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The molecular cloning of the chromogranin A-like precursor of β-granin and pancreastatin from the endocrine pancreas

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The cDNA encoding the precursor form of the chromogranin A-related proteins, β-granin and pancreastatin, was obtained by immune screening of rat insulinoma and pancreatic islet cDNA libraries. The sequence was virtually identical to that of rat adrenal chromogranin A, suggesting that the different molecular forms of chromogranin A immunoreactivity found in adrenal medulla and endocrine pancreas are related to differences in post-translational proteolytic processing. The rat chromogranin A, unlike its bovine and human counterparts, contained a 20-residue glutamine sequence inserted within the N-terminal β-granin sequence. Although the encoding CA(G/A) repeat recurs frequently in the rat genome, the rat chromogranin A molecule appears to be the product of a single gene and mRNA transcript.

Chromogranin A; β-Granin; Pancreastatin; cDNA; OPA repeat; (Pancreatic islet)

1. INTRODUCTION

The islets of Langerhans contain a number of peptides which are related in structure to chromogranin A, a 48 kDa acidic glycoprotein which is the major secretory granule protein of the bovine adrenal chromaffin cell [1]. For example, pancreastatin, a peptide inhibitor of glucose-induced insulin and somatostatin secretion, is homologous to residues 251-294 of bovine chromogranin A [2,3]. Similarly a 21 kDa peptide, β-granin, which is co-secreted with insulin from the pancreatic β-cell [4], resembles the N-terminus of the molecule [5].

Studies in pancreatic islets and insulinoma cells [6,7] indicate that β-granin is derived from a chromogranin A-like precursor by excision of pairs of basic amino acids from the molecule. The primary aim of the present investigation was to determine the structure of the precursor and so to establish whether variations in the molecular sizes of chromogranin A-immunoreactive peptides in different cells of the neuroendocrine system are related to differences in the precursor molecules or to differential proteolytic processing of a common precursor.

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2. MATERIALS AND METHODS

Antibodies were raised in guinea pigs to β-granin purified from a transplantable insulinoma propagated in NEDH rats [8]. Antibodies which reacted with *E. coli* or λ phage proteins were preabsorbed for 1 h at 4°C with a sonicate (25 mg protein/ml serum) of BNN97 *E. coli* grown for 16 h at 42°C with 0.5 mM isopropylthiogalactopyranose (IPTG). cDNA libraries were constructed [9] in the expression vector λgt11 from transplantable NEDH rat insulinoma [10] and neonatal Wistar rat islets isolated by collagenase digestion [11]. Nitrocellulose filter replicas from approx. 10^5 insulinoma library clones were incubated with the preabsorbed antisera (1:500) and then with horseradish peroxidase-conjugated rabbit anti-guinea pig immunoglobulins (1:500) following a protocol for Western blotting analyses [8]. Positive clones were mapped, subcloned into puC18 and suitable restriction fragments sequenced in m13mp18/19 phage by the dideoxychain-termination procedure. Restriction fragments from the cloned insert were purified by 7.5% acrylamide gel electrophoresis and used to screen the islet cell library after ^{32}P radiolabelling by the random primer technique.

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Southern blot analyses used Wistar rat liver nuclear DNA (40 μg) which was digested for 16 h at 37°C, electrophoresed in 40 mM Tris, 1 mM EDTA, 20 mM acetate buffer on 1% agarose gels and blotted onto nitrocellulose. Hybridization was for 16 h at 42°C in 50% (v/v) formamide, 5 × SSC, 5 × Denhardt's, 0.1 mg/ml salmon sperm DNA, 50 mM phosphate (pH 6.5) containing 5×10^5 dpm/ml ^{32}P-labelled DNA and the blots subsequently washed at 65°C in 0.1 × SSC with 0.1% SDS [12].

Northern blot analyses used total RNA (20 μg) prepared from 100–200 mg tissue by guanidine thiocyanate denaturation, CsCl gradient centrifugation and DNase digestion [12]. Samples were glyoxylated, electrophoresed in 50 mM Na phosphate (pH 6.5) on 1.2% agarose gels and blotted onto nitrocellulose [13]. Hybridization and washing (52°C) were as above.

