Biosynthesis and Post-Translational Processing of Site-Directed Endoproteolytic Cleavage Mutants of Pro-CCK in AtT-20 Cells †

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Biosynthesis and Post-Translational Processing of Site-Directed Endoproteolytic Cleavage Mutants of Pro-CCK in AtT-20 Cells†

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ABSTRACT: Site-directed mutagenesis in which individual cleavage site P1 amino acids were changed to Ala was performed to delineate their importance in the processing of pro-CCK in mouse pituitary tumor AtT-20 cells. Individual substitution of cleavage sites on pro-CCK, viz., CCK 58 cleavage site R/A to A/A, CCK 33 cleavage site R/K to A/K, CCK 22 cleavage site K/N to A/N, and CCK 8 cleavage site R/D to A/D, did not inhibit pro-CCK expression or the production of some form of amidated CCK. Wild-type CCK cDNA expression in these cells results in production and secretion of CCK 8 and CCK 22. Substitution of the 58R/A cleavage site with A/A produces only CCK 33; 33A/K and 22A/N produce only CCK 8, whereas 8A/D produces CCK 12 and some CCK 22. Where the GRR residues on the C-terminus of CCK 8 were mutated to GAA, no amidated CCK was produced. Significant amounts of the pro-CCK, C-terminal peptide S9S was found in the medium of cells transfected with GAA mutant cDNA, indicating that this pro-CCK was cleaved at the GAA site probably by a nonprohormone convertase enzyme. Further analysis of the cells expressing the GAA mutant demonstrated that it is not extensively cleaved at other sites to produce CCK 8 GAA or larger peptides. In the mutant where the entire pro-CCK, C-terminal S9S was deleted, CCK 8 is processed and secreted normally. Thus, the cleavage at the C-terminal GRR site is essential for subsequent cleavages, and modification of other cleavage sites (58, 33, 22, and 8) has a major impact on pro-CCK processing. These results suggest that there is a temporal order of cleavages, and the structure of pro-CCK has a strong influence on where and whether pro-CCK is processed.

Cholecystokinin (CCK)[1] is produced by both nerves and endocrine cells in the gut and by neurons. CCK is found in higher concentrations in the brain than in the gut, where it serves as a neurotransmitter or a neuromodulator. Pro-CCK passes through the regulated secretory pathway where it is processed to smaller bioactive forms and is secreted in response to specific stimuli (*1*). In the brain, the predominant form is CCK 8 (*2*), and in the gut, equal amounts of CCK 8 and CCK 33 are found (*3*). CCK 58 has been shown to be the major stored and circulating form of CCK in humans and dogs (*4*, *5*). These differences are thought to be due to tissue specific post-translational processing since the sequences of cDNA clones derived from brain and gut CCK mRNA demonstrate that the same precursor polypeptide must be synthesized in both tissues.

Proteolytic processing is an important regulatory mechanism used by cells to control gene expression at the post-translational level. Many biologically active proteins are synthesized as large precursors that remain inactive until they are proteolytically processed. The cleavage of protein precursors to yield peptides with bioactivity occurs at specific sites that have one or more basic amino acids. However, there are no apparent consensus sequences that serve as signals in targeting these sites for cleavage. Thus, it is not clear how the cellular processing machinery distinguishes between sites to be cleaved and sites that are to be left intact.

Biologically active hormones and neuropeptides are generally produced from inactive precursors by post-translational modifications that typically occur before secretion and include cleavage of the peptide chain, tyrosine sulfation, serine phosphorylation, glycosylation, C-terminal amidation, and N-terminal glutamine cyclization. The precursor of cholecystokinin is a good model for studying the post-translational processing mechanisms because it is relatively small and simple and also because several common post-translational modifications; namely, cleavage, sulfation, and amidation occur in a single octapeptide sequence which include the biologically important region of the hormone. In a recent study, it was shown that Tyr sulfation and serine phosphorylation do not affect the processing, though the amounts of CCK secreted becomes altered (*6*).

Pro-CCK has three dibasic and nine monobasic residues (Figure 1), some of which may be cleavage sites for endoproteases. Of the nine monobasic sites, only five (CCK 58, CCK 39, CCK 22, CCK 12, and CCK 8) are observed cleavages. Dibasic sites occur at positions CCK 61 and CCK 33 and at the C-terminal end. On the basis of identified products, dibasic cleavages should occur only at CCK 33 and the C-terminal end. The cleavage that gives rise to CCK 33 is between an Arg−Lys bond, instead of after the bond as in the case of other dibasic residue cleavage sites.

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[1] Abbreviations: CCK, cholecystokinin; POMC, pro-opiomelanocortin; RIA, radioimmunoassay.

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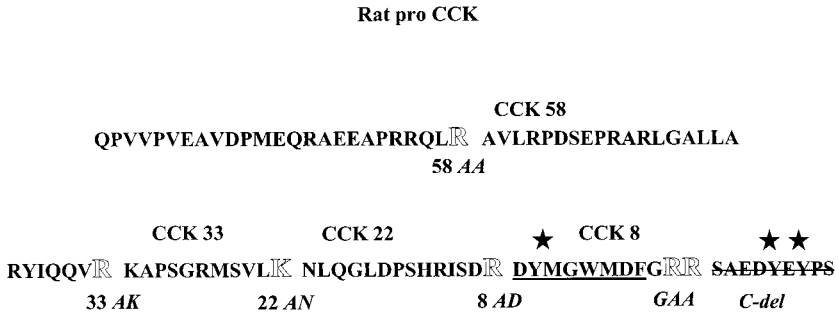

FIGURE 1: Rat pro-CCK sequence in the single-amino acid code with cleavage sites indicated by spaces. CCK peptides are numbered backward from the C-terminus of CCK 8. The amino-terminal ends of major forms of amidated CCK peptides are indicated. Stars denote the locations of the sulfated tyrosine residues, and CCK 8 is underlined. The cleavage site P1 amino acids are shown in outline letters, and the corresponding mutants that were generated were labeled below the cleavage sites. In the mutant C-del, the whole C-terminal S9S peptide region was deleted, shown with a strike through.

Cleavage after the first pair of dibasic residues would give rise to CCK 61, a peptide that never has been isolated. Thus, five of the nine monobasic sites and two of the three dibasic sites are cleaved. From studies with CCK (*1*), it can be concluded that all of the peptides characterized as arising from basic cleavages correspond to processing at predicted sites, but not all of the predicted sites are utilized.

