Phosphorylation and O-Glycosylation Sites of Human Chromogranin A (CGA<sub>79–439</sub>) from Urine of Patients with Carcinoid Tumors*

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Because of their water-soluble properties, chromogranins (CGs) and chromogranin-derived fragments are released together with catecholamines from adrenal chromaffin cells during stress situations and can be detected in the blood by radiochemical and enzyme assays. It is well known that chromogranins can serve as immunocytochemical markers for neuroendocrine tissues and as a diagnostic tool for neuroendocrine tumors. In 1993, large CGA-derived fragments have been shown to be excreted into the urine in patients with carcinoid tumors and the present study deals with the characterization of the post-translational modifications (phosphorylation and O-glycosylation) located along the largest natural CGA-derived fragment CGA<sub>79–439</sub>. Using mild proteolysis of peptidic material, high performance liquid chromatography, sequencing, and mass spectrometry analysis, six post-translational modifications were detected along the C-terminal CGA-derived fragment CGA<sub>286–439</sub>. Three O-linked glycosylation sites were located in the core of the protein on Thr<sub>165</sub>, Thr<sub>165</sub>, and Thr<sub>235</sub>, consisting in di-, tri-, and tetrasaccharides. Three phosphorylation sites were located in the middle domain, on serine residues Ser<sub>200</sub>, Ser<sub>252</sub>, and Ser<sub>315</sub>. These modified sites were compared with sequences of other species and discussed in relation with the post-translational modifications that we have reported previously for bovine CGA.

Chromogranins/secretogranins (CGs/Sgs) constitute a family of acidic secretory glycoproteins widely expressed in a large number of endocrine and neuroendocrine cells and in neurons (1–4). Chromogranin A (CGA), the major member (40% of total soluble granule proteins) of this family, has been studied extensively. At the subcellular level, chromogranins are exclusively found in the soluble core of hormone and neurotransmitter storage vesicles and are released during exocytosis. Chromogranins have been proposed to play multiple roles in the secretory process. An intracellular function as a “helper” protein in the packaging of peptides, hormones, and neuropeptides by virtue of their ability to aggregate in the low pH and high calcium environment of the trans-Golgi network and as modulators of the processing of these components has been suggested (3). Extracellularly, different members of the chromogranin family are now considered as precursor proteins, which are actively processed into peptides within the secretory granules (see Refs. 1 and 5 for reviews). Previously, we reported a detailed study of the intracellular and extracellular processing of CGA and CGB/SgI (6, 7) and a preliminary analysis of the post-translational proteolysis of CGC/SgII (8) in bovine chromaffin granules.

The proteolytic processing of CGA is a topic of growing interest, as biological activities have been attributed to peptides located along the sequence of CGA. For example, in the N-terminal domain, a peptide corresponding to the sequence 1–113 has been shown to inhibit hormone secretion in the bovine parathyroid gland (9); a homologous peptide, β-granin, corresponding to the sequence 1–115 has been isolated from rat pancreas, but its function has not yet been defined (10). Vasostatins are peptides containing the N-terminal sequence (1–76/113) (11) that exhibit vaso-inhibitory activity of isolated human blood vessels (12, 13). As early as 1988, it was established that CGA is the precursor of a peptide that inhibits the secretory activity on chromaffin cells (14), and recently, catestatin, a novel CGA fragment (344–364), was characterized as a non-competitive nicotinic cholinergic antagonist (15). In addition, pancrestatin (248–293) is a peptide with multiple properties, since it negatively modulates insulin secretion from endocrine pancreatic islets (16, 17), amylase release from exocrine pancreas (18), and acid secretion from parietal cells (19). Parasatistin (347–419) is another CGA-derived peptide located in the C-terminal domain of CGA that inhibits parathyroid cell secretion (20). In addition to the autocrine or paracrine role in hormone secretion of these CGA-derived peptides, we have shown recently that numerous peptides present as water-soluble components of bovine chromaffin granules and released during secretion display antibacterial activity (7, 21–24).

