Phosphorylation and O-Glycosylation Sites of Bovine Chromogranin A from Adrenal Medullary Chromaffin Granules and Their Relationship with Biological Activities*

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Bovine adrenal medullary chromogranin A, the major soluble component of chromaffin granulas, is a phosphorylated glycoprotein. In the present work, phosphorylation and glycosylation sites were determined using mild proteolysis, peptide separation, microsequencing, and mass analysis by electrospray and matrix-assisted laser desorption ionization time-of-flight techniques. Seven post-translational modification sites were detected. Two O-linked glycosylation sites, each consisting of the trisaccharide NeuAcα2-3Galβ1-3GalNAcα1, were located in the middle part of the protein, on Ser186 and on Thr231. The former residue is present in the antibacterial peptide named chromacin. Four phosphorylation sites were located on serine residues at positions Ser81 in the N-terminal region of the protein and Ser307, Ser372, and Ser376 in the C-terminal end. One additional phosphorylation site was found on the tyrosine residue at position Tyr172, the N-terminal amino acid of chromacin. With the exception of the phosphorylation on Tyr172, all of the other post-translational modifications are located on highly conserved chromogranin A regions, implying some biological importance.

In bovine adrenal medulla, secretory granules from chromaffin cells contain a complex mixture of secretory products, which include low molecular mass constituents such as catecholamines, ascorbate, nucleotides, calcium, enkephalins, and several water-soluble proteins. Among the latter, chromogranins/secretogranins, a family of acidic secretory proteins, have been extensively studied. CGA is the major member (40% of total soluble granule proteins) of this family, and these glycoproteins are expressed in a large number of endocrine and neuroendocrine cells and in neurons (1–4). In addition to the autocrine or paracrine role in hormone secretion of these CGA-derived peptides, we have hypothesized that CGA itself can also play multiple biological roles. 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 that are actively processed into peptides within the secretory granules (for reviews, see Refs. 1 and 5).

Recently, we reported a detailed study of the intracellular and extracellular processing of CGA and chromogranin B in bovine chromaffin granules from intact gland and from cultured chromaffin cells (6, 7).

The proteolytic processing of CGA is a topic of growing interest, since biological activities have been attributed to specific domains located along its sequence. 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 (8); a homologous peptide, betagranin, corresponding to the sequence 1–115 has been isolated from rat pancreas, but its function has not yet been defined (9). Vasostatin I is a peptide containing the N-terminal sequence 1–76/113 (10) and has been found to exhibit vasoinhibitory activity of isolated human blood vessels (11, 12). As early as 1988, it was established that CGA is the precursor of a peptide that inhibits the secretory activity of chromaffin cells (13). In addition, pancreastatin (248–293) is a peptide with multiple properties, since it negatively modulates insulin secretion from endocrine pancreatic islets (14, 15), amylase release from exocrine pancreas (16), and acid secretion from pial cells (17). Another CGA-derived peptide, located in the C-terminal domain of CGA, parastatin (347–419), inhibits parathyroid cell secretion (18). In addition to the autocrine or paracrine role in hormone secretion of these CGA-derived peptides, we have recently shown that the sequence 201–211 is a major domain found in the domain of CGA-derived peptides. For several antibacterial peptides derived from CGA and enkephalin-A, structural features and more particularly post-translational modifications have been directly related to biological activity (20, 21).

CGA is a single polypeptide chain of 431 residues, with an apparent molecular mass of 70 kDa as estimated by SDS-polyacrylamide gel electrophoresis and a pI of 4.7–5.2 (6). The amino acid sequence of bovine CGA (23, 24) indicates a real molecular mass of 48 kDa for the unmodified form of this peptide. This paper is available on line at http://www-jbc.stanford.edu/jbc/
Post-translational Modifications of Bovine Chromogranin A

The sequence of purified CGA-derived peptides was determined in our laboratory by automatic Edman degradation on an Applied Biosystems 473 A microsequencer. Samples (100 pmol) were loaded onto polybrene-treated and precycled glass fiber filters (6). To identify phosphorylated residues, samples were modified with ethanethiol according to the method previously described (32). Before sequencing, reagents were removed, using the ProSorb® sample preparation cartridge (Applied Biosystems, division of Perkin-Elmer).

