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Chromogranin B (secretogranin I), a putative precursor of two novel pituitary peptides through processing at paired basic residues

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During the course of reversed-phase high-pressure liquid chromatography (RP-HPLC) purification of the 7B2 peptide originally isolated in our laboratory from human pituitary gland extracts, two novel peptides were identified and purified to homogeneity. The complete amino acid sequence of the first one was established in 1985 and recently found to be entirely homologous to positions 420–493 of the just published chromogranin B sequence. This peptide, denoted GAWK, could originate from chromogranin B following specific cleavage at the basic amino acids flanking both termini of GAWK. Moreover, another peptide isolated in our laboratory from the same source and denoted CCB has been discovered and its sequence is also part of the same chromogranin B molecule. Here again, this peptide, occupying positions 597–653 and located at the COOH-terminal region of chromogranin B, could derive from specific processing at basic amino acids, Arg-Lys-Lys, present at positions 594–596. In a manner reminiscent of the relationship between pancreastatin and chromogranin A, it is proposed that both GAWK and CCB are produced from chromogranin B after specific processing at basic amino acids. These data are thus in favor of a putative role of chromogranins as precursors to potentially bioactive peptides.

Chromogranin; Secretogranin; Prohormone precursor; Peptide processing; GAWK; CCB

1. INTRODUCTION

The recent availability of the complete amino acid sequence derived from sequencing of cDNA of both chromogranin A [1,2] and secretogranin I (hereafter referred to as chromogranin B) [3] has stirred considerable interest in the field of neuroendocrinology. Together with chromogranin C, for which the primary structure is still unknown, these high molecular mass proteins are known to be highly acidic and to be extensively posttranslationally modified. Furthermore, they have been localized in a great number of secretory granules derived from endocrine and neuroendocrine cells [4–6]. On the other hand, apart from the reported calcium-binding activity of chromogranin A [7], their biological role has puzzled and eluded scientists for a number of years. Due mainly to their low p*I* and their localization, some possible roles have been proposed even though unascertained due to lack of purified products. These include involvement in the packaging and sorting of regulatory peptides, in the organization of secretory granule contents, as carrier for peptides after secretion and as precursors for biologically active peptides [1–6]. Moreover, chromogranin A which contains an Arg-Gly-Asp sequence, could per se interact with

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cellular membranes and thus act upon target cells [1,2]. Finally, we have recently reported that chromogranin A could also act as a reversible inhibitor of a putative processing enzyme [8].

In 1985, our laboratory reported the isolation, immunocytochemical localization and complete sequence of a novel peptide, denoted as GAWK, found in human pituitary extracts [9]. We have since found that this 74 amino acid peptide is entirely homologous to positions 420–493 of the just reported sequence of chromogranin B [3]. Even more interestingly, this peptide is flanked at both COOH- and NH_2-termini by pairs of basic amino acids in the chromogranin B sequence. Furthermore, we noticed that GAWK 1–74, itself, is converted into two smaller fragments, GAWK 1–17 and 20–74, following cleavage at an Arg-Arg pair occupying position 18–19.

The present report demonstrates that, in addition, another peptide from human pituitary extracts could be derived from cleavage at a pair of basic residues, from the chromogranin B molecule. This peptide, hereafter referred to as CCB (COOH-terminal region of chromogranin B), is entirely homologous to positions 597–653 of chromogranin B. We also describe preliminary results pertaining to tissue, cellular and subcellular distribution of GAWK and CCB.

2. MATERIALS AND METHODS

2.1. *Purification of CCB*

Initial results were obtained using whole human pituitary extracts fractionated on a (2.5 × 30 cm) C_{18} semi-preparative HPLC column. The peptides, coeluting with the COOH-terminal peptide of propressophysin (CPP) [10], were pooled and repurified on a Waters μ-Bondapak C_{18} (0.78 × 30 cm) using an aqueous phase containing either 0.1% trifluoroacetic acid (TFA) or 0.13% heptafluorobutyric acid (HFBA) and a gradient of acetonitrile at a flow rate of 2 ml/min. The purified fraction was used for amino acid sequence screening and the information so obtained allowed us to prepare an antiserum.

