Role of Tyrosine Sulfation and Serine Phosphorylation in the Processing of Procholecystokinin to Amidated Cholecystokinin and Its Secretion in Transfected AtT-20 Cells†

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ABSTRACT: Mammalian procholecystokinin (pro-CCK) is known to have three sulfated tyrosine residues, one of which is present in the CCK 8 moiety and two additional residues present in the carboxyl-terminal extension. In the present study, inhibition of tyrosine sulfation by sodium chlorate decreased the secretion of processed CCK 8 in CCK-expressing endocrine cells in culture. It was then demonstrated that when each of these tyrosines individually, as well as all three together, was mutated to phenylalanine and expressed in endocrine cells, CCK was still processed and secreted. However, the amount of CCK secreted varied with the type of mutation. Substitution of Phe to Tyr in CCK 8 reduced the quantity of secreted CCK 8 by 50%, and when all the sulfated Tyr were mutated to Phe the quantity of secreted CCK was reduced by about 70%, similar to what is observed with chlorate treatment. Changing of the putative phosphorylation site serine to alanine does not affect the processing. Serine phosphorylation at this site may play a functional role in regulatory events. Our results demonstrate that tyrosine sulfation alters the amount of secretion but is not an absolute requirement for the processing and secretion of CCK in this cell line. Tyrosine sulfation of CCK may still be important for its solubility, stabilization, and/or functional interaction.

Work during the past 10 years has established that tyrosine sulfation is a posttranslational modification that occurs in essentially all eukaryotic cells containing a Golgi apparatus (*1*). As compared to other various posttranslational covalent modifications of proteins, O-sulfation on tyrosine residues has until recently attracted relatively little attention because it was considered a rare modification. The presence of a sulfated tyrosine residue was first detected in fibrinopeptide B (*2*, *3*), then on gastrin (*4*) and CCK[1] (*5*), and was more recently shown to occur in a rather large number of secretory proteins such as immunoglobulin G (*6*), fibronectin (*7*), and procollagens (*8*). A quantitative study has shown that although most (65−95%) of the total protein-bound tyrosine sulfate synthesized by cells is recovered in secreted proteins, significant amounts of tyrosine sulfate are also found in nonsecretory proteins (*9*). The frequency of tyrosine sulfate in secretory proteins is demonstrated by in vivo studies with rats in which plasma proteins were shown to contain much more tyrosine sulfate than tissue proteins (*10*). Tyrosine sulfation was found to take place in the trans Golgi shortly after terminal glycosylation reactions, the last known covalent modification of proteins before their exit from the trans Golgi proceeding the sorting of secretory proteins (*1*). Two members of sulfotransferases responsible for peptide sulfation localized in the trans-Golgi network were recently cloned (*11*, *12*). In the case of many neuropeptides, which are sorted as precursors and processed proteolytically in secretory granules, sulfation precedes and thus could influence proteolytic processing of these precursors.

For many proteins, tyrosine sulfation appears to be important for biological activity and correct cellular processing. The loss of sulfated tyrosine residues decreases the interactions between factor VIII and von Willebrand factor (*13*, *14*), hirudin and thrombin (*15*, *16*), fibronectin and fibrin (*17*), complement C4 and C1s (*18*), and leuserpin 2 and thrombin (*19*). Studies with P-selectin glycoprotein ligand (PSGL) have shown that a sulfated peptide segment of the amino terminus of PSGL-1 is critical for P-selectin binding (*20*). Tyrosine sulfation of chemokine receptor CCR5 facilitates HIV-1 entry (*21*). The proinflammatory cytokine tumor necrosis factor α was found to convert CD44 from its inactive, nonbinding form to its active form by inducing the sulfation of CD44. Sulfation was thus shown as a potential means of regulating CD44-mediated leukocyte adhesion at inflammatory sites (*22*). Correlative studies on the degree of gastrin sulfation and its processing suggest that sulfated gastrin 34 is more readily processed to gastrin 17 (*23*, *24*). Mutational analysis of tyrosine sulfation of gastrin demonstrated that substitution of the alanyl residue N-terminal to the sulfated tyrosine with an acidic residue promotes sulfation and complete sulfation increases the endoproteolytic processing of progastrin (*25*). On the basis of this observation, it was also suggested that tyrosine sulfation is an important regulator of phenotypic gene expression. In mammals, only half of the gastrin is sulfated (*4*, *26*), in contrast to the homologous hormone CCK, which is completely sulfated (*5*).

