Cleavage-Site Mutagenesis Alters Post-translation Processing of Pro-CCK in AtT-20 Cells

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ABSTRACT: Cholecystokinin (CCK) is expressed in the central and peripheral nervous systems and functions as a neurotransmitter and neuroendocrine hormone. The in vivo forms of CCK include CCK-83, -58, -39, -33, -22, -12, and -8. Tissues in the periphery produce the larger forms of CCK, such as CCK-58, whereas the brain primarily produces CCK-8. The different biologically active forms of CCK observed in vivo may result from cell-specific differences in endoproteolytic cleavage during post-translational processing. Evidence suggests that cleavages of pro-CCK occur in a specific sequential order. To further delineate the progression of cleavages during pro-CCK maturation, mutagenesis was used to disrupt putative mono- and dibasic cleavage sites. AtT-20 cells transfected with wild-type rat prepro-CCK secret CCK-22 and -8. Mutagenesis of the cleavage sites of pro-CCK had profound effects on the products that were produced. Substitution of basic cleavage sites with nonbasic amino acids inhibits cleavage and leads to the secretion of pathway intermediates such as CCK-83, -33, and -12. These results suggest that CCK-58 is cleaved to both CCK-33 and -22. Furthermore, CCK-8 and -12 are likely derived from cleavage of CCK-33 but not CCK-22. Alanine substitution at the same site completely blocked production of amidated products, whereas serine substitution did not. The cleavages observed at nonbasic residues in this study may represent the activity of enzymes other than PC1 and carboxypeptidase E, such as the enzyme SKI-1. A model for the progression of pro-CCK processing in AtT-20 cells is proposed. The findings in this study further supports the hypothesis that pro-CCK undergoes parallel pathways of proteolytic cleavages.

Cholecystokinin (CCK)[1] is a peptide expressed in cells of both the central and peripheral nervous systems. Intracellularly, CCK is stored in dense-core secretory vesicles of the regulated secretory pathway and is released upon cellular stimulation. Upon secretion, CCK acts as a neurotransmitter, neuromodulator, neuroendocrine hormone, and growth factor (*1*−*3*). The secreted forms of CCK include CCK-58, -39, -33, -22, -12, and -8. CCK-8 is the primary form found in the brain, whereas both CCK-33 and -8 are found in the intestine. CCK-58 has been shown to be the primary circulating form of CCK in humans (*4*), dogs (*5*), and rats (*6*). The different forms of CCK are generated from the post-translational processing of a common precursor peptide, pro-CCK. The forms of mature CCK secreted in vivo are probably dependent on tissue-specific variations in post-translational processing of pro-CCK, rather than alternate splicing of pro-CCK mRNA or other mechanisms (*3*).

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[1] Abbreviations: @, stop codon; CCK, cholecystokinin peptide; CPE, carboxypeptidase E; CT, carboxyl terminus; ER, endoplasmic reticulum; HPLC, high-performance liquid chromatography; IR, immunoreactivity; PAM, amidating enzyme; PC, proprotein or prohormone convertase; Prepro-CCK, preprocholecystokinin peptide; Pro-CCK, procholecystokinin peptide; RIA, radioimmunoassay; SEC, size-exclusion chromatography; SKI-1, subtilisin/kexin-isozyme-1; SV, synaptic vesicle; TGN, trans-Golgi network; TSN, tyrosyl sulfotransferase; WT, wild type; αCCK-NH_2, anti-CCK amide antibody; αV9M, anti-V9M antibody.

Evidence suggests that post-translational processing events occur in a timely and sequential fashion (*3*). After insertion of the growing peptide chain into the ER, cleavage of the N-terminal signal sequence occurs. After the completion of synthesis, the full-length pro-CCK peptide is transported through the trans-Golgi network, where the C-terminal tyrosine sulfation reactions likely occur. During transit, pro-CCK is sorted for regulated secretion and is stored in dense-core secretory vesicles. In the vesicles, proteolytic enzymes digest pro-CCK at specific basic residues, resulting in the various lengths of secreted CCK seen in vivo. Finally, a carboxypeptidase enzyme removes C-terminal arginine residues, and the remaining glycine is converted to an amide by the amidating enzyme.

Post-translational processing and modifications of pro-CCK may be a mechanism for modulating the biological activity of mature CCK. Amidation and sulfation affect the affinity and potency of CCK at its receptors (*1*). Furthermore, neither amidation nor sulfation is required for processing and secretion of pro-CCK from AtT-20 cells (*7*). Proteolytic cleavages of pro-CCK may be another mechanism for modulating CCK biological activity. CCK-58 sulfate has a 3-fold greater binding potency and resistance to degradation (*8*), as well as a wider range of biological activity, compared to CCK-8 sulfate (*9*). Thus, in the periphery, larger forms of CCK may be produced to counterbalance degradation and increase residency time at target cells. In the complex and fast-paced neurons of the central nervous system, shorter

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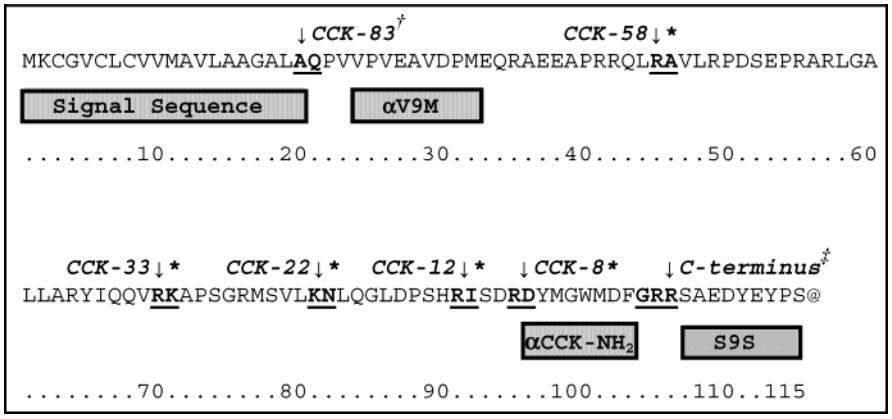

FIGURE 1: Map of the rat prepro-CCK peptide. Bold, underlined residues are the putative sites where endoproteolytic cleavage occurs during post-translational processing of prepro-CCK. Labeled rectangular boxes indicate the regions of the N-terminal signal sequence and the C-terminal S9S sequence, as well as the binding sites of the pro-CCK-specific antibody (αV9M) and the mature CCK-specific antibody (αCCK-NH_2). The symbol "@" represents a stop codon. A single dagger denotes the site cleaved by signalase enzymes; asterisks denote the sites cleaved by PC enzymes; and a double dagger denotes the site cleaved by carboxypeptidase enzymes.

forms of CCK may be favored because of their ease of clearance.