A larger preparation of CCB was made starting with 2000 frozen pituitaries (1034 g). The extraction was carried out using 4585 ml of 0.1 M ammonium acetate, pH 6.8, containing 2.5 mM EDTA, 0.1 μM pepstatin A and 1 mM PMSF. After centrifugation at low speed (8000 rpm) for 30 min and filtration, the supernatant is adsorbed onto C_{18}-silica which had been previously equilibrated in 0.1% TFA containing 5% acetonitrile. After removal of unadsorbed material and washing with starting buffer by filtration, the adsorbed peptides are extracted in batch-wise manner using 0.1% TFA containing 45% acetonitrile. The eluted fraction after lyophilization is then loaded on a μ-Bondapak-C_{18} column (0.78 × 30 cm) eluted with a linear gradient from 20% to 80% acetonitrile containing 0.1% TFA in 120 min at a flow rate of 2 ml/min. The major immunoreactive peak was repurified on an Aquapore RP-300 (0.7 × 25 cm) column eluted with a linear gradient from 30% to 80% 2-propanol containing 0.1% HFBA in 100 min at a flow rate of 2 ml/min. The immunoreactive material was further purified using an Ultrasphere-ODS (1 × 25 cm) column eluted with a linear gradient from 40% to 90% organic phase in 100 min at a flow rate of 2 ml/min: the aqueous phase was made with 0.2 M triethylamine phosphate (TEAP), pH 3.0, while the organic phase was acetonitrile: 0.175 M TEAP (70:30, v/v). The fractions were desalted on an RP-300 Aquapore column using a linear gradient from 20% to 60% acetonitrile in 80 min in the presence of 0.1% TFA at a flow rate of 2 ml/min. The immunoreactive peaks were further purified by molecular sieving using a Bio Sil TSK-250 column (0.75 × 60 cm) eluted with 0.1 M ammonium acetate, pH 6.8, at a flow rate of 1 ml/min. Each purified fraction was analysed by gel electrophoresis using either SDS-urea polyacrylamide or 15% polyacrylamide.

2.2. *Amino acid and sequence analysis*

The HPLC-purified peptides were hydrolyzed in vacuo using 5.7 N HCl at 110°C for 24 h. The amino acids were separated and quantitated on a modified Beckman 120C autoanalyser equipped with a Varian DS604 plotter/integrator: detection was carried out using post-column ninhydrin.

The amino acid sequence determinations were carried out on two automatic sequenators. The liquid-phase Beckman 890M and the gas-phase Applied Biosystem model 470A sequenators were operated as described in [11]. The released thiazolinones, after conversion into PTH

derivatives, were separated and quantitated by HPLC as described [12].

2.3. *Enzymatic and chemical cleavages*

Carboxypeptidase Y digestion was performed on 4.8 nmol of the purified fraction dissolved in 60 μl of 0.1 M ammonium acetate buffer, pH 5.5, to which 1.7 μg exopeptidase was added and the incubation was allowed to proceed for various time intervals (1, 5, 10, 15, 30 and 60 min). Amino acid analysis was carried out on 10 μl acidified (pH 2.2) aliquot in the presence of added norleucine acting as an internal standard.

The purified peptide was also cleaved with cyanogen bromide (CNBr) in 70% formic acid in the dark for 24 h. After lyophilizing the reaction mixture, the fragments produced were sequenced directly without prior separation.

2.4. *Immunological methods*

The information obtained following the first NH_2-terminal Edman degradation allowed us to develop an antiserum against a synthetic fragment corresponding to positions 6–21 of the CCB peptide. However, this synthetic peptide contained a substitution at position 5 where a Phe residue was put instead of a Ser residue as determined by later sequencing of CCB. A radioimmunoassay procedure using this ^{125}I-labeled synthetic peptide as tracer was also developed; the ED_{50} obtained from the displacement curve corresponds to 2.2 ng/ml. The antibody did not cross-react to any appreciable extent with known peptides tested; these included β-endorphin, α-MSH, human β-LPH, human NH_2-terminal of POMC, ACTH and human 7B2 (positions 117–128). It also proved unable to recognize CCB in species other than human such as rat, monkey, porcine and ovine. Immunocytochemical localization studies were conducted essentially as described in [9].

