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Peptide repertoire of human cerebrospinal fluid: novel proteolytic fragments of neuroendocrine proteins

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Abstract

Polypeptides in human cerebrospinal fluid (CSF), isolated by phase separation in chloroform–methanol–water and reversed-phase HPLC, were characterised by sequence analysis and mass spectrometry. This identified the presence of peptide fragments of testican, neuroendocrine specific protein VGF, neuroendocrine protein 7B2, chromogranin B/secretogranin I, chromogranin A, osteopontin, IGF-II E-peptide and proenkephalin. The majority of these fragments were generated by proteolysis at dibasic sites, suggesting that they are derived by activities related to prohormone convertase(s). Several of the fragments have previously not been detected, and their functions in CSF or elsewhere are unknown. A characteristic feature of all these fragments is a very high content of acidic residues, in particular glutamic acid. In addition to the fragments of neuroendocrine proteins, endothelin-binding receptor-like protein 2, ribonuclease 1, IGF-binding protein 6, albumin, α_1-acid glycoprotein 1, prostaglandin-H2 D-isomerase, apolipoprotein A1, transthyretin, β_2-microglobulin, ubiquitin, fibrinopeptide A, and C4A anaphylatoxin were found. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Polypeptides in cerebrospinal fluid (CSF) are derived mainly from the blood or from the central nervous system (CNS), and transport between blood and CNS is restricted by tight junctions at the blood–brain and the blood–CSF barriers. The protein repertoire of CSF is similar to that of serum [1], but the protein concentration is lower. The relative content of low-molecular-mass proteins is higher in CSF than in plasma, because of the diffusion restriction provided by the barriers, which are impermeable to large molecules. The protein composition of CSF is to some extent affected by disease states of the CNS. The immunoglobulin pattern is changed in patients with multiple sclerosis [2], and the β_2-microglobulin level is elevated in several disorders, i.e., lymphoblastic leukemia, malignant brain tumours, neurosarcoidosis and multiple sclerosis [3,4].

Traditionally, proteins and peptides in CSF or nervous tissue have been characterised by immunoassays or studies of mRNA expression levels. Although sensitive, these methods have limitations in studies of proteolytic fragments of proteins. Thus, antibodies may fail to react with protein fragments,

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and mRNA expression is unrelated to post-translational events. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a high-resolution technique useful for separation of polypeptides in complex mixtures. 2D-PAGE of CSF has shown differences between protein patterns in patients suffering from diseases such as schizophrenia and Creuzfeldt–Jakob disease [5]. Mass spectrometry (MS) is useful in identification of post-translational modifications of proteins, and recent developments in MS, alone or in combination with other techniques, have made it possible to identify several proteins in CSF, and also to study the processing of neuropeptides [6–8]. However, in identification of low-abundance polypeptides, which may be of importance in brain disorders, the relatively large amounts of albumin and immunoglobulins limits both 2D electrophoresis and MS. In addition, in 2D electrophoresis, the resolution is low for polypeptides smaller than M_r 10 000.

In isolation of hydrophobic peptides from lipid-rich tissues and body fluids, organic solvent extraction in a two-phase system of chloroform–methanol–water with subsequent chromatographies in organic solvents, has been useful [9–11]. Further separation of very unpolar peptides by reversed-phase high-performance liquid chromatography (RP-HPLC) in methanol or ethanol–propanol has been successful [12]. By these purification methods, hydrophobic peptides in bile [13], and prophenin fragments in pulmonary surfactant preparations [14] have been identified.

This phase separation technique has now been used for isolation of polar proteins and peptides from CSF. We have studied the peptide repertoire of human CSF by direct protein isolation and identification. This approach unexpectedly revealed a number of peptides, originating from chromogranin A and B, VGF, 7B2, IGF-II E-peptide, osteopontin, testican and proenkephalin.

2. Materials and methods

2.1. Materials

All chemicals and solvents were of analytical grade or higher grade. Precast Tricine gels, Mark 12 molecular mass standard and poly(vinylidene fluoride) (PVDF) membranes (0.2 μm) were from Novex. Porcine trypsin, sequanal grade, was from Promega. Sequazyme Peptide Mass Calibration Mixture 2, was from PerSeptive Biosystems. All sequencer chemicals employed were obtained from PE Applied Biosystems.

2.2. CSF and organic solvent extraction

Human CSF samples were obtained from subjects without known neurological disorders (with permission from the local ethical committee). Sample I (53 ml) was derived from CSF drainage of a patient, undergoing thoracic surgery, and sample II (35 ml), was obtained by pooling several lumbar puncture specimens. Cells and insoluble materials were removed by centrifugation at 2000 *g* for 10 min and the samples were stored at −70°C until processed.