Proteolytic cleavage of pro-CCK is thought to be mediated in vivo by prohormone convertase enzymes (PC1, PC2, and PC5) of the furin/Kex2 family of subtilase-like serine proteases (*3*). These enzymes prefer dibasic substrate residues at P1 and P1′ but may also recognize monobasic P1 sites (*10*). Additionally, up- and downstream basic residues may contribute to the recognition of the substrate cleavage site. However, PC enzyme specificity and activity is complex in comparison to Kex2 or furin because of alternate splicing, post-translational processing differences, and chaperones (*11*). Cleavages of pro-CCK have been observed at one of three dibasic residues yielding CCK-33. Five of the nine monobasic residues are cleaved yielding CCK-58, -39, -22, -12, and -8. The possibility exists that the PC enzymes are not the only enzymes involved in pro-CCK processing.

Previous work in our laboratory has partially clarified the role of each PC enzyme in pro-CCK processing. Roles for PC1, PC2, and/or PC5 in rat prepro-CCK processing have been demonstrated in vitro, in various cell lines, and through histological co-localization (*3*). We found that mutation of the putative basic cleavage residues of pro-CCK alters the secretion products produced by transfected AtT-20 cells in a manner suggestive of a complex temporal and sequential process (*12*).

The goal of the present study was to further investigate the relative importance of each proteolytic cleavage site in pro-CCK processing. The AtT-20 cell line is a superior model system for investigating pro-CCK proteolytic processing because the cells only express the PC1 convertase (*13*), exhibit regulated secretion, are easy to transfect, and have very low levels of endogenous pro-CCK. AtT-20 cells secrete sulfated/amidated CCK-22 and -8 after stable transfection with rat prepro-CCK. Mutagenesis was conducted on rat prepro-CCK cDNA to target and inhibit putative mono- and dibasic cleavage sites. The order of prohormone cleavages was deduced by associating the position of the introduced mutation with the profile of CCK secretion products. The results suggest that, in AtT-20 cells, proteolytic processing of pro-CCK branches into two mutually exclusive pathways, while progressing from the N terminus to the C terminus of the prohormone.

Table 1: Prepro-CCK Mutants

mutant designation	mutation site relative to the N terminus
CCK-58$^{RA/SA}$	R45S
CCK-58$^{RA/AA}$	R45A
CCK-33$^{RK/SS}$	R70S/K71S
CCK-33$^{RK/AA}$	R70A/K71A
CCK-22$^{KN/AN}$	K81A
CCK-12$^{RI/AI}$	R91A
CCK-8$^{DR/SS}$	D94S/R95S
CCK-8$^{DR/AA}$	D94A/R95A
CT$^{GRRS/GSSS}$	R105S/R106S
CT$^{GRRS/GRR@}$	S107@
CT$^{GRRS/GAA@}$	R105A/R106A/S107@
CT$^{GRRS/GAAS}$	R105A/R106A

MATERIALS AND METHODS

Construction of Rat Pro-CCK Mutants. Substitution of putative basic cleavage residues with nonbasic amino acids was performed on the rat prepro-CCK cDNA (Figure 1). Plasmids that express wild-type rat prepro-CCK and cleavage-site mutants CCK-58$^{RA/AA}$ and CT$^{GRRS/GRR@}$ were constructed as previously described (*12*). CT$^{GRRS/GRR@}$ previously referred to as "CDEL" is a mutant prepro-CCK with a premature stop codon (@) placed after the carboxyl-terminus cleavage sequence GRR. Additional mutants were generated using the QuickChange site-directed mutagenesis kit (Stratagene) with the appropriate oligonucleotide primers according to the recommendations of the manufacturer. The following sense primers were used to generate the indicated mutants: CCK-58$^{RA/SA}$ (GAGCACAGCCGACAGCTGCCTTCGTCG), CCK-33$^{RK/SS}$ (CAGCAGGTCTCCTCGGCTCCCTCTGGC), CCK-33$^{RK/AA}$ (CAGCAGGTCGCAGCAGCTCCCTCTGGC), CCK-22$^{KN/AN}$ (GTCCGTTCTTGCAAACCTGCAGGGCCC), CCK-12$^{RI/AI}$ (CCTAGCCACGCGATAAGTGACC), CCK-8$^{DR/SS}$ (GATAAGTGACAGCAGCTACATGGGCTGG), CCK-8$^{DR/AA}$ (GATAAGTGACGCGGCCTACATGGGCTG), CT$^{RRS/SSS}$ (GATTTCGGCAGCAGCAGTGCTGAGGAC), and CT$^{RRS/AA@}$ (GATTTCGGCGCGGCCTAGGCTGAGGACTAC). The corresponding alterations in the wild-type sequence for each of these mutant constructs are shown in Table 1. All plasmid DNAs were sequenced to confirm the introduced mutations by the Tufts University Core Sequencing Facility.

Stable Transfection of AtT-20 Cells. AtT-20 mouse pituitary tumor cells were transfected using Lipofectamine Plus (Invitrogen) according to the recommendations of the manufacturer. Single colonies of cells were selected for stable transfection with G418 (700 mg/L). As a control for transfection, nontransfected AtT-20 cells were simultaneously plated at the same density as transfected cells and treated with 700 mg/L of G418. Nontransfected cells died within a week in this medium.

Chromatographic Analysis of Media. Media from 75-cm^2 flasks ($2-3 \times 10^7$ cells/plate) were concentrated and partially purified with Sep-Pak C18 cartridges (Waters Associates). Concentrated media were separated by gel-filtration chromatography (SEC) on a 200-mL column of Bio-Gel P-10 (Biorad) run at 4 °C in 50 mM Tris and 200 mM NaCl (pH 7.8), containing 1 g/L BSA and 0.02% sodium azide. A low concentration of myoglobin and vitamin B12 (Sigma) were added to each sample as molecular-weight markers to control for the quality of the gel bed. Concentrated media were also analyzed by reverse-phase HPLC (Waters Alliance 2690 system) with elution buffer A (0.09% trifluoroacetic acid) and buffer B (90% acetonitrile in 0.09% trifluoroacetic acid). Samples were injected into a 5-μm Symmetry 300 C_{18} column (4.6 × 250 mm) and eluted at a rate of 1 mL/min in a gradient of 25−35% B over 60 min. For both chromatography procedures, the elution profiles of media and samples were compared to the elution profiles of synthetic CCK peptide standards. The peptide standards included CCK-8 amide sulfate (Neosystems) and sulfated/amidated forms of CCK-12, -22, and -33 (generously donated by Kouki Kitagawa, Niigata College of Pharmacy, Japan) (*14*). Fractions were collected, and aliquots were removed for the radioimmunoassay (RIA).