3. RESULTS

At the time we reported the isolation of GAWK, we noticed while purifying 7B2 [13] that numerous fractions were contaminated by a peptide containing an unusually high amount of acidic residues. After purification by HPLC, enough material was isolated in order to deduce the amino acid sequence of the first 40 residues (not shown). It was thus possible (i) to confirm its acidic character, (ii) to confirm its novelty since computer data bank search (NBRF) failed to reveal any significant homology to known proteins or fragments thereof and (iii) to synthesize chemically a segment comprising positions 6–21 for the production of an antibody. This fragment was chosen in order to minimize any cross-reactivity with the NH_2-terminal fragment of human POMC [14] which contains in positions 29–35 a sequence similar to the first six NH_2-terminal amino acids of CCB.

As can be seen in fig.1A, the purification of this peptide starting from a batch-eluted fraction (see section 2) proved especially difficult since the majority of the immunoreactive material elutes between 40 and 48 min together with a number of other peptides. On the other hand, it is worth mentioning that inclusion of an adsorptive step on C_{18}-silica allowed the extraction of material on a large scale and thus minimized the number of passages through separative or semi-preparative columns. Following a number of chromatographic steps, utilizing various ion-pairing agents or organic solvents, it was possible to obtain a sufficiently pure product (fig.1B) to extend the NH_2-terminal sequence past the first 40 residues. The results from the NH_2-terminal Edman degradation are shown in fig.2 and allow the identification of 57 residues. The characterization of the cleavage products at the single Met residue occupying position 46 confirms that the last identifiable residue belonging to the peptide starting at residue 47 is a Phe (not shown). The amino acid composition (table 1) agrees with the identification of the Phe as the COOH-terminal residue and indicates that the last 4 residues, Ser-Gln-Arg-Gly, are missing. The digestion by carboxypeptidase Y yields in all cases a complex pattern of released amino acids from which Gly and Arg were always absent (not shown). Thus, the major form of CCB as identified here does appear to be COOH-terminal truncated; this, together with the observed heterogeneity during HPLC purification, could be explained by proteolysis occurring after the autopsy and prior to storage. It must also be said that analysis of the immunoreactive fractions isolated by HPLC reveals that this peptide migrates abnormally during gel electrophoresis. Thus, for example, material analyzed by amino acid sequencing migrates routinely as a 15–16 kDa

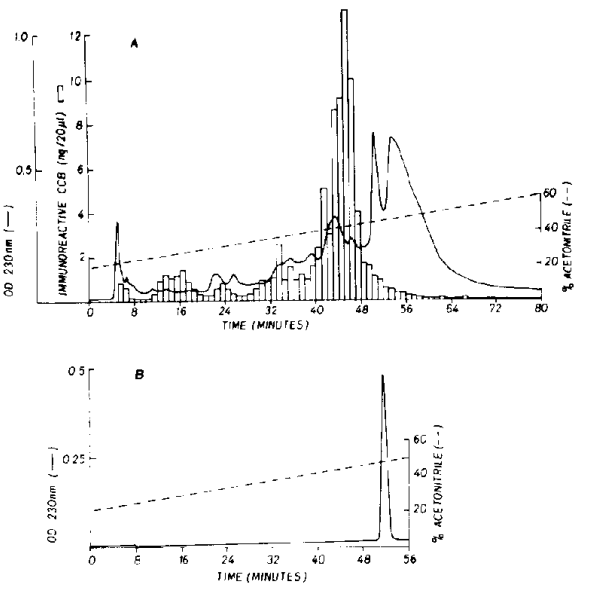

Fig.1. Reverse-phase HPLC purification of the polypeptide from whole human pituitary extracts. Chromatogram A depicts the HPLC purification of immunoreactive CCB previously enriched by C_{18}-batch extraction. The immunoreactive fractions (shown as histograms) were pooled and repurified (see section 2) to yield the fraction (chromatogram B) submitted to amino acid sequencing. The dashed lines represent the linear gradient of organic phase used in each case.

peptide on 15% polyacrylamide gels and as 13–14 kDa on SDS-urea gels which is almost twice its expected molecular mass. It is tempting to speculate that such a behavior could at least be explained by the very high number (28% of the total residues) of acidic residues distributed throughout the molecule which might prevent the formation of stable peptide-SDS complex. This abnormal migration on gel electrophoresis has already been observed in the case of both chromogranin molecules [1–3].