The cell-free CSF was mixed with four volumes of chloroform–methanol (2:1, v/v) to yield a two-phase system of chloroform–methanol–CSF (8:4:3, v/v). The phases were allowed to separate overnight at ambient temperature and were then collected and evaporated to dryness.

2.3. Gel filtration on Sephadex LH-60

The dried material from the unpolar chloroform–methanol phase after phase separation was redissolved in chloroform–methanol–0.1 *M* HCl (19:19:2, v/v) and subjected to size-exclusion chromatography on Sephadex LH-60 in the same solvent system. The column size was 85×1.1 cm, 3 mg was applied and the flow-rate was 4.5 ml/h.

2.4. RP-HPLC

The material in the other phase after phase separation, the polar water–methanol phase, was dissolved in water. Before injection onto the column, trifluoroacetic acid was added to 0.1%, and the samples were centrifuged for 10 min at 19 000 *g*. Ten or 15% of the total material was applied onto the column.

Separation was performed on a Vydac C_{18} column (250×4.6 mm, 5 μm particle size) using a Waters instrument. The solvent system consisted of 0.1%

trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). Absorbance was monitored at 214 nm. Elution was performed at a flow-rate of 1 ml/min. After injection of the sample, 100% solvent A was used for elution until the baseline was stabilised, followed by a gradient of 0–60% solvent B over 60 min. Fractions were collected manually, and dried by evaporation under a stream of nitrogen. The material eluting at 0–20% acetonitrile did not contain any amino acids after hydrolysis, and was therefore not considered to contain polypeptides.

2.5. Electrophoresis and electrophoretic blotting

Sodium dodecyl sulfate (SDS)–PAGE was performed in precast 10–20% Tricine gels [15] under reducing conditions. The gels were stained with silver [16], Coomassie R-250 (0.1% CBB R250–40% methanol–10% acetic acid), or transferred electrophoretically [17] to a PVDF membrane for 2 h at a constant voltage of 25 V, using Towbin half-strength buffer with 20% methanol. After the transfer was completed, the PVDF membrane was stained for 1 min with 0.1% CBB R250, in 45% methanol–2% acetic acid, and then destained in 90% methanol–2% acetic acid [18].

2.6. Protein analysis

Acid hydrolysis of eluates from RP-HPLC was performed with 6 *M* HCl–0.5% phenol in evacuated glass tubes for 20–24 h at 110°C. For Sephadex LH-60, eluate protein was determined by phenylthiocarbamyl amino acid analysis [11]. HPLC eluates were analysed with a ninhydrin-based LKB 4151 Plus Instrument.

N-Terminal sequence analysis was performed by Edman degradation in protein sequencers 494 or 494 cLC (PE Applied Biosystems). In estimation of the concentration of each polypeptide in CSF, the initial yield from the sequencers was set to 50%.

2.7. Tryptic digestion and separation of fragments

For tryptic digestion, 8 μg of protein was dissolved in 0.25 *M* ammonium hydrogencarbonate, pH 8.0, 0.5 μg trypsin was added, and the mixture was incubated at 37°C for 4 h. The proteolysis was halted by addition of formic acid to 5% (v/v).

The tryptic fragments obtained were separated by RP-HPLC on a Brownlee C_{18} column (150×0.5 mm, 5 μm particle size) using a PE Applied Biosystems microblotter system. The solvent system consisted of 0.1% trifluoroacetic acid in water (solvent A), and 0.1% trifluoroacetic acid in acetonitrile (solvent B). The sample was reconstituted and injected in solvent A. Elution was performed onto a PVDF membrane at a flow-rate of 5 μl/min, using a gradient of 5–45% solvent B over 200 min followed by 45–85% solvent B over 60 min. Absorbance was monitored at 214 nm.

2.8. Mass spectrometry

For matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) MS, samples were dried on a stainless steel plate together with α-cyano-4-hydroxycinnamic acid (≈5 μg) as matrix. Molecular masses were determined with a Voyager-DE PRO Biospectrometry Workstation (PerSeptive Biosystems) operated in the positive ion mode. The instrument is equipped with a 335-nm nitrogen laser, the flight tube is 1.3 m in the linear mode and 2.0 m in the reflectron mode. The spectra were acquired in the linear or the reflectron mode, with delayed extraction. Acquisition mass range was 500–10 000 u for linear mode, and 1000–6500 u for the reflectron mode. The spectra represented an average of 100–500 acquisitions. Calibration was performed using external calibration, in the m/z range 1297–5735.

