β-Granins: 21 kDa co-secreted peptides of the insulin granule closely related to adrenal medullary chromogranin A

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Three closely related forms of a 21 kDa protein which is co-secreted with insulin have been purified and analysed. These differed in behaviour on ion-exchange chromatography but were indistinguishable by their susceptibility to staphylococcal V8 proteinase digestion, amino acid composition or N-terminal amino acid sequence. Their amino acid composition and N-terminal sequences were remarkably similar to adrenal medullary chromogranin A, a much larger protein (72 kDa). Antibodies to chromogranin A also reacted strongly with the 21 kDa protein in isolated insulin granules. It is concluded that the 21 kDa proteins either represent a repeated domain within the chromogranin molecule or a closely related gene product. The name β-granin is proposed for these proteins.

Pancreas *Insulin* *Chromaffin* *Secretory granule*

1. INTRODUCTION

Studies of a number of endocrine and neural tissues have shown that the exocytotic release of the principal hormone or neurotransmitter is often accompanied by other biologically active molecules. These co-secreted components potentially act as modulators of the activity of the principal hormone, mediate in cellular autoregulation or are involved in the regulation of adjacent tissues.

The insulin-secreting B cell of the pancreas, for example, contains biogenic amines [1], thyrotropin releasing hormone [2] and prolactin [3]. Perifusion experiments with radiolabelled islet and insulinoma cells have also shown that at least 7 other proteins are co-secreted with insulin [4]. One of these, a molecule of 21 kDa is a prominent component of isolated insulinoma secretory granules [5] and we report here the purification and analysis of a series of proteins of this molecular size from this source.

2. MATERIALS AND METHODS

Insulin secretory granules were prepared from transplantable rat insulinomas and stored at −70°C [5]. Approx. 25 mg granule protein was sonicated in 20 ml of 20 mM Tris-Cl, pH 9.0, containing 1 mM PMSF, 1 mM EDTA, 0.1 mM PCMB, 0.1 mM TLCK, 0.1 mM TPCK and 15 μg/ml pepstatin and centrifuged at 4°C for 30 min at 100000 × *g*. The supernatant was adjusted to pH 5.3 with acetic acid and the sample centrifuged for 10 min at 10000 × *g* and the supernatant dialysed against 50 mM Tris-Cl, pH 8.7, containing 150 mM NaCl, 1 mM DTT, 1 mM EDTA and 0.1 mM PMSF. 10-ml aliquots were chromatographed at 15 ml/h on a 35 × 2.5 cm column of AcA44 resin (LKB, Stockholm)

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equilibrated with the same buffer. Fractions containing the 21 kDa proteins were dialysed against 20 mM Bistris, pH 6.0, containing 0.1 mM PMSF and 0.4 mM DTT and chromatographed at 1 ml/min on a 5 × 0.5 cm Mono Q column (Pharmacia, Uppsala) using a 35 ml, 0–0.5 M NaCl gradient in the same buffer. Collected fractions (0.1–0.5 ml) were injected directly onto a 10 × 0.5 cm Pro RPC reverse-phase column (Pharmacia) which was eluted at 0.7 ml/min with a 30 ml linear 25–50% (v/v) acetonitrile gradient in 0.1% trifluoroacetic acid. Fractions were lyophilized and stored at −20°C.

The purification procedure was monitored by SDS-polyacrylamide gel electrophoresis [6] using a silver staining procedure [7]. Gels were polymerized from 17.5% (w/v) acrylamide 0.125% *N,N'*-bisacrylamide. Protein amino acid composition was determined with a Durram analyser (Dionex, Sunnyvale, CA) after 24 h hydrolysis at 100°C in constant-boiling HCl containing 1% phenol. N-terminal sequences were determined with an Applied Biosystems 470A protein sequencer (Foster City, CA) the PTH-amino acids being analysed on an IBM CN column (IBM, CT) using a Hewlett Packard HP1084B HPLC. Protein determinations were by dye binding [8] or from the A_{214nm} using bovine serum albumin as standard. Partial digests of proteins with staphylococcal V8 protease (Miles Scientific, Slough, England) were performed and analysed by SDS-polyacrylamide gel electrophoresis according to Cleveland et al. [9]. Western blotting was performed as described by Towbin et al. [10], i.e. using antisera at a 1:500 dilution and 3-amino 9-ethylcarbazole as the chromogenic substrate for the peroxidase reaction.