RIA for Quantitation of Peptides. RIA was used to quantify the levels of prohormone and amidated CCK in cells, media, and fractions from SEC or HPLC. The RIA that is specific for CCK was performed as previously described (*15*), using the rabbit polyclonal antiserum 5 (R5), which was raised against CCK-8 amide. Gastrin-17 was iodinated utilizing the chloramine-T method (*16*) and was used as a tracer for the CCK (R5) RIA. Rabbit antiserum 79 (R79) was generated against the V9M peptide (rat prepro-CCK residues 24−32) with a carboxyl-terminal multiple-antigenic peptide tail. R79 was used in RIAs to assess pro-CCK levels and forms. Iodinated Y10M (tyrosine-extended V9M) was used as a tracer in the pro-CCK (R79) RIA. Known dilutions of CCK-8 sulfate (0−1000 pg/100 μL) and V9M (0−10 ng/100 μL) were used as a standard curve for quantification of unknown samples. A nonlinear one-site competitive-binding model was fit to the standard curve data (GraphPad Prism version 3.0), and the concentrations of unknowns were calculated from the model. To measure the cellular content of CCK, cells grown to a density of $1.5-2.0 \times 10^6$ cells/plate were lysed with 0.1 N HCl and clarified by centrifugation and aliquots were neutralized with 0.1 N NaOH and assayed by RIA. Total protein concentrations were estimated by a BCA protein assay (Pierce). The CCK concentrations in medium and cell lysates of control and mutant transfected cells were compared by one-way ANOVA with the Newman-Keuls post-hoc test. SEC fractions were quantified by RIA. Bell-shaped peaks were identified in the resulting elution profile and were modeled as a Gaussian distribution (GraphPad Prism version 3.0).

RESULTS

To determine the effects of mutation on CCK expression and secretion, the expression levels of pro-CCK and amidated CCK were determined in both media and cell lysates by RIA. Intracellular pro-CCK levels for most mutants were comparable to wild-type pro-CCK transfected cells (Figure 2A). CCK-58$^{RA/AA}$, CCK-12$^{RI/AI}$, and CT$^{GRRS/GSSS}$ transfected cells had lower levels of intracellular pro-CCK than wild-type transfected cells. CCK-8$^{DR/AA}$ transfected and the control, nontransfected AtT-20 cells did not express detectable levels of pro-CCK intracellularly. Intracellular levels of CCK were comparable in wild-type, CT$^{GRRS/GRR@}$, and CCK-8$^{DR/SS}$ transfected cells (Figure 2C). Mutants CCK-58$^{RA/SA}$, -58$^{RA/AA}$, -33$^{RK/SS}$, -33$^{RK/AA}$, -22$^{KN/AN}$, and -12$^{RI/AI}$ had comparable intracellular CCK levels that were lower than the wild-type levels. CCK was not detected in the CT$^{GRRS/GAA@}$ and CCK-8$^{DR/AA}$ transfected cells or in the control, nontransfected AtT-20 cells.

Secreted levels of pro-CCK were comparable between wild-type and mutant transfected cells, with the exception of CCK-8$^{DR/AA}$, which had no detectable pro-CCK expression, similar to the control, nontransfected AtT-20 cells (Figure 2B). Extracellular levels of CCK were comparable for cells transfected with wild-type pro-CCK and mutants CCK-58$^{RA/SA}$, -58$^{RA/AA}$, -33$^{RK/SS}$, and -33$^{RK/AA}$ (Figure 2D). Secreted levels of CCK from CT$^{GRRS/GRR@}$, CCK-12$^{RI/AI}$, GSS, and CCK-8$^{DR/SS}$ transfected cells were lower than the levels from wild-type transfected cells. CCK-22$^{KN/AN}$ transfected cells had very low levels of CCK secreted into media. CT$^{GRRS/GAA@}$ and CCK-8$^{DR/AA}$ transfected cells did not differ from nontransfected AtT-20 cells in that secreted CCK was not detected in the media.

The estimated concentrations of unprocessed pro-CCK in lysates of cells transfected with wild-type pro-CCK, CCK-58$^{RA/SA}$, -33$^{RK/SS}$, -33$^{RK/AA}$, -22$^{KN/AN}$, -8$^{DR/SS}$, CT$^{GRRS/GRR@}$, and CT$^{GRRS/GAA@}$ plasmids were not different (one-way ANOVA with Newman-Keuls test). Pro-CCK levels in cells transfected with CCK-58$^{RA/AA}$, -12$^{RI/AI}$, and CT$^{GRRS/GSSS}$ plasmids were 51, 40, and 42% lower than wild-type levels, respectively. Lysates of CCK-8$^{DR/AA}$ transfected cells and the control, nontransfected AtT-20 cells did not express detectable levels of either pro-CCK or amidated CCK. Therefore, most of the mutations of the pro-CCK sequence (64%) in this study had no overall effect on pro-CCK expression, and 27% had a reduced yet quantifiable expression levels. Extracellular pro-CCK levels were similar between wild-type prepro-CCK-containing cells and each of the other mutants, except for CCK-8$^{DR/AA}$ and control cells, which had no detectable expression.

Levels of amidated CCK that were detected by an RIA specific for amidated CCK were different for many of the mutants. Wild-type prepro-CCK transfected cells contained and secreted the highest levels of mature CCK compared to the mutants. CCK expression in lysates and media from cells transfected with CCK-58$^{RA/SA}$, -58$^{RA/AA}$, -33$^{RK/SS}$, and -33$^{RK/AA}$ was similar. Of note, CCK amide was not detected in media or cells of the CT$^{GRRS/GAA@}$ mutant, despite normal expression levels of unprocessed pro-CCK. Similarly, expression of