Positive-ion nano-electrospray mass spectra were recorded using a quadrupole time-of-flight (Q-TOF) tandem mass spectrometer equipped with a Z-spray nano-electrospray interface (Micromass). Samples were sprayed from gold coated borosilicate capillaries, and mass spectra were acquired as described previously [19].

2.9. Protein data base searches

N-Terminal sequences were compared with the structures in the Karolinska Institutet Nonredundant Database, which covers the databases Swissprot, PIR, GenPept and TrEMBL [20], for identification.

The database is found at ftp://ftp.mbb.ki.se/pub/KIND.

Signal peptides and their cleavage sites were predicted using the program SignalP [21], and transmembrane segments were predicted using the program TMHMM [22], respectively. The programs are available at the Centre for Biological Sequence Analysis, http://www.cbs.dtu.dk/.

3. Results

Polypeptides present in the water–methanol phase after chloroform–methanol–water phase separation of CSF was resolved by SDS–PAGE. This showed bands with apparent molecular masses of 60 000, 48 000, 33 000, 30 000, 25 000, 22 000, 15 000, 9000, and 6000 (Fig. 1). *N*-Terminal sequence analysis of these bands identified albumin, prostaglandin-H2 D-isomerase, apolipoprotein A1, transthyretin, β_2-microglobulin, ubiquitin and a fragment of chromogranin B/secretogranin I starting at position 368. The M_r 48 000 protein was refractory to Edman degradation and was identified by trypsin digestion and sequence analysis of the fragments obtained. This revealed α_1-acid glycoprotein 1, which contains an *N*-terminal pyrrolidone carboxylic acid, explaining the resistance to Edman degradation.

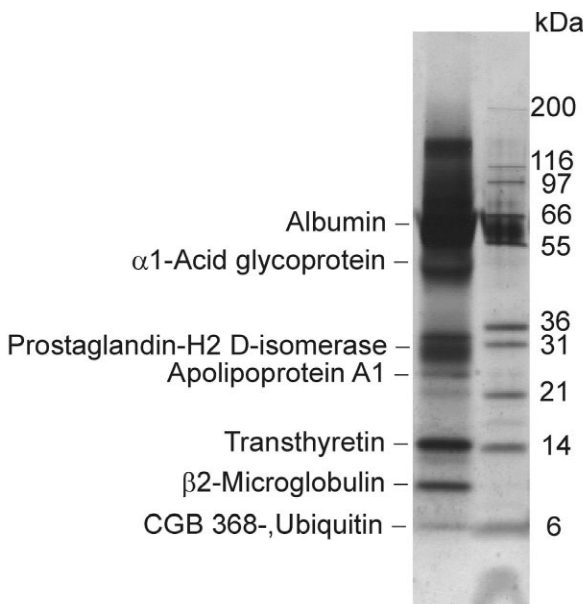

Fig. 1. SDS–PAGE of the water–methanol phase after organic solvent extraction of human CSF. Left lane contains material from the water–methanol phase after organic solvent extraction, right lane, molecular mass standards with respective masses in kDa. The gel was stained with silver. The proteins and peptides were identified after electrophoretic transfer to a PVDF membrane, with subsequent *N*-terminal sequence analysis.

Peptides in the polar phase were separated by RP-HPLC (Fig. 2). Several peptides eluted between 20 and 40% acetonitrile, while larger proteins started to elute at 45% acetonitrile. The fractions were analysed by amino acid analysis, SDS–PAGE, *N*-terminal sequence analysis, MALDI-TOF and Q-TOF mass spectrometry. The results of these analyses are summarised in Table 1. Several peptides and peptide fragments derived from larger proteins were found: fibrinopeptide A, C4A anaphylatoxin; *N*-terminal fragments of neuroendocrine specific protein VGF, ribonuclease 1; internal fragments of endothelin-binding receptor-like protein 2, chromogranin B/secretogranin I, chromogranin A, proenkephalin; and *C*-terminal fragments of neuroendocrine protein 7B2, pro IGF-II, testican and osteopontin. The amounts of the peptides in CSF were estimated from the amounts found by sequence analysis (Table 2).

The contents of the chloroform phase after phase separation were separated by gel filtration on Sephadex LH-60. Two peaks were obtained, one eluting at 30–40% of one column volume, containing amino acids after acid hydrolysis, and one eluting at 60–85% of one column volume, containing lipids. *N*-Terminal sequence analysis and MS of amino acid-containing fractions revealed no peptide sequences.