Mass Spectrometry Analysis

ES/MS—ES/MS analysis was done on a VG Bio-Q quadrupole mass spectrometer (Fisons Bio-Q; VG Bio-Tech) with a mass range of 4000 Da and operating in the positive ion mode (33). The peptide was dissolved in water/acetonitrile/acetic acid (49/50/1; v/v/v) at a concentration of about 2–5 pmol/μl. Aliquots (10 μl) were introduced into the ion source at a flow rate of 4 μl/min. Scanning was usually performed from m/z 500 to m/z 1500 in 10 with the resolution adjusted so that the peak at m/z = 998 from heart myoglobin was 1.5–2 wide on the base. Calibration was performed using the multiply charged ions produced by a separate introduction of horse heart myoglobin (16950.4 Da).

LC/MS—In order 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-proteinase weight ratio of 1000:1 in 100 m M Tris-HCl, pH 8.3. The peptides were separated with an HPLC system (Applied Biosystems 140 A solvent delivery system) equipped with a UV detector (UV Waters detector 386) 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/water (solvent A) and 0.1% trifluoroacetic acid/acetonitrile (solvent B). Material was eluted at a flow rate of 250 μl with a gradient of 0–80% B in 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 (34). The mass spectrometer was calibrated under conditions using a mixture of polyethylene glycols (average masses 400 and 2000 Da). Spectra were scanned over m/z 320–1800 for 6 s, and the total ion current was recorded.

MALDI-TOF Mass Spectrometry—This mass spectrometry analysis was carried out on a Brucker BIFLEX® matrix-assisted laser time-of-flight mass spectrometer equipped with the Scout™ high resolution optics with an X-Y multisample probe, a gridless reflector, and the HIMAS™ linear detector. This instrument has a maximum accelerating potential of 30 kV and may be operated either in the linear or reflector mode. Ionization was accomplished with a 337-nm beam from a nitrogen laser with a repetition rate of 3 Hz. The output signal from the detector was digitized at a sampling rate of 250 MHz 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 (21).

Sequence Comparisons

Sequence alignment of bovine CGA sequences with corresponding fragments of CGA from different species was performed using the Clustal V multiple sequence alignment program (35). Chromogranin sequences were retrieved from the Swiss-Prot data base.

RESULTS

In order to determine phosphorylation and glycosylation sites included within the bovine CGA sequence, the protein was purified according to the procedure previously described (21) and digested by endoproteinase Lys-C (see “Experimental Procedures”). The generated fragments were separated by HPLC on a reverse-phase C18 column. Our purpose was to identify peaks eluting at different times but containing peptides sharing identical peptidic sequences. The various elution times of these peptides indicated differences due to post-translational modifications. Taking into account these parameters, the chromatogram was divided into five regions from I to V (Fig. 1).

EXPERIMENTAL PROCEDURES

Purification of CGA- and CGA-derived Peptides

Secretory granules were isolated from bovine adrenal medulla (30), and soluble proteins were separated from membranes after osmotic shock-induced lysis and high speed centrifugation (31). CGA was purified by reverse phase HPLC on a Macherey Nagel Nucleosil 300–5C18 column (4 × 250 mm; particle size 5 mm and pore size 100 nm) with the Applied Biosystems HPLC system 140 B as described previously (7). Then CGA (10 nmol) was digested for 2 h at 37 °C with endoproteinase Lys-C at a protein:proteinase weight ratio of 1000:1 in 100 mM Tris-HCl, pH 8.3. Generated peptides were then separated on a Macherey Nagel 300–5C18 column. Absorbance was monitored at 214 nm, and the solvent system consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid, 30% water, 69.9% acetonitrile (solvent B). Material was eluted at a flow rate of 0.7 ml/min using, successively, a gradient of 0–25% B in A over 10 min followed by a gradient of 25–75% over 50 min. Each peak fraction was manually collected and concentrated by evaporation, but not to dryness.

