Journal of Neurochemistry, 2002, **81**, 565–574

Isolation and characterization of VGF peptides in rat brain. Role of PC1/3 and PC2 in the maturation of VGF precursor

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Abstract

The neurotrophin responsive gene vgf is widely expressed in central and peripheral neurones, and in certain neuroendocrine cell populations. Its encoded VGF precursor protein (proVGF1: 617 amino acids in rat, 615 in man, >85% homology) gives rise to several low molecular weight species. We studied a range of neuroendocrine and neuronal cells, in which VGF-processing products were prominent with an apparent molecular weight of 20 and 10 kDa (VGF20 and VGF10, respectively). Such peptides were recognized by antibodies specific for the C-terminal rat VGF nonapeptide, thus indicating that they included the C-terminus of proVGF. Ectopic expression of the neuroendocrine-specific prohormone convertases PC1/3 or PC2 in GH3 cells showed that both could generate VGF20, while VGF10 was preferentially produced by PC1/3. Site-directed mutagenesis was used to identify the $KRKRKK^{488}$ motif as the target within VGF sequence which leads to the production of VGF20. Molecular characterization of rat VGF10, on the other hand, revealed that this peptide is produced by cleavage at the RPR^{555} site. By the combined use of high-resolution separation techniques, matrix-assisted laser desorption/ionization time of flight (MALDI-ToF) mass spectrometry and manual Edman degradation we identified in rat brain a VGF fragment analogous to bovine peptide V and two novel peptides also derived from the C-terminal region of proVGF.

Keywords: endoproteolytic processing, MALDI-ToF mass spectrometry, rat brain, prohormone convertase, VGF peptides.

J. Neurochem. (2002) **81,** 565–574.

The gene named vgf was originally identified because its mRNA is considerably and selectively induced by nerve growth factor (NGF) in PC12 cells (Levi *et al*. 1985). Vgf is a rapidly responsive gene, which in the rat brain is mainly regulated by the neurotrophins BDNF and NT3 (Bonni *et al*. 1995; Snyder *et al*. 1997). VGF expression appears to occur in different areas of the central and peripheral nervous system during critical periods of morphological and functional development, both pre- and post-natally (Salton *et al*. 1991; Lombardo *et al*. 1995; Snyder *et al*. 1998). In the adult rat, VGF is detected in subpopulations of neurones within the CNS and in subsets of neuroendocrine cells (Ferri and Possenti 1996; Salton *et al*. 2000), including pancreatic islet cells (Possenti *et al*. 1999). VGF is highly expressed in female rat pituitary gonadotropes (and to a lower extent lactotropes) and the level of expression varies during the estrous cycle (Ferri *et al*. 1995). VGF knock-out mice showed striking alterations in energy homeostasis, with hyperactivity and a persistent hypermetabolic state (Hahm *et al*. 1999; Salton *et al*. 2000). In spite of normal or increased food intake, knock-out mice showed a significant reduction in body weight

Received October 9, 2001; revised manuscript received January 18, 2002; accepted January 24, 2002.

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The accession number for the rat VGF protein sequence is P20156

Abbreviations used: BDNF, brain-derived neurotrophic factor; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; MALDI-ToF, matrix-assisted laser desorption-ionization time of flight (mass spectrometry); NGF, nerve growth factor NT3, neurotrophin 3; PCs, prohormone convertases; RSV, Rous-sarcoma virus; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

and, even more so, in body fat deposition (Hahm *et al*. 1999). Sexual maturation and behaviour were severely affected, to such an extent that VGF-deficient female were almost infertile (Hahm *et al*. 1999).

Different lines of evidence suggest that the primary translation product, or VGF protein, is the precursor of biologically active VGF-peptides, which are released and play a role in intercellular communication (Trani *et al*. 1995; Possenti *et al*. 1999). Conceptual translation of VGF cDNA shows a secretory leader sequence in the VGF protein, as required for translocation into the endoplasmic reticulum, and numerous short stretches of basic amino acid residues, potential targets for cleavage by endopeptidases (Salton *et al*. 1991; Canu *et al*. 1997). In both neurones and pancreatic beta cells, the VGF protein was shown to be routed to the secretory cell compartment, and to be proteolytically processed to polypeptide fragments (Trani *et al*. 1995; Possenti *et al*. 1999). Ibidem, antibodies specific for the C-terminus of VGF showed two bands with an apparent molecular weight of 90 kDa (compatible with whole VGF, hence hereafter named proVGF), and of 80 kDa (VGF80). A number of smaller peptides were also prominent, including two species with an apparent molecular mass of 20 and 10 kDa (VGF20 and VGF10, respectively). The latter peptides were enriched in secretory granules and released upon stimulation, thus suggesting their involvement in cell to cell communication (Trani *et al*. 1995; Possenti *et al*. 1999). A peptide corresponding to the last 30 amino acids of VGF has been isolated from bovine posterior pituitary, and was named peptide V (for 'VGF-derived peptide': Liu *et al*. 1994). Conversely, some tissues, such as the adrenal medulla, or cell lines, such as pituitary-derived GH3 cells, contained substantial amounts of proVGF and VGF80, and hardly any low molecular weight VGF-peptides (Trani *et al*. 1995).