Human CGA is a single polypeptide chain of 439 residues, with an apparent molecular mass of 70 kDa as estimated by SDS-polyacrylamide gel electrophoresis gel and a pI of 4.7–5.2. The amino acid sequence of human CGA (25, 26) indicates a real molecular mass of 48 kDa for the unmodified form of this protein. The difference between the apparent (70 kDa) and theoretical molecular mass (48 kDa) probably results from post-translational modifications (i.e. glycosylation, phosphorylation) (27, 28) and the abundance of acidic residues (25%).
which cause a slower migration during electrophoresis in the presence of sodium dodecyl sulfate (see Ref. 1, for review).

In 1997, using mild proteolysis, peptide separation, microsequencing, and mass analysis techniques, seven post-translational modification sites were detected in bovine CGA (29). Two glycosylation sites, each consisting of the triasarchide NeuAcα2-3Galβ1-3GalNAcα-1-O-linked to Ser186 and Thr231. The former residue is present in the antibacterial peptide named chromacin (22). Five phosphorylation sites were located on serine residues at positions Ser81, Ser207, Ser372, Ser376, and on Tyr173, this latter residue being the N-terminal amino acid of chromacin. Furthermore, studying the new antibacterial bovine CGA-derived peptides G- and PG-chromacin (CGA172–194), we demonstrated that the two post-translational modifications (Tyr173 and Ser186) are both necessary for the antibacterial activity of chromacin.

Since 1989, it is well known that chromogranins can serve as immunocytochemical markers for neuroendocrine tissues and as a diagnostic tool for neuroendocrine tumors (3, 4, 30–32). Because of their water-soluble properties, chromogranins and chromogranin-derived fragments are released together with catecholamines from adrenal chromaffin cells during stress situations and can be detected in the blood by radioimmunoassay techniques and enzyme assays (33–38). Previously, we have shown that large fragments of CGA are excreted into the urine in some patients with carcinoid tumors (38). The present paper deals with the determination of the phosphorylation and carbohydrate binding sites of a large natural C-terminal CGA-derived fragment, CGA79–439, present into the urine of these patients. The strategy consists in characterizing the primary structure of modified phosphorylated and O-glycosylated peptides, which were isolated after proteolytic cleavage of CGA79–439 with endoprotease Lys-C. Then, using successively separation by reverse phase HPLC, enzymatic modification of phosphorylated peptides, and complete analysis by sequencing and mass spectrometry (liquid chromatography/mass spectrometry and matrix-assisted laser desorption ionization time-of-flight), a detailed study was carried out. These post-translational modifications were located along the polypeptidic chain, compared with sequences of others species and discussed in relation with biological activity of natural CGA-derived fragments.

**EXPERIMENTAL PROCEDURES**

**Isolation of Excreted CGA-derived Fragments**

Urine was collected during a 24-h period from a patient with a histologically verified carcinoid tumor and multiple liver metastasis. The sample was collected, after informed consent, when the patient was on a clinical trial at the Endocrine Unit of Uppsala University Hospital. The study was also approved by the local Ethical Committee. The urine sample was first filtered through a 0.22-μm membrane and then concentrated about 100 times in a dialysis tube (Spectra/Por; cutoff value, 6–8 kDa). CGA-derived fragments were isolated in a one-step separation on an anion exchange column (Mono Q, FPLC; Amersham Pharmacia Biotech) using a linear gradient of 0.2 M ammonium acetate buffer at pH 6.0 to 1.0 M ammonium acetate buffer at pH 6.0 containing 1.0 M sodium chloride. Fractions containing CGA-derived fragments were concentrated on a Minicon concentrator (Amicon) and stored in −70 °C before further analysis.

**Purification of CGA-derived Peptides after Digestion with Endoprotease Lys-C**

CGA79–439 (2.5 nmol) was digested for 18 h at 37 °C with endoprotease Lys-C at a protein to enzyme weight ratio of 1000:1 in 100 mM Tris-HCl, pH 8.3. Generated peptides were then separated by HPLC, using the SMART system (Amersham Pharmacia Biotech) on a Machery-Nagel Nucleosil 300–5 C18 column (250 mm; particle size 5 μm) with a linear gradient, at a flow rate of 250 μl/min using a gradient of 0–80% solvent B in solvent A over 80 min. A major part of the eluent (90%) was analyzed by UV detection and an aliquot (10%) was measured by LC-MS. The mass spectrometer was calibrated under total ion current was recorded. The mass spectrometer was calibrated under total ion current was recorded.