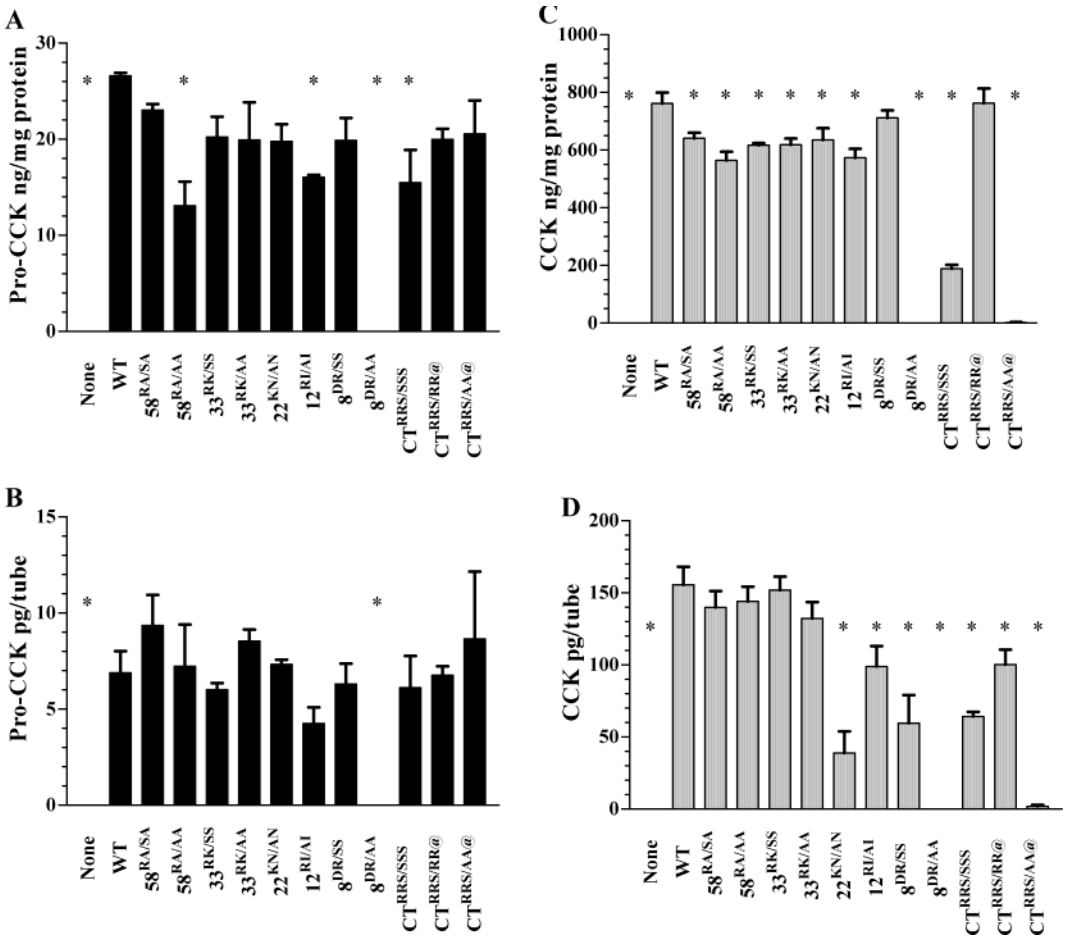

FIGURE 2: Effects of cleavage-site mutations on expression levels. For each mutant, intracellular expression levels of pro-CCK are shown in A, the extracellular levels of pro-CCK are shown in B, the intracellular expression of CCK amide is shown in C, and the extracellular levels of CCK amide are shown in D (asterisks = $p < 0.05$ compared to that of the wild type, with $n = 3-5$).

CCK was low in $CT^{GRRS/GSSS}$ transfected cells and media, despite normal levels of prohormone. The CCK-$8^{DR/SS}$ mutant transfected cells contained high levels of CCK intracellularly but secreted low levels of CCK in the media. CCK-$8^{DR/AA}$ cells had no detectable expression of CCK in the media or cells. The control nontransfected AtT-20 cells and media did not have detectable levels of CCK or pro-CCK.

Prohormone elution profiles were determined using HPLC and SEC fractions of media from cells transfected with either wild-type or mutant plasmids. The prohormone was not resolved clearly by either the HPLC or the SEC columns. In the HPLC, the prohormone eluted within the first 10 fractions and thus was present in or close to the void volume (Supplementary Figure 1 in the Supporting Information). On the SEC column, the prohormone eluted after the myoglobin standard (Supplementary Figure 2 in the Supporting Information), and therefore was determined to be less that 17 kDa. However, because of the nonlinear nature of the SEC elution and the lack of appropriate synthetic standards for the prohormone, an accurate determination of the prohormone molecular weight could not be determined in this study. Control AtT-20 cell media and CCK-$8^{DR/AA}$ cell media had no detectable prohormone immunoreactivity (IR).

Representative elution profiles of the media from wild-type CCK and the CCK-58 and CCK-33 mutants are shown in Figures 3−5. Media from wild-type rat prepro-CCK-expressing cells were fractionated by SEC and analyzed by RIA for CCK (Figure 3A). Fractions containing the highest concentrations of CCK IR corresponded to migration of sulfated CCK-22 and -8 synthetic standards.

Concentrated media from wild-type rat prepro-CCK-expressing cells were also fractionated by HPLC (Figure 3B). As with SEC, RIA of the HPLC fractions revealed CCK IR corresponding to the migration of sulfated CCK-8 and -22 synthetic standards. Additional HPLC fractions contained CCK IR that did not correspond in retention time to the available standards. These fractions were observed as a result of the high sample concentrations achieved with HPLC and may represent nonspecific binding, trace amounts of CCK intermediates, nonsulfated forms of CCK, or CCK degradation products. Unidentifiable peaks were also observed in the media from cells containing mutant constructs.

The media from cells transfected with CCK-$58^{RA/SA}$ or -$58^{RA/AA}$ (Figure 4) was remarkably different from media of cells expressing wild-type rat pro-CCK. Both CCK-$58^{RA/SA}$ and -$58^{RA/AA}$ led to the secretion of CCK-33 and -12. CCK-$58^{RA/SA}$ also led to the secretion of CCK-22 and -8 (parts A and B of Figure 4). HPLC analysis of the CCK-$58^{RA/AA}$ mutant revealed only CCK-12 and not -33. This discrepancy between HPLC and SEC data resulted from variability in the secretion of CCK-33 relative to CCK-12, possibly because of the formation of CCK-12 from CCK-33.

Mutants CCK-$33^{RK/SS}$ and -$33^{RK/AA}$ had similar elution profiles on SEC and HPLC (Figure 5). Both mutants led to

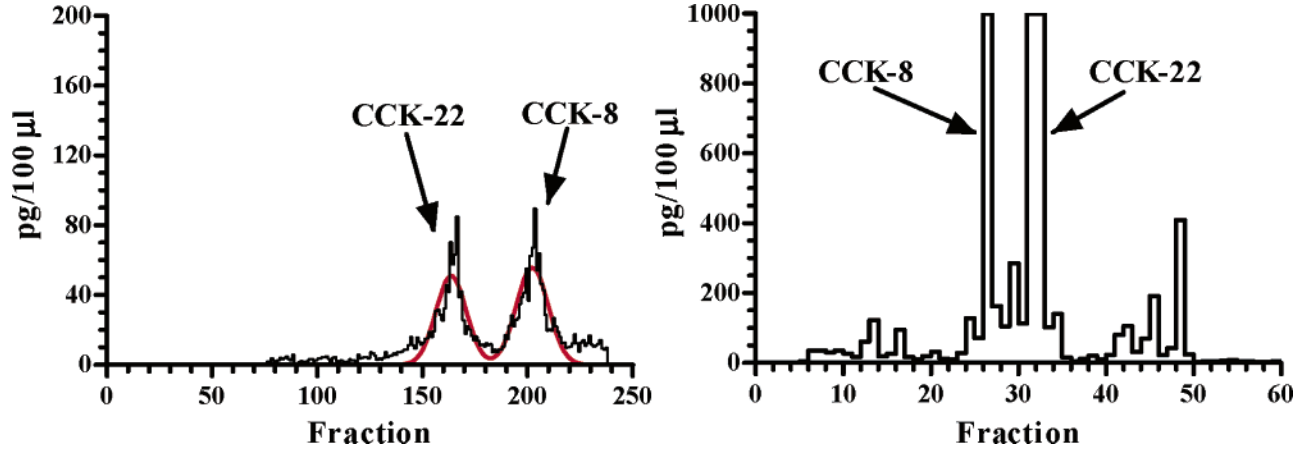

FIGURE 3: Forms of CCK secreted from wild-type rat prepro-CCK transfected AtT-20 cells. Media from transfected cells were separated by (A) SEC or (B) HPLC. Fractions were collected and analyzed for amidated CCK IR. Black lines represent the actual RIA data values. The red curves in the graph of (A) the SEC data represent the Gaussian distribution of the RIA peaks.