3. RESULTS

The above methods for the purification of the 21 kDa co-secreted component of rat insulinoma secretory granules yielded 3 major electrophoretically homogeneous proteins with unequivocal N-terminal sequences. On the initial gel filtration step the proteins eluted as a narrow band with an apparent M_r of 55000. On ion-exchange chromatography (fig.1) the protein eluted as 3 distinct peaks at NaCl concentrations of 0.19, 0.23 and 0.26 M. These fractions, designated A–C, were subjected to final purification by reverse-phase chromatography, the major components then eluting at acetonitrile concentrations of 36.5, 37.5 and 36%, respectively (fig.2). Fractions A and B also contained smaller quantities of proteins of 19 and 22 kDa which were also resolved by the reverse-phase step (fig.2).

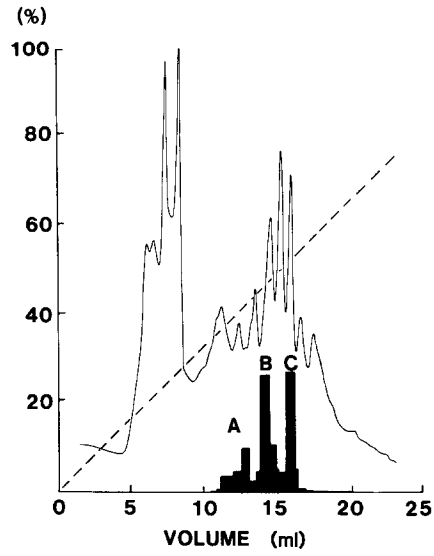

Fig.1. Ion-exchange chromatography of the 21 kDa proteins of insulin secretory granules. The histogram depicting the elution of the 21 kDa proteins was derived from analyses of each fraction by reverse-phase chromatography. The 100% value of the vertical axis corresponds to an A_{280nm} of 0.2 (solid trace), an NaCl concentration of 0.5 M (dotted trace) and a 21 kDa protein concentration of 0.17 mg/ml.

The insulin granule content of the 21 kDa proteins estimated by densitometric scanning of electrophoretograms was 0.5–1% of the total protein. From 25 mg granule protein 17, 30 and 30 μg of the A, B and C forms of the proteins were recovered. Thus the overall yield was 30–60%.

Partial digestion of the purified A, B and C forms of the peptides with staphylococcal V8 protease produced, in every case, major peptides of 15 and 17.5 kDa (fig.3). Compositional analyses revealed that around 37% of the amino acid residues were Glx or Asx, 8% proline and that the content of aromatic residues was low (table 1). No differences in amino acid composition were evident between the A, B and C forms of the protein. The first 14 residues of their N-termini were also identical (table 2).

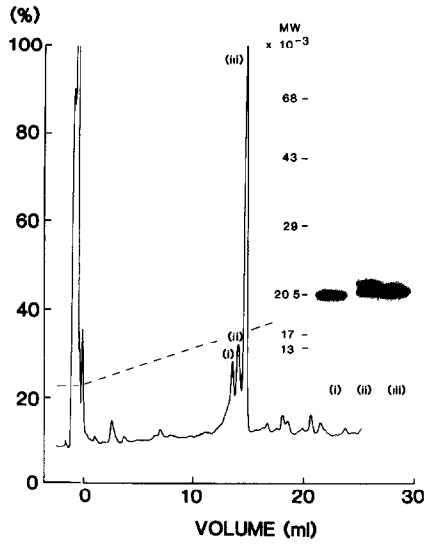

Fig.2. Reverse-phase chromatography of the 21 kDa protein fraction A. Equivalent quantities of the eluted fractions i–iii (approx. 1 μg) were analysed by SDS-polyacrylamide gel electrophoresis as shown in the inset. The 100% value of the vertical axis corresponds to an A_{214nm} of 0.5 (——) and an acetonitrile concentration of 99.9% (w/v) (– – –).