Prohormone cleavages occur mainly at specific paired basic sites, although some single and tetrabasic sites are also cleaved. The cleavages appear to take place in a strict temporal order and may involve more than one endoprotease (*7*). A major advance in the prohormone processing field was the discovery of the protein convertase family of enzymes, which now numbers eight (*8*−*10*). Most of these enzymes cleave at dibasic pairs such as the Arg-Lys pair, although some single basic sites are also cleaved (*11*, *12*). The question of which of these enzymes are responsible for individual prohormone cleavages in tissues is difficult to answer. The possibility that there is considerable redundancy in their activities and that different enzymes are active on the same prohormone in different tissues cannot be excluded. We have undertaken this study to investigate the role of cleavage sites in the processing and to delineate the temporal order of processing.

The AtT-20 neuroendocrine cell line derived from a mouse anterior pituitary tumor has often served as a useful system for studying processing of neuropeptide precursors. AtT-20 cells, which possess a regulated secretory pathway, contain all the necessary components required for the processing of proenkephalin (*13*), POMC (*14*), proinsulin (*15*), prorenin (*16*), prosomatostatin (*17*), proneuropeptide Y (*18*), and pro-CCK (*19*, *20*). AtT-20 cells have been shown to contain carboxypeptidase E (*21*), peptidyl α-amidating enzyme (*22*), and a subtilisin-like prohormone convertase PC1 (*23*). We have used these cells to express site-directed mutant CCK cDNAs to examine how they are processed.

MATERIALS AND METHODS

Construction and Expression of Rat Pro-CCK Mutants. To construct the expression plasmid pcDNA3.1(−)pCCK, which expresses control wild-type prepro-CCK, the rat cDNA fragment encoding prepro-CCK was amplified by PCR using appropriate oligonucleotides which insert an *Eco*RI site at the 5′-end and a *Hin*dIII site at the 3′-end of the sequence. The PCR product amplified using Vent Polymerase (New England Biolabs) was cloned into pCR-Blunt (Invitrogen) according to the manufacturer's instructions and digested with *Eco*RI and *Hin*dIII to release the fragment encompassing the complete coding region of prepro-CCK. It was subsequently cloned into expression plasmid pcDNA3.1(−) which was also digested with *Eco*RI and *Hin*dIII. Site-directed mutants where alanine was substituted for the basic amino acid at the P1 position of the cleavage site were generated by overlap extension PCR (*24*) using appropriate oligonucleotides (Table 1). Mutant CCK GAA was generated using the QuickChange site-directed mutagenesis kit (Stratagene). These mutants (Figure 1) were designated as follows: (1) CCK 58AA, where the Arg of the CCK 58 cleavage site was mutated to Ala; (2) CCK 33AK, where the Arg of the CCK 33 cleavage site was changed to Ala; (3) CCK 22AN, where the Leu of the CCK 22 cleavage site was mutated to Ala; (4) CCK 8AD, where the Arg of the CCK 8 cleavage site was changed to Ala; (5) CCK GAA, where the Arg-Arg pair present at the C-terminus of the CCK 8 cleavage site was changed to an Ala-Ala pair; and (6) C-del, where the entire pro-CCK, C-terminal S9S sequence is deleted, where GRR is followed by a stop codon. All the DNA fragments were sequenced to confirm the introduced mutations. AtT-20 mouse pituitary tumor cells were transfected using Lipofectamine plus (Gibco BRL). Stable transfected cells were selected with Geneticin (700 μg/mL) for further analysis.

Table 1: Sequences of Oligonucleotide Primers

control sense	5′-CCGGAATTCCGGATGAAGTGCGGCGTG-3′
control antisense	5′-CCCAAGCTTCTACGATGGGTATTCGTAG-3′
58AA sense	5′-CGAAGGCAGCTGGCAGCTGTGCTC-3′
58AA antisense	5′-GAGCACAGCTGCCAGCTGCCTTCG-3′
33AK sense	5′-CAGCAGGTCGCAAAAGCTCCCTCT-3′
33AK antisense	5′-AGAGGGAGCTTTTGCGACCTGCTG-3′
22AN sense	5′-ATGTCCGTTCTTGCAAACCTGCAGGGC-3′
22AN antisense	5′-GCCCTGCAGGTTTGCAAGAACGGACAT-3′
8AD sense	5′-CACAGGATAAGTGACGCAGACTACATG-3′
8AD antisense	5′-CATGTAGTCTGCGTCACTTATCCTGTG-3′
GAA sense	5′-ATGGATTTCGGCGCAGCAAGTGCTGAG-3′
GAA antisense	5′-CTCAGCACTTGCTGCGCCGAAATCCAT-3′
C-del antisense	5′-CCCAAGCTTCTAGCGCCGGCCGAAATC-3′

Chromatographic Analyses. Medium from five 10 cm plates ($1.4-1.8 \times 10^7$ cells/plate) was concentrated using Sep-Pak C18 cartridges (Waters Associates) and further concentrated to ∼1 mL in a Speedvac concentrator. Medium was separated by gel filtration chromatography on a 50 cm × 2.5 cm column of Spherilose GCL-90 (Isco) run at 4 °C in 50 mM Tris and 200 mM NaCl (pH 7.8) containing 0.1%