The sequence between the apparent (70 kDa) and theoretical molecular mass (48 kDa) probably results from post-translational modifications (i.e. glycosylation, phosphorylation, and sulfation) and the abundance of acidic residues (25%) that cause a slower migration during electrophoresis in the presence of sodium dodecyl sulfate (for a review, see Ref. 5). Previously, CGA has been described as a glycoprotein containing 5.4% carbohydrate (mass/mass), consisting of small glycan moieties (25); consensus sequences for N-glycosylation (NX(S/T)) are, however, not present in the primary structure (26). Furthermore, CGA has been described to be a phosphoprotein, with a ratio of five phosphorylated serine residues per protein molecule (27). There is a modest incorporation of sulfate into the protein. The carbohydrate content of CGA is relatively low, consisting of characterizing the structure of modified phosphorylated and O-glycosylated peptides that were isolated after proteolytic cleavage of CGA with endoproteinase Lys-C. We have performed a detailed study using separation by reverse phase HPLC, chemical modification of phosphorylated peptides, and complete analysis by sequencing and mass spectrometry using electrospray mass spectrometry (ES/MS), liquid chromatography/mass spectrometry (LC/MS), and matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry. Bovine CGA contains five phosphorylated residues located in four endoproteinase Lys-C-generated peptides (CGA-(173–194), CGA-(297–314), CGA-(78/79–109), and CGA-(331–420/421)) and two polysaccharide attachment sites linked to serine and threonine residues located within the CGA-(173–186) and CGA-(222–243) regions, respectively. The location of these sugar- and phosphate-rich peptides in the whole protein is specified in relation to the primary structure of bovine CGA. Sequence alignment of these modified peptides with CGA fragments of different species has led us to discuss these results in relation with phylogenetic features and specific physiological processing.

The sequence of purified CGA-derived peptides was determined in our laboratory by automatic Edman degradation on an Applied Biosystems 473 A microsequencer. Samples (100 pmol) were loaded onto polybrene-treated and precycled glass fiber filters (6). To identify phosphorylated residues, samples were modified with ethanethiol according to the method previously described (32). Before sequencing, reagents were removed, using the ProSorb® sample preparation cartridge (Applied Biosystems, division of Perkin-Elmer).
Identification of Phosphorylation Sites on CGA Protein

Structural Characterization of Phosphorylated Peptides Present in Area I Peaks—The four peaks present in region I corresponding to chromacin-derived peptides previously characterized as CGA-(173–194) (YPGPQAKEDSEGPSQGPASREK) (21). The N-terminal Tyr173 has already been identified as one of the phosphorylation sites (21).

Structural Characterization of Phosphorylated Peptides Present in Area III Peaks—After automatic Edman degradation of peptide material recovered in peaks 1 and 2 of region III, we identified a major fragment with the sequence SGEPEQEEQLSKEWEDAK that corresponds to CGA-(297–314). To determine the post-translational modification of the more polar peptide (peak 1), comparative mass spectra analysis was performed by ES/MS (Fig. 2). Using the MacPro Mass program, the theoretical molecular mass of this peptide was estimated to be 2118.8 Da, corresponding to the experimental mass of the peptide recovered in peak 2. In contrast, the molecular mass of peptides in peaks 1 and 2 was evaluated to be 2198.8 Da, showing an additional mass of 80 Da. CGA fragment 78–109 included seven potential phosphorylation sites (Ser80, Ser81, Tyr82, Ser87, Thr103, Ser107, and Ser108).

Upon treatment with alkaline phosphatase, peptide 1 lost 80 Da, confirming the presence of a phosphate group. Location of the phosphorylated residue was determined after ethanethiol derivatization and microsequencing; a phenylthiohydantoin-ethylcysteine was identified in position 81.

Structural Characterization of Phosphorylated Peptides Present in Area IV Peaks—Sequencing and mass spectrometry analysis of the material in the peaks of region IV revealed two groups of two peptides. After sequencing, the four peptides (numbered 1–4; Fig. 1) were identified as CGA fragments corresponding, respectively, to CGA-(78–109) (KHSSYEDELSEVLEKPNDQAEPKEVTEEVSSK) (peaks 1 and 3) and CGA-(79–109) (HSSYEDELSEVLEKPNDQAEPKEVTEEVSSK) (peaks 2 and 4). To characterize the structural differences between the peptides in peaks 1 and 3 and those in peaks 2 and 4, they were analyzed by mass spectrometry (Fig. 3). The molecular mass of fragments 78–109 and 79–109 was calculated to be 3662.9 and 3534.7 Da, respectively. Experimental molecular masses of peptides in peaks 2 and 4 fitted with these values, indicating that these were unmodified peptides. In contrast, the molecular mass of peptides in peaks 1 and 3 were evaluated to be 3741.63 Da and 3613.50 Da, showing an additional mass of 80 Da. CGA fragment 78–109 included seven potential phosphorylation sites (Ser80, Ser81, Tyr82, Ser87, Thr103, Ser107, and Ser108).