**Mass Spectrometry Analysis**

Liquid Chromatography/Mass Spectrometry (LC/MS)—To isolate and characterize glycopeptides, we have performed LC/MS analysis of CGA-derived peptides obtained after endoproteinase Lys-C digestion of CGA. Then, CGA (500 pmol) was digested for 2 h at 37 °C with endoproteinase Lys-C at a protein-to-enzyme weight ratio of 1000:1 in 100 mM Tris-HCl, pH 8.3. Then, peptides were separated with an HPLC system (Applied Biosystems 410A Solvent Delivery System) equipped with a UV detector (UV Waters Detector 380) on a Narrowbore Macherey Nagel Nucleosil 300–5C18 column (2 × 150 mm). Absorbance was monitored at 214 nm, and the solvent system consisted of 0.1% trifluoroacetic acid/acetone (solvent A) and 0.1% trifluoroacetic acid/acetonitrile (solvent B). Elution was performed at a flow rate of 250 μl/min using a gradient of 0–80% solvent B in solvent A over 80 min. The output signal from the detector was digitized at a sampling rate of 250 samples per second.
MH in linear mode and 500 MHz in reflector mode using a 1-GHz digital oscilloscope (Lecroy model). The instrument control and data processing were accomplished with software supplied by Brucker using a Sun Sparc workstation. These studies were realized according to the procedure previously described (22).

Sequence Comparisons

Sequence alignment of bovine CGA sequences with corresponding fragments of CGA from different species was performed using the Cameleon sequence alignment program using default parameters (41). Chromogranin sequences were retrieved from the Swiss-Prot data base.

RESULTS

In this study, we have isolated a major fragment of human CGA corresponding predominantly to the domain CGA116–439 and a minor larger CGA-derived fragment CGA242–439, which are both excreted in urine of patients with carcinoid tumors (38). To determine phosphorylation and O-glycosylation sites included within tumoral CGA, the large C-terminal fragment was digested by endoprotease Lys-C and the generated fragments were separated by HPLC on a reverse phase C18 column (Fig. 1A). The different peaks of the chromatogram were directly submitted to automatic Edman degradation and mass spectra analysis to detect post-translational modifications. It is important to note that all the sequences determined in this study are in accordance with the primary structure proposed by Konecki et al. (26); in contrast, they diverge on 15 points (Fig. 2) from the primary structure reported by Helman et al. (25).

Identification of O-Glycosylation Sites

Carbohydrate analysis was performed using gas chromatography (22), and the carbohydrate content was evaluated to 5% (m/m, carbohydrate/protein): NeuAc, Gal, and GalNAc were detected in a molar ratio 1.2: 1.7: 1, suggesting short glycans with different structures.

Fragments resulting from endoprotease Lys-C digestion of CGA78–439 were analyzed by LC-MS to detect areas containing O-glycosylated peptides (Fig. 1B). The HPLC chromatogram (a), the single ion recording of specific ions characteristic of glycosylation sites (b), and the total ionic current of the chromatogram (c) are indicated. In b, the presence of glycans was recovered in peaks of regions I–IV (linedate in Fig. 1A), and each area was analyzed to characterize the O-glycosylated sites. Area I included peaks corresponding to free saccharides, since areas II–IV contained glycopeptides.

