The primary structure of human secretogranin I (chromogranin B): comparison with chromogranin A reveals homologous terminal domains and a large intervening variable region

Ulrich M.Benedum, Annie Lamouroux²,
David S.Konecki, Patrizia Rosa, Annette Hille,
Patrick A.Baeuerle, Rainer Frank¹, Friedrich Lottspeich³,
Jacques Mallet² and Wieland B.Huttner

Cell Biology Programme and ¹Biochemical Instrumentation Programme,
European Molecular Biology Laboratory, Postfach 102209, D-6900
Heidelberg, FRG, ²Department of Neurobiology, Centre National de la
Recherche Scientifique, F-91190 Gif-sur-Yvette, France, and ³Genzentrum,
Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG

Communicated by B.Dobberstein

We have determined and analyzed the primary structure of human secretogranin I (chromogranin B), a tyrosine-sulfated secretory protein found in a wide variety of peptidergic endocrine cells. A 2.5-kb cDNA clone, hybridizing to an mRNA of similar length, was isolated from a cDNA library of human pheochromocytoma. The identity of the clone was established by comparison of its deduced amino acid sequence with N-terminal and several internal secretogranin I sequences as well as by immunoprecipitation of the protein produced by *in vitro* transcription–translation of the cloned cDNA. Secretogranin I is a 657 amino acid long polypeptide of 76 kd and is preceded by a cleaved N-terminal signal peptide of 20 residues. Comparison of the predicted amino acid sequence of human secretogranin I with that of bovine chromogranin A reveals significant homologies near the N termini and at the C termini. The N-terminal homologous domains contain the only two cysteine residues of both proteins and form disulfide-stabilized loop structures. The sequences between the homologous terminal domains in both proteins differ but are characterized by a remarkable hydrophilicity, an abundance of acidic amino acids and potential dibasic cleavage sites for the generation of smaller, perhaps hormone-like, peptides.

Key words: adrenal medulla/cDNA sequence/peptide hormones/sorting/tyrosine sulfation

Introduction

Many eucaryotic cells contain more than one pathway of protein secretion (see Kelly, 1985). Cells which release specific proteins in response to an appropriate stimulus are capable of sorting them from the constitutively secreted proteins into specialized organelles, the secretory granules. This 'regulated' pathway of protein secretion is found in cells secreting into external fluids (exocrine cells) as well as in cells secreting into internal fluids (endocrine cells and neurons). In studying protein tyrosine sulfation (see Huttner and Baeuerle, 1987), we described several years ago an acidic protein doublet (p113/p105) that was secreted by the regulated pathway from a rat neuroendocrine cell line (Huttner and Lee, 1982; Lee and Huttner, 1983). Subsequent studies revealed the presence of this or a very similar protein in many, but not all, secretory granules of a wide variety of peptidergic endocrine cells and neurons (Fischer-Colbrrie *et al.*, 1985; Rosa

et al., 1985b; Lassmann *et al.*, 1986; Rindi *et al.*, 1986). This protein has been called secretogranin I (Rosa *et al.*, 1985b; see also Hille *et al.*, 1984; Lee and Huttner, 1985; Rosa *et al.*, 1985a) or chromogranin B (Falkensammer *et al.*, 1985a,b). Secretogranin I (chromogranin B, see note on nomenclature in Materials and methods) has not been found in exocrine cells and is therefore characteristic for a large proportion of endocrine secretory granules.

Since secretogranin I is an abundant protein which is sorted to secretory granules in a large variety of endocrine cells and neurons, we consider it ideal for identifying protein structural features involved in sorting to endocrine secretory granules. The search for such structural features is greatly facilitated by the fact that secretogranin I is just one member of a class of proteins that all show a widespread distribution in secretory granules of peptidergic endocrine cells and neurons (Rosa *et al.*, 1985b). Such a cellular and subcellular distribution has been shown for at least two other proteins, chromogranin A (Lloyd and Wilson, 1983; O'Connor *et al.*, 1983; Cohn *et al.*, 1984; Somogyi *et al.*, 1984) and secretogranin II (Rosa *et al.*, 1985a,b; Lassmann *et al.*, 1986; Rindi *et al.*, 1986). These proteins may share certain structural features required for their sorting. It is therefore of interest to determine the primary structures of these proteins and to identify common domains.

The determination of the primary structure of these proteins will also be of great value in defining their, as yet unknown, function. The amino acid sequence of chromogranin A, the major secretory protein of the bovine adrenal medulla, has recently been described (Benedum *et al.*, 1986; Iacangelo *et al.*, 1986) and suggests that one of several possible functions of chromogranin A may be a precursor for regulatory peptides. Consistent with this possibility, we (Huttner and Benedum, 1987) and others (Eiden, 1987) have recently reported that chromogranin A is likely to be the precursor for pancreastatin, a novel peptide inhibiting insulin secretion (Tatemoto *et al.*, 1986). In the present study we describe and analyze the primary structure of secretogranin I, the major secretory protein of the human adrenal medulla (Hagn *et al.*, 1986), and present the first detailed molecular comparison between two proteins that are characteristic for a major population of endocrine secretory granules.

Results

Purification and protein sequencing of secretogranin I (chromogranin B)

Secretogranin I was purified to homogeneity from a mixture of [³⁵S]sulfate-labeled and unlabeled PC12 cells by heat treatment, anion exchange chromatography and either preparative SDS–PAGE or two-dimensional PAGE (Figure 1, top). Purified secretogranin I from one-dimensional gels was used to obtain the sequence of the first 14 amino acids of the N terminus of the rat protein (Figure 1, top). The sequence obtained was different from that reported for the corresponding M_r 100 000 protein from bovine chromaffin granules (Settleman *et al.*, 1985).