Proteolytic processing of protein precursors in the secretory pathway occurs largely via endoproteases, that act upon recognition of specific stretches of basic amino acid residues and their surrounding sequence. This is often followed by removal of N- and C-terminal residues due to the action of exopeptidases (Fricker 1988; Cool *et al*. 1997; Yasothornsrikul *et al*. 1998). The best characterized prohormone processing enzymes are mammalian members of the family of subtilisin/kexin-like serine proteinases, commonly known as prohormone convertases (PCs; Halban and Irminger 1994; Seidah *et al*. 1994; Steiner 1998; Muller and Lindberg 1999; Seidah and Chretien 1999). Presently, seven PCs are known, PC1/3 and PC2, furin, PACE4, PC4, PC5/6 and PC7/8. Of these, PC1/3 and PC2 are expressed in endocrine and neuroendocrine cells and act specifically in secretory granules.

To get insight onto which convertase/s may be responsible for *in vivo* processing of the VGF precursor, we investigated a range of cell types in their profile of VGF-peptide fragments, and of PC1/3 and PC2. In addition we ectopically expressed PC1/3 and PC2 in the pituitary cell line GH3 and studied the differential processing of endogenous VGF by the two enzymes. Finally, the identity of VGF peptides present within the rat CNS was addressed using MALDI-ToF mass spectrometric analysis and microsequencing of immunopurified VGF species.

Experimental procedures

Media and reagents

Culture media, sera, lipofectamine transfection reagent and phosphate-buffered saline (PBS) were purchased from Gibco-BRL (Grand Island, NY, USA). The Enhanced Chemiluminescence System (ECL) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other chemicals were from Sigma/Aldrich (St Louis, MA, USA) unless otherwise indicated.

Cell lines

These were kindly provided as follows. The GT1-7 cell line by S. S. Stojilkovic (Bethesda, MD, USA), the beta TC-1 line by S. Efrat (Tel Aviv, Israel), the alpha TC1-6 line by A. L. Notkins (Bethesda, MD, USA), all other lines by the American Type Culture Collection (Rockville, MD, USA).

Antisera

Anti-human PC1/3$_{113-158}$ and antihuman PC2$_{112-159}$ sera were purchased from Chemicon International Inc. (Temecula, CA, USA); these antisera recognize a sequence within the catalytic domain conserved in the human, rat and mouse convertases. Anti-VGF$_{573-617}$ and anti-VGF$_{609-617}$ sera, that recognise: the C-terminal 45 amino acid region and the C-terminal nonapeptide of rat VGF, respectively, have been previously described in detail (Ferri *et al*. 1995; Possenti *et al*. 1999). For isolation of VGF peptides from tissue extracts, immunoglobulins from 4 mL of anti-VGF$_{573-617}$ were purified using protein A-Sepharose, then coupled to Sepharose (4B FastFlow, Sigma) using the standard cyanogen bromide method. Before use, the preparation was tested for its ability to bind recombinant VGF10.

Recombinant plasmids

Expression vectors for mouse PC1/3 and human PC2 under the control of the cytomegalovirus promoter (CMV-mPC1/3 and CMV-hPC2, respectively) have been previously described (Vollenweider *et al*. 1995). The beta galactosidase expression vector used as a control (pcDNA3.1/His/lacZ) was purchased from Invitrogen (San Diego, CA, USA).

The expression vector for VGF was constructed by subcloning in pCDNA3 (Invitrogen) a Pvu II – Xba I genomic fragment containing the entire transcribed sequence of rat VGF. The VGFm20 VGF mutant was also cloned in pCDNA3. To construct such a mutant, the RKRKKNA sequence, corresponding to residues 484–490 of rat VGF, was removed using PCR-based procedures. Briefly, DNA encoding the protein sequences upstream residue 484 (fragment 1) and downstream residue 490 (fragment 2) were amplified separately. The oligonucleotides were designed so that a XhoI site was present at the 3′ of fragment 1 and at the 5′ of fragment 2. Following digestion with XhoI and ligation sequence a second round of PCR with oligonucleotides complementary to the 5′ of fragment 1 and to the 3′ of fragment 2 was used to amplify the correct fragment.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