Structural Characterization of O-Glycosylated Peptides Contained in Area II Peaks—Sequencing of material included in the two major peaks (30, 31) eluting in region II (Fig. 1A) indicates the presence of peptides with N-terminal end located in position 145 and 124, respectively (Table I). MALDI-TOF MS analysis (negative mode) of peptide included in peak 31 reveals an experimental molecular mass of 2216 Da corresponding to the oxidized form of 124–144 (oxygenation of Met146; calculated molecular mass 2200 Da). MALDI-TOF MS analysis of material included in peak 30 (Fig. 3A) shows four different major molecular species with respective molecular masses of 3682, 3974, 4048, and 4264 Da (Table I), indicating the presence of several different glycans. By comparison with the expected molecular mass of CGA145–175 (3983 Da), these three values might be attributed to the O-glycosylated peptide CGA210–245 with the disaccharide Galβ1–3GalNAcα1, named antigen T (42), the trisaccharide NeuAcα2–3Galβ1–3GalNAcα1 (29), and the tetrasaccharide with an additional NeuAc linked in 2–6 on GalNAc (Fig. 3A) suggested previously for fetuin (43). To obtain confirmation of the structure of these glycans, material included in peak 30 was slot-blotted onto nitrocellulose sheet and immunodetected with a panel of three lectins (MAA, SNA, and PNA), the specificity of which was reported previously (22). Experimental data have shown unambiguously the simultaneous presence of the following linkages: Galβ1–3GalNAcα1-O (PNA), NeuAcα2–3Galβ (MAA), and NeuAcα2–6GalNAcα. Thus, our data revealed the presence of four O-glycosylated moities on the peptide CGA145–175, including complete forms with a tri- or a tetrasaccharide and two truncated glycans corresponding to disaccharides (Fig. 3A).

Primary structure of CGA145–175 (Fig. 3A) includes four potential O-glycosylated residues corresponding to Thr163, Thr165, Ser170, and Ser173. The sequence in the vicinity of the serine residues Ser170 and Ser173 (PPAS170LPS173QKYPGP) fits with the sequence patterns described by Wilson for O-glycosylation sites and characterized by high proline, serine, and threonine content (44). In addition, the presence of clusters of several closely spaced glycosylated residues was reported by these authors. In contrast, predictions of mucin type O-glycosylation sites in mammalian proteins, according to Hansen method (45) suggest the presence of glycans on residues Thr165, Ser170, and Ser173. To determine the two O-glycosylation sites we have submitted the peptide material included in peak 30 to a proteolytic digestion with endoprotease Glu-C and automatic Edman degradation. Sequencing of the derived peptides indicates the presence of two unmodified serine residues Ser170 and Ser173, while threonine residues Thr163 and Thr165 were undetected. Furthermore, the cleavage of the fragment CGA145–175 by endoprotease Glu-C was very weak, indicating that the glycans are located in the vicinity of the cleavage point Glu161. In contrast, the cleavage by endoprotease Lys-C of the peptide linkage Lys175-Tyr176 is achieved with a good yield, suggesting that polysaccharide chains are distant. Then, we proposed that two glycans are linked on residues Thr163 and Thr165.

Structural Characterization of O-Glycosylated Peptides Contained in Area III Peaks—Sequencing of material included in peak 50 eluting in region III (Fig. 1A) indicates the presence of a CGA-derived peptide beginning at position 210 (Table I). To characterize its primary structure, this peptide was subjected to MALDI-TOF MS analysis (negative mode, Fig. 3B). The mass spectra shows three molecular masses of 4345, 4636, and 4928 Da (Table I). By comparison with the expected molecular mass of CGA210–245 (3983 Da), these three values might be attributed to the O-glycosylated peptide CGA210–245 with the disaccharide Galβ1–3GalNAcα1, named antigen T (42), the trisaccharide NeuAcα2–3Galβ1–3GalNAcα1 (29), and the tetrasaccharide with an additional NeuAc linked in 2–6 on GalNAc (43). Primary structure of CGA210–245 (Fig. 3B) includes two potential O-glycosylated residues Thr233 and Ser241 (PSEE'GPT233VVLNPHPS241LYKEI); Wilson and Hansen predictions (44, 45) indicated that threonine residue Thr233 is likely to be glycosylated, whereas serine residue Ser241 is unmodified.

Structural Characterization of O-Glycosylated Peptides Contained in Area IV Peaks—Sequencing of material included in peak 63 eluting in region IV (Fig. 1A) indicates the presence of CGA-derived peptide beginning at position 198 (Table I), and MALDI-TOF MS analysis gives three different molecular masses of 5497, 6019, and 6212 Da (Fig. 3C). By comparison with the expected molecular mass of CGA198–245 (5264 Da), the experimental mass of 6212 Da corresponded to the peptide CGA198–245 O-glycosylated with the tetrasaccharide similar to...
the glycan present in the glycopeptide CGA_{145-175}. The molecular mass of 5497 Da might be attributed to the di-oxidized form (oxidation of Trp^{205} and Tyr^{243}) of the O-glycosylated peptide CGA_{198-245} with GalNAc_{a1} corresponding to antigen Tn (42). In addition, the experimental molecular mass of 6019 Da corresponds to the mono-oxidized O-glycosylated peptide CGA_{198-245} with the trisaccharide NeuAc_{a2-3}Gal_{b1-3}GalNAc_{a1}, including an additional molecular mass of 80 Da.