Fig. 1. (Top) Purification of rat secretogranin I from [^{35}S]sulfate-labeled PC12 cells. **Left:** aliquots of the soluble fraction (S) of the cell homogenate, of the heat-stable fraction (HS), and of the DEAE peak (DE) were analyzed by SDS-PAGE followed by staining with Coomassie Blue (CB) and autoradiography (AR). The M_r 113 000/105 000 components of secretogranin I are indicated by the bracket. **Right:** proteins of the DEAE peak were separated by two-dimensional PAGE (basic side, left; acidic side, right). The M_r 113 000/105 000 components of secretogranin I used for tryptic digestion and h.p.l.c. (see bottom) are indicated by the dotted line. The inset in the right panel shows the N-terminal sequence (referred to as sequence I, see Figure 2B) of rat secretogranin I isolated after SDS-PAGE. The positions of molecular mass markers are given in kd. (Middle) Immunoblots of a total homogenate of rat PC12 cells (**left**) and of the heat-stable protein fraction of a human pheochromocytoma (**right**) using affinity-purified anti-rat secretogranin I antibodies (3 and 6 $\mu\text{g}/\text{ml}$ respectively) followed by [^{125}I]protein A and autoradiography (AR). The middle panel shows the protein staining of a two-dimensional gel run in parallel with the immunoblot shown in the right panel. The M_r 113 000/105 000 components of rat secretogranin I and the M_r 120 000 human secretogranin I are indicated by the bracket and the filled arrowheads respectively. (Bottom) h.p.l.c. of tryptic peptides of [^{35}S]sulfate-labeled two-dimensionally pure rat secretogranin I (see top right panel). Fractions were collected as indicated by the marks on the top abscissa and analyzed for ^{35}S -radioactivity (upper part). Selected peaks were subjected to N-terminal sequencing. The sequences obtained (referred to as IIa, IIb and III, see Figure 2B) are shown in the boxes. The dashed line indicates the acetonitrile gradient.

Fig. 2. (A) Restriction map of clone hSGI/19. The thick line indicates the 2031-bp open reading frame. Only some of the restriction sites used for subcloning are shown. Arrows indicate the length and location of the sequences derived from M13 clones. (B) The complete nucleotide and deduced amino acid sequence of human pre-secretogranin I. The negative numbers above the amino acid sequence indicate the signal peptide. The lines with Roman numbers above the amino acid sequence show the positions of the sequences determined for rat secretogranin I (see Figure 1, top and bottom). Asterisks, sites with two or more basic amino acids; open arrows, cysteine residues; filled triangle, potential N-glycosylation site; circled 'S', identified tyrosine sulfate site; filled diamond, potential tyrosine sulfation site; open triangles, polyadenylation signal.

Purified secretogranin I from two-dimensional gels was digested with trypsin, followed by h.p.l.c. separation of the resulting peptides and N-terminal sequencing of selected peaks (Figure 1, bottom). Partially purified secretogranin I was used for the affinity purification of polyclonal anti-rat secretogranin I antibodies raised previously against pure rat PC12 cell secretogranin I (Rosa *et al.*, 1985b). The affinity-purified anti-rat secretogranin I antibodies recognized the M_r 113 000/105 000 protein from rat PC12 cells as well as the corresponding M_r 120 000 protein from a human pheochromocytoma (Figure 1, middle), but not the M_r 100 000 protein from bovine chromaffin granules (data not shown).

Cloning of a human secretogranin I (chromogranin B) cDNA

Putative cDNA clones for secretogranin I were selected from a human pheochromocytoma λ gt11 library on the basis of (i) the presence and abundance of the corresponding mRNA in endocrine tissue and (ii) the reaction of the fusion protein with the affinity-purified anti-rat secretogranin I antibodies (data not shown). The sequence of one such clone revealed high homologies with the sequences of tryptic peptides of rat secretogranin I but did not contain the N terminus of the protein. Fragments of this cDNA insert were used for screening a size-selected λ gt10 cDNA library of the same human pheochromocytoma. From > 100 hybridizing clones, 30 were plaque-purified and screened for inserts containing the N terminus of secretogranin I with a 42-mer oligonucleotide. This oligonucleotide had been synthesized according to the N-terminus sequence of rat secretogranin I (Figure 1, top), applying the codon usage table for mammals (Grantham *et al.*, 1980). Two clones were found, of which one (hSgl/19) turned out to be full-length after sequencing.

Complete nucleotide and deduced amino acid sequence of human secretogranin I (chromogranin B)

The complete nucleotide sequence of the mRNA derived from the cDNA clone hSgl/19 was obtained as illustrated in Figure 2A and is shown in Figure 2B. It is 2454 bp long [not including the poly(A) tail] and contains a 112-bp untranslated region at the 5' end, a 2031-bp coding region, and a 311-bp untranslated region at the 3' end which includes the polyadenylation signal AAUAAA. The open reading frame codes for a 677 amino acid long protein. The first 20 amino acids constitute a typical signal sequence responsible for targeting the nascent polypeptide to the membrane of the rough endoplasmic reticulum. The signal sequence ends with a serine residue which is a suitable cleavage site for signal peptidase (Hortsch and Meyer, 1986). The sequence of the following 14 amino acids is highly homologous to the N-terminal sequence of rat PC12 cell secretogranin I (Figure 1, top), the three differences being almost certainly due to species variation. This indicates that the mature form of secretogranin I (M_r 120 000 in man) is not proteolytically processed at the N terminus after signal peptide removal. Three of the tryptic peptides predicted by the cDNA sequence show high homology to the three tryptic peptide sequences determined for rat PC12 cell secretogranin I (compare Figure 1, bottom, and bars in Figure 2B).