7B2 mRNA levels were assessed by semiquantitative RT-PCR. For this purpose, 20 μg of total RNA were retrotranscribed, using random examers, by Moloney murine leukaemia virus reverse transcriptase. The resulting cDNAs were amplified with primers specific for 7B2 and β-actin sequences, this last used as an internal control. PCR was carried out in a reaction volume of 100 μL containing 0.2 mM dNTP (Amersham Pharmacia Biotech), 25 pmol of each primers, 2.5 μCi 32P dCTP and 2 U of Taq DNA polymerase (Promega Corporation, Madison, WI, USA) at 94°C for 60 s, 50°C for 60 s, 72°C for 90 s. The number of cycles necessary for obtaining signals of comparable intensity in the linear range of the amplification reaction, was determined empirically to be 30 and 25 for 7B2 and β-actin, respectively. The PCR products were electophoretically separated on 1.8% agarose gel that was then dried and exposed to Kodak X-film overnight. The sequence of the primers used to amplify 7B2 were: 5′-CACCAGGCCATGAATC-TT-3′ sense and 5′-TCTTGTTCCACTTGCCCT-3′ antisense; those for β-actin were: 5′-TGCTCGACAACGGCTCCGGCATGT-3′ and 5′-CCAGCCAGGTCCAGACGCGGAT-3′.

Cell culture and transfection

GT1-7, AtT20, alpha T3-1, GH3 and COS-7 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum. Alpha TC1-6 and beta TC-1 cells were maintained in culture in DMEM containing 1 mg/mL glucose, 15% horse serum, 5% foetal calf serum, and 25 mM HEPES. Primary cultures of cerebellar granule cells were set up as described (Levi and Ciotti 1983). Briefly, cells dissociated from cerebella of 8 days old-rats were plated on poly-L-lysine-coated dishes at a density of 2.5–2.8 × 10^6 cells per 35-mm dish in basal modified Eagle's medium supplemented with 10% heat-inactivated foetal calf serum. The medium was renewed after 2, 5 and 8 days in culture. All cells were cultured in a 95% air, 5% CO_2 humidified atmosphere, at 37°C. Cell transfection was performed using the lipofectamine transfection reagent, according to the supplier's protocol (Gibco-BRL). COS-7 cells were seeded at 1/5 of confluence in 35 mm tissue culture dishes (day 0), transfected on day 1, replated in 60 mm dishes (day 2) and processed on day 3 or 4.

Stable transfectants of GH3 cells expressing PC1/3 or PC2 were obtained by cotransfecting (at a 1 : 10 ratio) RSVneo (Gorman *et al*. 1983) together with either: CMV-mPC1/3, or: CMV-hPC2. Stable transfectants were selected by addition of 0.5 mg/mL G418 in culture media. After 3–4 weeks, resistant colonies were pooled and amplified. The pools were analyzed for expression of the relevant convertase by indirect immunofluorescence, using antihuman $PC1/3_{113-158}$ and antihuman $PC2_{112-159}$ sera, followed by fluorescein isothiocyanate-coniugated secondary antibodies. Approximately 25% of transfected, G418 resistant GH3 cells were immunostained for the relevant convertase. Immunostaining controls included substitution of each layer, in turn, with PBS and resulted in no signal.

Cell extraction

Cell lysis was carried out in hypotonic buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 0.2% Nonidet *p*-40, 1 mM EDTA and the following protease inhibitors: phenylmethylsulfonylfluoride (1 mM), leupeptin (20 μg/mL), and pepstatin A (0.2 mg/mL). Cell extracts were centrifuged at 400 *g* for 10 min, in order to remove nuclei, and the protein content was determined according to the method of Lowry (Lowry *et al*. 1951). The amount and composition of VGF peptides extracted from the cells with Laemmli sample buffer or hypotonic buffer were compared and didn't show significant differences. To examine the profile of secreted VGF peptides, cultures were washed twice with prewarmed serum-free DMEM and incubated in the same medium (1.5 mL, for a 3-h incubation at 37°C). Culture media were then harvested. Proteins were precipitated using 10% trichloracetic acid and redissolved in SDS sample buffer.

Western blotting

SDS–PAGE was carried out using a Tris-glycine 10–15% acrylamide gradient, according to Laemmli (Laemmli 1970). For the two-dimentional analysis, proteins were seperated by isoelectrofocusing (Eboli *et al*. 1994), then subjected to electrophoresis, as above. For analysis with PC1/3 and PC2 antisera, proteins were run on a Tris-glycine 7.5% acrylamide gel. Gels were analysed by the western blot procedure according to Burnette (Burnette 1981). Blotted membranes were incubated in PBS containing 0.1% Tween-20 with either of: anti-$VGF_{609-617}$ (1 : 4000 dilution; Ferri *et al*. 1995; Trani *et al*. 1995), antihuman $PC1/3_{113-158}$ (1 : 2000), or antihuman $PC2_{112-159}$ (1 : 2000), followed by horseradish peroxidase-conjugated protein A (1 : 10000). Immunoblots were developed using the ECL System. Protein size was determined by comparison with appropriate molecular weight standards (Amersham Pharmacia Biotech).