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[1] Abbreviations: CCK, cholecystokinin; PSGL, P-selectin glycoprotein; DMEM, Dulbecco's modified Eagle's minimal essential medium; RIA, radioimmunoassay.

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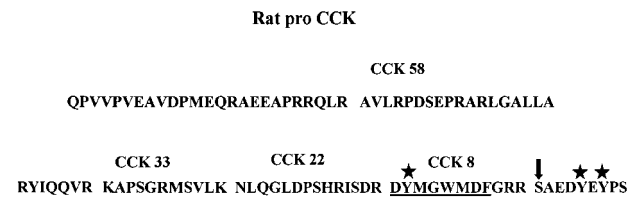

FIGURE 1: Rat pro-CCK sequence is written in the single amino acid code with cleavage sites indicated by spaces. CCK peptides are numbered backward from the C terminus of CCK 8. The amino-terminal ends of the major forms of amidated CCK peptides are indicated. Stars mark locations of the sulfated tyrosine residues, while the potential casein kinase II serine phosphorylation site is marked by a vertical arrow.

Mammalian pro-CCK is known to have three sulfated tyrosine residues, one of which is present in the CCK 8 moiety and two additional ones present in the carboxyl-terminal extension (Figure 1). Pro-CCK has a M_r of 12,826 kDa and contains one copy of CCK 8 flanked by a large amino-terminal segment and a short carboxyl-terminal segment. During posttranslational processing, pro-CCK is tyrosine-sulfated at three out of its four tyrosine residues in the trans-Golgi network, undergoes a series of endoproteolytic cleavages, followed by the action of carboxypeptidase, and finally C-terminal amidation of the final products (*27*). CCK 8 is the predominant form of the peptide in the brain while CCK 58, CCK 33, and CCK 22 are more abundant in the gut. This sulfation of the tyrosine on CCK is required for maximal biological activity at CCK-A-type receptors. Sulfated CCK has >500-fold higher affinity toward CCK-A receptor than the nonsulfated CCK. By use of three-dimensional modeling, arginine 197 and methionine 195 of CCK-A receptor were identified as the putative amino acids that interact with the sulfate and the aromatic ring of tyrosine of CCK. The two amino acids Arg 197 and Met 195 that interact with sulfated Tyr were proposed to regulate the conformational change of the CCK-A receptor that occurs during its activation in physiological conditions (*28*, *29*). An earlier study on the effect of inhibition of sulfation with sodium chlorate supported a role for these sulfated tyrosines in the processing of pro-CCK. Inhibition of tyrosine sulfation by sodium chlorate dramatically decreased the secretion and partially reduced the cellular content of processed CCK 8 amide in CCK-expressing endocrine cells in culture (*30*). In this report, we examine the ability of AtT-20 cells to process site-directed mutants of pro-CCK in which specific tyrosine residues were changed to phenylalanine to determine the importance of tyrosine sulfation for the processing and secretion of CCK. The neuroendocrine AtT-20 cells are known to contain carboxypeptidase E (*31*), peptidyl α-amidating enzyme (*32*), and subtilisin-like prohormone convertases PC1 (*33*) and PC2 (*34*). These cells can be grown easily, can be stably transfected, and are shown to process and secrete amidated CCK (*35*−*37*).