The mol. wt of unmodified human secretogranin I deduced from the cDNA is 76 295, which differs from the apparent mol. wt of the protein after two-dimensional PAGE (M_r 120 000; see Figure 1, middle). We examined the possibility of an anomalous electrophoretic mobility of secretogranin I by analyzing the electrophoretic mobility of the polypeptide produced from the cloned cDNA *in vitro*. For this purpose, the *Eco*RI fragment of the λ gt10 clone hSgl/19, containing the entire insert for

secretogranin I, was cloned into the expression vector pDS6 (Stueber *et al.*, 1984). The construct was transcribed *in vitro*, and the resulting capped mRNA was translated in a cell-free wheat germ system. The polypeptide synthesized (i) was recognized by a monoclonal anti-secretogranin I antibody, (ii) was heat-stable as authentic secretogranin I, and (iii) had an electrophoretic mobility (M_r ~ 110 000) similar to the mature form of human secretogranin I. [The slightly slower electrophoretic mobility of mature secretogranin I from human pheochromocytoma tissue compared to the *in vitro* transcribed-translated secretogranin I precursor is most likely due to post-translational modifications (see Rosa *et al.*, 1985b).] Since the electrophoretic mobility of pre-secretogranin I produced *in vitro* is markedly higher than expected from its real mol. wt of 78 246, secretogranin I behaves anomalously upon SDS-PAGE. When the *in vitro* translation was performed in the presence of rough microsomes from dog pancreas, secretogranin I was protected from digestion by added protease (Figure 3). This indicated that the pre-secretogranin I produced *in vitro* was competent to be translocated across the membrane of the rough endoplasmic reticulum and thus to enter the secretory pathway. The agreement between the rat secretogranin I peptide sequences and the present human cDNA sequence as well as the results of the coupled *in vitro* transcription-translation experiments lead us to con-

Fig. 3. *In vitro* transcription-translation of the human secretogranin I precursor. Plasmid pDS6-Sgl was transcribed *in vitro* and the resulting capped mRNA translated in a cell-free wheat germ system using [35 S]-methionine, followed by SDS-PAGE and autoradiography (lanes 2–8). **Panel A:** immunoprecipitation of the translation products with monoclonal antibodies recognizing human secretogranin I (lane 2) or bovine chromogranin A (lane 3). **Lane 1** shows the Coomassie blue staining of crude bovine chromaffin granules electrophoresed adjacent to the sample shown in lane 2. **Panel B:** transcription-translation in the presence (lane 4) and absence (lane 5) of pDS6-Sgl DNA. Translation products were subjected to heat treatment, and the heat-stable proteins analyzed. **Panel C:** transcription-translation in the presence of rough microsomes. **Lane 6:** no treatment after translation; **lane 7:** addition of proteinase K after translation; **lane 8:** addition of proteinase K and Triton X-100 after translation. The positions of secretogranin I are indicated by the filled arrowheads; open triangle, chromogranin A. The positions of mol. wt markers are shown on the left.

clude that the predicted amino acid sequence shown in Figure 2B represents the complete primary structure of human secretogranin I.

The human secretogranin I (chromogranin B) mRNA

Northern blot analysis of poly(A)⁺ RNA from human adrenal medulla and a human pheochromocytoma indicated that the human secretogranin I mRNA had a length of 2.5–2.6 kb. Under stringent conditions no other bands were detected in these tissues (Figure 4). Secretogranin I mRNA was not detectable in human liver, as expected from previous data on the tissue distribution of the protein (Rosa *et al.*, 1985b).

Analysis of the human secretogranin I (chromogranin B) sequence

The hydropathy plot according to Kyte and Doolittle (1982) shows that secretogranin I is a highly hydrophilic protein and does not contain a long hydrophobic domain indicative of a membrane spanning protein (Figure 5). Its hydrophilic character is also apparent from the deduced amino acid composition which is high

in charged amino acids and similar to chromogranin A (Table I).

Secretogranin I contains several sites for post-translational modification. Two of the 21 tyrosine residues (Tyr-153 and Tyr-321) are contained in sequences that fulfill the consensus features for tyrosine sulfation sites (Huttner and Baeuerle, 1987). Tyr-321 is expected to be the better sulfation site since an acidic amino acid is directly preceding the tyrosine residue. Secretogranin I is known to be sulfated, containing most of the sulfate as tyrosine sulfate (Lee and Huttner, 1983; Rosa *et al.*, 1985b). The major sulfate-labeled, tyrosine-containing tryptic peptide of rat PC12 cell secretogranin I corresponds to the predicted tryptic peptide of human secretogranin I starting at Ala-314 (see Figures 1 and 2). Hence, Tyr-321 is the major site for tyrosine sulfation in secretogranin I. Secretogranin I contains up to 7% of its tyrosine residues in sulfated form (Huttner and Baeuerle, 1987), which equals 1.47 mol of tyrosine sulfate per mol of polypeptide (see Figure 2B). Thus, in addition to Tyr-321, Tyr-153 may also be sulfated, but only partially. This is consistent with our previous proposal that tyrosine sulfation sites lacking acidic amino acids at position -1 of the tyrosine may be subject to substoichiometric sulfation (Huttner and Baeuerle, 1987). Asp-295 constitutes a potential N-glycosylation site. Neither bovine nor rat secretogranin I produced by cells in culture alter their electrophoretic mobility in one- and two-dimensional gels after inhibition of N-glycosylation by tunicamycin (Rosa *et al.*, 1985a; W.B.Huttner, unpublished data). It remains to be shown whether Asp-295 is actually used for N-glycosylation in the human protein. Human secretogranin I contains 15 sites with two or more adjacent basic amino acids (see Figure 2B). As discussed below, these may be potential sites for the proteolytic generation of smaller polypeptides. In contrast to bovine chromogranin A, human secretogranin I does not contain -Arg-Gly-Asp-, a sequence implicated in the interaction of certain proteins with cell membranes (see Ruoslahti and Pierschbacher, 1986).

A comparison of the secretogranin I sequence with the protein sequence data libraries of the National Biomedical Research Foundation (NBRF) and the EMBL showed no significant homologies with sequenced proteins. (The limited homology between secretogranin I and chromogranin A is described below.) A sequence homologous to pancreastatin (Tatemoto *et al.*, 1986) which occurs in bovine chromogranin A (Eiden, 1987; Huttner and Benedum, 1987) is not found in human secretogranin I. It has previously been reported that antisera raised against bombesin recognize bovine secretogranin I (chromogranin B; Fischer-Colbrie *et al.*, 1986). However, no significant homology between bombesin and human secretogranin I was found.