Tissue extraction

Whole rat brains (2nd−3rd postnatal week) were frozen immediately after dissection and stored at −70°C. Frozen tissues were homogenized at 4°C in 5 mM EDTA pH 7 (approximately 5 mL per gram of tissue). Tubes were immediately moved to a vigorously boiling water bath and kept there for 10 min, quickly chilled on ice, and centrifuged at 100 000 *g* for 30 min. Addition of phenylmethylsulfonylfluoride (1 mM), leupeptin (20 μg/mL), and pepstatin A (0.2 mg/mL) did not affect the pattern of immunoreactive bands, so that the protein inhibitors could be omitted. From supernatants, proteins were passed through an Amicon membrane with a molecular weight cut-off point at 100 kDa. After addition of Tris-HCl pH 7 (10 mM final concentration) samples were stored at −70°C.

Purification of VGF peptides

An affinity column containing Sepharose-coupled anti-$VGF_{573-617}$ antibodies was equilibrated with 10 mM Tris-HCl (pH 7). Size-fractionated brain extracts were applied at a flow rate of 1 mL/min at 4°C. The column was then washed with 40 mL of 10 mM Tris-HCl (pH 7), followed by 20 mL of double-distilled water. Bound peptides were eluted with 10 mM HCl (pH 2.5), followed by 100 mM NH_4HCO_3 (pH 8.1). Fractions of 1 mL were lyophilised (after sampling for Western blotting) and then subjected to reverse phase-HPLC using a LabService Analytica apparatus and a Vydac C18 column (2.1 × 150 mm, 5 μm), eluted with a linear gradient from 7% to 50% acetonitrile in 0.05% trifluoroacetic acid (TFA), at a flow rate of 150 μL/min followed by a 10-min wash with 70% acetonitrile. The effluent was monitored at 220 nm, and peptide

fractions were collected on the basis of the UV trace. Two independent purifications were processed, as described below, with the same results.

Mass spectrometric analysis – HPLC fractions were lyophilized and redissolved in 10 μL of an aqueous acetonitrile solution containing 0.1% TFA. One microliter of each sample was combined with a 3,5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid) matrix solution and deposited on a stainless steel target. MALDI-ToF analyses were performed on a Voyager DE mass spectrometer (Applied Biosystems) equipped with a nitrogen laser (337 nm). An external standard based on a mixture of bovine insulin (m/z 5734.6, MH +), E. coli thioredoxin (m/z 11 674.5, MH +) and horse apomyoglobin (m/z 16 952.6, MH +) was used for molecular mass determinations (SequazimeTM Peptide Mass Standards kit). Data were acquired over 250 shots.

Manual Edman degradation

One-microliter sample of the selected HPLC fraction was mixed with the same volume of a 50% pyridine solution in H2O and 10% phenylisothiocyanate in pyridine. The coupling mixture was incubated at 50°C for 30 min and then vacuum-dried. The cleavage step was performed by adding 10 μL of anhydrous TFA and heating at 50°C for 10 min under nitrogen. After vacuum-drying, the peptide mixture was redissolved in 1 μL of the MALDI-ToF matrix solution and applied to a stainless steel target for spectrometry.

Results

PC1/3 and PC2 content correlates with specific patterns of VGF-derived peptides

VGF-expressing endocrine and neuronal cell lines store and secrete different repertoires of low molecular weight VGF species, which are produced by endoproteolytic cleavage in a late compartment of the regulated secretory pathway (Trani *et al*. 1995; Possenti *et al*. 1999). A number of mammalian subtilisin-like proteases process precursor proteins (Halban and Irminger 1994; Seidah *et al*. 1994; Steiner 1998; Muller and Lindberg 1999; Seidah and Chretien 1999), PC1/3 and PC2 being the major convertases that operate in secretory granules of the regulated secretory pathway of neuroendocrine cells. To get insight on the role of the latter proteases in VGF maturation, we examined a range of cell types for VGF-peptide profile (Fig. 1, top panel) and expression of PC1/3 and PC2 (ibid., middle and bottom panels).