4. Discussion

Globular proteins like immunoglobulins and albumin quantitatively dominate proteins in CSF. During purification of neuropeptides from CSF, which in many cases are present at low concentrations, the large globular proteins may cause problems. In the purification procedure described here, the organic solvents, in particular chloroform, will precipitate much of the globular proteins, while smaller peptides remain soluble in the methanol–

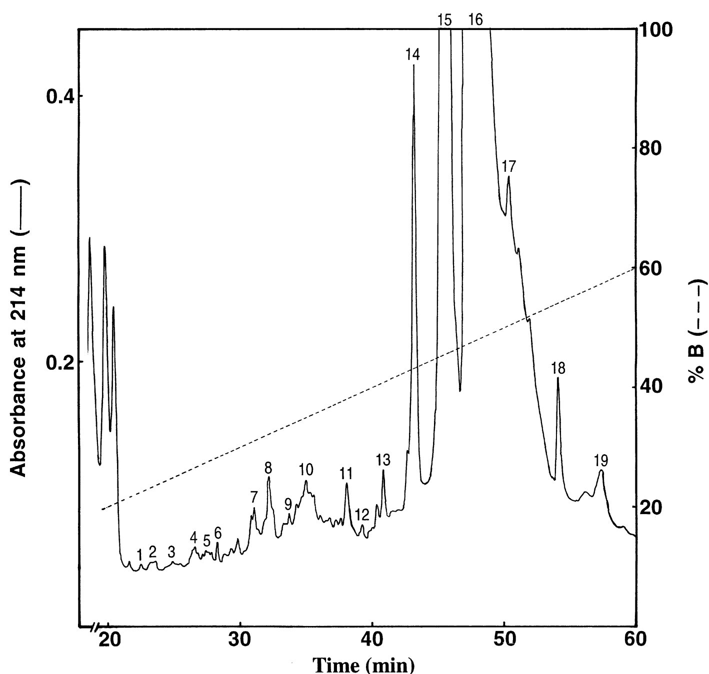

Fig. 2. RP-HPLC of polar peptides and proteins from CSF. The contents of the polar water–methanol phase were separated by RP-HPLC using a C_{18} column. The elution profile monitored at 214 nm is shown. Elution was performed with a linear gradient of acetonitrile as shown. The fractions were analysed by *N*-terminal sequencing and mass spectrometry (Table 1).

water phase. After reversed-phase chromatography, peptides/proteins were pure enough for analysis by SDS–PAGE, MS and Edman degradation. We then found that the human CSF contains several peptides not earlier described. The peptide locations in the proforms and the cleavage sites are summarised in Fig. 3.

The amount of the peptide fragments now found (Table 2) were consistent with previously reported polypeptide levels in human CSF. β_2-Microglobulin analysed by radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) is approximately 70–100 pmol/ml in healthy controls [3,23], and the levels now found are similar (24 and 95 pmol/ml). IGFBP6 analysed by RIA ranges 1–4 pmol/ml [24], to be compared with the level now found, 1 pmol/ml. All peptides found here were in the low pmol/ml range (0.5–8 pmol/ml).

The peptides may be products from unspecific processing of the proteins. However, the peptides found were all processed after dibasic sites, or after single Arg residues. In addition, no peptide fragments were found from any of the plasma proteins present. Thus, is not likely that the polypeptides are unspecifically processed.

In addition to the peptide fragments, known constituents of CSF were found, for example albumin, α_1-acid glycoprotein, prostaglandin-H2 D-isomerase, transthyretin, β_2-microglobulin [1], apolipoprotein A1 [25], ubiquitin [26], fibrinopeptide A, and C4A anaphylatoxin [27].

Secretory granules of neuroendocrine and/or endocrine cells contain chromogranins [28], 7B2 [29], VGF protein [30,31] and proenkephalin [32]. Each of these proteins contains several dibasic sites, which are potential cleavage sites for prohormone convertases [33,34]. In line with this, several peptide fragments of chromogranin A [35] and chromogranin B/secretogranin I [36] in bovine chromaffin granules, chromogranin/secretogranin fragments in human cerebrospinal fluid [37] and VGF in nerve cells and hypophysis from rat [38] have been described. This suggest that these proteins contain short neuropeptide or hormone fragments which are re-

Table 1
Results from *N*-terminal sequence analysis, mass spectrometry and SDS–PAGE of polypeptides in individual HPLC fractions (note: X denotes unidentified residues, HPLC fraction numbers refer to the peaks in Fig. 2, the MS data were obtained by MALDI-TOF analysis unless otherwise specified and the SDS–PAGE data refer to the results shown in Fig. 1)