Western blot analyses used guinea pig antisera (1:500) raised to the β-galactosidase hybrid protein detected during the initial screening (clone 218/38 incorporating amino acids 60–234; fig.5). The protein was recovered by $(NH_4)_2SO_4$ precipitation (30–40% saturated pellet; pH 7.4) from lysed bacteria (Y1089 derivative) grown at 42°C in LB media containing 1 mM IPTG. The precipitated protein was then chromatographed in 20 mM Tris-Cl (pH 7.4) containing 100 mM NaCl on a Superose 6 HR10/30 column (Pharmacia) and the hybrid recovered as a 145 kDa (SDS-PAGE) homogeneous product (>90% purity; 5–10 mg/l culture).

Purified native β-granin [5] (30 μg) was incubated for 3 h a 37°C in 400 μl of 0.5% NH_4HCO_3 containing 0.3 μg TPCK-treated trypsin (Sigma). Digests were lyophilized and chromatographed at 1 ml/min on a C-18 reverse-phase HPLC column (Waters μ-bondapak) using a 9–45% (v/v) linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Peak fractions ($A_{214\ nm}$) were subjected to automated gas-phase sequencing (Applied Biosystems 470A sequencer).

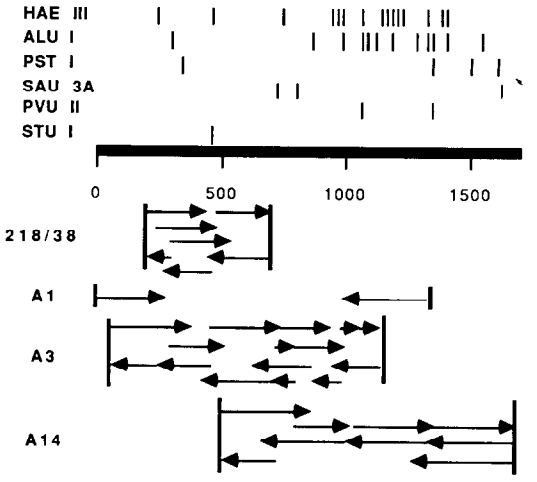

Fig.1. Relationship of the cDNA clones obtained and the strategy used for sequencing. The indicated restriction sites were used for subcloning into m13; the direction of sequencing and length of readings are indicated.

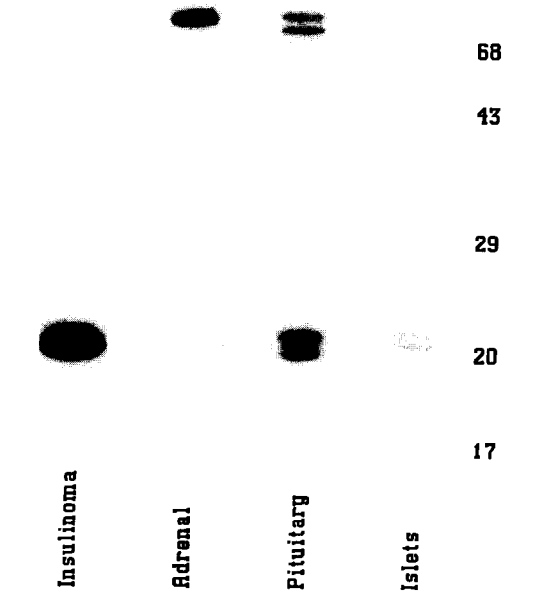

Fig.2. Western blot analysis of different rat tissues using antisera raised to the cloned β-galactosidase/β-granin hybrid protein. Samples of 100 μg (islets) or 300 μg protein were subjected to SDS-PAGE on 7.5–15% acrylamide gradient gels. The migration of protein molecular size standards is indicated (molecular mass expressed in kDa).

3. RESULTS

Immune screening of the rat insulinoma library yielded two clones having *Eco*RI inserts of approx. 520 bp encoded within β-galactosidase hybrid proteins of 130 and 145 kDa. Both clones had similar restriction maps and only one (218/38, fig.1) was examined further. The β-galactosidase hybrid protein encoded by this recombinant when used as an immunogen yielded an antibody with a reactivity on Western blotting which was similar in specificity to that of the original antisera used in the screening. It recognised proteins of 21 and 22 kDa (β-granins) in insulinoma and rat islet tissue, of 21, 22, 90 and 100 kDa in rat pituitary and a 100 kDa protein in rat adrenal 9 (fig.2). The clone 218/38 was therefore considered to be authentic.