Structural Characterization of Phosphorylated Peptides Contained in Area V Peaks—The first 23 amino acids of the major peptide (>90%) recovered in region V were sequenced as RLEGGEEEEEEDPRSMRLSFRAR.
the CGA fragment beginning at position 331 and results from a cleavage of the Lys 330-Arg331 residues. Mass spectra analysis showed the presence of four molecular species with molecular masses of 2741.26, 7859.43, 10578.03, and 10677.50 Da (Fig. 4). The lower molecular mass may be assigned to the fragment 332–354, with a theoretical molecular mass of 2738.90 Da. We then focused on the higher molecular mass components. The calculated mass difference between 10677.50 and 10578.03 Da suggested the presence of a valine residue (99 Da) at the C-terminal end of the peptide. Taking into account the N-terminal sequence, the molecular mass, and the presence of C-terminal valine, we speculated that the CGA fragments 331–420/421 with calculated molecular masses of 10418.3 and 10517.5 Da probably correspond to the unmodified peptides eluting in peaks of region V. Comparing the experimental masses (10578.03 and 10677.50 Da) with theoretical values, a difference of 160 Da was obtained, suggesting the presence of two phosphorylated residues. Indeed, this fragment CGA-(331–420/421) possesses nine potential phosphorylation sites Ser345, Ser349, Tyr355, Ser372, Ser376, Tyr388, Ser398, Ser410, and Ser412. To characterize the two sites with post-translational modifications, a mixture of peptides 331–420/421 was treated with alkaline phosphatase. After this treatment, mass spectra analysis revealed the presence of two peptides with respective molecular masses of 10418.30 Da and 10517.50 Da instead of 10578.03 and 10677.50 Da. This observed mass loss of 160 Da confirmed the presence of two phosphate groups on both peptides.

After tryptic digestion of the peptides 331–420 and 331–421, the two phosphorylated residues were identified. The generated peptides were separated by HPLC (Fig. 5), and their primary structure was determined by microsequencing and MALDI-TOF analysis (Fig. 5B). By comparing the experimental and calculated molecular mass of peptide CGA-(367–386) (2430.2 versus 2267.5 Da), the addition of 160 Da indicated the phosphorylated residues on this peptide. After derivatization of the GWRPNSREDSEVLEAGLPVR peptide with ethanethiol, it was unambiguously demonstrated that the phosphorylated residues were located in positions Ser372 and Ser376.

Thus, the peptide with the experimental molecular mass of 7859.43 Da (Fig. 4A) probably corresponds to the bisphosphorylated CGA fragment 357–421 (calculated molecular mass 7698.40 Da).

Location of the Five Phosphorylated Residues of Bovine CGA along the Polypeptide Chain

The location of the observed phosphorylated residues of CGA along the CGA backbone are represented in Fig. 6. The present data indicate that (i) four of the phosphorylated residues are located on serines at positions Ser81, Ser307, Ser372, and Ser376; (ii) two of those serine residues at positions Ser311 and Ser317 belong to glutamic acid-rich sequences (SSYEDELESEVLE and EPEQEEQLSKEWE), (iii) one phosphorylation site is present on a tyrosine residue at position Tyr173; (iv) this phosphorylated Tyr173 is included in the proline-rich sequence LPSPKY177PGPQAKDSEGPGSQAQP, (v) as far as can be determined with the technology used in the present work, there are no more than five phosphorylation sites per bovine CGA molecule.
Identification of O-Glycosylation Sites

From structural properties of unmodified and modified CGA-(173–194) chromacin peptide, we have previously shown that bovine CGA contains at least one trisaccharide moiety with the NeuAc<sub>a</sub>2–3Gal<sub>b</sub>1–3GalNAc<sub>a</sub>1 sugar sequence (21). G- and PG-chromacin (glycosylated and phosphorylated/glycosylated chromacin) are O-glycosylated peptides in which Ser 186 is the residue to which the sugars are linked.