HPLC fraction	Sequence determined	Protein identified	Location	Molecular mass		SDS–PAGE
				Calculated[e,f]	Mass spectrometry[g,h]	
1	SETHAAGHSQEKTHSREKS	Chromogranin B	197–255	6437[e]	6436[g]	
2	AVTEDDEDEDDDKEDEVGY	Testican	419–437	2187.8	2186.8[h]	
3	SSSQGGSLPSEEKGH	Chromogranin B	273–			
4	APPGRPEAQPPPLSSEHKEP	VGF	23–62	3951.0[f]	3951.6[h]	
5	GRPEAQPPPLSSEHKEPVAG	VGF	26–62	3685.8[f]	3686.5[h]	
	APPGRPEAQPPLSSEHKEP	VGF	23–59	3666.8[f]	3668.2[h]	
	SSQGGSLPSEEKGHPQEESE	Chromogranin B	273–303	3202.4[f]	3219.7[h](oxidised)	
	ADSGEGDFLAEGGGVR	Fibrinopeptide A	1–15	1535.7[f]	1536.8[h]	
6	ADSGEGDFLAEGGGVR	Fibrinopeptide A	1–15		1536.5[h]	
	DSGEGDFLAEGGGVR	Fibrinopeptide A	2–15	1464.7[f]	1465.5[h]	
7	GTEDEEAKGVQQYVPEEXA	ET_BR-LP-2	47–			
	SVNPYLQGQRLDNVVAKKSV	7B2 (CT peptide)	164–185[a]	3510.7[f]	3511.5[h]	
8	NYPSLESDKMAHGYGEESEE	Chromogranin B	368–417	5749[e]	6503[g,i]+oxidised forms	6000
	DHHSTHYRASEEEPEYGEEI	Chromogranin B	306–365	7182[e]	7181[g,i]+oxidised forms	
	EDSKEAEKSGEATDGARPQU	Chromogranin A	116–207	9730[e]	9760[g,i] (possibly oxidised)	
9	NVNFQKAINEKLGQYASPTA	Anaphylatoxin A	1–			
	KANDESNEHSDVIDSQELSK	Ostepontin	249–314	7658[e]	7675[g,i]	
10	KANDESNEHSDVIDSQELSK	Ostepontin	249–314		7680[g,i]	
	GHVLAKELEAFREAKRHRPL					
	LESRAKKFQRQHMDSDSSPS	ProlGFII	115–			
		Ribonuclease 1	1–			
11	RXPGXGQGVQAGXPGGXVEE	IGF binding protein 6	1–			
	HSGFEDELSEVLENQSSQAE	Chromogranin A	79–113[a]	3905.8[f]	3907.0[h]	
	EEEEEEEEEAEAGEEAVPEE	Chromogranin A	210–			
12	DAEEDDSLANSSDLKELLLE	Proenkephalin	119–159	4586[e]	4587[g]	
	AEEDDSLANSSELKELLET		120–159	4472[e]	4471[g]	
13	MQIFVKTLTGKTITLEVEPS	Ubiquitin				6000
14	IQRTPKIQVYSRHPAENGKS	β_2-Microglobulin				9000
15	SAEFPBFYDSEE	Chromogranin B (CBB peptide)	597–656	6974.5[e]	6972.5[b]	
	TEDTIFLR[c]	α_1-Acid glycoprotein 1[c]				48 000
	TYMLAFDVNDEK[c]					
16	DAHKSEVAHRFKDLGEENFK	Albumin				61 000
17	DAHKSEVAHRFKD	Albumin[d]				61 000
	APEAQVSVQPNFQQDKFLGR	Prostaglandin-D-isomerase[d]				30 000
	GPTGTGESKZPLMVK	Transthyretin[d]				15 000
18, 19	DAHKSEVAHRFKD	Albumin				61 000
	GPTGTGESKXPLMVK	Transthyretin				15 000

[a] Confirmed by Q-TOF-MS and sequence analysis by collision-induced dissociation analysis.
[b] Q-TOF-MS.
[c] Identified after tryptic digestion and subsequent analysis of the tryptic fragments obtained.
[d] Identified by an SDS–PAGE migration, identical to that of the corresponding proteins in Fig. 1.
[e] Average molecular mass.
[f] Monoisotopic molecular mass.
[g] Linear mode.
[h] Reflectron mode.
[i] Broad peak.