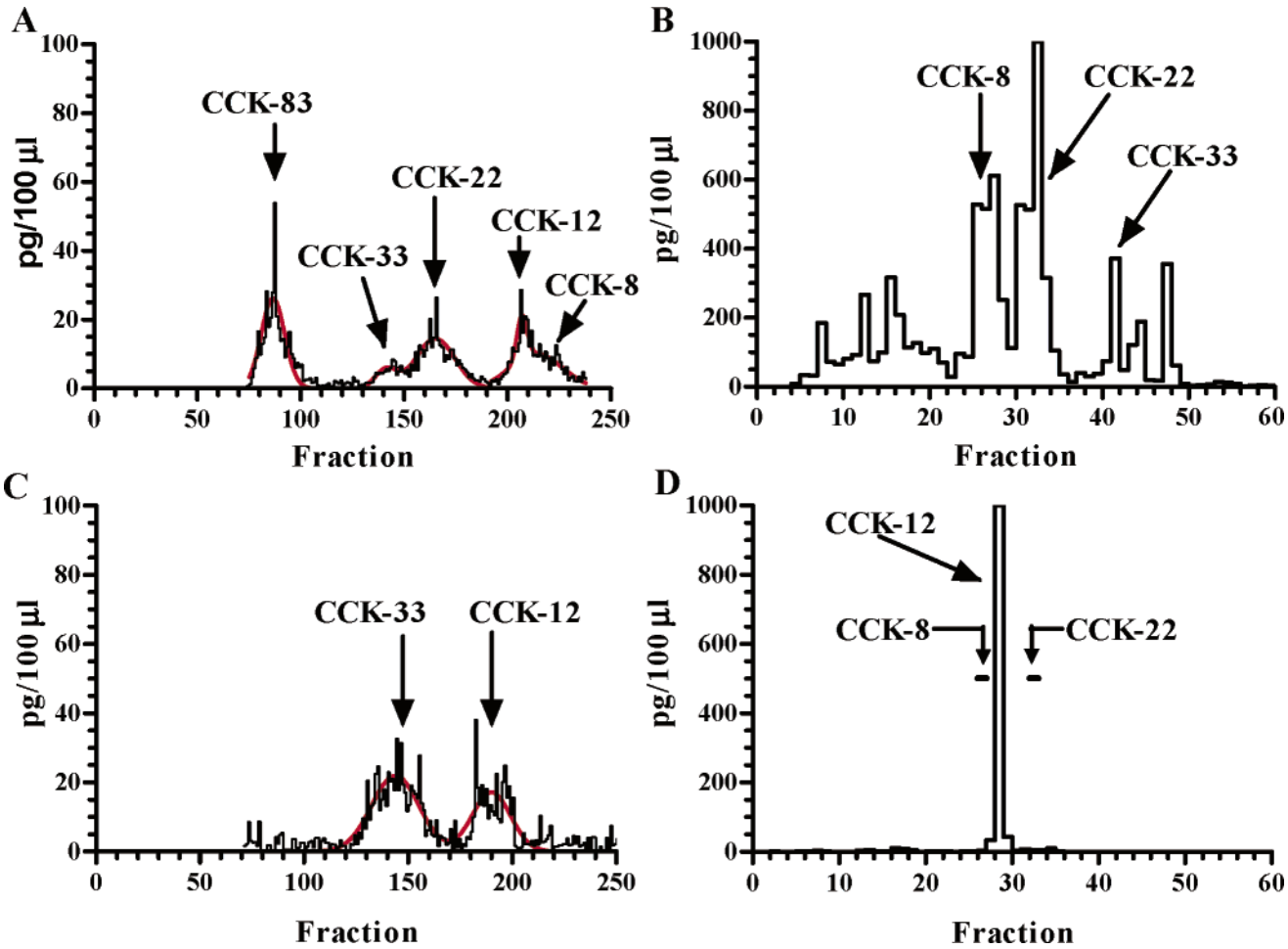

FIGURE 4: Forms of CCK secreted from mutant CCK-58$^{RA/SA}$ and -58$^{RA/AA}$ transfected AtT-20 cells. Separation profiles are shown as follows: (A) SEC of CCK-58$^{RA/SA}$, (B) HPLC of CCK-58$^{RA/SA}$, (C) SEC of CCK-58$^{RA/AA}$, and (D) HPLC of CCK-58$^{RA/AA}$. Fractions were collected and analyzed for amidated CCK IR. Black lines represent the actual RIA data values. The red curves in the graphs of (A and C) the SEC data represent the Gaussian distribution of the RIA peaks.

the secretion of amidated CCK-33, -22, and -8. The CCK-33$^{RK/AA}$ mutant also led to the secretion of CCK-12 (Figure 5C).

Table 2 summarizes the chromatographic elution profiles for each mutant. The quantity of each peptide eluted is represented as a percentage of the total quantity of all elution products. The mutant CCK-22$^{KN/AN}$ had a similar elution profile to wild-type CCK. On HPLC, both CCK-8 and -22 were observed in media from cells expressing CCK-22$^{KN/AN}$. However, SEC of CCK-22$^{KN/AN}$ cell media had low levels of CCK-8 and no observable CCK-22, because sample dilution reduces the sensitivity of this method (data not shown).

Both SEC and HPLC showed that media from cells transfected with the CCK-12$^{RI/AI}$ mutant contained CCK-33 and -12.