Homologous domains in secretogranin I (chromogranin B) and chromogranin A

Figure 6 shows a diagonal plot of human secretogranin I and bovine chromogranin A using the program developed by Argos

Fig. 4. Northern blot analysis of human secretogranin I. Poly(A)⁺ RNA from a human pheochromocytoma (lane 1, 1 μ g RNA), human adrenal medulla (lane 2, 1 μ g RNA) and human liver (lane 3, 3 μ g RNA) was hybridized with a 1.8-kb fragment of the secretogranin I clone extending from the poly(A) tail approximately to the 5' *Bal*I site (see Figure 2A). The positions of molecular size markers (*Hind*III-digested lambda DNA) are given in kb on the right.

Fig. 5. Hydropathy plot of human secretogranin I precursor according to Kyte and Doolittle (1982). The numbering of amino acids is the same as in Figure 2B. HPhobic, hydrophobic; HPhilic, hydrophilic.

(1987). Two domains of significant amino acid sequence homology are observed. The strongest homology is very close to the N termini of both proteins (see values in the legend to Figure 6). The other domain of high homology is located near the C termini. Secretogranin I and chromogranin A each con-

Table I. Amino acid composition of bovine chromogranin A and human secretogranin I deduced from nucleotide sequences (Benedum *et al.*, 1986; Figure 2B) without signal peptides

	Human secretogranin I	Bovine chromogranin A
Ala	5.6	8.1
Arg	8.4	6.5
Asn	4.3	2.1
Asp	5.9	5.1
Cys	0.3	0.5
Glu	17.7	18.8
Gln	5.3	5.3
Gly	7.0	7.7
His	4.1	1.6
Ile	1.1	1.2
Leu	5.5	7.4
Lys	7.1	8.1
Met	1.2	1.6
Phe	2.1	1.2
Pro	5.0	8.8
Ser	8.5	8.1
Thr	3.4	2.1
Trp	1.5	1.4
Tyr	3.2	1.0
Val	2.7	3.5

tain only two cysteine residues which are located in the homologous N-terminal domains. Their sequence positions differ by one residue and their relative spacing (20 amino acids) is identical. Secondary structure prediction (Argos, 1985) indicates that in secretogranin I and chromogranin A the sequences between the top cysteines have a high turn conformational potential and a tendency to form a loop structure.

Intramolecular disulfide bonds in secretogranin I (chromogranin B) and chromogranin A

Neither secretogranin I (Figure 7, left) nor chromogranin A (Figure 7, left; Hörtnagl *et al.*, 1974) form disulfide-bonded oligomers, as shown by their identical electrophoretic mobility in reducing and non-reducing SDS gels. Labeling of reduced and non-reduced secretogranin I and chromogranin A with radioactive *N*-ethyl maleimide (NEM) was used to determine whether the cysteines in each of the two proteins form intramolecular disulfide bonds. As shown in Figure 7 (right), both secretogranin I and chromogranin A from secretory granules were not labeled with NEM unless the proteins had been reduced, indicating that the cysteine residues were disulfide-bonded *in vivo*.

Discussion

The cDNA sequence of secretogranin I reveals the primary structure of the major secretory protein of the human adrenal medulla. Furthermore, we have identified structural features which are common to secretogranin I and chromogranin A and therefore likely to be biologically significant (see Figure 8).

The homologous domains at the N termini and the C termini

The most striking observation made by comparing the secretogranin I and chromogranin A sequences is the homology

Fig. 6. Comparison of human secretogranin I (hSgI) and bovine chromogranin A (bCgA) according to Argos (1985, 1987), using window lengths of 7–27 residues. The significance of homology is given in decreasing order as follows: bars, $\sigma > 5.5$; open circles, $\sigma = 5.0–5.5$; thick lines, $\sigma = 4.5–5.0$; thin lines, $\sigma = 4.0–4.5$. σ refers to the SD of the search matrix values. For details, see Argos (1985, 1987). Within the regions corresponding to the two bars, homologies with a $\sigma > 6.0$ were found which are shown in sequence. Asterisks indicate sequence identities. In the N-terminal domains, the sequences from cysteine to cysteine are $>54\%$ identical. In the C-terminal domains, the sequences are $>44\%$ identical. Non-identical amino acids are mostly conservative changes. The numbering of amino acids of human secretogranin I and bovine chromogranin A is the same as in Figure 2B and in Benedum *et al.* (1986) respectively.

between the domains near the N termini and at the C termini of the two proteins. It is probably not a coincidence (i) that both proteins contain only two cysteine residues, (ii) that in both proteins the cysteines are located in nearly identical sequence positions and are separated from each other by the same number of amino acids, and (iii) that the domain of highest homology between the two proteins is found between these cysteines. This domain contains several residues with high turn conformational potential (Argos, 1985). Since these two cysteine residues form an intramolecular disulfide bond, this highly homologous domain is probably exposed in a disulfide bond-stabilized loop structure. Whether this domain or the C-terminal domain are epitopes involved in the intracellular transport of secretogranin I and chromogranin A to endocrine secretory granules is currently under investigation.

Fig. 7. Intramolecular disulfide bonds in secretogranin I and chromogranin A. **Left panel:** crude bovine chromaffin granules were analyzed by SDS-PAGE under non-reducing (NR) and reducing (R) conditions, followed by staining with Coomassie Blue (CB). **Right panel:** crude bovine chromaffin granules were incubated in the absence (-) and presence (+) of DTT. After removal of DTT, samples were labeled with [3 H]NEM and analyzed by SDS-PAGE under reducing conditions followed by fluorography. Filled arrowheads, secretogranin I; open arrows, chromogranin A. The positions of mol. wt markers are given in kd.