All members of the CCK/gastrin family sequenced so far have a putative casein kinase II serine phosphorylation site in the carboxy-terminal extension. Gastrin is known to be phosphorylated at this site (*38*) and a physiological casein kinase-like enzyme in Golgi membranes has been shown to phosphorylate this peptide (*39*). Similar putative phosphorylation sites are found adjacent to cleavage sites in a variety of regulatory peptide precursors (*40*). The phosphorylation site is of special interest because it is immediately adjacent to the cleavage site that subsequently yields the important biologically active C-terminally amidated form of CCK. The importance of the putative casein kinase II serine phosphorylation site was also determined by expressing a mutant in which the serine was replaced by an alanine.

MATERIALS AND METHODS

Sodium Chlorate Inhibition Assays. Mouse pituitary tumor AtT-20 cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% newborn calf serum, 10% horse serum supplemented with 50 μg/mL streptomycin sulfate, and 50 units/mL penicillin. AtT-20 cells were transfected with 25 μg of pcDNA 3 containing the rat CCK cDNA by the calcium phosphate method. Stably transfected cells were selected with Geneticin (G418). Sodium chlorate was neutralized, filter-sterilized, and added in sulfate-free medium at a final concentration of 10 mM to cells, which had been previously rinsed with sterile Tris-buffered saline (25 mM Tris, 0.14 M NaCl, 0.6 mM KCl, and 0.7 mM sodium phosphate, pH 7.5), when they reached about 80% confluence. Sulfate-free medium utilized DMEM base-modified medium from Gibco BRL, to which was added 2.23 g of glucose, 55 mg of sodium pyruvate, 0.1 g of $CaCl_2$, and 50 mg of $MgCl_2$/500 mL prior to filter sterilization. Control untreated cells were incubated with normal growth medium. After 24 h the medium was collected and the cells were extracted by scraping into 0.1 N HCl, sonicated, and clarified by centrifugation. CCK content of the medium and cell extracts were determined by CCK RIA. The results from control vs treated cells were compared with the paired Student's *t*-test.

Construction and Expression of Rat Pro-CCK Mutants. To construct the expression plasmid pcDNA3.1(−)pCCK, the rat cDNA fragment encoding pro-CCK was amplified by PCR with appropriate oligonucleotides that 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 by use of 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 pro-CCK. It was subsequently cloned into expression plasmid pcDNA3.1(−), which was also digested with *Eco*RI and *Hin*dIII. Site-directed mutants where the sulfated Tyr is replaced by Phe, and Ser to Ala, were generated by PCR with appropriate oligonucleotides. These mutants were designated as (1) CCK F77Y, where the tyrosine of CCK 8 was mutated to Phe [generated by overlap extension PCR (*41*)]; (2) CCK F91Y, where the second tyrosine at the carboxyl terminus was mutated to Phe; (3) CCK F93Y, where the last tyrosine at the carboxyl terminus was mutated to Phe; and (4) CCK F77/91/93Y, where all three sulfated tyrosines were mutated to Phe, generated by use of CCK F77Y DNA as template and a 3′ mutagenic primer where both Tyr residues were substituted with Phe; (5) CCK A87S, where the consensus phosphorylation-site serine was mutated to Ala. All the DNA fragments were sequenced in order to confirm the introduced mutations. AtT-20 mouse pituitary tumor cells were transfected with 25 μg of plasmid DNAs by the calcium phosphate method. Stably transfected cells were selected with Geneticin for further analysis.