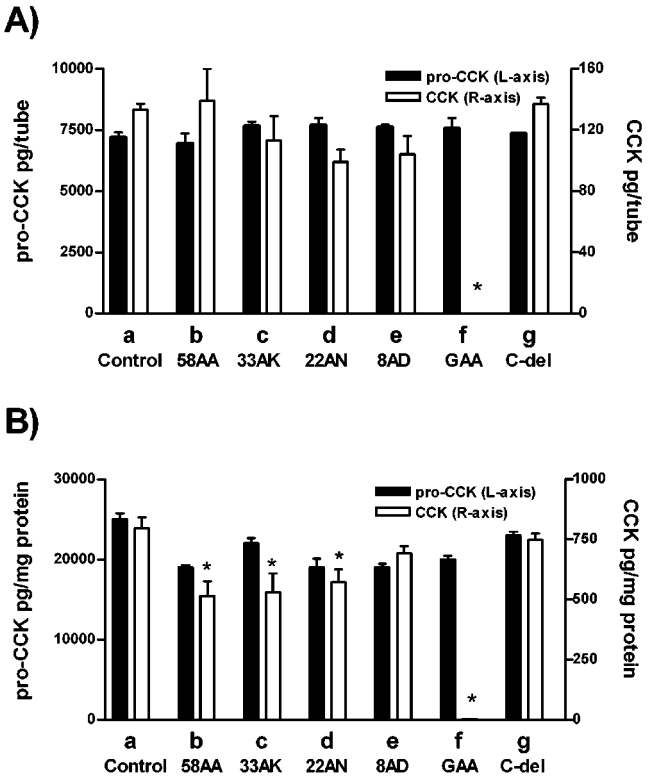

FIGURE 2: (A) Effect of Ala substitution at the P1 cleavage site on the secretion of pro-CCK and amidated CCKs in different mutants. Medium (100 μL/assay) was aliquoted for RIA from 1.4 to 1.8 $\times$ 10^7 cells. (B) Effect of Ala substitution at the P1 cleavage site on the cellular content of pro-CCK and amidated CCKs in different mutants: (A) control, (B) 58AA, (C) 33AK, (D) 22AN, (E) 8AD, (F) GAA, and (G) C-del. The bar graph shows means $\pm$ SE. The asterisk indicates that $p < 0.05$.

BSA and 0.02% sodium azide. Fractions (1.0 mL) were collected and aliquots removed for the radioimmunoassay (RIA). Elution of media and samples was compared to the elution of synthetic nonsulfated amidated CCK 8, CCK 12, CCK 22, and CCK 33 as well as nonsulfated CCK 8 Gly, CCK 22 Gly, and S9S standards. Graphically presented data representative of at least three independent experiments provide a qualitative assessment of the elution profile.

Radioimmunoassay. The CCK 8 RIA was performed as previously described (*25*), using the rabbit polyclonal CCK 8 antibody (R5) that is specific for amidated forms of CCK. The RIA used [^{125}I]gastrin-17 as a tracer, produced by iodination with chloramine-T (*26*). Antiserum 79, used to assess pro-CCK, was generated against V9M (VPVEAVDPM) synthesized with a carboxyl-terminal multiple-antigenic peptide tail. Iodinated Y10M (tyrosine-extended V9M) was used as a tracer. S9S RIA used ^{125}I-labeled S9S (SAEDYE-YPS) as a tracer and antibody (121) generated against S9S conjugated to keyhole limpet hemocyanin with carbodiimide (EDC). This antiserum detects S9S without the sulfated tyrosine residues. Natural S9S is doubly tyrosine sulfated (*27*). Its detection requires treatment of samples with arylsulfatase. CCK Gly RIA used [^{125}I]gastrin-13 Gly, and antiserum (22) generated against CCK 8 Gly. When the cellular content was measured, cells grown to a density of 1.4$-$1.8 $\times$ 10^7 cells/plate was lysed with 0.1 N HCl and clarified by centrifugation, and aliquots were assayed by a RIA. Protein concentrations were estimated by a BCA protein assay (Pierce). The CCK concentrations in medium and cell lysates of mutants were compared to control with the paired Student's *t* test.

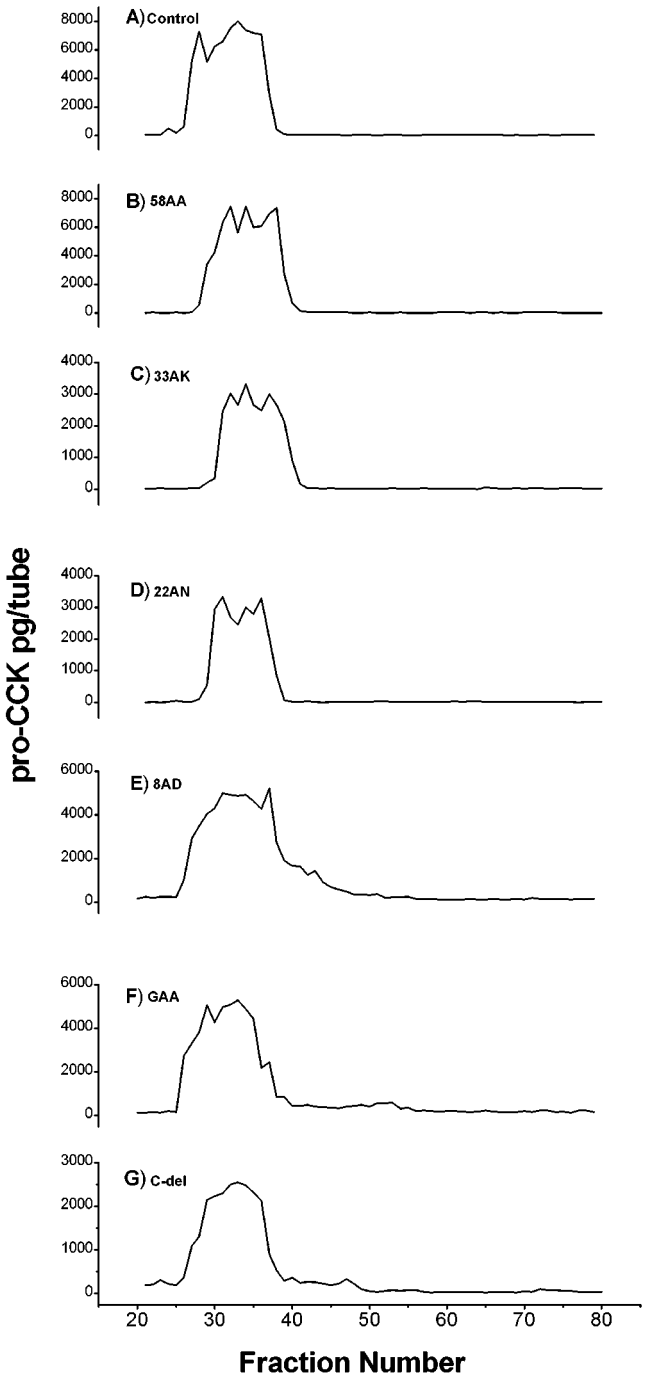

FIGURE 3: Spherilose GCL-90 chromatography fractions of medium from AtT-20 cells transfected with different mutants assayed for immunoreactive pro-CCK: (A) control rat prepro-CCK, (B) 58AA, (C) 33AK, (D) 22AN, (E) 8AD, (F) GAA, and (G) C-del.