Attempts to rescreen the insulinoma cDNA library for longer cDNAs using the 523 bp *Eco*RI insert from 218/38 as a probe produced a series of

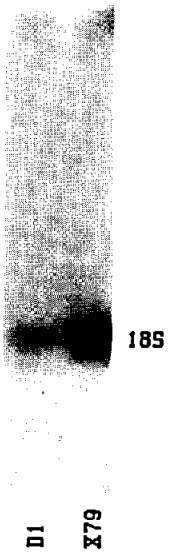

Fig.3. Southern blot analysis. DNA samples (40 μg/track) were digested overnight with the indicated enzymes, electrophoresed on 1% agarose gels, blotted and hybridized with: (A) a nick-translated cDNA (6×10^7 dpm/μg) representing bases 368–890 of rat chromogranin A [15]; (B) an m13 probe produced by primer extension (2×10^7 dpm/μg) representing bases 368–477 and; (C) an m13 probe (2×10^7 dpm/μg) representing bases 478–890. The latter probe avoids the $(CAG)_{16}\cdot(CAA)_4$ sequence between bases 410 and 469. Exposure times of autoradiographs at −70°C: 3, 18 and 120 h, respectively. Migration of λ *Hin*dIII fragments is indicated.

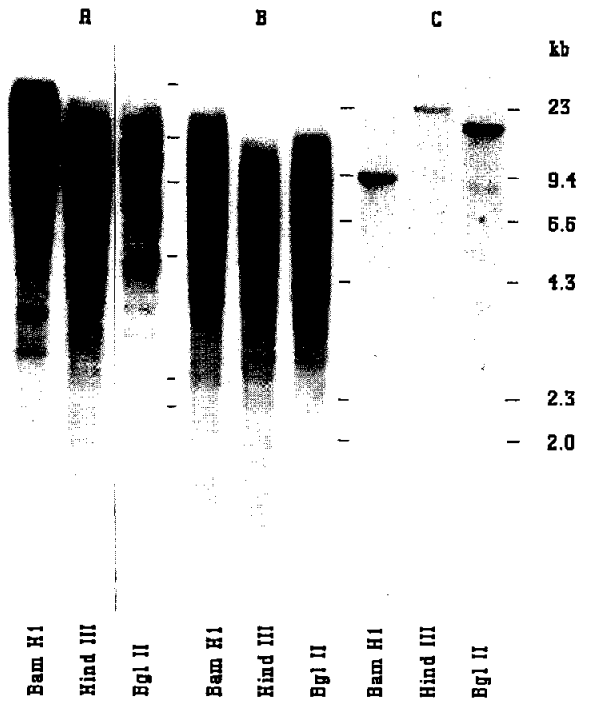

Fig.4. Northern blot analysis of insulinoma cell lines. Total RNA (20 μg/track) was glyoxylated and electrophoresed on 1.2% agarose gels, blotted and hybridized to an m13 probe (2×10^7 dpm/μg) representing the reverse complement of bases 478–890 of rat chromogranin A. Insulinoma line D1, although of the same origin as X-79 [10], is less well granulated and has 1/5–1/10 the content of insulin and β-granin. The exposure time of the autoradiograph at −70°C was 24 h. The migration of a 18 S ribosomal RNA is indicated.

spurious clones related to the original sequence only in having long tracts of a repeated CA(G/A) sequence. A 413 bp fragment of clone 218/38 extending from a single *Alu*I site to its 3′-end which omitted the CA(G/A) repeat, however, yielded 3 overlapping clones from the islet library. These covered 1696 bp of sequence comprised of an open reading frame of 1360 bp and 336 bp of 3′-untranslated sequence incorporating a concensus sequence for poly(A) addition, 23 bp from the 3′-terminus. The cDNA encoded the sequence of a 448 amino acid, 50.2 kDa precursor form of β-granin and 5 amino acids of an incomplete presequence. Six tryptic fragments of β-granin subjected to amino acid sequencing were all represented in the protein, the most C-terminal of these extending within two residues of the probable proteolytic processing site at $K_{129}R_{130}$. An amino acid analysis of native β-granin [5] was in excellent agreement with the predicted composition of the 14.3 kDa protein encoded by amino acids 1–128. The observed molecular masses of β-granin and its precursor on SDS-PAGE [6], however, are almost double those predicted by the sequence, a discrepancy probably related to anomalous electrophoretic behaviour of the molecule.

Northern blot analysis of insulinoma tissue showed the presence of a single mRNA species of approx. 2 kb (fig.3). mRNAs of a similar size were found in rat adrenal and pituitary but not in rat liver, cerebellum and exocrine pancreas. The presence of the 2 kb transcript in these tissues correlated with their immunoreactivity with anti-β-granin antisera as revealed by Western blotting.