The tissue distribution of CCB reveals that human pituitary gland and human adrenal are the richest tissues containing 152 and 18 ng/mg of protein, respectively: human testes, liver and plasma did not contain any whereas various brain regions contain different amounts of immunoreactive material ranging from 0.7 ng/mg to 6.9 ng/mg of protein. Furthermore, no im-

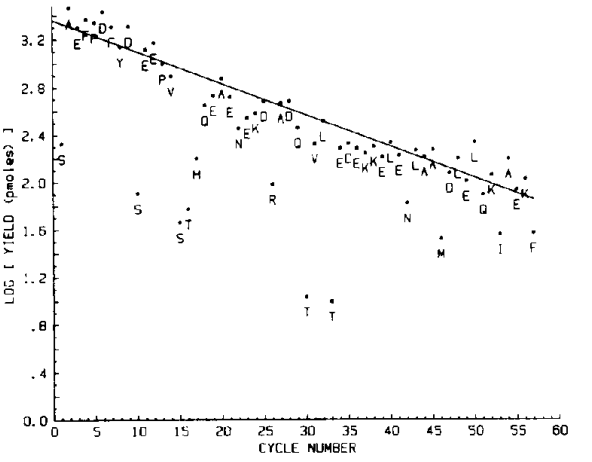

Fig.2. Automatic NH_2-terminal degradation of CCB. Quantitative yields of PTH-amino acids normalized to a PTH-norleucine internal standard are illustrated as a function of residue numbers. The slope and intercept were obtained by a linear regression analysis on selected stable PTH-amino acids giving the repetitive yield (94.1%) and initial yield (2.28 nmol), respectively.

Table 1

Amino acid composition of CCB

Amino acid	CCB[a]	Chromogranin B[b] (597–657)
Asx	7.93 (8)	8
Thr	2.84 (3)	3
Ser	3.07 (3)	4
Glx	15.16 (15)	16
Pro	2.05 (2)	2
Gly	0.41 (–)	1
Ala	5.76 (6)	6
Val	1.98 (2)	2
Met	0.84 (1)	1
Ile	0.97 (1)	1
Leu	5.17 (5)	5
Tyr	1.06 (1)	1
Phe	3.13 (3)	3
His	1.02 (1)	1
Lys	4.84 (5)	5
Arg	1.16 (1)	2
Total	57	61

[a] The computed values represent the average of three analyses. Values in parentheses represent the nearest integer

[b] These values are taken from the cDNA sequence [3]

munoreactive CCB was detected in the pig anterior or neurointermediate pituitary lobes and in ovine or rat whole pituitaries. This result could be seen as evidence that either CCB is absent in these species or, in all likelihood, it is not recognized by this antiserum. Similar results were also obtained while using two antibodies raised against GAWK 1–17 and GAWK 20–38 which appeared to display species selectivity (not shown). Thus, the tissue distribution of CCB follows very closely that of the GAWK peptide [9]. Displacement curves using various amounts of tissue extracts always yielded a similar profile to that obtained with the synthetic peptide. Moreover, as shown in fig.3, cellular localization of GAWK reveals that this peptide is present, using the antiserum raised against positions 20–38, in somatotrophs and in thyrotrophs. Indeed, colocalization using antibodies to human growth hormone and human TSH can clearly be seen: no such colocalization can be seen with antisera against prolactin, ACTH, LH and FSH. Moreover, GAWK was localized by immunoelectron microscopy in spherical secretory granules whose sizes were estimated to be about 450–500 nm in agreement with those associated with somatotrophs. Such studies have not yet been conducted on CCB but in view of their similar origins and tissue distribution, it is reasonable to propose that CCB will also be present in identical granules.