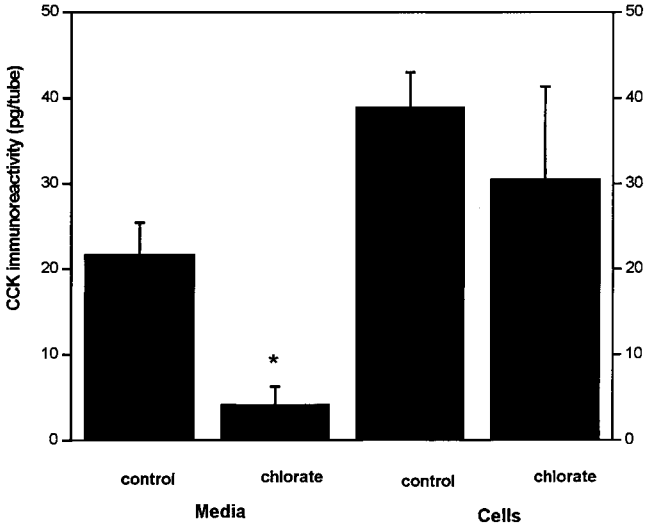

FIGURE 2: Effect of 10 mM sodium chlorate on the secretion and cellular content of immunoreactive CCK. Medium (50 μL/assay) was aliquoted for RIA from the untreated control cells and chlorate-treated cells. Cellular CCK content (picograms) present in 50 μL aliquots of cell lysates from control untreated and chlorate cells is shown. Bar graph shows mean ± SD. The asterisk indicates $p < 0.05$.

Chromatographic Analyses. Medium from four to eight 10 cm plates at 80% confluence was concentrated in Sep-Pak C18 cartridges (Waters Associates) and further concentrated to about 1 mL in a Speedvac concentrator. Medium was separated by gel-filtration chromatography on a 50 × 2.5-cm column of Spherilose GCL-90 (Isco) in a run at 4 °C in 50 mM Tris and 200 mM NaCl, pH 7.8, containing 0.1% BSA and 0.02% sodium azide. Fractions of 1.0 mL were collected and aliquots were removed for the radioimmunoassay (RIA). 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 (*42*), with the rabbit polyclonal CCK 8 antibody (R5) that is specific for amidated forms of CCK. The RIA used [^{125}I]gastrin-17 as tracer, produced by iodination with chloramine T (*43*). Antiserum 79, used to measure pro-CCK, was generated against V9M (VPVEAVDPM) synthesized with a carboxyl-terminal multiple antigenic peptide tail. Iodinated Y10M (tyrosine-extended V9M) was used as tracer.

RESULTS

The amount of endogenous CCK secreted in the untransfected AtT-20 cells is negligible in comparison with the transfected ones (<1%, results not shown). Incubation of transfected AtT-20 cells that process pro-CCK to CCK 8 amide with 10 mM chlorate causes an 80% decrease in the amount of immunoreactive CCK 8 secreted, while the cellular content did not change in comparison with untreated control cells (Figure 2). These results demonstrate that chlorate inhibition of tyrosine sulfation of pro-CCK reduces the quantity of CCK secreted. This result is similar to an earlier observation on a different CCK-expressing endocrine cell line (*30*). This result, along with several other reports suggesting the role of tyrosine sulfation on processing and secretion of proteins, prompted us study the role of individual tyrosine residues in pro-CCK processing and secretion.