Table 2
Amount polypeptide in CSF[a]

Protein	Sample I	Sample II
Anaphylatoxin	1	2
CGA_{97-}	2	5
CGA_{134-}	2	6
CGA_{228-}	–	3
CGB_{197-}	–	4
CGB_{273-}	0.5	5
CGB_{306-}	2	3
CGB_{368-}	6	6
CGB_{597-} (CCB peptide)	3	n.d.
$ET_BR\text{-}LP\text{-}2_{47-}$	3	8
Fibrinopeptide A	3	3
Fibrinopeptide A_{2-}	3	2
$proIGF\text{-}II_{139-}$	5	5
$IGFBP6_{28-}$	1	1
$OSTP_{249-}$	1	8
RNase 1	3	2
$PENK_{143-}$	0.5	2
7B2, CT-peptide$_{164-}$	2	8
Testican$_{419-}$	–	8
Ubiquitin	3	7
VGF_{23-}	1	2
VGF_{26-}	n.d.	2
β_2-Microglobulin	24	95

[a] The amounts of each protein/peptide were estimated from the amounts found by sequence analysis, expressed as pmol per ml of starting material. Sample I and II are described under Materials and methods. For comparison, β_2-microglobulin is included. Normal levels of β_2-microglobulin in human CSF are 70–100 pmol/ml.

n.d., Not determined; –, not found in sample I.

leased by proteolysis. Whether the remaining parts of the precursors have specific functions is unknown.

Testican mRNA is expressed in most human organs, predominantly in the brain, but also in tissues as prostate, placenta and heart [39], and the protein is thought to play a role in interactions between cell surfaces and extracellular matrix molecules. The testican gene is located in a region containing a number of genes encoding neurotransmitters and growth factors [40]. The protein fragment now found in CSF, derived from the *C*-terminal part of the protein, has not been previously described.

Proteolytic processing of neuroendocrine specific protein VGF has been reported in rat nervous tissues. The M_r 90 000 protein is processed to M_r 10 000–20 000 *C*-terminal forms, which are stored in mature secretory granules [38]. Using the program SignalP [21], residues 1–22 of VGF are predicted to constitute a signal peptide, (cleavage site LGA–APP), which is consistent with the fragment now found in CSF, starting at position 23. Thus, the fragments now found are all derived from the *N*-terminal part of the corresponding protein, and have not been reported previously.

Endothelin receptor type B like protein 2 (ET_BR-LP-2) is highly expressed at the mRNA level in human CNS [41]. It is homologous to the G protein-coupled receptor, human endothelin type B receptor, and is considered to be a member of this family of proteins. However, ET_BR-LP-2 does not bind endothelin [41], and when analysed for transmembrane helices using the program TMHMM [22], it does not display the seven transmembrane stretches that are characteristic for the G protein-coupled receptors (data not shown). The protein fragment found in CSF is cleaved after a Lys–Arg site, suggesting processing by a specific protease. Our results suggest that ET_BR-LP-2 may be a precursor of secretory polypeptides.

Neuroendocrine protein 7B2 is an M_r 27 000 protein present in neurons and endocrine cells [42]. It is cleaved within the Golgi apparatus into an M_r 21 000 protein and a 31-residue carboxy-terminal (CT) peptide, which both are stored in the secretory granules [43]. The M_r 21 000 protein has previously been identified in CSF [44]. The CT-peptide was now found in CSF. In vitro, the CT-peptide inhibits the prohormone convertase PC2, but its inhibitory effect disappears by cleavage at an internal Lys–Lys site [45].

The chromogranins/secretogranins are precursor proteins stored in secretory granules. Within the granules, the precursors are processed to several smaller peptides. Peptides derived from chromogranins A and B, and secretogranin II have been described in CSF from results obtained by immunoassays [37]. The peptides now found in CSF, i.e., chromogranin A positions 116–209 and position 210– have not been described earlier. A fragment similar to the third chromogranin A fragment found in CSF, covering positions 79–113, has earlier been reported to be secreted from bovine chromaffin cells [35], there covering the positions 79–115, where the last two residues are Lys and Arg. These residues are not found in the human form of the peptide now found in CSF, most likely because of further pro-