Southern blot analyses performed with the nick-translated *Eco*RI insert from 218/38 showed hybridization to a very large number of restriction fragments including a number of well-defined bands (fig.4). The use of primer-extended m13 probes, however, indicated that this pattern arose from the same CA(G/A) repeat in the molecule which had been incriminated as the source of spurious clones obtained when initially rescreening the insulinoma cDNA library. Accordingly, when probes were used which avoided this sequence a single major hybridizing species was observed.

4. DISCUSSION

The cDNA sequence of the β-granin precursor which we obtained from rat insulinoma and pancreatic islet libraries corresponded to bases 174–1871 of the recently published rat adrenal chromogranin A cDNA (1871 bp) [15] and was identical except for a deletion of a cytosine at position 1862. This difference lies at the extreme 3′-end of the untranslated region and, even if real, is unlikely to affect the expression of the protein in a tissue-specific manner. Our results therefore do not support previous suggestions [16,17] that tissue-specific variants of chromogranin A mRNA exist. Rather, the heterogeneity of immunoreactive forms of chromogranin A in different tissues would appear to arise from proteolytic processing of the molecule. In the case of β-granin, *N*-linked glycosylation at Asn_{107} could also contribute to the observed small variations in size and charge of the molecule [5,8].

Whereas chromogranin A is a stable product of adrenal medullary cells [18], it is rapidly processed in pancreatic β-cells [6] by proteases with properties similar to those responsible for proinsulin conversion [19]. It is likely that several of the other dibasic sites in chromogranin A are susceptible to attack by the same enzymes. Of 10 such sites in the rat sequence, 7 are conserved in bovine [20,21] and human [22] chromogranins A (fig.5). Many of the peptides which would be produced (e.g. WE 14, EN 7 and AL 26) have a highly conserved sequence suggestive of conserved biological function.

These results suggest that chromogranin A is the actual precursor of pancreastatin. A C-terminally amidated peptide equivalent in size to the porcine molecule, however, cannot be generated simply by excision of paired basic sequences from the rat, human or bovine chromogranin A sequences. The conservation of the Gly-Lys sequence following the C-terminal glycine amide of pancreastatin suggests that initial endoproteolytic attack is directed on the C-terminal side of the lysine, the basic residue is then removed by carboxypeptidase H and the new C-terminal glycine then acts as the amide donor. Enzymes which could carry out such conversions are present in the endocrine pancreas. How and where cleavage might occur on the N-terminal end of the pancreastatin sequence is not clear as the homology in this region is weak.

Other postulated functions of chromogranin A include its involvement in the segregation of proteins to the regulated secretory pathway [20], binding of intragranular Ca^{2+} [21] and regulation of post-translational proteolysis [23]. Since the molecule is subject to cleavage at dibasic residues [7] it ought to be a competitive inhibitor of prohormone conversion, however, it is not particularly abundant in the pancreatic β-cell (1–2% of insulin). A role in protein segregation is suggested by the appearance of the fibronectin cell-binding domain sequence, Arg-Gly-Asp, at amino acids 43–45 in the bovine and human chromogranin A. In rat chromogranin A, this site is lost (Arg_{43}→Gln) though an equivalent sequence appears at residues 403–405. It remains to be seen if either site is functional, especially as the membrane adhesion properties in fibronectin depend on additional domains within the molecule [24]. The calmodulin-like Ca^{2+}-binding domains in bovine chromogranin A are poorly conserved in the rat molecule, thus arguing against this function.

Searches of the Doolittle, PIR, Claverie and Swiss protein sequence databases using the FASTP alignment program revealed a number of alignments of the polyglutamine sequence (amino acids 74–93) with diverse proteins such as wheat gliadin, the OPA repeat of *Drosophila* homeotic proteins, interleukin 2 and the rat glucocorticoid receptor. As in the case of the chromogranins, the polyglutamine tract in the rat glucorticoid receptor [25] is not present in the human sequence and interrupts an otherwise closely homologous region of the molecule. It is clear from Southern blot analyses and from our experience with cDNA library screening that the encoding CA(G/A) sequence is extensively repeated in the rat genome