The large variable region

The least hydrophilic regions of secretogranin I and chromogranin A coincide with the homologous N-terminal and C-terminal domains. In contrast, the intervening regions of both proteins, comprising 85–90% of the entire sequence, vary. However, in both proteins this variable region is characterized by a remarkable hydrophilicity and a high proportion of amino acids providing free carboxyl groups, notably glutamic acid residues. We have previously suggested that the abundance of acidic residues in the secretogranins/chromogranins may indicate a helper function in endocrine secretion, such as (i) in the packaging of peptide hormone precursors into secretory granules, (ii) in the processing of peptide hormone precursors, and/or (iii) in the solubilization of the granule content after exocytosis (Rosa *et al.*, 1985a,b; Huttner *et al.*, 1987). Any such helper function probably resides in the variable region of secretogranin I and chromogranin A. The variation in sequence of the negatively charged region may indicate either that this variation reflects different specific demands on the sequence (such as specific peptide recognition) or that it is the simple presence of negative charge in this region which is essential for function.

Is secretogranin I (chromogranin B) a precursor for regulatory peptides?

Secretogranin I, secretogranin II and chromogranin B undergo partial proteolytic processing during storage in secretory granules and give rise to multiple proteolytic products (Rosa *et al.*, 1985b). The biological significance of this processing is not yet fully understood. We (Benedum *et al.*, 1986) and others (Iacangelo *et al.*, 1986) have previously pointed out for chromogranin A that the presence of eight dibasic sites, which are potential signals for proteolytic processing of prohormones (see Docherty and Steiner, 1982), may indicate a role of this protein as precursor for regulatory peptides. In addition, it has recently been noted (Eiden, 1987; Huttner and Benedum, 1987) that pancreastatin, a pancreatic peptide that inhibits glucose-induced insulin release (Tatemoto *et al.*, 1986), is highly homologous to a portion of chromogranin A and may well be one of the proteolytic products of chromogranin A produced in endocrine pancreatic cells. The observation that secretogranin I contains as many as 15 sites with two or more adjacent basic amino acids would be consistent with secretogranin I being a multi-peptide precursor and may explain why – at least in certain tissue such as the bovine adrenal medulla – the number of proteolytic products generated from secretogranin I is greater than that generated from chromogranin A (Rosa *et al.*, 1985b). If secretogranin I and chromogranin A should indeed prove to be hormone precursors, the number of biologically active peptides potentially generated may be very large since each of the sequences between the potential dibasic cleavage sites in secretogranin I and chromogranin A is unique.

Fig. 8. Scheme comparing the secretogranin I (SgI) and chromogranin A (CgA) molecules. Bars represent the mature proteins and lines the cleaved signal peptides. Black areas represent the highly homologous disulfide-bonded (S–S) loop structures and the homologous domains at the C termini. The parts of the molecules between the black areas represent the variable regions. The shaded area indicates the location of the pancreastatin sequence (see Eiden, 1987; Huttner and Benedum, 1987). RGD box, -Arg-Gly-Asp- site; trident, potential N-glycosylation site; SO_3^- and (SO_3^-) , identified and potential tyrosine sulfation site respectively; open triangles, sites with two or more dibasic amino acids.

On the other hand, the available evidence that secretogranin I and chromogranin A are physiological precursors of peptide hormones and neuropeptides is not yet conclusive. The limited proteolysis of secretogranin I and chromogranin A (and even the C-terminal amidation of fragments with C-terminal glycines) *in vivo* may simply be due to co-storage with prohormone processing enzymes in endocrine secretory granules but may have other roles than the generation of biologically active peptides. One promising approach to solve this issue for secretogranin I will be to test peptides synthesized according to the sequence reported here for biological activity.

Materials and methods

Purification of rat secretogranin I (chromogranin B) from PC12 cells

To monitor the purification, a mixture of unlabeled and [³⁵S]sulfate-labeled PC12 cells (Lee and Huttner, 1983) were used. Frozen pellets of PC12 cells (1 g) were thawed into 10 ml of ice-cold solution containing 20 mM Tris-HCl, pH 7.5; 10 mM EDTA; 5 mM 2-mercaptoethanol; 0.1 mM phenylmethylsulfonyl fluoride. The suspension was sonicated on ice (Branson Sonifier, 3 × 15 s, full power) and the resulting homogenate was centrifuged at 4°C for 30 min at 100 000 g. To the supernatant (S) obtained, 2 M NaCl was added to a final concentration of 0.1 M, followed by boiling for 5 min and centrifugation as above. The resulting supernatant containing heat-stable proteins (referred to as heat-stable fraction, HS) was adjusted to pH 6.0 with 1 N HCl and loaded on a DEAE-Sephacel column (Pharmacia) equilibrated with 0.1 M NaCl in buffer A (20 mM MES-NaOH, pH 6.0; 5 mM 2-mercaptoethanol). The column was washed with 0.2 M NaCl in buffer A and eluted with a linear gradient from 0.2 M to 0.4 M NaCl in buffer A. The peak containing secretogranin I (eluting around 0.3 M NaCl and referred to as DEAE peak, DE) was subjected to acetone precipitation prior to SDS-PAGE (Laemmli, 1970) or two-dimensional PAGE (O'Farrell, 1975), using the ampholyte mixture described previously (Rosa *et al.*, 1985a). Secretogranin I from two-dimensional gels is referred to as 'two-dimensionally pure' secretogranin I.

N terminus of secretogranin I (chromogranin B)

After SDS-PAGE of 1.5 mg of protein purified from PC12 cells through the DEAE chromatography step (see above), the gel was stained and secretogranin I eluted as described by Benedum *et al.* (1986). Eluted secretogranin I was lyophilized. The residue was suspended and washed at room temperature in 1 ml 100% methanol and centrifuged for 5 min in a microfuge. The pellet was dissolved in trifluoroacetic acid (TFA) and subjected to N-terminal sequencing in a gas-phase sequenator as described by Frank and Trosin (1985).

Affinity purification of antibodies and immunoblotting

Rabbit antisera previously raised against two-dimensionally pure rat secretogranin I from PC12 cells were affinity-purified on a Sepharose 4B (Pharmacia) column bearing rat secretogranin I purified from adrenal glands through the DEAE chromatography step as described above. Preparation of the affinity resin was performed according to the instructions of the supplier. Bound antibodies were eluted by low pH. Immunoblotting and detection of bound antibody by [¹²⁵I]-protein A was performed as described previously (Rosa *et al.*, 1985b). The heat-stable protein fraction from frozen and crushed human pheochromocytoma tissue (kindly provided by Dr B. Wiedenmann) was prepared as described previously for other tissues (Rosa *et al.*, 1985b).