Desulfation and Carboxypeptidase Y Treatment. Ten units of type V limpet sulfatase (Sigma) was added to concentrated medium in 200 mM sodium acetate (pH 5.0) and 0.1% NaCl and incubated for 2 h at 37 °C (*27*). The reaction mixture was centrifuged, and the supernatant was purified by Sep-Pak treatment before loading onto the GCL-90 column. Concentrated medium after sulfatase treatment was digested with carboxypeptidase Y (Cp-Y) (Calbiochem) in 50 mM sodium citrate buffer (pH 6.0). One hundred units of Cp-Y was used for treating 500 μL of 10-fold concentrated medium

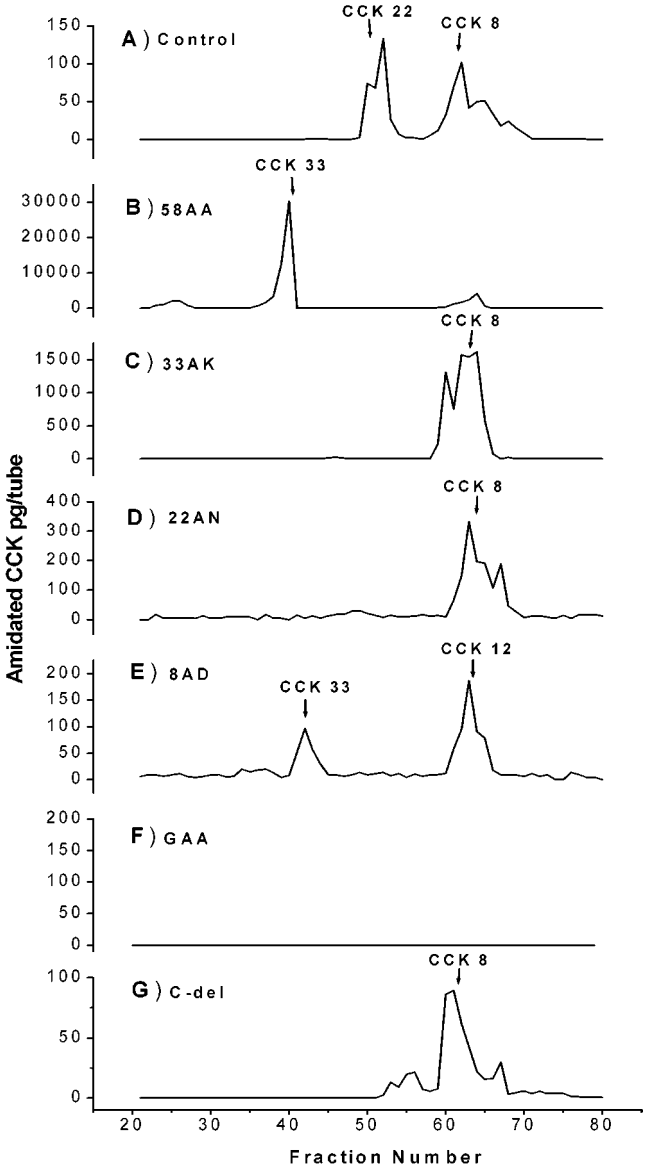

FIGURE 4: Spherilose GCL-90 chromatography fractions of medium from AtT-20 cells transfected with different mutants assayed for immunoreactive amidated CCKs: (A) control rat prepro-CCK, (B) 58AA, (C) 33AK, (D) 22AN, (E) 8AD, (F) GAA, and (G) C-del. Peaks were identified with reference to synthetic standards.

for 10 min at 25 °C, and the reaction was stopped by acidification with glacial acetic acid.

HPLC. S9S and desulfated medium samples were analyzed by HPLC (Waters 600, multisolvent delivery system from Millipore) with elution buffer A [0.09% trifluoroacetic acid (TFA)] and buffer B (90% acetonitrile in 0.09% TFA). Reverse-phase HPLC was carried out using a 5 μm, 90 Å Vydac C_{18} column (4.6 mm × 250 mm) eluted at a rate of 1 mL/min. The column was first eluted with 10% B for 10 min, and then a gradient was developed from 10 to 40% B over the course of 40 min. Fractions (1 mL) were collected; an aliquot was dried down, and the S9S concentration was determined by a RIA.

RESULTS

AtT-20 cells express very small amounts of endogenous CCK. The amount of pro-CCK and amidated CCK in cell lysates and medium in the untransfected AtT-20 cells was

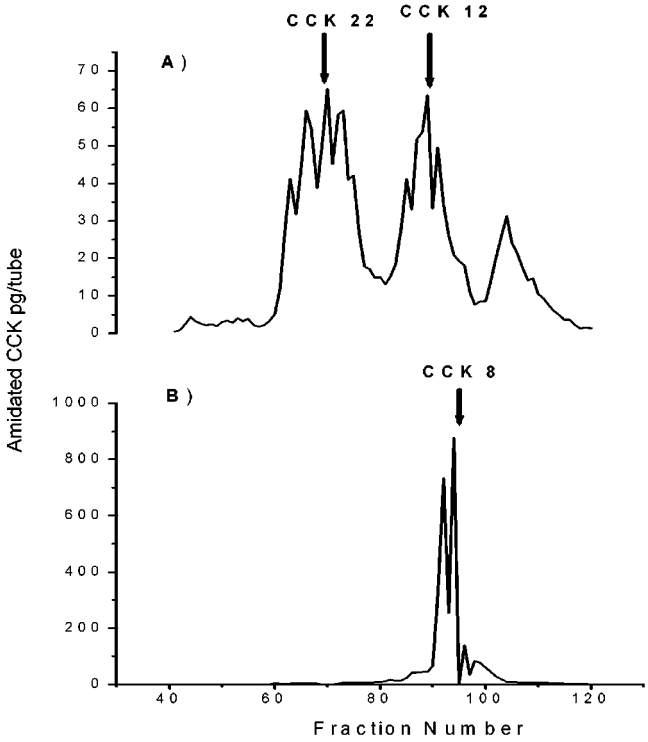

FIGURE 5: Spherilose GCL-90 chromatography fractions assayed for immunoreactive amidated CCKs. (A) Medium from AtT-20 cells transfected with 8AD and (B) CCK 8 synthetic standard.