As described above for the CGA_{210-245} glycopeptide, we propose the O-glycosylated site to be located on residue Thr^{233}. To
verify the location of the O-glycosylation site, a mixture of CGA209/210–245 peptides has been treated with endoproteinase Glu-C.

Digestion of CGA209/210–245 with Endoproteinase Glu-C—After digestion, the generated CGA-derived peptides were isolated by HPLC (Fig. 4A). Three peaks were isolated, sequenced, and submitted to MALDI-TOF MS analysis. Material included in peak 1 contained a mixture of unmodified CGA225/228/230–245, while material included in peak 3 contained nondigested CGA209/210–245. Sequencing of peptidic material included in peak 2 shows the presence of two CGA-derived peptides with the N-terminal ends located at residues 219 and 221 (Table II). The six peaks obtained by MALDI-TOF MS (Fig. 4B) might be attributed to different states of O-glycosylated CGA221–245 (2985, 3276, and 3567 Da) and CGA219–245 (3185, 3476, and 3766 Da) (Table II). By comparison with the expected molecular masses of CGA219–245 (2821 Da) and CGA221–245 (2821 Da), the experimental values of 2985 and 3185 Da correspond to the disaccharide Galβ1–3GalNAcα1, whereas 3276 and 3476 Da fit with the trisaccharide NeuAcα2–3Galβ1–3GalNAcα1, and the last values, 3567 and 3766 Da, correspond to the tetrasaccharide with an additional NeuAc linked in 2–6 on GalNAcα (43). In addition, we have observed that endoproteinase Glu-C is unactive toward the linkage Glu230-Glu231, in contrast to endoproteinase Lys-C, which cleaves the large CGA-derived fragment CGA79–439 after the lysine residue Lys245, which suggests that glycans are located close to Glu230–Glu231. Thus, we propose that threonine Thr233 is glycosylated.

At this stage, we have determined three O-linked carbohydrate attachment sites on residues Thr163, Thr165, and Thr233 located in the middle part of the whole protein (Fig. 5). It is important to note that a fraction (25%) of carcinoid CGA was not completely O-glycosylated since unmodified forms CGA145–175 and CGA210–245 were recovered in peaks 38 and 59, respectively (Fig. 1A).

Identification of Phosphorylation Sites

Structural analysis by sequencing and MALDI-TOF MS of each HPLC peak (Fig. 1A; Table I) indicates the presence of...
MALDI-TOF mass spectrometry of the modified glycopeptides. A, the glycopeptide included in peak 30 (Fig. 1A) has been identified as CGA_{145-175}. B, the glycopeptide included in peak 50 (Fig. 1A) has been identified as CGA_{210-245}. C, glycopeptide included in peak 63 (Fig. 1A) corresponds to CGA_{198-245}. "O"-glycosylated threonine; \( \ominus \), GalNAc; inverted pentagon, Gal; \( \triangle \), NeuAc.
modifications with a molecular mass of 80 in peaks 37 and 63. Sequencing of peptides included in peak 37 shows fragments beginning at positions 246, 250, 277, and 304. The experimental molecular mass of 1611 and 3317 Da might be attributed to phosphorylated or sulfated forms of CGA246–277 (Fig. 6B). After alkaline phosphatase treatment we observed the removal of a mass of 80 Da on each peptide, confirming the presence of a phosphate group on either Ser252 or Ser254 and Ser304 or Ser315. To characterize the exact location of the two phosphorylated residues, peptidic material included in peak 37 was digested by trypsin and analyzed by MALDI-TOF. The two modified serine residues were identified as Ser252 and Ser315 (data not shown).