Sequencing of tryptic peptides of rat secretogranin I (chromogranin B)

After two-dimensional PAGE of ~0.2 mg of protein purified from [³⁵S]-sulfate-labeled PC12 cells through the DEAE chromatography step (see above), the gel was stained with ice-cold 0.25 M KCl (Hager and Burgess, 1980). Secretogranin I was excised (see Figure 1, top right) and the protein fixed in 10% acetic acid, 50% methanol. After several washes in distilled water, the gel pieces (~2 ml of wet gel) were minced, lyophilized, re-swollen in 5 ml of a solution containing 50 mM ammonium bicarbonate, 0.5 mM CaCl₂ and 5 µg TPCK-trypsin (Worthington), and incubated for 26 h at 37°C. The eluate was collected and the gel piece re-eluted for a further 25 h with an additional 2.5 µg of TPCK-trypsin. The 12-ml eluate was lyophilized, and the residue was dissolved in 10% (v/v) acetic acid and subjected to h.p.l.c. on a LKB system equipped with a Vydac RP18 column using an acetonitrile gradient in TFA as described (Benedum *et al.*, 1986). Individual peaks were collected, concentrated in a Speed-vac, and subjected to N-terminal sequencing in a gas-phase sequenator as described (Lottspeich, 1985). ³⁵S-Radioactivity in aliquots of the fractions was determined by liquid scintillation counting.

Cloning of secretogranin I (chromogranin B) cDNA

cDNA libraries from a human pheochromocytoma (kindly provided by Dr X. Bertania) had been constructed in λgt10 and λgt11 according to Young and Davies

(1983). For the λgt10 cDNA library, size-selected cDNA has been used for the ligation with λ-vector arms prior to transfection of *Escherichia coli* Q358. The amplified λgt10 cDNA library was screened with a 250-bp fragment from the 5' end of an insert of a λgt11 clone which produced a fusion protein recognized by affinity-purified anti-secretogranin I antibodies (see above). This 250-bp DNA fragment was radioactively labeled according to Feinberg and Vogelstein (1983). λgt10 plaques (1.5×10^6) were transferred onto nitrocellulose filters as described in Maniatis *et al.* (1982). Filters were prehybridized at 65°C for 2 h in hybridization buffer [6 × SSC; 5 × Denhardt's; 50 mM sodium phosphate, pH 6.5; 100 µg/ml boiled and sonicated herring DNA; 20% (v/v) deionized formamide; 0.1 g/ml dextran sulfate (Pharmacia, mol. wt 3.5×10^6)]. The filters were then hybridized at 65°C for 12 h with the radioactive probe (500 000 c.p.m./ml) in hybridization buffer. The nitrocellulose filters were washed at 60°C for 30 min in 1 × SSC/0.1% SDS with several buffer changes and exposed overnight with an intensifying screen at -70°C. Positive plaques were purified by additional rounds of screening.

The 42-mer oligonucleotide:

(5'-GCCCGCTGGACAACAGGGACCACAACGAGAGATGGTGACC-3') corresponding to the N-terminal sequence of rat secretogranin I was synthesized according to the codon usage table for mammals (Grantham *et al.*, 1980) with an Applied Biosystems 380B synthesizer by the phosphorcyanethyl method (Sinha *et al.*, 1984) and purified on a 10% denaturing polyacrylamide gel (Maniatis *et al.*, 1982). The oligonucleotide was electro-eluted from the gel with a Schleicher and Schuell electron-elution chamber and further purified by binding to a C₁₈-cartridge (Sep-Pak, Waters) followed by elution with 60% methanol (Atkinson and Zoller, 1984). Fifty nanograms of oligonucleotide were labeled and purified as described in Benedum *et al.* (1986). Filters with plaque-pure clones from the screening with the 250-bp fragment were prehybridized at 37°C for 4 h in hybridization buffer and then hybridized with the oligonucleotide (1.5×10^6 c.p.m./ml) at 38–40°C for 12 h. The nitrocellulose filters were washed at 37°C for 30 min in 1 × SSC/0.1% SDS with several buffer changes and exposed overnight with an intensifying screen at -70°C. DNA was isolated from positive clones according to Huynh *et al.* (1985).

Sequencing of secretogranin I (chromogranin B) cDNA

The 2.5-kb cDNA insert (EcoRI fragment) from the λgt10 clone hSg1/19 was isolated and subcloned in both orientations into M13mp8 (Messing, 1983). This cDNA insert was mapped with several restriction enzymes and fragments to be used for sequencing were isolated and subcloned into M13. The M13 subclones obtained were subjected to DNA sequence analysis according to the method of Sanger *et al.* (1977) incorporating the modifications reported by Biggin *et al.* (1983).

Northern blots

Poly(A)⁺ RNA was isolated from various human tissues and subjected to Northern blot analysis as described in Benedum *et al.* (1986).

In vitro transcription-translation of human secretogranin I (chromogranin B) cDNA

In vitro transcription-translation was performed as described by Stueber *et al.* (1984). The 2.5-kb cDNA insert (EcoRI fragment) of clone hSg1/19 was subcloned into the expression vector pDS6. The orientation of the insert in the recombinant was analyzed by restriction mapping. The clone containing the cDNA of human secretogranin I in the correct orientation, designated pDS6-SgI, was propagated in LB medium in the presence of 100 µg/ml ampicillin. Plasmid DNA was isolated according to the rapid alkaline extraction method of Birnboim and Doly (1979). This DNA was linearized with *Hind*III and further purified by low-melting agarose gel electrophoresis prior to *in vitro* transcription-translation. Translation with [³⁵S]methionine was carried out using the wheat germ system (Warren and Dobberstein, 1978). Some translation reactions were performed in the presence of dog pancreas rough microsomes, followed by treatment with proteinase K and Triton X-100 as indicated in Figure 3 (Stueber *et al.*, 1984; Dobberstein *et al.*, 1979). In some cases, translation products were subjected to immunoprecipitation using monoclonal antibodies recognizing human secretogranin I or bovine chromogranin A (kindly provided by U. Holzer and C. Niehrs respectively) and anti-mouse IgG agarose (Sigma). In other cases, translation products were subjected to heat treatment (Benedum *et al.*, 1986), except that the supplementation with soluble proteins before boiling was omitted. All samples were analyzed by SDS-PAGE followed by autoradiography, using standard methods described previously (Lee and Huttner, 1983).