Table 2: Summary of Gel Filtration Analysis[a]

	pro-CCK	CCK 33	CCK 22	CCK 12	CCK 8
control	X	–	X	–	X
58AA	X	X	–	–	X
33AK	X	–	–	X	X
22AN	X	–	–	X	X
8AD	X	X	–	X	–
GAA	X	–	–	–	–
C-del	X	–	–	–	X

[a] Summary of gel filtration analysis on medium from different mutants and control pro-CCK-transfected AtT-20 cells for pro-CCK and different forms of amidated CCKs identified by a RIA.

negligible in comparison with the amount in the transfected ones (results not shown). When transfected with the CCK cDNA where expression is driven by the CMV promoter, large amounts of amidated CCK were found in cell lysates and in media. This was accompanied by large amounts of pro-CCK found in both cell lysates and media. These amounts of amidated CCK are similar to levels of CCK in rodent brain (*25*). This high level of pro-CCK probably indicates that the secretory and processing pathway has finite capacity and cannot process all the expressed pro-CCK. This secreted pro-CCK serves as a useful marker for CCK expression and indicates that the mutant pro-CCKs are being made and secreted like the wild type, and are not being degraded due to structural modifications introduced with the mutations. Similar amounts of pro-CCK were secreted in all the mutants where the P1 position of the endoproteolytic cleavage site was changed to alanine in comparison with the AtT-20 cells transfected with the wild-type prepro-CCK construct (Figure 2A). The amount of pro-CCK secreted is also comparable in the cells transfected with the GAA mutant. No difference was observed in the cellular content of pro-CCK in any of the different mutants (Figure 2B).

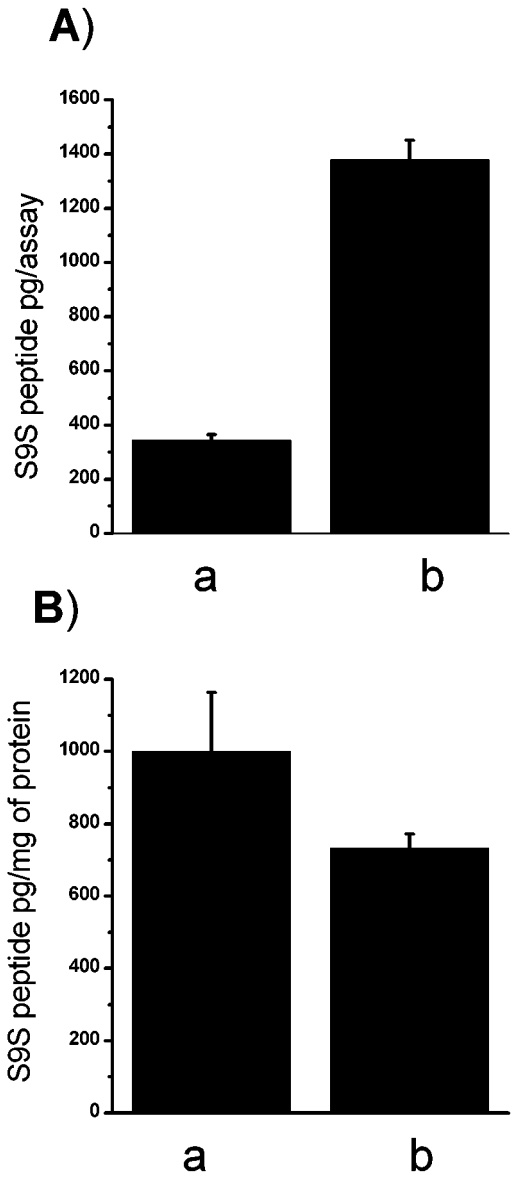

FIGURE 6: (A) Concentration of S9S released in medium. Medium (100 μL/assay) was aliquoted for RIA from 1.4 to 1.8 $\times$ 10^7 cells. (B) Cellular content of S9S: (a) control and (b) GAA.

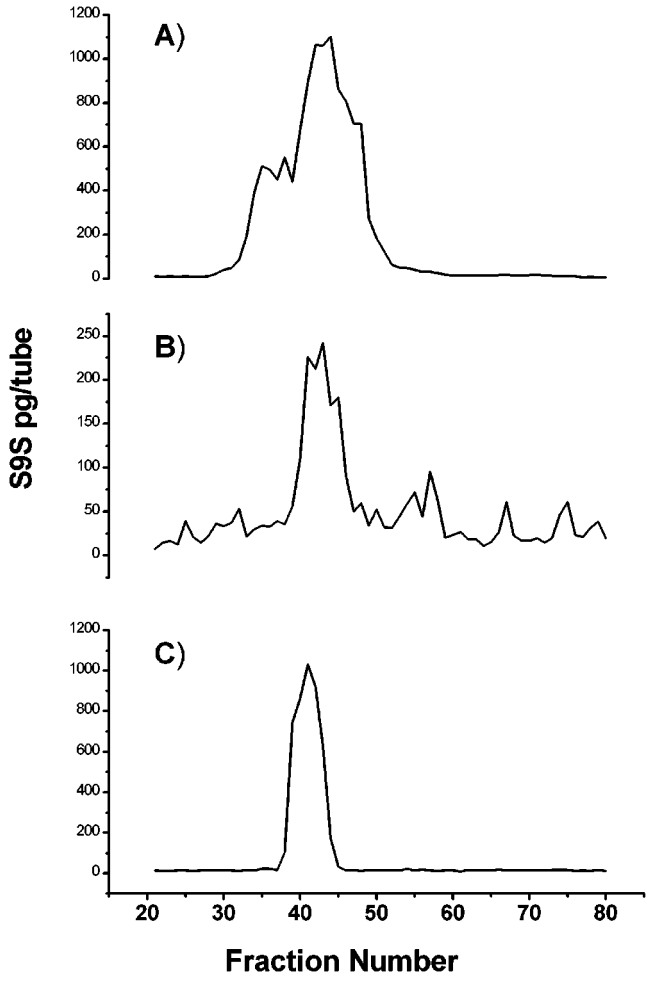

FIGURE 7: Spherilose GCL-90 chromatography fractions of desulfated medium assayed for immunoreactive S9S: (A) GAA, (B) control, and (C) S9S synthetic standard.