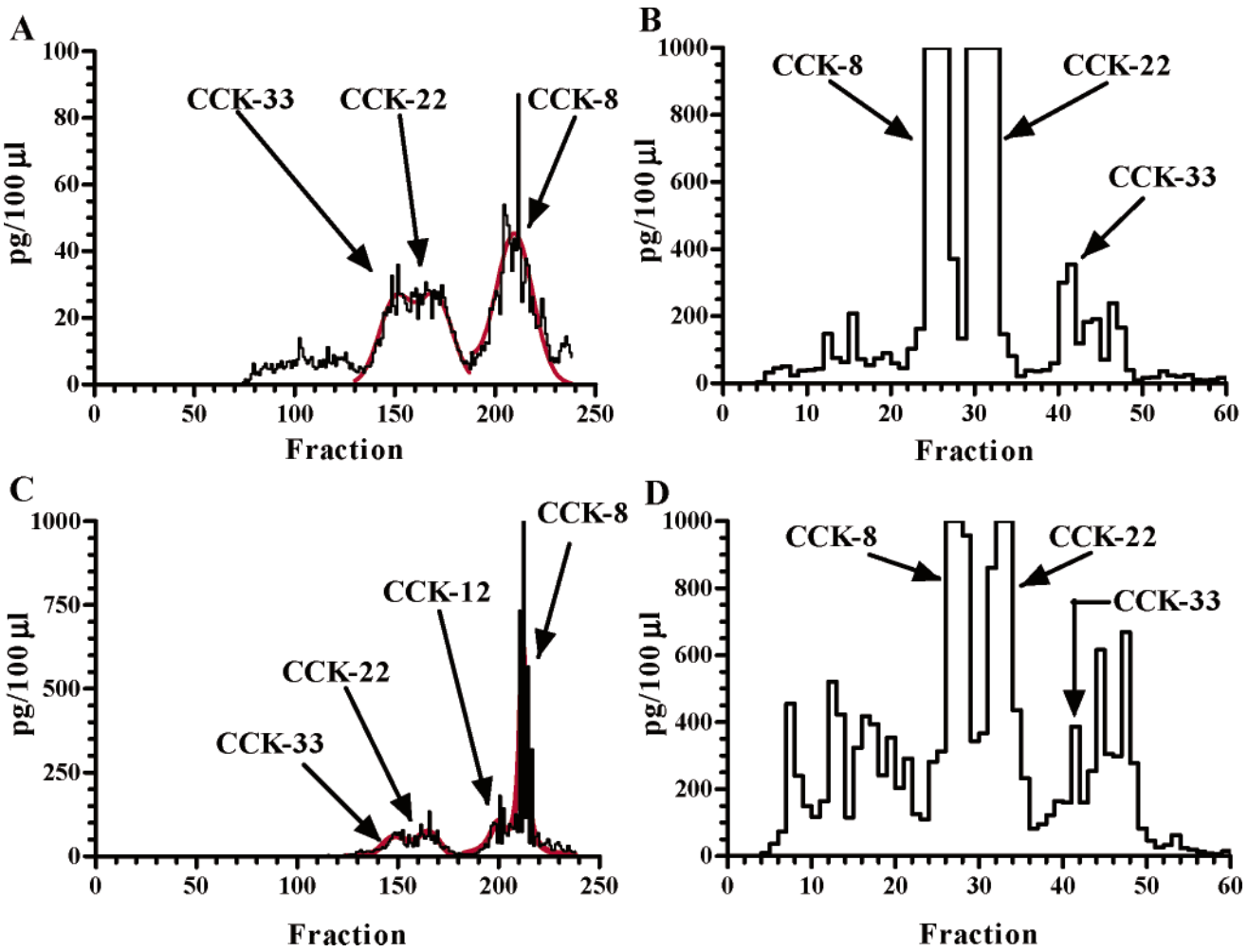

FIGURE 5: Forms of CCK secreted from CCK-33$^{RK/SS}$ (A and B) and CCK-33$^{RK/AA}$ (C and D) transfected AtT-20 cells. Media from transfected cells were separated by (A and C) SEC or (B and D) HPLC. Fractions were collected and analyzed for amidated CCK IR. Black lines represent the actual RIA data values. The red curves in the graphs of (A and C) the SEC data represent the Gaussian distribution of the RIA peaks.

Table 2: Summary of Chromatographic Analysis of Cleavage Products[a]

plasmid	CCK-83 (%)	CCK-58 (%)	CCK-33 (%)	CCK-22 (%)	CCK-12 (%)	CCK-8 (%)
wild type				58		42
CCK-58$^{RA/SA}$	30		5	29	22	14
CCK-58$^{RA/AA}$			63		37	
CCK-33$^{RK/SS}$			23	31		46
CCK-33$^{RK/AA}$			15	14	19	52
CCK-22$^{KN/AN}$				54		46
CCK-12$^{RI/AI}$			86		14	
CCK-8$^{DR/SS}$				76	24	
CCK-8$^{DR/AA}$						
CT$^{GRRS/GRR@}$				46		54
CT$^{GRRS/GAA@}$						
CT$^{GRRS/GSSS}$						100

[a] Summary of amidated cleavage products identified in the media of wild-type and mutant transfected cells separated by SEC and/or HPLC. The estimated peak area for each elution product is represented as a percentage of the total area of all peaks present in a given chromatogram.

CCK-8 formation was dramatically effected by mutagenesis of the CCK-8 cleavage site. CCK-8$^{DR/SS}$ transfected cells produced CCK-22 and -12. CCK-8$^{DR/AA}$ cells did not have detectable CCK amide IR in the media.

The CT$^{GRRS/GRR@}$ mutant produced CCK-22 and -8, thus had full wild-type characteristics (data not shown), and was identical to previous results obtained in our laboratory (see the CDEL mutant in ref *12*). Transfection of the CT$^{GRRS/GAA@}$ mutant did not lead to detectable expression of amidated CCK in the media. However, prohormone expression was normal for this mutant. The CT$^{GRRS/GSSS}$ mutant led to the secretion of low levels of CCK-8. No other forms of CCK amide were detected in media from these cells.

DISCUSSION

In this study, putative cleavage sites within the rat prepro-CCK sequence were analyzed by site-directed mutagenesis. The results further support the hypothesis that rat pro-CCK cleavage in AtT-20 cells occurs in a defined temporal sequence and follows at least two branching pathways. The results of these experiments demonstrate that proteolytic processing is highly sensitive to small modifications of the pro-CCK sequence. Previous studies have suggested that enzymes of the prohormone convertase family can cleave pro-CCK at various residues. Mutation of the basic amino acids in the putative cleavage sites of pro-CCK was anticipated to lead to the disruption of proteolytic cleavage by the PC enzymes. However, the data from this study are

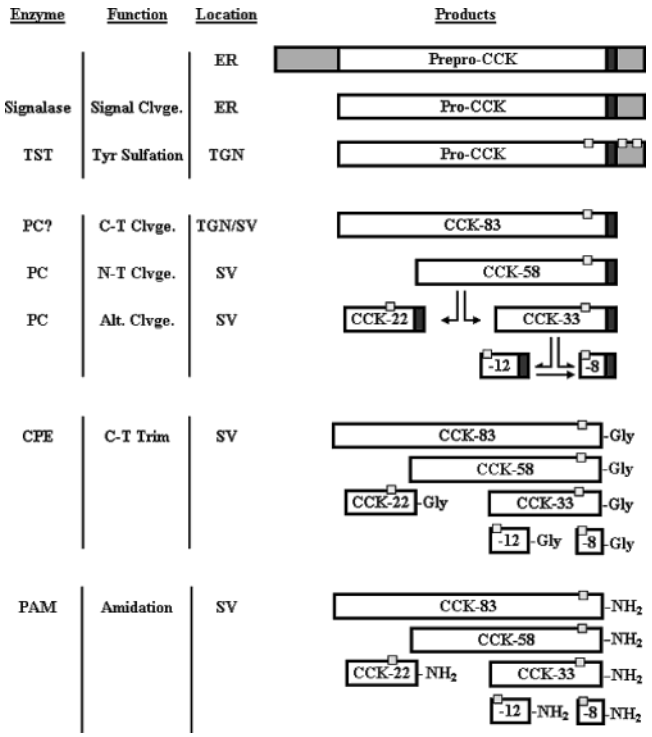

FIGURE 6: Model of post-translational processing of prepro-CCK. Prepro-CCK is synthesized in the endoplasmic reticulum (ER). The N-terminal signal sequence cleavage (Signal Clvge.) is mediated by the signalase enzyme. Tyrosine sulfotransferase (TSN) sulfates pro-CCK (small light gray squares) in the trans-Golgi network (TGN), where the C-terminal (CT) S9S sequence is also cleaved. In the secretory vesicles (SVs), endoproteolytic cleavages are mediated by prohormone convertases (PCs) progressing from the N terminus (NT). The pathway branches after CCK-58. In the SVs, any form of CCK with the C-terminal S9S removed can be trimmed by carboxypeptidase E (CPE), amidated by the amidating enzyme (PAM), and secreted.

indicative of complex effects of the mutations. Many of these mutations also affected expression, intracellular sorting, and/or secretion of CCK peptides. Furthermore, cleavage is seen in mutants containing serine and alanine in putative cleavage sites. These mutants may be cleaved by novel proteases, such as the recently described SKI-1 enzyme. On the basis of the results presented here, a temporal/special model of pro-CCK processing in AtT-20 cells can be deduced. The model is presented in Figure 6. This study of pro-CCK processing implies complex effects can be anticipated in the development of therapeutics that target prohormones or PC enzymes.