NEM labeling

Crude granules from bovine adrenal medulla were obtained in the absence of added reducing agents as described in Trifaró and Duerr (1976) and resuspended in 0.3 M sucrose at a concentration of 10 mg/ml. For the determination of intermolecular disulfide bonding, aliquots of the crude granule fraction were analyzed by reducing and non-reducing SDS-PAGE followed by Coomassie Blue staining. For the determination of intramolecular disulfide bonding, crude chromaffin granules were lysed by freezing and thawing, and aliquots containing 50 µg of

protein were incubated for 10 min at 20°C in the absence and presence of 1 mM DTT in 100 µl of 10 mM Hepes, pH 7.7. After acetone precipitation of proteins to remove the DTT, samples were dissolved in 100 µl of 10 mM Hepes, pH 7.7, added to 20 µCi of [2-³H]NEM (21.5 Ci/mmol, New England Nuclear) from which the organic solvent had been removed by evaporation, and incubated for 30 min at 37°C. The reaction was terminated by the addition of DTT to a final concentration of 10 mM. Samples were analyzed by SDS-PAGE under reducing conditions followed by fluorography.

Nomenclature

The name chromogranin B was introduced for a proteolytic fragment of chromogranin A (O'Connor and Frigon, 1984) and for a soluble M_r 100 000 protein of bovine chromaffin granules (Falkensammer *et al.*, 1985a,b). The latter seemed to be identical to the polypeptide doublet of M_r 113 000/105 000 first described in rat PC12 cells (Huttner and Lee, 1982; Lee and Huttner, 1983) and called secretogranin I (Rosa *et al.*, 1985b). In an attempt to simplify the nomenclature, it has been suggested to abandon the name chromogranin B in connection with the chromogranin A fragment and to use it for the bovine M_r 100 000 protein as well as for the rat M_r 113 000/105 000 protein (Eiden *et al.*, 1987). However, the relationship between the latter proteins may be more complex than anticipated. The results of the present study show that the N-terminal sequence of the rat M_r 113 000/105 000 protein (Figure 1, top right panel) is strikingly different from that reported for the bovine M_r 100 000 protein from chromaffin granules (Settleman *et al.*, 1985) but almost identical to that of the human M_r 120 000 protein (Figure 2B) which also contains all the internal sequences determined for the rat protein. Moreover, our affinity-purified polyclonal antibodies and a monoclonal antibody, raised against the rat M_r 113 000/105 000 protein, recognize the human M_r 120 000 protein from pheochromocytoma tissue (Figure 1B) and the corresponding protein produced from the cloned cDNA (Figure 3), respectively, but not the M_r 100 000 protein from bovine chromaffin granules. On the other hand, some antisera raised against the bovine M_r 100 000 protein recognize the human M_r 120 000 protein (Hagn *et al.*, 1986). Thus, the relationship of the rat M_r 113 000/105 000 protein to the human M_r 120 000 protein is obvious while that to the bovine M_r 100 000 protein is – at least at present – less well established. In view of these recent data, the use of a simplified nomenclature awaits clarification of this issue at the level of cDNA sequences in various species as well as the genomic level. To indicate the potential complexity of this issue, the term 'secretogranin I (chromogranin B)' is used in this paper in the title and in the subheadings. However, for the ease of presentation only the term 'secretogranin I' is used throughout the text.

Acknowledgements

U.M.B. and W.B.H. are grateful to Professors E.Fanning and H.Thoenen for their support of the doctoral thesis of U.M.B. P.R. is grateful to Professor F.Clementi for his support. We thank Drs M.-T.Haeuptle and J.Lipp for help with *in vitro* transcription-translation, Dr B.Sprout and P.Neuner for synthesizing the 42-mer oligonucleotide, Drs X.Bertania and B.Wiedenmann for human pheochromocytoma tissues, U.Holzer and C.Niehrs for monoclonal antibodies, Drs P.Argos and P.McCaldon for help in computer sequence analysis, and Drs B.Dobberstein and S.Fuller for many helpful discussions and comments on the manuscript. J.M. was supported by the Centre National de la Recherche Scientifique and W.B.H. by grants from the Deutsche Forschungsgemeinschaft (Hu 275/3-2, Hu 275/3-3).

