Rin5F and STC-1 cells. We observed at least three different effects on target mRNA by Northern analysis in PC1 and PC2-transfected Rin5F and STC-1 cells. We noted that the target protein level was dramatically reduced when measured by Western blot analysis. These observations suggest that the effect of antisense on the RNA level is complex and may be affected at multiple levels to produce inhibition of the protein product. In fact, many mechanisms of action have been proposed previously for antisense including inhibition of primary transcript processing (42, 51), produce inhibition of the protein product. In fact, many mechanisms of action have been proposed previously for antisense including inhibition of primary transcript processing (42, 51), block of nuclear transport (43), degradation of target mRNA (52), and block of translation (53, 54).

Another control that was performed was to measure the effect of PC2 message and protein level in antisense PC1-transfected Rin5F cells and vice versa. Although PC1 and PC2 are homologous genes within the same family of endoproteases, the effect of antisense was specific for each. These observations suggest that inhibition by antisense in these cells was highly specific. Further detailed analysis of pro-CCK processing intermediates produced by these transfected cells should yield more information about the nature of pro-CCK processing and possibly identify the site at which PC1 is acting. The successful antisense inhibition of PC1 or PC2 in Rin5F and STC-1 cells identifies a possible method to study pro-CCK processing in other systems as well, including in living animals. Furthermore, since PC1 and PC2 are known to cleave a large number of other biologically active peptides, this approach may be helpful in studies of processing pathways of these peptides as well.

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Prohormone Convertase 1 Is Necessary for the Formation of Cholecystokinin 8 in Rin5F and STC-1 Cells
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