The amount of amidated CCK secreted is the same in all the cleavage site mutants in comparison with the wild-type control cells (Figure 2A,a). However, there is no CCK secreted in the medium of cells expressing the GAA mutant cDNA (Figure 2A,f). The cellular content of amidated CCK was decreased by ~40% in mutants 58AA, 33AK, and 22AN (Figure 2B,b−d), whereas the amidated CCK cellular levels in the mutant 8AD (Figure 2B,e) and C-del (Figure 2B,g) were similar to those in the control AtT-20 cells transfected with the wild-type construct (Figure 2B,a). The cellular CCK level was undetectable in GAA (Figure 2B,f), similar to the case in the untransfected AtT-20 cells.

Media from different mutants were further analyzed by gel filtration chromatography to investigate the role of each cleavage site on the products of pro-CCK processing. Medium from all the mutants when assayed for pro-CCK after separation on a gel filtration column shows an elution pattern similar to that of control cells transfected with the wild-type construct (Figure 3). The antibody used to detect CCK 8 also cross-reacts with all the major forms of CCK like CCK 12, CCK 22, and CCK 33, allowing us to identify different amidated forms of CCK. The exact quantities of CCK immunoreactive peptides and pro-CCK shown in the figures differ between mutants because of the differences in the amount of separated fraction used for the RIA to achieve a clear separation profile. These chromatographic analysis were carried out at different times. Each sample was calibrated independently with synthetic standards.

The AtT-20 cells expressing wild-type CCK secrete forms of CCK that coelute with CCK 8 and CCK 22 (Figure 4A). Medium from the 58AA mutant shows mainly a peptide coeluting with CCK 33 and a very small amount of one coeluting with CCK 8 (Figure 4B). Media from mutants 33AK (Figure 4C) and 22AN (Figure 4D) show products coeluting with CCK 12 and CCK 8. These forms are not separated by this chromatographic system. Mutant 8AD secreted products coeluting with CCK 33 and CCK 12 (Figure 4E). Medium from AtT-20 cells expressing the C-del mutant separated on the gel filtration column shows the presence of mainly CCK 8 (Figure 4G). Further separation of 8AD medium on a longer column that can resolve CCK 8 and CCK 12 confirms the identity of one of the secreted forms as CCK 12 (Figure 5). Results from the gel filtration analysis are summarized in Table 2.

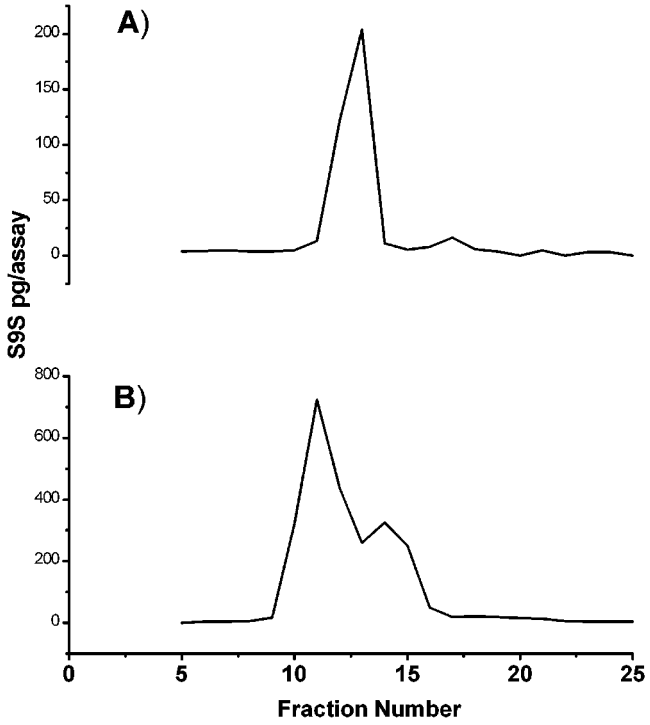

FIGURE 8: HPLC characterization of desulfated media for S9S: (A) control and (B) GAA. Eluted peaks were identified as S9S by coeluting with a synthetic standard. Different amounts of separated fractions were used for RIA to achieve a clear elution pattern.

Media pooled from several plates of the GAA mutant when analyzed by gel filtration chromatography and the CCK RIA did not show the presence of any amidated CCK (Figure 4F). This is logical because the carboxypeptidase in AtT-20 cells cannot remove the alanines due to its specificity for C-terminal basic residues. Without exposure of the glycine, the amidation reaction to make the C-terminal amide cannot take place. Since the antibody used to detect CCK 8 and other larger forms is specific for the amidated forms, it is still possible that the cleavage at GAA does take place at other sites. Such cleavage could generate the C-terminal S9S peptide as well as C-terminal GAA-extended peptides such as CCK 8GAA or larger forms. These forms probably cannot be further processed by carboxypeptidase and amidating enzyme activity.

Other methods are available for evaluating whether the expressed GAA mutant is cleaved at these sites. Cleavage at the GAA site would yield the S9S peptide (SAEDYEYPS), and an additional cleavage toward the N-terminus would be required to generate CCK 8, CCK 12, CCK 22, or CCK 33 with a GAA extension. The S9S peptide can be detected with the S9S RIA after treatment with aryl sulfatase to remove the two sulfates on the tyrosines. The GAA-extended peptides can be detected with the CCK Gly RIA after treatment with carboxypeptidase Y. This enzyme removes alanine residues easily but cannot easily remove glycine (*28*).

The presence of the S9S peptide was examined with an antiserum against unsulfated S9S. When the desulfated medium of GAA was analyzed for the presence of S9S, the GAA mutant secretes >3 times more S9S than the control cells transfected with wild-type CCK (Figure 6A). The cellular content of S9S in GAA is decreased by ~25% in comparison with control cells transfected with the wild type (Figure 6B). The coelution of the peptide from sulfatase-