The quantification of pro-CCK and amidated CCK in cell lysates and media showed that mutation of any sort had some effect on the expression, sorting, or secretion of the hormone. Cells expressing wild-type pro-CCK had the highest expression levels of prohormone and amidated peptide intra and extracellularly. The most dramatic effect of mutation was seen with mutant CCK-$8^{DR/AA}$, which did not lead to detectable expression of pro-CCK or CCK. This mutant may be degraded early after translation as an unrecognized or improperly folded protein.

Mutants CCK-$12^{RI/AI}$ and $CT^{GRRS/GSSS}$ had normal levels of secreted prohormone but had decreased levels of intracellular prohormone and peptide and decreased levels of secreted peptide compared to that of the wild type. This may be explained by the shunting of the prohormone to the constitutive secretory pathway, where it would not be processed.

The CCK-$58^{RA/AA}$ mutant may also be bypassing the regulatory secretion pathway to some degree, because reduced cellular contents of prohormone and amidated peptides were observed. However, CCK-$58^{RA/AA}$ media levels were not different from those of the wild type. This may indicate that CCK-$58^{RA/AA}$ has a shorter half-life intracellularly but is relatively stable when secreted.

In comparison to the wild type, mutants CCK-$22^{KN/AN}$, -$8^{DR/SS}$, and $CT^{GRRS/GRR@}$ had reduced levels of CCK amide in the media but not in the cell lysates. Additionally, these mutants expressed near wild-type levels of prohormone. These results suggest that mutants CCK-$22^{KN/AN}$, -$8^{DR/SS}$, and $CT^{GRRS/GRR@}$ are sorted correctly for regulated secretion but have defective interactions with the regulated secretory machinery that prevent the release into the media. Alternatively, these mutants may be less stable in the media than in the cells.

The mutant $CT^{GRRS/GAA@}$ had wild-type levels of prohormone expression, but amidated CCK was not detected. This result was expected because the alanine substitution for arginines should eliminate processing by carboxypeptidase and prevent amidation.

The least dramatic effect on expression was seen with mutants CCK-$58^{RA/SA}$, -$33^{RK/SS}$, and -$33^{RK/AA}$. These mutants had approximately a 20% decrease in intracellular amidated CCK but had normal levels of extracellular amidated CCK and pro-CCK. These results may indicate that the vesicular forms of these mutants have an increased rate of intracellular degradation, possibly because of the incorrect processing within the vesicles.

The profile of biologically active secreted forms of CCK is dependent on the rate or efficiency of proteolytic processing. AtT-20 cells transfected with wild-type rat prepro-CCK secreted large amounts of unprocessed prohormone as well as amidated/sulfated CCK-22 and -8. Substitution of putative basic/amphipathic cleavage sites with hydrophobic alanines or hydrophilic (partially acidic) serines inhibited normal cleavages and led to the secretion of pathway intermediates such as CCK-83, -33, and -12. When the position of the introduced mutation was correlated to the lengths of the corresponding secretion products, the temporal order of cleavages can be deduced.

Mutant CCK-$58^{RA/SA}$ secreted CCK-83, -33, and -12 in addition to the expected secretion of CCK-22 and -8. Replacement of the basic arginine with the polar serine at the 58 site caused a reduced rate of proteolytic cleavage and led to the accumulation of precursor and intermediate cleavage products. The CCK-83 precursor was observed in CCK-$58^{RA/SA}$ mutant media because of inefficient cleavage, increased residency of CCK-83 in the secretory vesicles, and availability of CCK-83 for amidation and secretion. The CCK-83 precursor was absent in CCK-$58^{RA/AA}$ mutant media probably because the lower expression level of this mutant did not lead to saturation of the 58 cleavage-site enzyme and the subsequent buildup of precursor molecules. These results are consistent with a previous study (*12*) that demonstrated CCK-33 in media from CCK-$58^{RA/AA}$ transfected cells.

Interesting results were obtained from 58-site mutant media in comparison to CCK-$33^{RK/SS}$, -$33^{RK/AA}$, and -$12^{RI/AI}$

mutant media. In media from both the CCK-$58^{RA/SA}$ and -$58^{RA/AA}$ mutants, the direct product of cleavage at the 58 site, CCK-58 itself, was not observed. CCK-58 was expected to be secreted because cleavage at 58 was partially hindered. An explanation for the absence of CCK-58 is that cleavage at additional sites occurs rapidly after cleavage at the 58 site. In contrast, data from the two 33-site mutants and the 12-site mutant indicated that the direct products of these cleavages were formed. The 33-site mutants, CCK-$33^{RK/SS}$ and -$33^{RK/AA}$, led to secretion of CCK-33. In a previous study (*12*), the 33AK mutant secreted CCK-12 and/or CCK-8, which could not be resolved on the chromatographic system used. Similarly, the CCK-$12^{RI/AI}$ mutant led to the secretion of CCK-12. This suggests that cleavages normally following cleavage of CCK-33 and -12 are slow enough to enable the buildup of these precursors.

CCK-12 is likely formed from cleavage of CCK-33 and not -22. In CCK-$58^{RA/SA}$ media, the peak area of CCK-33 was small compared to the CCK-22 peak area, which in turn was similar to the CCK-12 peak area. This profile suggests that CCK-33 is a precursor of a smaller form and that CCK-22 and -12 arise from separate precursors. CCK-33 cannot be a precursor for CCK-22, because CCK-33 was present in the CCK-$58^{RA/AA}$ mutant media and CCK-22 was absent. Likewise, in wild-type, $CT^{GRRS/GRR@}$, and CCK-$22^{KN/AN}$ media, CCK-22 was present and CCK-12 was absent. Furthermore, cosecretion of CCK-33 and -12 was observed in mutants CCK-$58^{RA/SA}$, -$58^{RA/AA}$, -$33^{RK/AA}$, -$12^{RI/AI}$, and possibly -$33^{RK/SS}$, where the CCK-12 and -8 peaks may have been overlapping. In the CCK-$12^{RI/AI}$ mutant, blockade of the 12 cleavage site would lead to accumulation of the precursor of CCK-12. Because CCK-33 was observed, CCK-12 likely arose from the cleavage of CCK-33. When taken together, these data suggest that cleavage at 58 is a prerequisite for CCK-22 and -12 formation and that cleavage at 58 is followed by two independent cleavages at 33 or 22. These results identify a branch point in the processing of pro-CCK.