All pituitary-derived cell lines tested (AtT20: corticotropes; alpha T3-1: gonadotropes; GH3: somatomammotropes: lanes 1, 2 and 7) expressed the 90 kDa form of VGF (proVGF), while VGF processing varied. Alpha T3-1 cells produced considerable amounts of 20 kDa and 10 kDa VGF (VGF20 and VGF10, respectively), AtT20 only revealed VGF10, and GH3 showed no low molecular weight VGF. As to pancreatic islet cell lines, alpha TC1-6 cells (glucagon producing: lane 8) contained more VGF20 than VGF10, while the opposite was true for beta TC-1 cells (insulin producing: lane 9). With respect to neurons, the GT1-7 mouse hypothalamic cell line (lane 3) and cerebellar granules on day 8 *in vitro* (lane 6) contained both VGF20 and VGF10. Conversely, proVGF processing was restricted to VGF80 in immature cerebellar granules (day 1 *in vitro*: lane 5).

Alpha T3-1 and GT1-7 cells revealed the mature form of both PC1/3 and PC2 (66 kDa: ibid., lanes 2, 3, middle and bottom panels), hence it was not immediately apparent which convertase would be responsible for cleavage of VGF10 and VGF20. However, AtT20 cells only expressed PC1/3 (lane 1), suggesting that PC1/3 was sufficient to produce VGF10. Interestingly out of the two pancreatic islet cell lines alpha TC1-6 (lane 8) which had a lower ratio between mature PC1/3 and mature PC2 contained VGF20 as prevailing form whereas beta TC-1 had large amount of VGF10. Neither early cultures of cerebellar granules (day 1 *in vitro*), nor GH3 cells contained either of the convertases, nor low molecular weight VGF (lanes 5 and 7). We have previously shown that cultures of cerebellar granules cells represent a convenient model system to study the processing of VGF and the regulated secretion of the low molecular weight forms (Trani *et al*. 1995). With *in vitro* maturation (day 8 in culture, lane 6), cerebellar granules started to produce PC1/3 (PC2 was still below detection), as well as VGF20 and VGF10 peptides. As expected, neither VGF, nor the PC1/3, or PC2 convertases were detected in the monkey kidney derived COS-7 cell line.

VGF processing in GH3 cells

GH3 cells, which express VGF (Fig. 1, lane 7), but do not contain PC1/3, nor PC2 (Fig. 1, lane 7) are a convenient cell line in which to investigate VGF processing by either convertase, ectopically expressed. Importantly GH3 cells express 7B2 (Fig. 2, left panel) which is required for maturation of PC2 into a fully active convertase (Muller and Lindberg 1999; Westphal *et al*. 1999). Stable transfectants expressing either convertase were obtained and their VGF-peptide repertoire was compared to the one produced by wild type cells. ProVGF and VGF80 prevailed in wild type, as well as in PC1/3-, or PC2- transfected GH3 cells (C lanes 1, 3 and 5). Conversely, VGF species released into culture media varied. No small molecular weight VGF peptide was found in the medium of control GH3 cells (wild type, lane 2). Both VGF20 and VGF10 were well represented in media from PC1/3-transfected cells (lane 4), while VGF20 was found in the medium of PC2-transfected cells (lane 6). The absence of small VGF forms within the cells suggests that VGF processing by prohormone convertases occurs in a late compartment of the secretory pathway and likely reflects limited capability of GH3 cells to accumulate secretory vesicles (Tougard *et al*. 1989; Galanopoulou *et al*. 1995).

It should be noted that transfected GH3 cells were only selected on the basis of their resistance to G418 encoded by a cotransfected RSVneo plasmid, and not upon actual