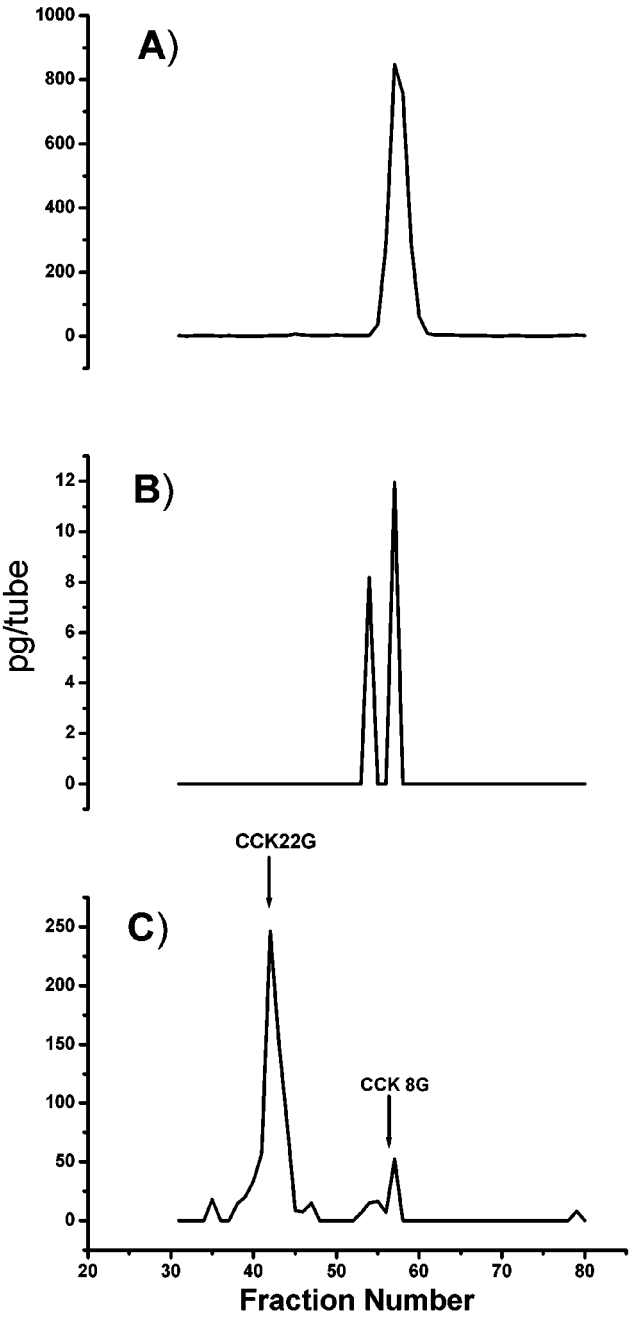

FIGURE 9: Spherilose GCL-90 chromatography fractions of desulfated and carboxypeptidase Y-treated medium assayed for immunoreactive G13Gly: (A) synthetic CCK 8 Gly standard, (B) GAA, and (C) control.

treated GAA medium with synthetic S9S was confirmed by both gel filtration (Figure 7) and by HPLC (Figure 8). Thus, the GAA mutant is cleaved liberating S9S.

Sulfatase treatment followed by carboxypeptidase Y treatment to remove the C-terminal AA was used to generate CCK Gly immunoreactive peptides. Little CCK Gly immunoreactivity was detected in media of GAA-transfected cells. When the medium from control cells transfected with the wild type was separated by gel filtration, CCK Gly immunoreactivity was observed coeluting with CCK 22 Gly and CCK 8 Gly (Figure 9). The media of GAA had very small amounts of CCK Gly immunoreactivity which coeluted with CCK 8 Gly. This demonstrates that although the S9S peptide was released, the GAA mutant was probably not extensively

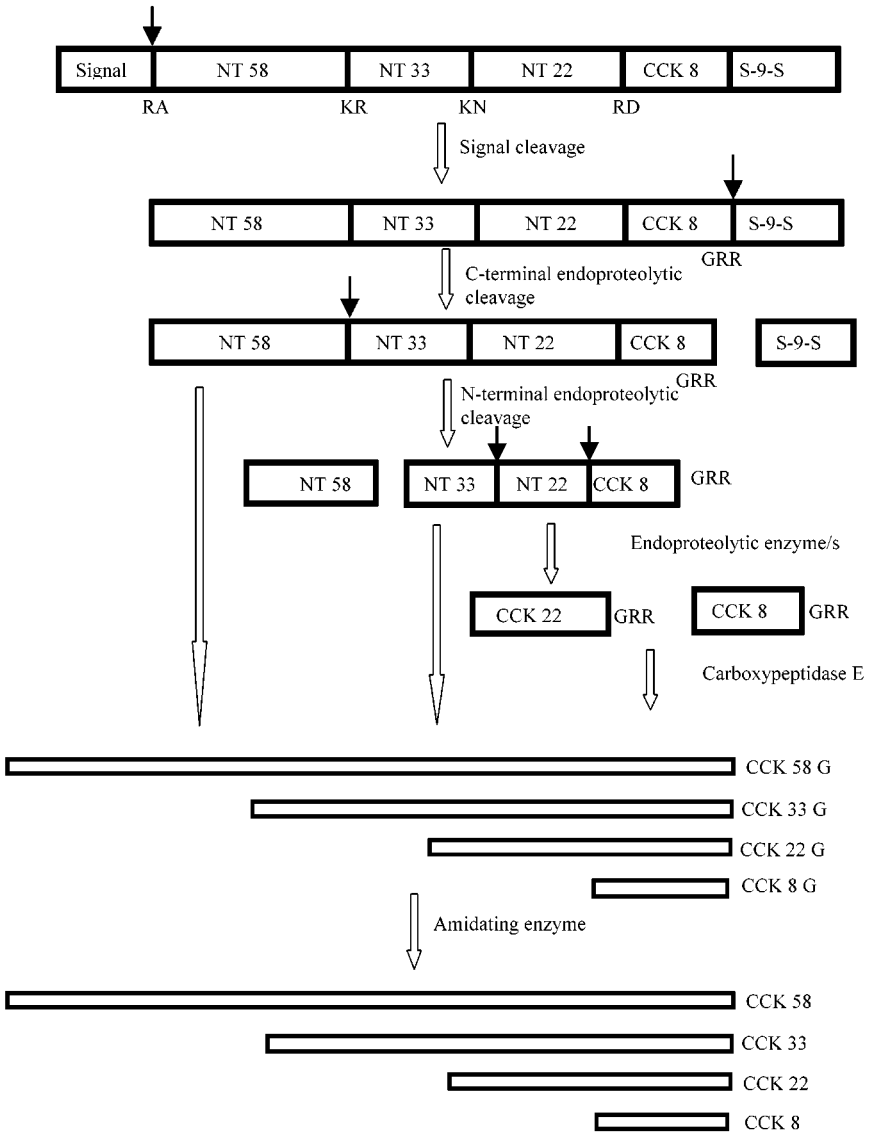

FIGURE 10: Model of prepro-CCK processing. The cleavage sites generating different CCKs are indicated by single-amino acid code below the box. NT 58, 33, and 22 are the amino-terminal portions of CCK 58, 33, and 22, respectively, while S-9-S is the carboxyl-terminal extension (SAEDYEYPS). Cleavages are shown in solid arrows, and the sequence of steps is followed in block arrows.

cleaved elsewhere to generate a peptide like CCK 8 GAA or another larger GAA-extended form of CCK.