References

- Argos, P. (1985) *EMBO J.*, **5**, 1351–1355.
- Argos, P. (1987) *J. Mol. Biol.*, **193**, 385–396.
- Atkinson, T. and Zoller, M. (1984) In Gait, M.J. (ed.), *Oligonucleotide Synthesis: A Practical Approach*. IRL Press, Oxford.
- Benedum, U.M., Baeuerle, P.A., Konecki, D.S., Frank, R., Powell, J., Mallet, J. and Huttner, W.B. (1986) *EMBO J.*, **5**, 1495–1502.
- Biggin, M.D., Gibson, T.J. and Hong, G.F. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3963–3965.
- Birnboim, H.C. and Doly, J. (1979) *Nucleic Acids Res.*, **7**, 1513–1523.
- Cohn, D.V., Elting, J.J., Frick, M. and Elde, R. (1984) *Endocrinology*, **114**, 1963–1973.
- Dobberstein, B., Garoff, H., Warren, G. and Robinson, P.J. (1979) *Cell*, **17**, 759–769.
- Docherty, K. and Steiner, D.F. (1982) *Annu. Rev. Physiol.*, **44**, 625–638.
- Eiden, L.E. (1987) *Nature*, **325**, 301.
- Eiden, L.E., Huttner, W.B., Mallet, J., O'Connor, D.T., Winkler, H. and Zanini, A. (1987) *Neuroscience*, in press.
- Falkensammer, G., Fischer-Colbrie, R., Richter, K. and Winkler, H. (1985a) *Neuroscience*, **14**, 735–746.
- Falkensammer, G., Fischer-Colbrie, R. and Winkler, H. (1985b) *J. Neurochem.*, **45**, 1475–1480.
- Fischer-Colbrie, R., Lassmann, H., Hagn, C. and Winkler, H. (1985) *Neuroscience*, **16**, 547–555.
- Fischer-Colbrie, R., Diez-Guerra, J., Emson, P.C. and Winkler, H. (1986) *Neuroscience*, **18**, 167–174.
- Feinberg, A.P. and Vogelstein, B. (1983) *Anal. Biochem.*, **132**, 6–13.
- Frank, R. and Trosin, M. (1985) In Tschesche, H. (ed.), *Modern Methods in Protein Chemistry*. De Gruyter, Berlin, pp. 287–302.
- Grantham, R., Gautier, C., Guoy, M., Mercier, R. and Pavé, A. (1980) *Nucleic Acids Res.*, **8**, 49–62.
- Hager, D.A. and Burgess, R.R. (1980) *Anal. Biochem.*, **109**, 76–86.
- Hagn, C., Schmid, K.W., Fischer-Colbrie, R. and Winkler, H. (1986) *Lab. Invest.*, **55**, 405–411.
- Hille, A., Rosa, P. and Huttner, W.B. (1984) *FEBS Lett.*, **177**, 129–134.
- Hörtnagl, H., Lochs, H. and Winkler, H. (1974) *J. Neurochem.*, **22**, 197–199.
- Hortsch, M. and Meyer, D.I. (1986) *Int. Rev. Cytol.*, **102**, 215–245.
- Huttner, W.B. and Baeuerle, P. (1987) In Satir, B. (ed.), *Modern Cell Biology*. Alan R. Liss, New York, Vol. 5, in press.
- Huttner, W.B., Benedum, U.M., Hille, A. and Rosa, P. (1987) *Ann. NY Acad. Sci.*, in press.
- Huttner, W.B. and Benedum, U.M. (1987) *Nature*, **325**, 305.
- Huttner, W.B. and Lee, R.W.H. (1982) *J. Cell Biol.*, **95**, 389a.
- Huynh, T.V., Young, R.A. and Davis, R.W. (1985) In Glover, D.M. (ed.), *DNA Cloning*. IRL Press, Oxford, Vol. 1, pp. 49–78.
- Iacangelo, A., Affolter, H.-U., Eiden, L.E., Herbert, E. and Grimes, M. (1986) *Nature*, **324**, 82–86.
- Kelly, R.B. (1985) *Science*, **230**, 25–32.
- Kyte, J. and Doolittle, R.F. (1982) *J. Mol. Biol.*, **157**, 105–132.
- Lassmann, H., Hagn, C., Fischer-Colbrie, R. and Winkler, H. (1986) *Histochem. J.*, **18**, 380–386.
- Laemmli, U.K. (1970) *Nature*, **227**, 680–685.
- Lee, R.W.H. and Huttner, W.B. (1983) *J. Biol. Chem.*, **258**, 11326–11334.
- Lee, R.W.H. and Huttner, W.B. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6143–6147.
- Lloyd, R.V. and Wilson, B.S. (1983) *Science*, **222**, 628–630.
- Lottspeich, J. (1985) *J. Chromatogr.*, **326**, 321–327.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, New York.
- Messing, J. (1983) *Methods Enzymol.*, **101**, 20–78.
- O'Connor, D.T. and Frigon, R.P. (1984) *J. Biol. Chem.*, **259**, 3237–3247.
- O'Connor, D.T., Burton, D. and Deftos, L.J. (1983) *Life Sci.*, **33**, 1657–1663.
- O'Farrell, P.H. (1975) *J. Biol. Chem.*, **250**, 4007–4021.
- Rindi, G., Buffa, R., Sessa, R., Tortora, O. and Solcia, E. (1986) *Histochemistry*, **85**, 19–28.
- Rosa, P., Fumagalli, G., Zanini, A., De Camilli, P. and Huttner, W.B. (1985a) *J. Cell Biol.*, **100**, 928–937.
- Rosa, P., Hille, A., Lee, R.W.H., Zanini, A., De Camilli, P. and Huttner, W.B. (1985b) *J. Cell Biol.*, **101**, 1999–2011.
- Ruoslahti, E. and Pierschbacher, M.D. (1986) *Cell*, **44**, 517–518.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
- Sinha, N.D., Biernat, J., McManus, J. and Köster, H. (1984) *Nucleic Acids Res.*, **12**, 4539–4556.
- Settleman, J., Fonseca, R., Nolan, J. and Hogue-Angeletti, R. (1985) *J. Biol. Chem.*, **260**, 1645–1651.
- Somogyi, P., Hodgson, A.J., De Potter, R.W., Fischer-Colbrie, R., Schober, M., Winkler, H. and Cubb, I.W. (1984) *Brain Res. Rev.*, **8**, 193–230.
- Stueber, D., Ibrahim, I., Cutler, D., Dobberstein, B. and Bujard, H. (1984) *EMBO J.*, **3**, 3143–3148.
- Tatemoto, K., Efendić, S., Mutt, V., Makk, G., Feistner, G.F. and Barchas, J.D. (1986) *Nature*, **324**, 476–478.
- Trifaró, J.M. and Duerr, A.C. (1976) *Biochim. Biophys. Acta*, **421**, 153–167.
- Warren, G. and Dobberstein, B. (1978) *Nature*, **273**, 569–571.
- Young, R.A. and Davis, R.W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1194–1198.

Received on February 6, 1987

Note added in proof

These sequence data have been submitted to the EMBL/GenBank data libraries under the accession number Y00064.