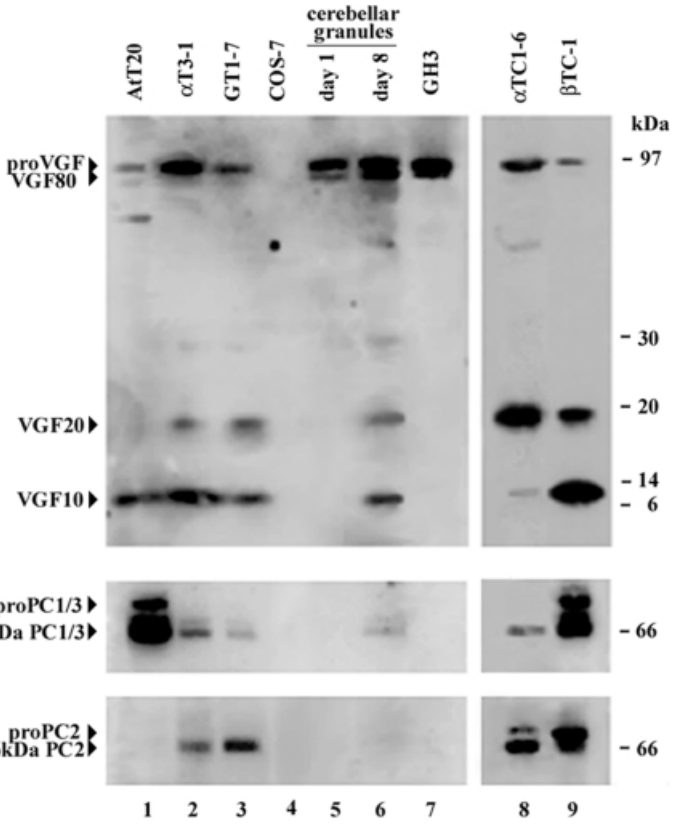


Fig. 1 VGF peptide profile vs. PC1/3 and PC2 convertases in different cell types. Western blot analysis of pituitary (AtT20, alpha T3-1, GH3), pancreatic islet (alpha TC1-6, beta TC-1), neuronal (GT1-7) and-non-endocrine (COS-7) cell lines. Primary cultures of cerebellar granule cells were also studied (immature and mature cells: day 1 and day 8 in culture, respectively). Cell proteins (200 μg of protein for upper lanes 3 and 4; 100 μg for all others) were run on a Tris-glycine 10–15% acrylamide gradient and analysed with anti-$VGF_{609–617}$ antibodies (see Experimental procedures). VGF precursor (proVGF) and the major processing products: VGF80, VGF20 and VGF10 are indicated in the upper panel. For the middle and lower panels, proteins were resolved with a 7.5% acrylamide gel and probed with antihuman $PC1/3_{113–158}$ (middle panel) and anti-human $PC2_{112–159}$ (bottom panel). Arrowheads indicate the immature and mature forms of either convertase, respectively. Molecular masses of size standards are indicated for each panel.

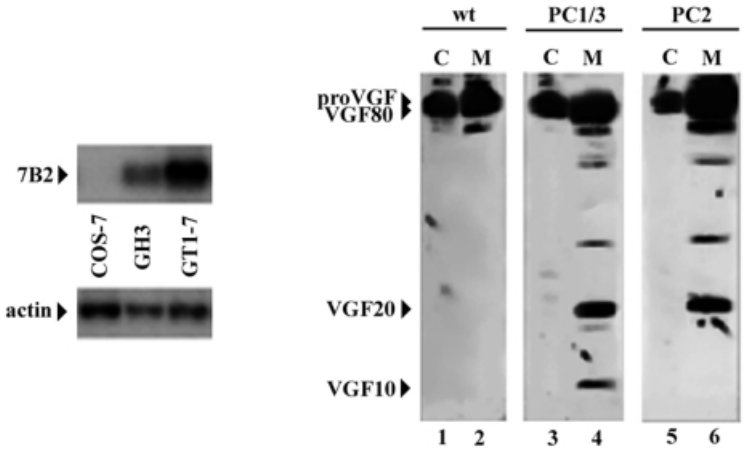


Fig. 2 Processing of VGF in wild type and transfected GH3. Left panel: 7B2 mRNA in COS-7, GH3 and GT1-7 cell lines. 20 μg of whole RNA from each cell line was reverse transcribed (see: Experimental Procedures). Right panel: western blot analysis of VGF peptides from cell extracts (C: 100 μg proteins, from about 10^6 cells) and culture media (M: proteins released by 5×10^6 cells in 3 h) from wild type (wt), PC1/3- or PC2-transfected GH3 cells (lanes 1–2, 3–4, 5–6, respectively).

expression of PC1/3 or PC2 convertase. In fact, approximately 25% of G418 resistant cells expressed immunostainable PC1/3, or PC2, as appropriate, hence the extent of processing is likely to be underestimated.

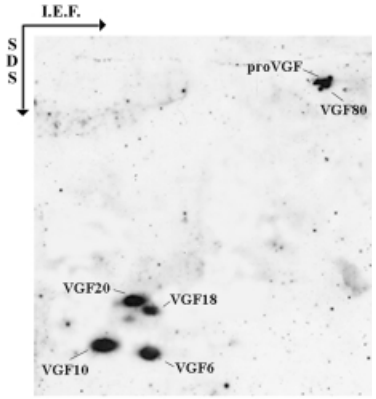
Isolation and characterisation of VGF peptides from rat CNS

In order to characterize tissue VGF peptides, we carried out their isolation from the rat brain, in which VGF is highly expressed (Lombardo *et al*. 1995; Trani *et al*. 1995).

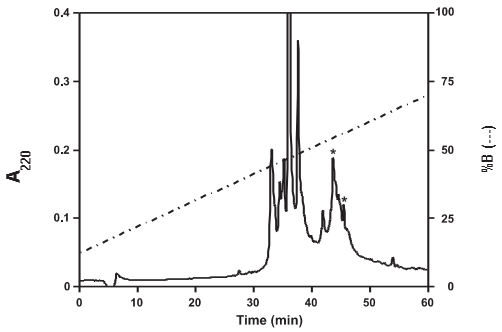
Pilot experiments indicated a considerable increase in brain VGF peptides between second and third postnatal week, hence this time point was selected for study. Western blot analysis of two dimensional gel electrophoresis (Fig. 3a) showed that brain extracts contain two major forms of 18 and 6 kDa apparent molecular weight (hence named: VGF18 and VGF6) in addition to VGF20 and VGF10 (see also Trani *et al*. 1995).