DISCUSSION

Prohormone processing is a complex process involving various enzymes and thought to occur in a strict temporal order. This temporal order is thought to reflect the influence of precursor structure on the cleavage. The most accessible sites are cleaved first, and this allows the precursor to assume a different conformation, exposing other sites. We have used site-directed mutagenesis of the CCK cDNA and expression in pituitary AtT-20 cells to examine the effect of mutation at specific cleavage sites on the production of amidated CCK. Irrespective of the mutation and their effect on processing, the pro-CCK cellular content and secretion in all the mutants remain high, indicating that all of the mutant pro-CCKs were expressed and secreted normally. In the case of GAA where there is no amidated CCK secreted, pro-CCK levels in the medium are similar to those in the wild type, suggesting that secretion of pro-CCK is independent of processing. As long as the intracellular levels are high, the secretory machinery tends to secrete, keeping the cellular levels relatively constant. In all the mutants, the secretion of amidated CCKs was similar, indicating that once the processing is complete the amidated forms is secreted and the secretory pathway does not favor any particular cleaved form. The significance of the decrease in the cellular content of amidated CCKs in 58AA, 33AK, and 22AN is not clear because the amount of secretion remains similar in all. Mutations at all these cleavage sites prevented cleavage at their respective sites. This result strongly supports the role of a prohormone convertase-like enzyme in AtT-20 cells in these cleavages. The most likely candidate in AtT-20 cells would be PC1, but other enzymes with similar specificities could be responsible.

Analysis of the different amidated CCK forms on gel filtration (Table 2) revealed that all the mutations have a specific effect on the products that were produced, suggesting that the sequence and structure of a pro-CCK have an influence on where it is cleaved. The cleavage site at CCK

58 had the most dramatic effect on the production of CCK 8. This site is known to be cleaved in rat brain where the propeptide is detected (*29*). This propeptide is concentrated in rat brain synaptosomes (*30*) and is released from rat brain slices and some endocrine cells by high levels of potassium or treatment with cAMP (*31*). We do not detect significant quantities of the propeptide in control AtT-20 cells, so it is unclear whether it is cleaved in these cells. It is conceivable that the propeptide is further cleaved to smaller forms that are not detected by the antiserum.

Mutating this cleavage site probably changes the conformation of pro-CCK, altering the accessibility of other cleavage sites to the processing enzymes so that mainly CCK 33 and low levels of CCK 8 are produced. The observed low levels of CCK 8 might be due to the further very slow kinetic cleavages of CCK 33 to smaller forms. Mutations of CCK 33 RK to AK, 22KN to AN, and 8RD to AD all prevented production of CCK 22, while allowing production of either CCK 8 or CCK 12. This supports the hypothesis that CCK 8 and CCK 22 are produced independently, a conclusion that was reached using other cell lines in several previous enzyme antisense studies (*32*−*34*).

The mutant in which GAA replaced GRR is very informative. Production of S9S immunoreactivity demonstrates that GAA is cleaved, and coelution of the desulfated product with synthetic S9S confirms that it is identical to S9S. This unexpected cleavage is probably being performed by an enzyme, which has a specificity different than those of the known prohormone convertase enzymes. One possible candidate enzyme for this cleavage is the newly discovered SKI-1 enzyme, which is abundant in rat anterior pituitary (*10*). It is interesting to note that while the secreted pro-CCK levels remain similar in both the control and GAA mutant the levels of S9S secreted are different. It is also possible that the processing and kinetics of processing in GAA leading to S9S affect the observed differences in secreted levels when compared to control cells. Though the cellular levels of S9S are correspondingly higher in control cells compared to GAA, the level of secretion of S9S is lower. As the S9S sequence is highly conserved among several species (*35*) and is sulfated in a manner similar to that of CCK, it is possible that this peptide has an important biological role yet to be discovered. The S9S peptide was shown to be present in aqueous rat brain extracts at levels almost similar to the levels of CCK 8 (*27*). The results presented in this study show that the S9S is processed and secreted despite the mutation, which supports a possible biological role for this peptide.

Mutation of the C-terminal GRR to GAA blocked production of amidated CCK products, while treatment with carboxypeptidase Y fails to release much Gly-extended CCK, indicating that this mutation also blocks other cleavages toward the N-terminal end. Expression of a mutant pro-CCK, C-del, in which the entire S9S peptide is deleted and a stop codon is placed immediately after GRR was processed to amidated CCK as the wild type indicating that the S9S peptide is not essential to subsequent cleavages. This mutation may have a minor effect on pro-CCK processing because mainly CCK 8 and no other CCK was produced. This supports the hypothesis that this is the first cleavage. The presence of Ala-Ala residues at C-terminal after Gly blocks their removal by carboxypeptidases and subsequent cleavages toward the N-terminal. These alanine residues must influence how pro-CCK is folded so that additional cleavages are not facilitated. Thus it appears that there is a strict temporal order of cleavages that starts with the cleavage at GRR. The other mutations all have specific effects on the products that are produced, suggesting that the sequence and structure of pro-CCK have an influence on where it is cleaved. It thus appears that cleavage at the GRR site is essential for subsequent production of CCK 8 GRR. Previous studies on CCK products in $Cpe^{fat/fat}$ mice and CCK-expressing endocrine cells have demonstrated that CCK 8 GRR and CCK 8 Gly are the immediate precursors of CCK 8 amide (*36*). Both the extreme C-terminal cleavage site at GRR and N-terminal cleavage site at CCK 58 strongly influence the rest of the processing. Taken together, these results suggest that the initial processing proceeds from both ends toward the center of the prohormone. On the basis of our results presented in this study and our understanding of processing from several studies, we propose a working model (Figure 10) elucidating the entire sequence of CCK processing.

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