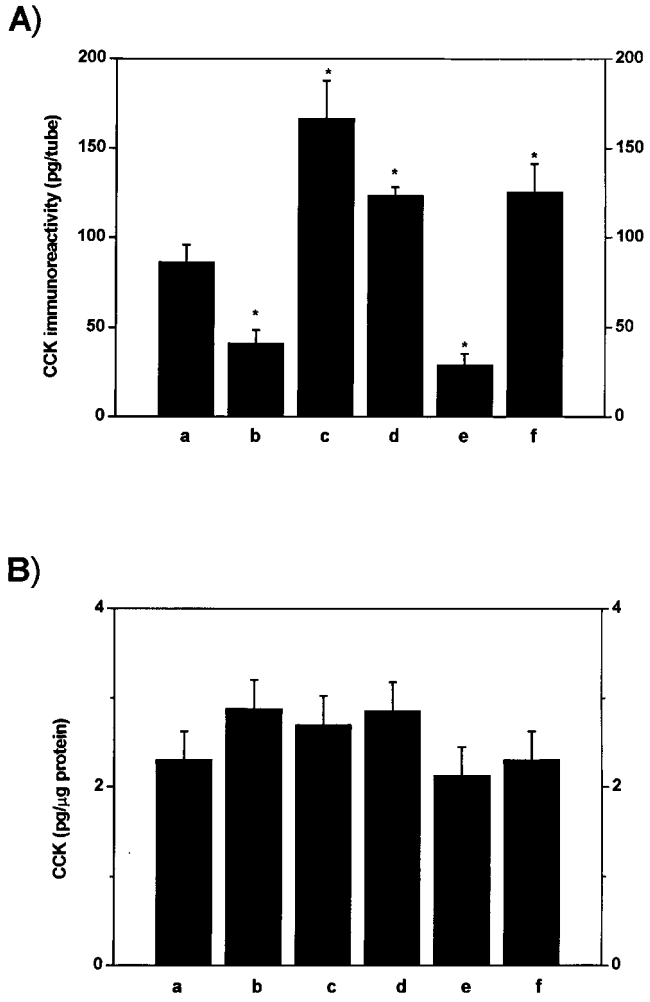

FIGURE 3: (A) Effect of Tyr/Phe substitution on the secretion of CCK 8 in different mutants. Medium (100 μL/assay) was aliquoted for RIA from the cells grown to 80% confluence. (B) Effect of Tyr/Phe substitution on the cellular content of CCK 8 (picograms of CCK 8 per microgram of total cellular protein) in different mutants. Cells grown to 80% confluence were lysed with 0.1 N HCl and clarified by centrifugation, and aliquots were assayed by RIA. Protein concentration was estimated by BCA protein assay (Pierce). (a) Control; (b) CCK F77Y; (c) CCK F91Y; (d) CCK F93Y; (e) CCK F77/91/93Y; (f) CCK A87S. Bar graph shows mean ± SD. Data comparisons with control wild type were made by one-way ANOVA with Dunnet's test.

The amount of immunoreactive CCK secreted in medium varies with the position of the mutation (Figure 3A). Medium from the CCK F77Y has less than half of CCK concentration in comparison with the wild-type control cells. CCK F91Y and CCK F93Y both secrete significantly higher amounts of CCK than the control cells. Cells transfected with CCK F91Y secreted almost twice the amount of CCK, whereas the CCK F93Y secretes about 50% more CCK than the control wild-type cells. When all the Tyr were substituted with Phe, the level of CCK secreted was reduced by two-thirds, similar to the CCK F77Y mutation. The level of increase in CCK secreted by CCK A87S transfected AtT-20 cells was comparable to that of CCK F93Y. The cellular content of the CCK remained unaltered in all the mutants (Figure 3B) in comparison with control cells, similar to the cellular content of sodium chlorate-treated cells.

Medium from different mutants was further analyzed by gel-filtration chromatography to investigate the role of each

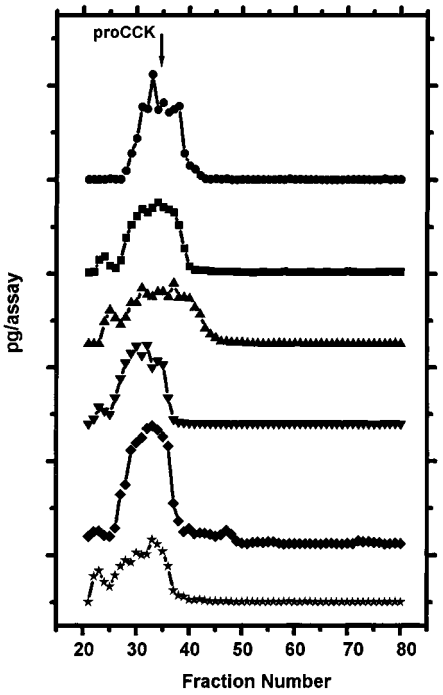

FIGURE 4: Spherilose GCL-90 chromatography fractions of medium from AtT-20 cells transfected with different mutants assayed for immunoreactive pro-CCK: (●) Control rat prepro-CCK; (■) CCK F77Y; (▲) CCK F91Y; (▼) CCK F93Y; (◆) CCK F77/91/93Y; (★) CCK A87S.