Sequence and mass spectra analysis of material included in peak 63 show the presence of an additional increment of 80 Da in the sequence CGA198–245 (Table I). As this modification is not present in CGA210–245, the location of the phosphorylated residue is likely to be in the region CGA198–209. A unique potential phosphorylated residue is Ser200 (Fig. 2).

In conclusion, we have identified three phosphorylated sites along the polypeptidic fragment CGA79–439 located on residues Ser200, Ser252, and Ser315 (Fig. 5).
been proved as a useful tool in the diagnosis of peptide-producing endocrine neoplasms. Recent investigation has shown that the 24-h urine measurement of catecholamine and their related metabolites, together with serum CGA and CGB values, are proportional to pheochromocytoma mass and provide reliable diagnosis markers (55).

Knowledge of CGA primary structure from tumoral tissue should provide information with regards to the use of this protein as a tumoral marker (32). In the present paper we report for the first time the characterization of six post-translational modifications of a large CGA fragment present in the urine of patients with carcinoid tumors. Three phosphorylated serine residues were identified as Ser200, Ser252, and Ser315 and three O-glycosylation sites were found on residues Thr163, Thr165, and Thr233. In a recent work, we have identified five phosphorylated residues on the polypeptidic chain of bovine CGA located on residues Ser81, Tyr173, Ser307, Ser372, and Ser376 (29). Comparison of human, bovine (56), pig (57), mouse (58), and rat (59) sequences in the vicinity of post-translational modified residues is shown in Fig. 7. Although regions are highly conserved, particularly those bearing phosphorylation modifications (Ser372, Ser376, and Ser376 in bovine sequence), differences can be pointed out. The two phosphorylated residues Ser200 and Ser315 are strictly conserved and included in homologous sequences with the typical pattern E-K/R-G-L/P-S 200-A-G/Y-E/R-Q- and E/Q-E/Q-E-E-R/Q- L-S315-R/K/E-E-W-E-D/N. In contrast, Ser252 is included in a nonconservative sequence. These differences may however result from the carcinoid nature of CGA, the modifications on the human normal protein yet remain to be studied. In addition, Ser315 in human protein, which corresponds to Ser370 in bovine sequence, is also phosphorylated. The other residues Ser80, Tyr176, Ser380, and Ser384 in human sequence are not modified. In contrast, the phosphorylated residue Ser200 in the human sequence is located within a conservative region, but the corresponding residue in bovine sequence is not phosphorylated. The recent characterization of the phosphorylated sites of bovine CGA has established the presence of a phosphorylated tyrosine residue Tyr173 (22). Tyrosine phosphorylation is not a common post-translational modification, since it represents only 0.03% of the phosphorylated amino acids in normal cells (60). The significance of this tyrosine phosphorylation is not known yet, although we have reported recently that chromacin, the CGA-derived peptide 173–194, displays antibacterial activity when the N-terminal fragment corresponding to residues 1–78 and 1–115 and with a synthetic peptide corresponding to the domain CA 1–78 (52). Furthermore, it is established that within the brain CGA is localized in neurodegenerative areas associated with reactive microglia, and it was found that both recombinant human CGA and natural bovine CGA were able to induce an activated phenotype in rat microglial cells maintained in primary culture (53). More recently, it was shown that the recombinant human N-terminal fragment CA 1–78 stimulated microglial cells in primary culture to secrete heat-stable diffusible neurotoxic agents (54). Furthermore, antibodies against chromogranins have been widely used for immunohistochemical staining of endocrine tissues and tumors of neuroendocrine origin, i.e. in pancreatic tumors, pheochromocytoma, midgut carcinoid tumors, prostate, etc. Antibodies have also been raised to develop specific radioimmunooassays for plasma measurements of the different chromogranins. Measurement of CGA level in plasma has