To further characterize other glycosylated fragments present on CGA, the protein was digested with endoproteinase Lys-C and analyzed by LC/MS.

LC/MS Analysis of Endoproteinase Lys-C-generated Fragments—In Fig. 7, the HPLC chromatogram (A), the single ion recording (SIR) of specific ions characteristic of glycosylation sites (B), and the total ionic current (TIC) of the chromatogram (C) are shown. In B, the presence of O-glycosylated peptides were recovered in peaks of regions I and II (Fig. 1). Analysis of region I has been previously reported with the characterization of G- and PG-chromacin (21).

Structural Characterization of O-Glycosylated Peptides Contained in Area II Peaks—Sequencing of material included in the three peaks eluting in region II indicates the presence of a peptide corresponding to CGA-(222–243); a component recovered in B3 and B4, with an experimental molecular mass of 7859.43 Da was identified as modified CGA-(354–421); a component recovered in D8–D14, with an experimental molecular mass of 10677.50 Da was identified as modified CGA-(331–421). B, primary structure of modified CGA-derived fragments 331–420/421; S*, phosphorylated serine residues.

Location of the Two O-Glycosylation Sites of Bovine CGA within the Polypeptidic Chain

A schematic representation of bovine CGA showing the two O-linked carbohydrate attachment sites Ser<sup>186</sup> and Thr<sup>231</sup> located in the middle part of the whole protein is given in Fig. 6. The sequence in the vicinity of the serine residue Ser<sup>186</sup> (PSQGP) fits with the two characteristic sequence patterns described by Wilson (36) for O-glycosylation sites. In contrast, the fragment 222–243 around threonine residue Thr<sup>231</sup> is a proline-rich sequence (40%) but lacks of the Wilson consensus pattern.

DISCUSSION

Chromogranins A and B occur in multiple secretory cell types of numerous species within the animal kingdom (1, 37–40). 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 roles for this protein. Despite the fact that CGA has been widely studied since its discovery 30 years ago, the characterization of possible functions is still an open question.

On the basis of secondary and tertiary structures predicted from its primary structure, CGA possesses a “random coil” structure (1). In addition, according to Kyte and Doolittle predictions (41), this protein is very hydrophilic throughout the length of its polypeptide (6). This is in accordance with its biochemical properties, and in particular with the observation that CGA remains soluble after boiling for several minutes (29). Concerning the post-translational modifications, CGA has previously been described to be a glycoprophosphoprotein containing small O-glycosidically linked carbohydrate moieties (25) and five phosphorylated residues (27). Besides the 70-kDa molecular species, several observations have reported the presence of an SDS 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 (42). The functional significance of this proteoglycan in hormone storage vesicles is unknown. Furthermore, several studies have documented the presence of other post-translational modifications on the CGA molecule including methylation via protein carboxymethylase (43, 44) and trans-glutamination (45, 46).

In the present paper, we report for the first time the full characterization of seven post-translational modifications that are present along the polypeptide chain of CGA from bovine chromaffin granules. Five phosphorylated residues were found to be on residues Ser81, Tyr173, Ser307, Ser372, and Ser376. This finding is in agreement with the quantification of the phosphorylated residues previously reported (27). The novel interesting observation was the presence of a phosphorylated tyrosine residue. Tyrosine phosphorylation is not a common post-translational modification, since it represents only 0.03% of the phosphorylated amino acids in normal cells (47). The significance of this tyrosine phosphorylation is not yet known, although we have recently reported that chromacin, the CGA-derived peptide 173–194, displays antibacterial activity when the N-terminal tyrosine 173 residue is phosphorylated (21). On the basis of a protein consensus domain specific to kinases, it is possible to predict that protein kinase C may introduce a phosphate group on residues Ser81, Ser173, Ser307, Ser372, and Ser376. GalNAc, Gal, and NeuAc O-glycosylated sites on positions Ser186 and Thr231 are shown. Panel B, chemical structure of the trisaccharide NeuAcα2–3Galβ1–3GalNAcα1–O-linked to Ser186 and Thr231.

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granule or within the secretory granule itself.