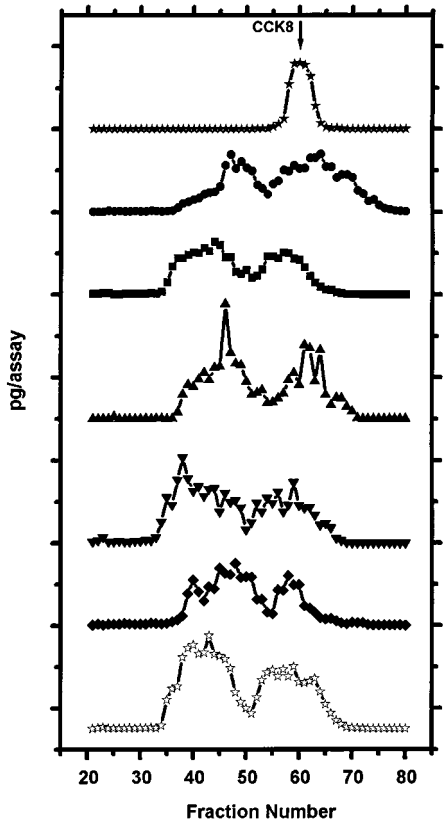

FIGURE 5: Spherilose GCL-90 chromatography fractions of medium from AtT-20 cells transfected with different mutants assayed for immunoreactive CCK 8: (★) CCK 8 synthetic standard; (●) control rat prepro-CCK; (■) CCK F77Y; (▲) CCK F91Y; (▼) CCK; (◆) F93Y CCK F77/91/93Y; (☆) CCK A87S.

of the sulfated Tyr on the processing of CCK. Medium from the CCK F77Y mutant, when analyzed by gel-permeation chromatography for the presence of both pro-CCK (Figure 4) and amidated CCK (Figure 5), showed a similar elution pattern in comparison to that of wild type without any mutations. The antibody used to detect CCK 8 also cross-reacts with larger forms like CCK 12, CCK 22, CCK 33, and CCK 58 and the additional peaks besides the CCK are these different forms, probably mainly CCK 22. The difficulty of chemically synthesizing these larger sulfated peptides of varying lengths has prevented us from having standards to identify each of them precisely. Both wild-type and mutant pro-CCK-expressing cells secreted large amounts of pro-CCK into the medium, indicating that their ability to express and process pro-CCK was saturated. The gel-permeation chromatographic separation carried out with CCK F91Y, CCK F93Y, CCK F77/91/93Y, and CCK A87S assayed for both pro-CCK (Figure 4) and CCK (Figure 5) was mostly similar to that of wild type. Slight differences in the various forms can be found up on careful observation; however, the overall elution profile remains unchanged. The exact quantity of CCK immunoreactive peptides and pro-CCK shown in these figures differs between mutants owing to the differences in the amount of concentrated medium loaded onto the column and on the amount of separated fractions used for the RIA assay to get a clear separation profile.

DISCUSSION

Our understanding of the physiological roles of sulfation has increased during the past decade. Since the 1970s, it has also become evident that sulfation is involved in the metabolism of numerous endogenous compounds including steroids, bile acids, monoamines, neurotransmitters, and thyroid hormones. Sulfation clearly is involved in the metabolism of small compounds, drugs, or xenobiotics and is generally accepted as a mechanism for increasing the water solubility and excretion of the products of oxidation reactions (*44*). The biological role of protein tyrosine sulfation has been studied only in very few cases, most of which concern small tyrosine-sulfated peptides. This is partially due to the fact that studies comparing sulfated and unsulfated forms of proteins (rather than peptides) are experimentally more difficult and also because the unsulfated form of a tyrosine-sulfated protein could not easily be obtained.