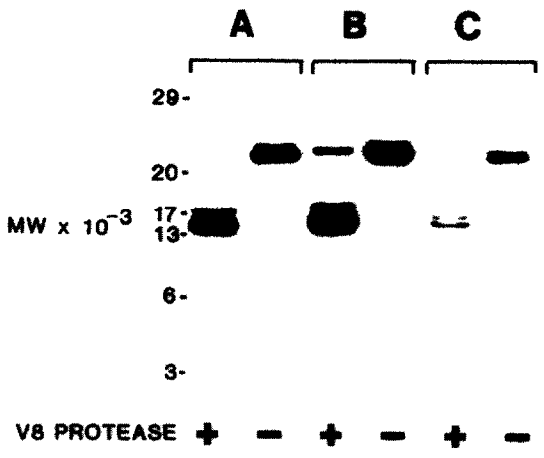

Fig.3. Staphylococcal V8 proteinase digestion of the predominant A, B and C forms of the 21 kDa proteins. Samples of each protein (2, 6 and 1 μg, respectively) were incubated for 40 min at 37°C with staphylococcal V8 proteinase as indicated and analysed by SDS-polyacrylamide gel electrophoresis.

Table 1

Amino acid composition of the A, B and C forms of the 21 kDa protein and of bovine adrenal medullary chromogranin A [11]

Amino acid	Residues per mole	Percentage composition (w/w)			
		21 kDa A	21 kDa B	21 kDa C	Chromogranin A
Asx	16	9.27	9.13	8.94	8.41
Thr	6	3.11	3.11	2.91	1.57
Ser	17	7.75	7.47	7.24	3.78
Glx	50	32.90	33.00	32.06	28.76
Pro	14	6.34	6.79	7.32	7.95
Gly	8	2.49	2.54	2.08	4.36
Ala	13	4.45	4.86	4.53	5.51
Val	9	4.38	4.72	4.34	4.51
Met	2	0.94	0.95	1.62	1.57
Ileu	2	1.29	1.21	1.31	1.40
Leu	18	10.47	10.10	10.56	8.06
Phe	3	2.38	2.06	2.69	2.03
His	5	3.65	3.71	3.57	1.74
Lys	12	6.76	6.43	6.66	9.62
Arg	6	3.87	3.98	4.20	9.64
Tyr	0	0.00	0.00	0.00	0.00

Results expressed as % (w/w) are the means of duplicate analyses performed on 2 different preparations. The molar composition was estimated from the mean composition of all 3 forms assuming a molecular mass of 21 kDa and an isoleucine content of 2 mol/mol

A survey of the amino acid composition of secretory granule proteins of a number of tissues revealed that the 21 kDa proteins were remarkably similar to the 72 kDa major protein of the adrenal medullary chromaffin granule, chromogranin A (table 1). Furthermore, with the exception of the exchange of a Thr for Asn at position 9, the N-termini of the 21 kDa protein and bovine chromogranin A were identical (table 2). The N-terminus of the 21 kDa peptides was also the same as that of secretory protein 1 (70–72 kDa) of the parathyroid gland [16] and showed extensive homology to a 32 kDa CNBr fragment of bovine chromogranin A [15].

A polyclonal antiserum directed against bovine adrenal medullary chromogranin A reacted to an equivalent extent on Western blots with the A, B and C forms of the 21 kDa protein (fig.4). The presence of minor components of 19 and 22 kDa in

Table 2

Amino-terminal sequence of the 21 kDa A, B and C proteins, bovine adrenal medullary chromogranin A, a chromogranin A CNBr fragment and bovine parathyroid gland secretory protein 1

Protein	Residue no.													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
21 kDa proteins	Leu	Pro	Val	Asn	Ser	Pro	Met	Thr	X	Gly	Asp	Thr	X	Val
Chromogranin A [12]	Leu	Pro	Val	Asn	Ser	Pro	Met	Asn	Lys	Gly	Asp	Thr	Glu	Val
Chromogranin A [13,14]	Leu	Arg	Val	Asn	Ser	Pro	Met	Asn	Lys	Gly	Asp	Thr	Glu	Val
Chromogranin 32 kDa fragment [15]	Leu	Pro	Val	Asn	Gln	Pro	Gly	Asn	Lys	Leu	Asp	Glu	Glu	Val
Secretory protein 1 [16]	Leu	Pro	Val	Asn	Ser	Pro	Met	Asn	Lys	Gly	Asp	Thr	Glu	Val

The 21 kDa sequence was compiled from duplicate analyses of each of the proteins with 2 different preparations. Residues denoted X could not be unequivocally assigned. Data for the other proteins are derived as indicated