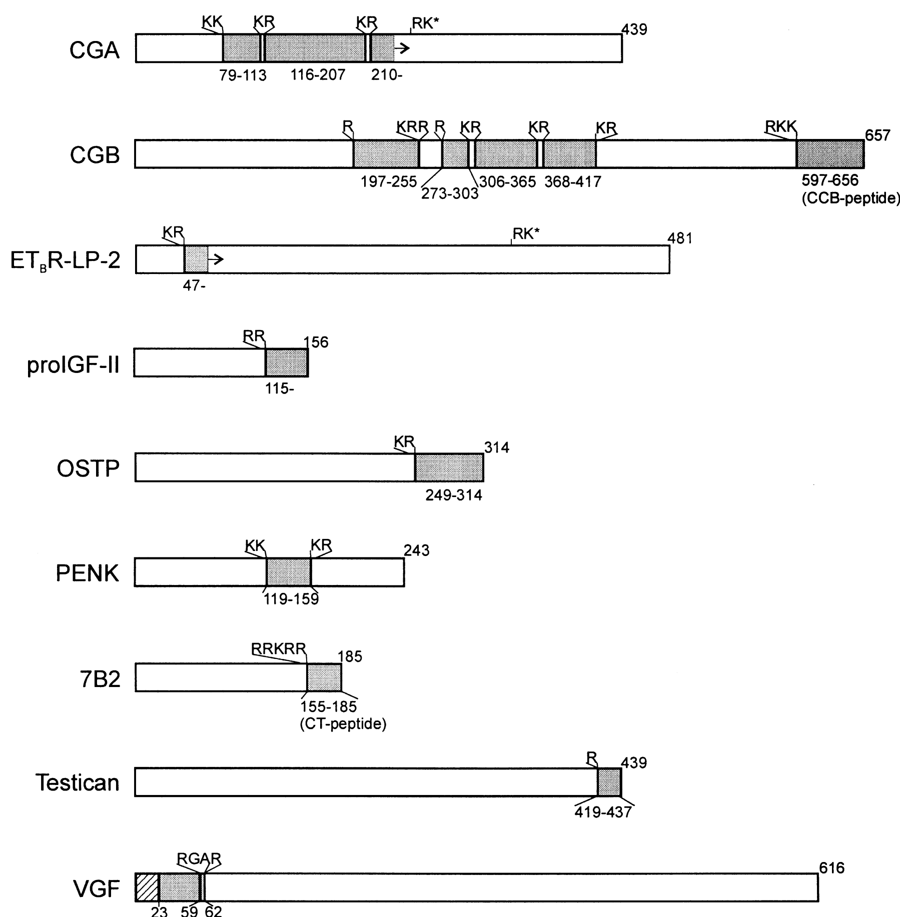

Fig. 3. Features of precursor proteins. The peptides identified by *N*-terminal sequencing and mass spectrometry are marked grey. Predicted signal peptides are hatched. Polypeptides without defined *C*-terminal ends are marked grey as far as the sequence was determined, followed by an arrow, * defines next dibasic site. The names of peptides previously reported are given within parentheses.

cessing by carboxypeptidases. The fragments of chromogranin B/secretogranin I (positions 197–255, 273–303, 306–365, 368–417) have also not been described before. The fragment covering positions 597–656, the CCB-peptide, has however been reported [46]. The fragments covering positions 197–255 and 273–303 are derived by cleavage after a single arginine at the N-terminal side, while the other two fragments are produced by cleavages after dibasic sites. Judged from MS and SDS–PAGE data, the latter peptides are concluded to end at the next dibasic site, i.e., 306–365 (theoretical mass 7182) and 368–417 (theoretical mass 5749). Post-translational modifications as glycosylation, phosphorylation and sulfation are known features of the chromogranins [47,48], which may explain the discrepancy between the theoretical and obtained mass values of the 368–417 fragment.

Osteopontin has a widespread tissue and body fluid distribution [49]. It is a glycosylated phosphoprotein and has been found as M_r 76 000 and 45 000 isoforms in CSF [50]. Our finding of a previously unknown 65-residue C-terminal peptide, cleaved after a Lys–Arg site, suggest that osteopontin is proteolytically processed into specific fragments.

IGF-II is a known constituent of CSF and brain, suggesting a possible regulatory or growth promoting role for IGF-II in CNS [51,52]. In maturation of proIGF-II, the carboxy-terminal fragment covering

positions 68–156, the E-peptide, is cleaved off [53]. A fragment of the E-peptide (positions 117–156) has been identified in rat serum and cell lines [54,55]. Possible functions of the E-peptide have been discussed, but so far data in support of a specific function have not been reported. The E-peptide fragment found here, starting at position position 115– of proIGF-II, by cleavage after an Arg–Arg site, has not previously been described. The carboxy-terminal end of this E-peptide fragment now found may be the C-terminal end of the E-peptide, i.e., position 156. The expected mass of a peptide covering positions 115–156 is 4598, but such a fragment was not found by MS. Post-translational processing of IGF-II precursor from human embryonic cell lines showed that *O*-linked glycosylation occurs at the E-peptide position Thr^{139} [56]. Thus, the difficulty in determining the molecular mass of the E-peptide fragment may be caused by glycosylation of the peptide. The finding in CSF of an E-peptide fragment of proIGF-II supports the speculations that this peptide, or a part of it, might have a biological function.

IGF-binding protein 6 (IGFBP6) has a selective affinity for IGF-II, is abundant in CSF [57], and is *O*-glycosylated [58]. Signal peptide prediction of IGFBP6 [21], suggest that residue 1–27 constitute a signal peptide (results not shown), which is consistent with a start at position 28 of the mature IGFBP6 as now found.