### DISCUSSION

Chromogranins A and B are present in multiple secretory cell types of numerous species within the animal kingdom (1, 46–49). On the basis of secondary and tertiary structures predicted from its sequence, CGA possesses a “random coil” structure (1). In addition, according to Kyte and Doolittle predictions (50), this protein is very hydrophilic throughout the length of its polypeptidic chain (6). Besides the 70-kDa molecular species, several observations have reported the presence of a molecular mass 80–90 kDa diffuse form of chromogranin A immunoreactivity, as full-length chromogranin A-core proteoglycan, in secretory granules from bovine adrenal medulla and from PC12 cells (51). Multiple neuroendocrine sources other than the adrenal medulla appear to contribute to the high basal circulating CGA concentration in man (1). The widespread occurrence of CGA is indicative of some important biological functions for this protein. Despite the fact that CGA has been largely studied since its discovery thirty years ago, the characterization of these functions is still an open question. However, in addition to the autocrine or paracrine role in hormone secretion of CGA-derived peptides, our recent work has shown that numerous peptides with autocrine or paracrine role in hormone secretion of CGA-derived fragments can inhibit fibroblast adhesion (52). In contrast, derived fragments corresponding to residues 1–78 and 1–115 and with a synthetic peptide corresponding to the domain CA 7–57 (52). Furthermore, antibodies against diffusible neurotoxic agents (54). Furthermore, antibodies against chromogranins have been widely used for immunohistochemical staining of endocrine tissues and tumors of neuroendocrine origin, i.e. in pancreatic tumors, pheochromocytoma, midgut carcinoid tumors, prostate, etc. Antibodies have also been raised to develop specific radioimmunooassays for plasma measurements of the different chromogranins. Measurement of CGA level in plasma has

### TABLE II

**Structural characterization of human CGA-derived glycopeptide CA 219–245 generated after endoproteinase Glu-C digestion of CA 210–245**

| Peak number | N-terminal sequence | Experimental molecular mass | Location | Calculated molecular mass |
|-------------|---------------------|----------------------------|----------|---------------------------|
| 2           | AGEAVPVEEGP'TTVLN   | 2885                       | 221–245  | 2621                      |
|             |                     | 3276                       |          |                           |
|             |                     | 3567                       |          |                           |
|             |                     | 3185                       | 219–245  | 2821                      |
|             |                     | 3476                       |          |                           |
|             |                     | 3766                       |          |                           |

![Fig. 5. Location of the post-translational modifications present in the natural C-terminal fragment CA 79–439 isolated from urine of patients with carcinoid tumors.](attachment:image)

| Amino Acid Composition | T | T | S | T | T | S | S |
|------------------------|---|---|---|---|---|---|---|
| G                      | G | G | P | G | P | P | P |

![Fig. 5. Location of the post-translational modifications present in the natural C-terminal fragment CA 79–439 isolated from urine of patients with carcinoid tumors.](attachment:image)
during the transfer of CGA\textsubscript{79–439} from chromaffin and entero-
chromaffin cells to plasma and urine. Along this line, processing of CGA
has been shown to be tissue-specific (62), and the carcinoïd nature of the biological material may induce variation in protein processing as compared with normal tissues (62, 63).

In the present study we have identified three \textit{O}-glycosylation sites located on Thr\textsubscript{163}, Thr\textsubscript{165}, and Thr\textsubscript{233}. The presence of the three glycans (Fig. 5) on human CGA\textsubscript{79–439} gives rise to a calculated sugar/protein ratio of 5\% in accordance to the sugar experimental content that we have obtained. The attachment \textit{O}-glycosylation sites Thr\textsubscript{163} and Thr\textsubscript{165} correspond to proline-
rich sequences. However, they are not used as such in bovine sequence, and it is not yet known whether they are genuine glycosylation sites in CGAs from rat, mouse, and pig. Therefore, it is probable that post-translational modifications in the human carcinoïd CGA are not representative of the modifications present on the non-carcinoïd normal protein. In contrast, the residue Thr\textsubscript{233} is present in bovine, human, mouse, and rat CGAs. In pig this threonine is changed into a serine residue, which might well represent a glycosylation site. With regard to the Wilson consensus sequence (44), the proline residues are present upstream and downstream in the bovine, human, and pig sequences but are scarce in rat and mouse CGAs. Concerning the \textit{O}-glycosylation sites included on bovine adrenal med-
ullary CGA, we had determined previously that an unique carbohydrate moiety composed of the trisaccharide NeuAc\textsubscript{2}–3Gal\textsubscript{b}1–3GalNAc\textsubscript{a}1 was found to be located on Ser\textsubscript{186} and Thr\textsubscript{231} (29). By comparison with the present study it appears that the glycosylation on Thr\textsubscript{233} is a conservative process, whereas the glycosylation located on residues Thr\textsubscript{163} and Thr\textsubscript{165} might be specific to the human protein or to the carcinoïd protein. The identification of the disaccharide Gal\textsubscript{b}1–3GalNAc\textsubscript{a}1 and the monosaccharide GalNAc\textsubscript{a}1, corresponding to the antigens T and Tn, respectively, is in agreement with the human adenocarcinoïd source (42).