To understand the role of protein sulfation in general, CCK represents an excellent model protein having all the desirable features: (1) of the three sulfated Tyr, the one present in CCK 8 has a functional role contributing to selectivity, while the other two at the C-terminal end have no identified function; (2) CCK 8 interacts with at least two different receptor types, the CCK-A receptor (peripheral) type, which has high affinity for the sulfated form, and the CCK-B (brain) receptor, which has approximately the same affinity for both sulfated and unsulfated forms; and (3) it undergoes extensive processing and exhibits a size heterogeneity in the processed forms that differs among different tissues and species.

Chlorate inhibition of cellular sulfation reactions has been used widely to assess the importance of this modification by incubating the culture of cells in chlorate-supplemented medium. Sodium chlorate treatment was shown to cause selective effects on the sulfation of heparin sulfate, as chlorate is known to decrease the synthesis of the sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (*45*). It is likely that

chlorate, besides inhibiting sulfation of CCK, also inhibits the tyrosine sulfation of other proteins involved in the processing and secretion of CCK. Though the cells did not show any cytotoxic effects, it cannot be concluded that the overall cellular metabolism is not altered. Chlorate inhibition experiments in this study and in an earlier study using rat medullary thyroid carcinoma cells (*30*) demonstrated that, in these two CCK-expressing cell lines, the secreted CCK 8 was drastically reduced while the cellular content of amidated CCK 8 was not decreased in transfected AtT-20 cells.

Reduced levels of secreted CCK 8 in mutant CCK F77Y and CCK F77/91/93Y may be due to reduced stability of the phenylalanine-substituted peptide in granules or in the medium or actual loss of the peptide from granules. Similar reduced levels of secreted CCK 8 observed in chlorate-treated control cells correlates the degree of sulfation with the amount of secretion. It also confirms that chlorate treatment specifically inhibits sulfation. In contrast, the higher levels of secreted CCK observed for CCK F91Y and CCK F93Y may expose the molecule in to a more relaxed conformation wherein the processing enzymes can readily access the cleavage sites so the prohormone is more readily processed and secreted. NMR studies have shown that sulfated CCK exists in a more folded conformation than the unsulfated form (*46*). Taken together, these results also suggest that it is the sulfated Tyr in the CCK 8 that may be more important for its stability and/or secretion than the other two sulfated Tyr at the C-terminal end. The sulfated Tyr content of thyroglobulin is shown to regulate the production of thyroid hormones (*47*), and thyrotropin regulates the number of sulfate residues on Tyr of thyroglobulin (*48*).

Inhibition of Tyr sulfation by site-directed mutagenesis of substituting Phe to Tyr and by chlorate treatment caused a 2-fold increase in the half-time of transport of the yolk protein of *Drosophila melanogaster* from trans-Golgi to cell surface (*49*). The shorter residence time of sulfated protein was suggested to induce structural change that facilitates its passage or may prevent a structural change that would slow the passage. In contrast, in a similar study of Tyr sulfation inhibition by chlorate treatment on the regulated secretory proteins in *Xenopus* melanotrope cells, it was concluded that intracellular transport, sorting, and proteolytic processing of regulated secretory proteins does not require protein sulfation (*50*).