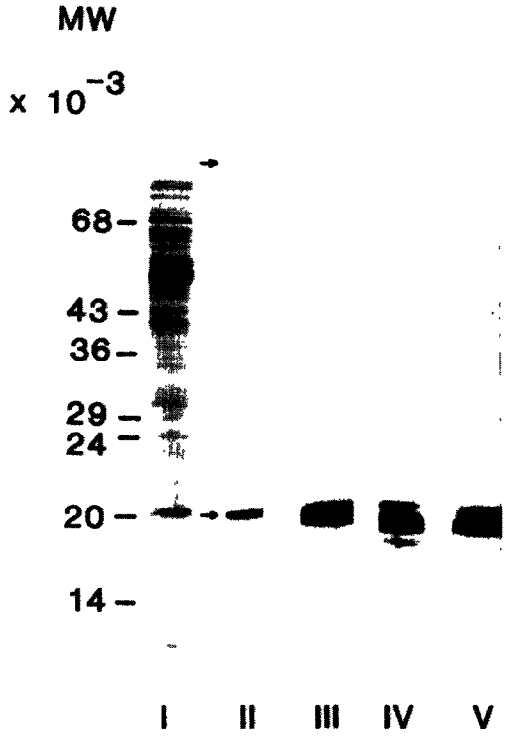

Fig.4. Chromogranin A immunoreactivity of insulin granules (lanes I and II, approx. 20 μg protein) and purified A, B and C forms of the 21 kDa proteins (lanes III–V, respectively, 0.5 μg protein). SDS-polyacrylamide gel electrophoretograms were transferred to nitrocellulose paper and either stained with naphthalene black (lane 1) or developed in an immunoperoxidase reaction using rabbit anti-bovine adrenal medullary chromogranin A as the primary antibody (lanes II–V). The positions of the major 21 kDa and minor 80 kDa immunoreactive forms are indicated.

fraction B was also revealed, corresponding to the minor components observed on reverse-phase chromatography (fig.2). The initial secretory granule preparation showed a 21 kDa immunoreactive component as the major form together with a minor 80 kDa constituent (< 5% by densitometry).

4. DISCUSSION

The 21 kDa co-secreted product of the insulin granule was isolated in these studies as 3 major proteins differing principally in their behaviour on ion-exchange chromatography. Two-dimensional electrophoretic analyses of isolated insulinoma granules [5] has previously revealed the presence of 4 peptides in the 21–22 kDa size range with isoelectric points of between 5.0 and 5.1. These 4 peptides crossreact with antisera raised to the isolated peptides (Hutton and Peshavaria, unpublished) and thus appear to be the native forms of these proteins.

The remarkable finding which emerged in the course of these investigations was the similarity of the isolated proteins to a major secreted protein of the adrenal medulla, chromogranin A. Except for a possible point mutation resulting in the replacement of an Asn at position 8 with Thr, the N-terminal sequence was indistinguishable from that reported for bovine chromogranin A [12–14]. The amino acid composition of the 21 kDa proteins and chromogranin A were also remarkably similar in spite of the 3–4-fold difference in size of those molecules on SDS-polyacrylamide gel elec-

trophoresis. This suggests that the 21 kDa proteins represent a domain which is repeated within the chromogranin molecule, a conclusion which is supported by the recent demonstration [15] that the N-termini of several CNBr fragments of chromogranin A show considerable homologies to the N-terminus of the native forms of the molecule.

Antisera raised to bovine adrenal medullary chromogranin A applied to Western blots of insulin secretory granules indicated that the 21 kDa proteins were the predominant (>95%) immunoreactive species in this organelle. A trace of an 80 kDa protein, however, was also observed which was identical in electrophoretic mobility to the major immunoreactive form in isolated rat adrenal chromaffin granules (Hutton and Peshavaria, unpublished). The insulin granule thus appears also to contain native chromogranin A. It may be that the 21 kDa proteins are derived from the larger precursor and that proteolytic processing in the insulin granule occurs by a different pathway or to a different extent than in the chromaffin granule. However, the size of the primary translation product of chromogranin mRNA in a number of tissues varies widely, suggesting that either multiple genes exist or that differential splicing of chromogranin mRNA occurs in different tisues [17].

Chromogranin A immunoreactivity appears to be a feature of many endocrine tissues [18], however, it is clear from the present study that the molecular form and the quantity stored in secretory granules vary with the tissue in question. The fact that the N-termini of the rat insulin granule 21 kDa proteins and bovine chromogranin A are highly conserved is indicative of a conserved biological function. If these proteins play a common intracellular role, however, it follows that the activity may reside on a smaller fragment of the molecule than on chromogranin A itself.

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