Concerning the \textit{O}-glycosylation and phosphorylation modifications, we have reported recently that they are necessary for the full antibacterial activity of chromacin peptides. The natural CGA-derived antibacterial peptide, pro-chromacin CGA\textsubscript{79–431} contains these seven modifications. The antibacte-
rial activity of chromacin CGA\textsubscript{173–194} is correlated with the presence of \textit{O}-glycosylation modification on Ser\textsubscript{186} or/and phos-
phorylation on Tyr\textsubscript{173} (22). In the present study we have estab-
ishled that these two modifications are missing in the human CGA sequence homologous to bovine chromacin. However, this absence may be related to the carcinoïd nature of CGA, and these modifications may be well present in the protein expressed in normal human tissues.

The three post-translational glycosylations of CGA probably have a structurally related function. As recently the most antigenic sites of recombinant human CGA have been characterized (64), it seems interesting to correlate their location with regard to the modified residues. The modified residues Thr\textsubscript{233}, Ser\textsubscript{252} and Ser\textsubscript{315} appear to be preferentially located into or near domains with high antigenic feature 222–230 and 315–330, whereas three modified residues in position 163, 165, and 200 are located in the 163–210 region described to possess low antigenicity. These results indicate that the two glycosylated residues clustered in the N-terminal moiety of the natural excreted CGA (Thr\textsubscript{163} and Thr\textsubscript{165}) may induce important structural modifications of the protein. For instance, these two moi-
eties may alter proteolytic cleavage of CGA in tumoral tissues and explain the recovery of the large CGA-derived fragment in
The urine of patients, whereas it is not detectable in normal fluids. In addition, it is important to discuss our present data in relation with a new and accurate sandwich immunoassay (32). To develop this assay, 24 monoclonal antibodies were raised against human CGA. The better combination was that involving two monoclonal antibodies directed against contiguous epitopes located in the mid CGA145–245 domain. Therefore, taking into account the location on Thr163, Thr165, and Thr233 of O-glycosylation sites, it is clear that these glycans prevent the natural proteolytic degradation of this central domain; the quantification of this central domain allows the accurate determination of circulating CGA level (32). This mid-molecule fragment has been shown to be the major circulating constituent in case of renal dysfunction, kidney representing the site where CGA immunoreactivity is removed or destroyed in normal subjects (65). Some years ago, one of us (38) determined four natural proteolytic cleavage sites of human CGA from carcinoid (38). Among them, the two major cleavage sites correspond to dibasic amino acid sites (115–116 and 209–210), whereas the two minor are located in position Asp-Pro (272–273) and Arg-Gly (394–395). These four proteolytic cleavage sites have also been described earlier for CGA isolated from neuroendocrine tumors (51, 62, 63, 66–68), and it has thus been suggested that this natural degradation occurs in tumors or serum but not in urine.

Mid-molecule fragments of CGA are recovered in the urine of patients with carcinoid tumors, whereas CGA is not detectable in the urine of normal subjects; nevertheless, plasma measurement of CGA has been shown to be a more reliable diagnostic marker than the corresponding urine analysis, since CGA was present in urine of one-third of patients (69). The presence of CGA in urine may result from pharmacological treatment (69), dysfunction (65), or structural abnormalities. The characterization of specific short carbohydrate structures (antigens T and Tn) from carcinoid CGA allow us to envisage the development of new tools, including MALDI-TOF or carbohydrate dot blotting, to detect and measure these CGA forms specific to tumors. Subsequently, these tools may also be used to establish corre-
lation between structural modifications and pathological states, including stress.

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