When the sulfated tyrosine is mutated to Phe, CCK 8 processing is not altered, though it has known functional significance in terms of receptor binding. Results with CCK F77Y have demonstrated that tyrosine sulfation in CCK 8 is not required for its processing. The short carboxyl-terminal peptide has two sulfated tyrosines and they could have a role in CCK processing. Analysis of these individual site-directed mutants show that, despite their lack of sulfated tyrosines, pro-CCK is processed to amidated CCK and secreted normally; in fact, the secretion is increased. It is intriguing to note that the short C-terminal peptide of pro-CCK with no function identified so far has two sulfated tyrosines and suggests its role more in terms of structure stabilization or solubility. The possibility of sulfation of all three tyrosine sites synergistically playing a role in the processing and secretion was excluded, as the mutant in which all the sulfated tyrosines are changed to Phe also is normally processed and secreted. It is possible that sulfation plays a role in stabilization and/or solubilization as in the case of drugs and xenobiotics. It may be of importance when these peptides gets processed and travel through the acidic environment of the secretory granules. The great sensitivity to hydrolysis of the sulfated peptides in acidic medium (*46*) may also partially explain the increase in secretion observed with the CCK F91Y and CCK secretion in control cells.

Analysis of the mutant changing the consensus serine sequence for phosphorylation shows that it also is processed and secreted normally. This result suggests that the putative phosphorylation is not required for processing and secretion of CCK and might serve another function. In contrast, in gastrin processing it has been shown that prohormone phosphorylation modulates the rate of cleavage at the adjacent dibasic site (*51*). In the mutant the secreted amount of CCK 8 is increased, similar to the effect of mutating the sulfated Tyr in CCK F93Y. It is likely that in the altered conformation caused by mutation the pro-CCK is processed more readily, resulting in the increase in secreted form.

Tyrosine sulfation can also be a means of creating functional diversity. Gastrin requires tyrosine sulfation for its pancreatic secretory activity (*52*), whereas its ability to stimulate gastric acid secretion is unaffected by sulfation (*53*). Considering the fact that gastrin is only partially sulfated itself suggests that sulfation, though not critical for its primary function, may be involved in creating functional diversity. P-Selectin glycoprotein ligand 1 also requires tyrosine sulfate to bind to P-selectin but not E-selectin (*54*). For a number of neuroactive steroids, sulfation at C-3 was shown to reverse the direction of modulation from positive to negative. In an interesting study it was shown that when hemisuccinate is substituted for sulfate at C-3, inhibitory activity was retained, suggesting that it is the negative charge rather than the sulfate group that confers the inhibitory efficacy (*55*).

Horse gastrin and turtle antral peptide, which are not sulfated in the native organism, when expressed in mammalian HT cells are shown to be sulfated (*56*). This result taken together with the results in this study suggest the sulfation as a process without a significant role in function. Though molecular modeling and manual docking of CCK onto CCK-A receptor shows strong contact points of sulfated Tyr interaction (*29*), whether it reflects the in vivo situation remains to be established. On the basis of our results and the fact that CCK does not require sulfated Tyr to interact with CCK-B receptor present in the brain, we hypothesize that sulfation of Tyr makes it more soluble for its interaction on the peripheral receptors, whereas the lipophilic, hydrophobic environment in brain sulfation is not necessary. Further to support our hypothesis, in a recent NMR study the authors suggest on the basis of their results that it is the membrane-associated conformation of CCK that first interacts with the receptor (*57*). NMR studies have shown that CCK exists preferentially in folded conformation, leading to a hydrophobic surrounding, while the Tyr (sulfated or unsulfated) is oriented opposite to the hydrophobic part of the molecule (*58*). These studies attribute an amphiphilic structure, and the presence of a sulfate group increases the folding tendency. Given the fact that nonsulfated CCK 8 peptides have very low solubility in aqueous medium, the tendency of sulfated CCK 8 to aggregate strongly suggests the role of sulfation in terms of solubility. Sulfation of

tyrosine can also prevent proteolysis; e.g., chymotryptic cleavage in vitro does not occur at the C-terminal side of sulfated tyrosine residues (*59*). Though the above possibilities remain open, our results for the first time conclusively demonstrate that tyrosine sulfation alters the amount of secretion and is not required for the processing of CCK in AtT-20 cells. Tyrosine sulfation of CCK may still be important for its solubility and for stabilization contributing to functional interaction.

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