The mutant CCK-$22^{KN/AN}$ had low expression levels and led to the secretion of both CCK-22 and -8 at very low concentrations. A previous study (*12*) showed using SEC that CCK-12 and -8 was formed at low concentrations from the CCK-$22^{KN/AN}$ mutant. The mutant used in the present study was resynthesized and analyzed by a higher resolution SEC and also HPLC. The SEC revealed only a minute amount of CCK-8. The HPLC concentrates the sample severalfold and revealed small amounts of CCK-8 and -22. The expected result of the 22-site mutation would be the secretion of precursors to CCK-22. Therefore, this cleavage-site mutation primarily affected expression and not processing. Expression would be affected by improper folding of the prohormone and premature degradation. Processing in this case may have been the result of inefficient cleavage by a PC enzyme or possibly a non-PC enzyme.

The CCK-$8^{DR/SS}$ mutant transfected cells secreted CCK-22 and -12 but not -8. In a previous study (*12*), a similar mutant, 8AD, had equivalent results to the CCK-$8^{DR/SS}$ mutant in this study. A possible explanation for this finding is that the substitution of the dibasic residues at the 8 cleavage site completely blocks cleavage. Therefore, CCK-12 may be a precursor of CCK-8. Another possibility is that cleavage at CCK-12 does not naturally occur in AtT-20 cells but is artificially favored by mutation of the 8 cleavage site. The CCK-$8^{DR/AA}$ mutant did not express, probably because of the degradation of the prohormone immediately after synthesis.

The difference between CCK-$8^{DR/SS}$ and -$8^{DR/AA}$ mutants may arise from differing effects on the prohormone secondary structure. Alanines are often found in α-helical formations, whereas serines are often found in β sheets. Nuclear magnetic resonance studies of CCK predict a pseudohelical conformation for CCK-8 (*17*). Therefore, substitution with two alanines may increase the helical nature of this portion of the molecule and have a dramatic impact on the overall structure of the prohormone.

The $CT^{GRRS/GSSS}$ mutant transfected AtT-20 cells secreted only CCK-8. Because the overall expression of the $CT^{GRRS/GSSS}$ mutant was low, undetectable levels of processing products other than CCK-8 may have been secreted. As previously mentioned, the $CT^{GRRS/GSSS}$ mutant prohormone may have preferentially entered the constitutive secretory pathway, thereby decreasing the concentration of pro-CCK entering the regulated secretory pathway. Because of low expression, cleavage products could not be detected by SEC (data not shown).

The $CT^{GRRS/GRR@}$ mutant was previously reported as a C-terminal deletion or "CDEL" mutant (*12*). The results from the current study are consistent with the results from the former study despite the difference in separation methods.

Previously, a mutant $CT^{GRRS/GAAS}$ without a premature stop codon yielded the same result as the $CT^{GRRS/GAA@}$ mutant in this study (*12*). Normal levels of prohormone were present, yet amidated CCK was absent. Further examination of the $CT^{GRRS/GAAS}$ mutant revealed that the flanking S9S peptide was produced, but the prohormone was not further cleaved (*12*). As mentioned with the 8 cleavage-site mutants, the alanines on the carboxyl terminus in $CT^{GRRS/GAA@}$ may favor a rigid helical conformation that changes the overall structure of the prohormone and thus prevents cleavage. In contrast, $CT^{GRRS/GSSS}$ was processed to amidated CCK-8 at levels much lower than those of the wild type. The serines at this site may form a less rigid structure than the alanines, because serines prefer a β-sheet conformation. This observation implies that cleavage occurred at some point in the GSS−S9S sequence and that the carboxyl terminal serines were removed by a carboxypeptidase at low efficiency.

Most biologically active peptides are formed from limited proteolysis of larger precursor peptides in the secretory pathway. For many peptides, endoproteolytic cleavages are mediated by prohormone convertases (PCs), an evolutionary-conserved family of subtilisin-like, calcium-dependent serine proteases (*18*). The seven PC enzymes thus far discovered have different substrate specificities, subcellular distributions, and tissue distributions in mammals. The presumed substrate specificity for PC enzymes includes four criteria: (1) a P1 basic residue; (2) a basic residue preference at P2, P4, and/or P6; (3) a preference for a hydrophobic/aliphatic residue at P2′; and (4) the absence of proline or cysteine residues (*18*). Pro-CCK has several known cleavage sites that are compatible with this motif. All four criteria are satisfied at cleavage sites 58 and 22. Three of the four criteria are met at cleavage site 8, and only two of the four criteria are met at sites 33 and 12. This discrepancy may result from incomplete knowledge of the PC enzyme substrate motif or

from the presence of additional enzymes that may participate in the processing of pro-CCK.

Evidence suggests that the PC enzymes are involved in pro-CCK post-translational processing. Rat insulinoma cells (Rin5F) and mouse AtT-20 cells were found to express and process CCK (*19*). Both pro-CCK mRNA expression and release of processed CCK peptides were increased after treatment with forskolin and 3-isobutyl-1-methyxanthine (IBMX). Subsequently, SK-N-MCIXC, a human cholinergic neuroepithelioma cell line, was found to express CCK as well as processing enzymes such as PC1, PC2, and neutral endopeptidase 24.11 (EC 3.4.24.11) (*20*). Stable transfection with antisense PC1 inhibited CCK-8 formation by approximately 70−80% in RIN5F cells (*21*) and STC-1, murine intestinal tumor cells, while sparing CCK-22 levels (*22*). Furthermore, antisense PC2 transfection reduced CCK-22 levels with a comparative sparing of CCK-8 secretion in Rin5F and STC-1 cells (*23*). In contrast, in vitro experiments showed that CCK-33 can be cleaved effectively by PC2 to form CCK-8, without formation of CCK-22 (*24*). PC2 could not cleave pro-CCK or a peptide equivalent to the sequence between the 33 and 22 cleavage sites. PC1 transfected into L cells lead to cleavage of pro-CCK into CCK-8 (*25*). Also, PC1 cleavage at the pro-CCK 58 site and at the C-terminal GRR sequence could be inferred from the data.

Additional enzymes may be expressed in AtT-20 cells that are capable of performing cleavages at internal and carboxyl-terminal serines and alanines. A recently characterized enzyme, SKI-1, is the first enzyme identified in the secretory pathway of mammalian cells that can cleave prohormones at nonbasic residues (*26*). The proposed substrate motif recognized by SKI-1 is R-X-(hydrophobic)-(L, T). This substrate motif is still preliminary and requires further investigation. CCK can potentially be cleaved in vitro by SKI-1 to form CCK-5 (*26*). SKI-1 activity on pro-CCK in AtT-20 cells has not been determined. Pro-CCK may also be processed by enzymes that have yet to be discovered.

Studies of prepro-CCK processing illustrate the complexity of the mechanisms of prohormone processing in mammalian systems. The finding that the length of secreted peptide is dependent on the rate of proteolysis has important implications for the investigation of tissue-specific differences in processing. These findings may also contribute to the design and development of peptide therapeutics that are resistant to proteolysis. On the basis of our findings, we suggest a model for the order of cleavages in the maturation of prepro-CCK to the various biologically active forms of CCK (Figure 6).

SUPPORTING INFORMATION AVAILABLE

Elution profiles of synthetic sulfated standards and prepro-CCK by HPLC (Supplementary Figure 1) and standards and pro-CCK by SEC (Supplementary Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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