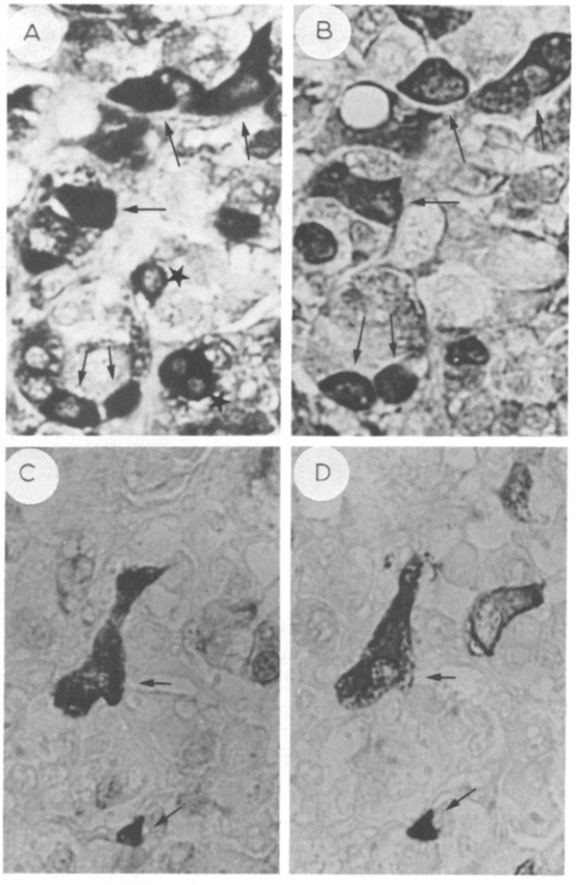

Fig.3. Immunocytochemical colocalization of GAWK (A,C) with growth hormone (B) and TSH (D) as observed by light microscopy on 3 μm serial paraffin sections of human adenohypophysis. Cells positively labeled are indicated by arrows. Magnification is × 365.

4. DISCUSSION

The present data clearly demonstrate that two peptides, GAWK and CCB, isolated from pituitary extracts represent two segments of chromogranin B (fig.4). The sequence of GAWK covers positions 420–493 while CCB corresponds to positions 597–653. In all likelihood, the full CCB probably extends to the last Gly residue which could thus yield an amidated peptide. However, according to Bradbury and Smyth [15], this should not be the case considering the slower kinetics of the amidation enzyme observed whenever a neutral residue occupying the penultimate position is replaced by a charged residue for instance, in this case, an Arg residue. However, due to the observed heterogeneity during the purification and amino acid analysis, the presence of a longer form having an amidated COOH-terminus, at least in some molecules, cannot be excluded.

Interestingly, other peptides were also reported and can now be related to the chromogranin B sequence [16,17]. Indeed, the occurrence in ovine adrenal medulla of two peptides, one derived from the other by cleavage at an Arg-Arg pair, and displaying very high homology to the GAWK peptide has been reported [16]. Similarly, other ovine peptides were isolated and their amino acid sequences [17] can now be localized to positions 568–593 as shown in fig.4. It is worth mentioning that all these peptides, GAWK 1–17 and 20–74, OA-24/OA-60, OA-8/OA-21 and CCB, are flanked in the chromogranin B sequence by pairs of basic amino acids which are well recognized