After immunoaffinity purification with anti-$VGF_{573–617}$ antibodies, reverse phase HPLC showed a majority of

(a)



(b)



(c)

	Sequence	MW expected	MW computed	Location
VGF 2	RLQEQEEL…	2545,84	2546,38	598-617
	LQEQEEL…	2389,65	2389,72	599-617
	EQEEL…	2148,36	2149,17	601-617
	QEEL…	2019,24	2020,27	602-617
Peptide V	RAQEEADA…	3830,94	3831,63	587-617
	AQEEADA…	3674,94	3675,82	588-617
VGF 6	HFHHALP…	6150,71	6151,85	567-617
VGF 10	TLQPPAS…	7401,13	7402,12	556-617

Fig. 3 Isolation and characterization of VGF peptides from rat brain. (a) two-dimentional SDS–PAGE of soluble proteins from rat brain. Western blot analysis was performed using anti-$VGF_{609–617}$ antibodies. (b) soluble proteins from rat brain extracts were size-fractionated, and VGF peptides were immunopurified using anti-$VGF_{579–617}$ antibodies coupled to Sepharose. Fractions richest in VGF peptides were chromatographed on a reverse phase Vydac C18 column. Peptides were eluted with a linear gradient of acetonitrile (7–50% in 0.05% TFA), followed by a 10-min wash with 70% acetonitrile. Absorbance was monitored at 220 nm. For sake of clarity, only the linear gradient pattern containing VGF peptide fractions (*), revealed by western blot analysis, is reported. (c) Biochemical characterization of VGF10, VGF6, peptide V (with its N-terminally extended processing intermediate), and of the four species found at the 2.6–2.0 kDa molecular mass interval. Peptide identity was determined by differential MALDI-ToF mass spectrometric analysis before and after single cycles of Edman degradation.

immunoreactive VGF to elute in two specific fractions (Fig. 3b). The corresponding peptide material was analysed by MALDI-ToF mass spectrometry, resulting in a number of high intensity peaks. Measurements of two independent purifications yielded reproducible mass spectra. The measured masses of: 7402.12 Da, 6151.85 Da, 3675.82 Da, and 2389.72 Da appeared to fit well with the calculated mass of expected VGF cleavage peptides derived from the C-terminal portion of proVGF (Fig. 5). The 7.4 kDa and 3.6 kDa moieties appeared to correspond to VGF10, and to the previously described peptide V (Liu *et al.* 1995), respectively. Cleavage following the sequences: 'RRR' at positions 564–566, and 'RR' at position 597–598 of rat VGF would produce VGF6 and a 2.3-kDa form which was named VGF2. These predictions were confirmed by mass spectrometry of the relevant peptides after two cycles of Edman degradation.

Single molecular species were revealed for VGF10 and VGF6, whereas an additional form was shown for peptide V, retaining the N-terminal Arg_{587} residue. In the VGF2 range of molecular mass, three further fragments were detected, of 2546.38, 2149.17 and 2020.27 Da. The 2.5 kDa form appeared to correspond to VGF2 retaining the N-terminal Arg_{598}, while the 2.1 and 2.0 kDa peptides proved to be VGF2 fragments with deletion of two, and three N-terminal aminoacid residues, respectively. Thus, initial cleavage at the $VGF_{586–587}$ or $VGF_{597–598}$ sites would be followed by progressive N-terminal 'trimming' of the resulting peptides. Biochemical characterization of VGF20 and VGF18 could not be addressed because these forms were not recovered following the purification steps. Firstly only limited amounts were recuperated by immunopurification (data not shown), possibly due to low affinity of the anti-$VGF_{579–617}$ antibodies for non-denatured VGF20 and VGF18 and, secondly, they were not eluted from the HPLC because the conditions employed were likely to favour elution of forms with low molecular weight.