Concerning the O-glycosylation sites included on bovine adrenal medullary CGA, we had previously located a unique carbohydrate moiety composed of the tri saccharide NeuAcα2–3Galβ1–3GalNAca1; a component recovered in B2 and B3 with an experimental mass of 2627.45 Da was identified as O-glycosylated CGA-(222–243) with the disaccharide Galβ1–3GalNAco1; a component recovered in C2 and C3 with an experimental mass of 2262.65 Da corresponds to the CGA-derived peptide CGA-(222–243). The two post-translational glycosylations of CGA probably have a structurally related function. The two tri saccharide moieties are attached on residues Ser186 and Thr231, which are present in the central domain of the protein; this modification may prevent natural cleavage in this domain. The closest proteolytic sites have been identified on Arg115, Asp116 and Arg247, A248 (6). This CGA proteolysis gives rise to a long glycopeptide of 132 residues containing the two sugar moieties.

In the C-terminal domain, the phosphorylated residues Ser372 and Ser376 can be assumed to prevent the natural cleavage on dibasic sites 314–315 and 366–367, since no corresponding peptides have been found (6). However, cleavage of the unphosphorylated form can generate low amounts of minor fragments beginning at residue 316.

**Location of the O-Glycosylation and Phosphorylation Sites on Bovine CGA in Relation to Biological Active Peptides**—Present results revealed that two of the post-translational modifications on bovine CGA are detected on derived peptides that inhibit hormone and neurotransmitter release: (i) CGA-(1–113) containing phosphorylated Ser81 corresponds to the vasostatin II sequence (10) and is homologous to pancreatic rat betagranin (9), and (ii) the natural fragment CGA-(347–419) bearing phosphorylated Ser372 and Ser376 is parastatin (18). Previously, both CGA and pancreastatin were shown to inhibit low Ca2+–stimulated parathyroid cell secretion (53, 54). The N-terminal fragment, named betagranin, corresponding to the CGA-(1–113) peptide has been shown to inhibit parathyroid hormone secretion stimulated by low calcium concentrations (8). This fragment is generated naturally in several endocrine tissues, notably the adrenal medulla (10, 12, 55), pituitary (56), endocrine pancreas (9), and parathyroid glands (8). Another CGA-derived fragment, named parastatin, located in the C-terminal domain and corresponding to CGA-(347–419), strongly inhibits low Ca2+–stimulated parathyroid hormone secretion. Vasostatins, and more particularly vasostatin I, corresponding to CGA-(1–76) were described to inhibit the potent vasoconstrictor peptide, endothelin-1. It is not yet known whether phosphorylation is important for the biological activity of these CGA-derived fragments.

Concerning the O-glycosylation and phosphorylation modifications, we have recently reported that they are necessary for the full antibacterial activity of chromacin peptides. The natural CGA-derived antibacterial peptide, prochromacin CGA-(79–431), included the seven modifications. We have previously established that the antibacterial activity of chromacin
Post-translational Modifications of Bovine Chromogranin A

Bovine CGA in Relation to Antigenic Sites of Human CGA—Recently, the most antigenic sites of recombinant human CGA have been characterized and have been correlated with the location of the different biological active fragments derived from human chromogranin (63). Taking into account the high degree of homology between the bovine and human CGA proteins, it was of interest to correlate the location of these antigenic sites with the modified residues. The post-translational modified residues in positions 81, 231, 307, 372, and 376 appear to be preferentially located in or near domains with high antigenicity (68–106, 222–230, 315–330, and 376–394), whereas only two modified residues in positions 173 and 186 are located in the 163–210 region described to have low antigenicity. This result indicates that post-translational modifications are located outside of highly antigenic domains, thus suggesting important, potential biological roles for the O-linked-trisaccharide and phosphorylated residues.

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CGA (173–186) is correlated with the presence of O-glycosylation modification on Ser186 and/or phosphorylation on Tyr173 (21).

In addition, the region CGA (305–309) contains a specific Ca2+-binding domain including the phosphorylated Ser307 residue. Again, the presence of phosphate group may affect the binding of calcium.