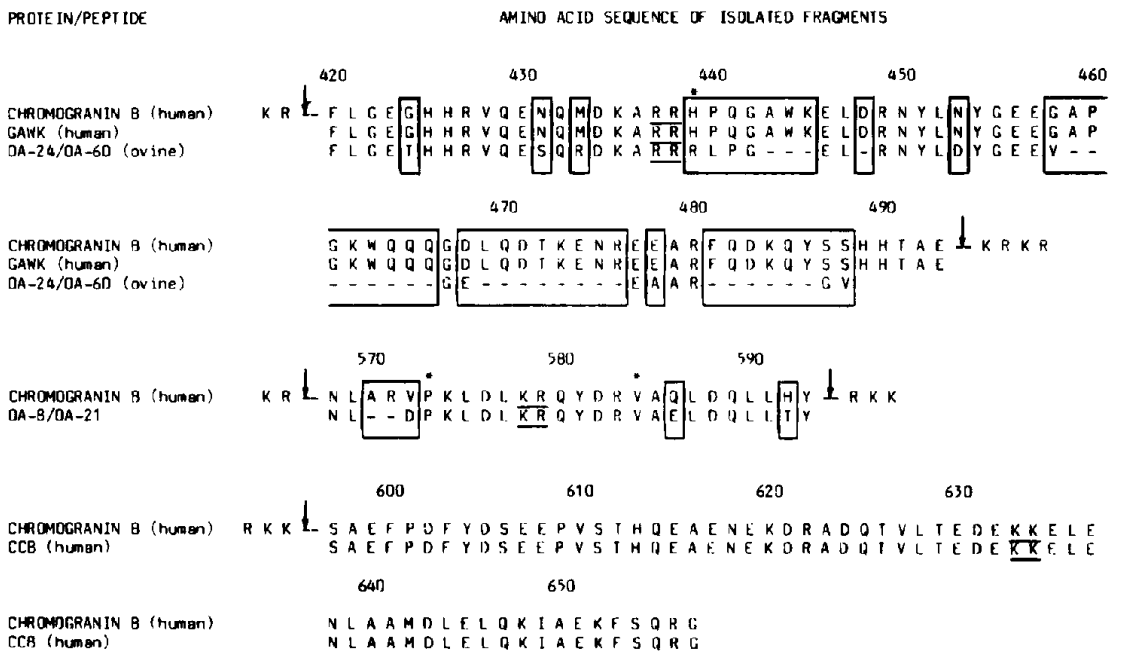

Fig.4. Sequence homology between human GAWK [9], human CCB and various ovine peptides [16,17] with segments of human chromogranin B. The alignment of ovine peptide OA-60 sequence with human GAWK was obtained by using the program ALIGN from the National Biomedical Research Foundation (Washington, DC). The arrows indicate the sites of cleavage necessary to generate the identified peptides whereas asterisks indicate the NH_2-termini of other isolated fragments.

sites of proteolytic cleavage in prohormones [18]. Furthermore, Tatemoto et al. [19] have described a novel porcine C-terminally amidated peptide, pancreastatin, which inhibits insulin secretion and displays a significant sequence homology to positions 261–314 of prechromogranin A [20,21]. These findings must also be related to numerous reports concerning the occurrence of a 21 kDa fragment, known as β-granin, which is cosecreted with insulin [22]: this fragment is structurally related to the NH_2-terminal region of chromogranin A and also appears to be produced after cleavage at a pair of basic residues [23]. Based upon these data, it appears that, indeed, chromogranin B and chromogranin A could represent precursors to smaller peptides whose significance remains to be established.

This report also describes evidence that fragments of chromogranin B, namely the GAWK-like immunoreactivity, are present in somatotrophs and in thyrotrophs. Thus, it is conceivable that secretion of GAWK may be stimulated by growth-hormone releasing factor (GRF) or inhibited by somatostatin. On the other hand, it is known to be released from chromaffin granules by common secretagogues such as nicotine and K^+ salts (not shown). However, chromogranin B per se was never shown to be present in these cells; indeed, it has been reported in gonadotrophs and in corticotrophs but is absent in thyrotrophs, in mammotrophs and in somatotrophs [24]. These apparently conflicting results could be explained by the differential epitope recognizing ability of the antibodies used in this and other studies or, more attractively, by variable amounts of chromogranin B in these cells. Therefore, chromogranins and/or their fragments can be found in many hormone producing cells though the processing rates of chromogranins appear to be low except for chromogranin B in adrenal chromaffin granules [25]. Considering the proposed involvement of chromogranin A in enzymatic activity regulation [8], one can wonder about their role as potential competitive substrates or inhibitors and thus as potential regulators of the maturation process.

Clearly more studies are required before any

conclusions concerning the biological role of chromogranins can be reached.

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