Ribonuclease 1 (RNase 1, also called pancreatic ribonuclease) mRNA is expressed in various human tissues, like pancreas, skeletal muscle, heart and brain, although expression in pancreas is greater than that in other tissues [59]. Rnase 1 has also been purified from human brain [60]. The widespread distribution of RNase 1 indicates that the enzyme may have other functions than as a digestive enzyme. Our finding of RNase 1 in CSF further support this.

Proenkephalin (PENK) is synthesised by neuroendocrine cells, and is proteolytically processed to 5–8 residue Met- and Leu-enkephalins, and several larger enkephalin-containing polypeptides, see, e.g., Ref. [61]. The proenkephalin fragment now identified in CSF, covering positions 119–159, has not previously been described. It is derived from a location in between two Met-enkephalins, and does not contain any enkephalin. Thus, $PENK_{119-159}$ is not an intermediate in enkephalin biosynthesis, and the finding of this peptide in CSF may suggest that proenkephalin can also be a precursor of neuropeptides other than the enkephalins.

The polypeptide fragments now identified are derived from precursors by proteolytic cleavages at sites mainly after two basic residues, in most cases Lys–Arg, although a single-Arg site also occurs (Fig. 3). This is consistent with processing of neuroendocrine precursors in general, see, e.g., Ref. [62]. At the C-terminal end of the peptides, the basic residues are removed, suggesting further processing by carboxypeptidases. This defined pattern, resembling that for established neuroendocrine peptides, suggest that also the novel peptides are defined products with specific neuroendocrine functions.

A distinguishing feature of most of the peptide fragments now found is their very high content of acidic amino acid residues, ranging from 19 to 63% Asp plus Glu (Table 3). In the cases of proenkephalin, and chromogranins A and B, both the entire protein and the peptide fragments are highly acidic. Regarding testican, the entire protein is not particularly acidic, but the fragment found is extremely acidic (Table 3). Also in the osteopontin fragment and the IGF-II E-peptide fragment, the contents of acidic residues are higher than in their precursors. It is tempting to suggest that the high contents of Asp and Glu residues in these peptides may have a physiological function. Notably, glutamic acid is a neurotransmitter, and peptides with terminal Glu residues can activate NMDA-types of glutamate receptors [63].

5. Nomenclature

CGA	Chromogranin A
CGB	Chromogranin B/secretogranin I
CNS	Central nervous system
CSF	Cerebrospinal fluid
ES	Electrospray
ET_BR-LP-2	Endothelin type B receptor-like protein-2
IGF-II	Insulin-like growth factor-II
IGFBP6	Insulin-like growth factor binding protein 6

Table 3
Contents of Asp and Glu in the CSF polypeptides identified[a]

Polypeptide	Asp (%)	Glu (%)	Net charge
CGA_{79-113}	6	26	−8
$CGA_{116-207}$	4	21	−15
$CGA_{210-247}$[b]	–	42	−13
Entire CGA	*4*	*21*	
$CGB_{197-255}$	6	47	−8
$CGB_{273-303}$	–	22	−5
$CGB_{306-365}$	7	25	−9
$CGB_{368-417}$	4	20	−1
$CGB_{597-656}$	10	20	−10
Entire CGB	*6*	*18*	
proIGF-$II_{115-156}$	2	10	+8
Entire proIGF-II	*4*	*6*	
$OSTP_{249-314}$	11	15	−3
Entire OSTP	*15*	*9*	
$PENK_{119-159}$	17	20	−10
Entire PENK	*6*	*14*	
$7B2_{165-185}$ (CT peptide)	13	6	−1
Entire 7B2	*8*	*8*	
$Testican_{419-437}$	37	26	−11
Entire Testican	*6*	*8*	
VGF_{23-62}	5	10	−1
Entire VGF	*4*	*16*	
ET_BR-LP-2_{1-481}	2	5	
$Anaphylatoxin_{1-77}$	4	5	
$IGFBP6_{1-213}$	5	7	
RNase 1_{1-128}	5	5	

[a] The lines in italics give the Asp and Glu contents of the entire precursor. The average contents of Asp and Glu in proteins are 5.3% and 6.2%, respectively [64]. The last three proteins are probably present in CSF in their uncleaved forms (see text for details) and do not have elevated contents of Asp plus Glu. The net charges of the peptides are calculated from the total amounts of basic residues (Lys, Arg and His), and acidic residues (Asp and Glu).

[b] Estimated length of the peptide from residue 210 to the next dibasic site.

MALDI	Matrix-assisted laser desorption/ionisation
OSTP	Osteopontin
PC	Proprotein convertase
PENK	Proenkephalin
PVDF	Polyvinylidene difluoride
Q	Quadrupole
RNase 1	Ribonuclease 1
RP	Reversed-phase
TOF	Time-of-flight

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