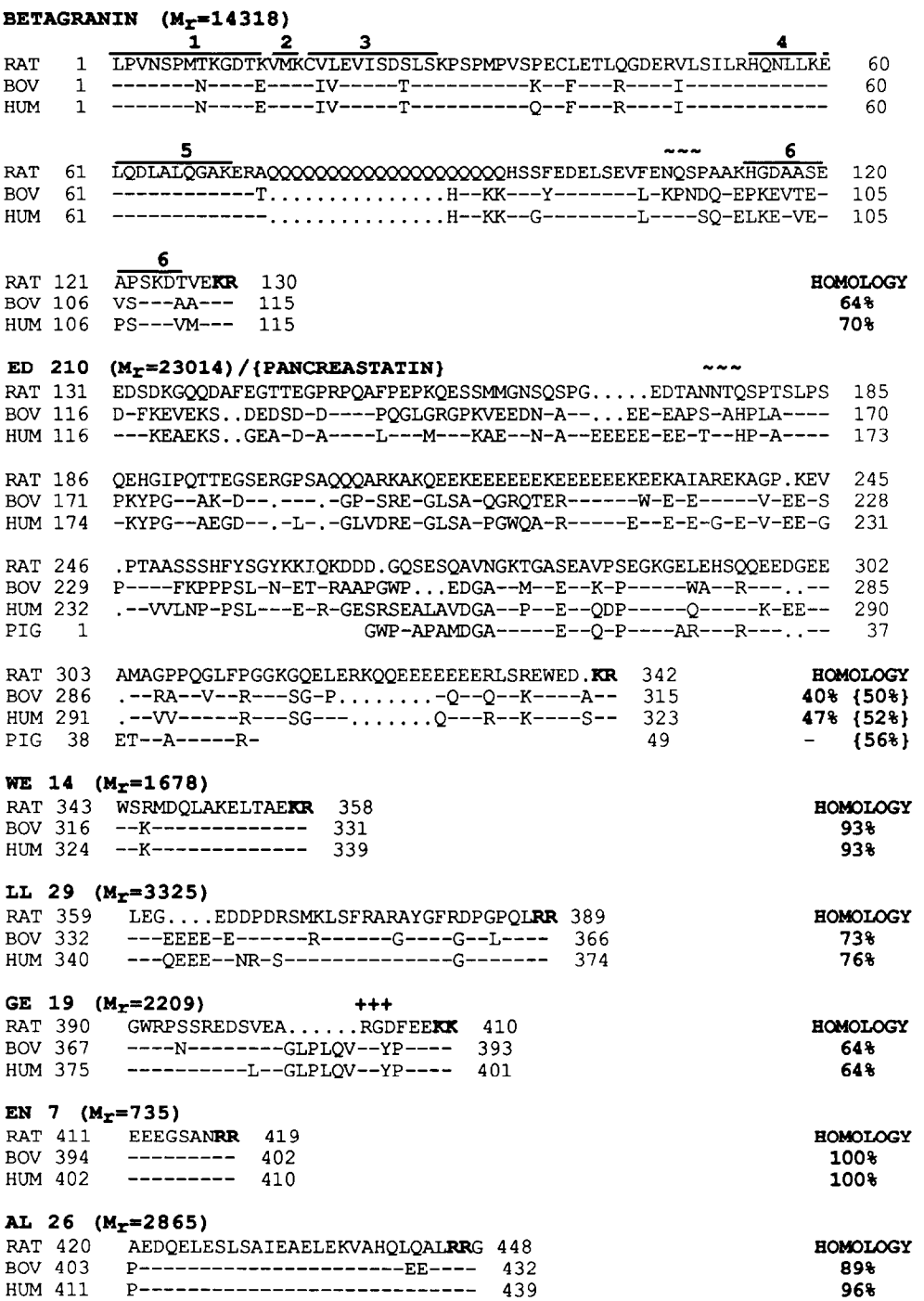

Fig.5. The deduced protein sequence of rat pancreatic chromogranin A and its homology with bovine adrenal [21] and human adrenal [22] chromogranin As and porcine pancreastatin [2]. The N-terminal peptide (1) and peptide sequences established from tryptic peptides (2-6) of β-granin are indicated, as are the 2 consensus sequences for *N*-linked glycosylation (~~~) and the tripeptide equivalent to the fibronectin receptor-binding site (+ + +). Alignments are oriented by 7 of the 10 dibasic sequences in the rat which are conserved and which form potential sites of endoproteolytic cleavage. The putative peptide products are designated by the N- and C-terminal residues followed by the peptide length. The molecular sizes of such peptides are shown in parentheses and the percentage homology to the rat sequence is indicated at the end of each segment.

and is transcribed. Since the polyglutamine sequence is absent in bovine and human chromogranin A, one assumes it to be neutral in its effect, possibly separating different domains of the protein, rather than having a specific functional role in tissue-specific expression or development.

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