Phylogenetic Features Related to the Location of O-Glycosylation and Phosphorylation Sites within Bovine CGA—In Table I, we report the bovine CGA regions (24) where post-translationally modified residues are present and compare them with the corresponding sequences of human (57, 58), pig (59), mouse (60), and rat (61) species and the N-terminal domain of the ostrich CGA sequence (62). The three phosphorylated residues Ser307, Ser372 and Ser376 are strictly conserved and included in homologous sequences with the typical pattern (E/Q)(E/Q)(E/E/E/E/E/E/E/D/N) and (R/P/R/S/N/G/S/R/P/S/N/S)(E/P/E/P/E/D/N)186. Furthermore, Ser81 is present in bovine, porcine, mouse, and rat CGA and changed in threonine residue in ostrich CGA sequence, whereas in the human CGA sequence Ser81 is changed into Gly on (Q/T)/Q/E/Q/R/K/H/Q/R/S/R/G/T186/E/E/D/D/Q/EL sequence. However, in the human CGA sequence the adjacent amino acid in position 80 is a serine residue that could represent the phosphorylation site. In contrast, the phosphorylated tyrosine residue Tyr173 is restricted to bovine and human CGAs; the sequence PS/F/Q/K/Y173. PPGQAK is homologous in both species. In pig, mouse, and rat, Tyr173 is changed into arginine or histidine, and no tyrosine residue is present in the immediate vicinity.

The attachment O-glycosylation site Ser186 is highly conserved in bovine, human, porcine, mouse, and rat CGAs, but the Wilson consensus pattern PS186/QGP is conservative in bovine and pig CGAs, whereas important variation is present in human, mouse, and rat protein. Concerning the second O-glycosylated site, the residue Thr231 is present in bovine, human, mouse, and rat CGAs. In pig, this threonine is changed into a serine residue, which might be a phosphorylation site. With regard to the Wilson consensus sequence (36), the importance of proline residues in the vicinity of Thr231 is not known. They are present upstream and downstream in the bovine, human, and pig sequences but are scarce in rat and mouse CGAs.

Location of O-Glycosylation and Phosphorylation Sites of CGA-sequences were retrieved from the Swiss-Prot database: CGAb (bovine; P05059; Ref. 24), CGAh, (human; P10645; Refs. 57 and 58), CGAp (pig; P04404; Ref. 59), CGAm (mouse; P26359; Ref. 60), CGAr (rat; P10834; Ref. 61), CGAo (ostrich; P23716; Ref. 62). Identical residues and conservative changes in positions corresponding to modified residues are in boldface type. The consensus O-glycosylated sequence according to Wilson (36) is underlined.

**Table I**

| CGAb | Q Q K K S Y E D E L P S P K Y P G P Q A K E D S E — G P S Q G P |
| CGAh | Q Q K K S G F E D E L P S Q K Y P G P Q A K G D S E — G L S Q G L |
| CGAp | Q Q K K S Y E D E L P S K R P G A Q A E E D E H — G P S Q G P |
| CGAm | Q Q Q H S F E D E L P S K R E D V P Q A T G D E R G L S A Q Q |
| CGAr | Q Q Q H S F E D E L P S Q E H G I P T Q T E G S E R G P S A Q Q |
| CGAo | T Q Q R E T D E Q E 231 |
| CGAb | A V P E E E S P P T A A F K P P P E P E Q E Q E Q L S K E W E D |
| CGAh | A V P E E E G — P T V V L N P H P L E Q E E E R L S K E W E D |
| CGAp | A V P E E E G P R S E A F D S H P A Q E E E R L S E W E N |
| CGAm | A G P K E E V — P T A A S S S H F E E E E E R L S R E W E D |
| CGAr | A G P K E V — P T A A S S S H F E E E E E R L S R E W E D |
| CGAh | G W R P N S R E D S V E A G L P L Q V |
| CGAm | Q L R G R S P R N S W E D S L E A G L |
| CGAr | G W R P S R E D S V E A R S D F E E |
| CGAo | G W R P S R E D S V E A R G D F E E 376 |

CGA sequences were compared with bovine CGA-derived peptides using the Swiss-Prot database: CGB (bovine; P05059; Ref. 24), CGAh, (human; P10645; Refs. 57 and 58), CGAp (pig; P04404; Ref. 59), CGAm (mouse; P26359; Ref. 60), CGAr (rat; P10834; Ref. 61), CGAo (ostrich; P23716; Ref. 62). Identical residues and conservative changes in positions corresponding to modified residues are in boldface type. The consensus O-glycosylated sequence according to Wilson (36) is underlined.
Phosphorylation and O-Glycosylation Sites of Bovine Chromogranin A from Adrenal Medullary Chromaffin Granules and Their Relationship with Biological Activities
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