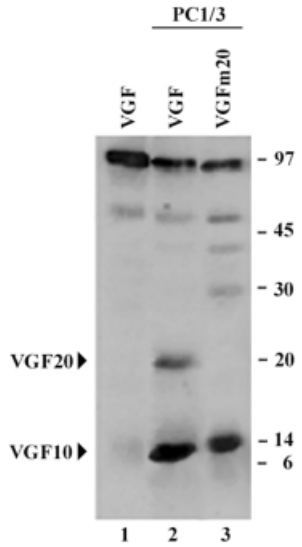


Fig. 4 VGF20 is generated by cleavage within the basic motif at position 483–488 of proVGF. Amino acids alteration at the rat $VGF_{483-488}$ (KRKRKK) cleavage site was created, in order to address the possible blockade of VGF20 biosynthesis. Rat VGF (lanes 1 and 2) and the mutated VGFm20 (lane 3) were expressed in COS-7 cells in the absence (lane 1) or in the presence of PC1/3 (lanes 2 and 3). Cotransfected cells were grown on 60 mm culture dishes until 90% confluence and proteins from 3 h incubation media were run on a Tris-glycine gel gradient (10–15% acrylamide) and analysed by Western blotting using anti-$VGF_{609-617}$ antibodies. VGF20 and VGF10 forms are indicated. Numbers at the left are molecular masses, in kDa, of size standards.

Identification of the VGF20 cleavage site

Being unable of directly establishing the cleavage sites from which VGF20 and VGF18 are generated, we then decided to utilize an indirect approach based on mutation of potential prohormone target sites within VGF sequence. The primary sequence of proVGF shows various consensus sequence for recognition by PC1/3 and PC2 (Fig. 5, top panel). Cleavage within one of these, the KRKRKK sequence from residue 483 to residue 488, was predicted to generate a fragment with molecular weight in the 20 kDa range. We produced a VGF mutant in which the entire sequence RKRKKNA from residue 484–490 was deleted (VGFm20). The plasmid encoding VGFm20 was cotransfected with an expression vector for PC1/3 in the monkey kidney derived COS-7 cells and processing of the mutant and wild type VGF was assessed by western blot analysis of the secreted proteins. PC1/3, as opposed to PC2, does not require the expression of helper proteins for full activity and several of its substrates are correctly processed also in cells devoid of the regulated secretory pathway (Galanopoulou *et al*. 1993; Zhou and Lindberg 1994; Jutras *et al*. 1997).

As shown in Fig. 4, COS-7 cells produced both VGF20 and VGF10 but only in the presence of PC1/3 (compare lanes 1 and 2); in addition deletion of the RKRKKNA sequence prevented generation of VGF20 by PC1/3, while production of VGF10 was unaffected (compare lanes 2 and 3). Although we cannot exclude that deletion of the RKRKKNA sequence results in an altered conformation of VGF so that a it is no

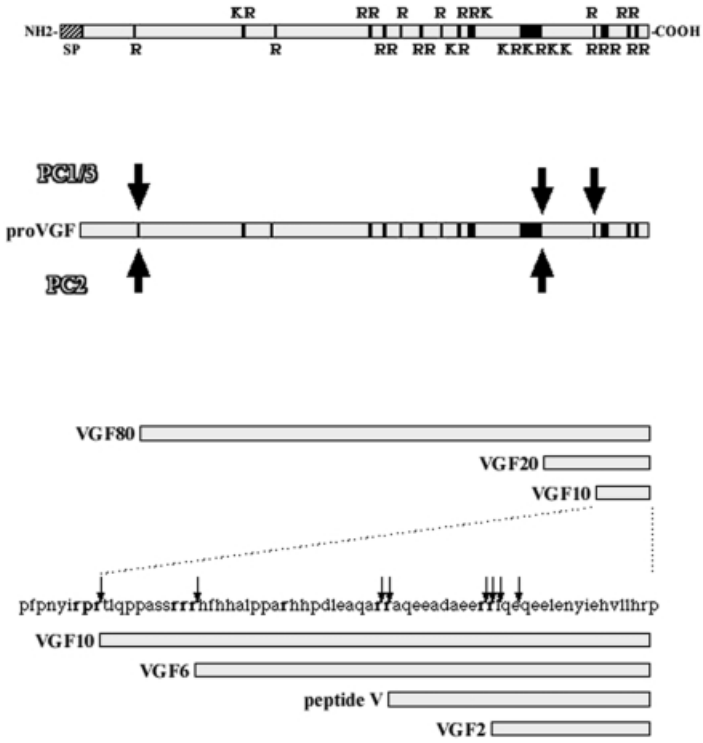


Fig. 5 Schematic representation of pro-VGF processing. Top panel: location of basic amino acid residues within VGF (K: lysine, R: arginine, indifferently indicated above/below the bar representing pre/proVGF; SP: signal peptide). Middle panel: processing sites utilized by PC1/3 and PC2 prohormone convertases, as demonstrated by the present study (downward and upward arrows, respectively). Bottom panel, VGF-processing products isolated from rat brain. Arrows upon the C-terminal region of proVGF indicate cleavage sites processed by PC1/3, as well as by endo- and/or exo-peptidases (yet to be characterized), to yield VGF10, VGF6, peptide V and VGF2 species, respectively.

longer a good substrate for PC1/3, this data strongly suggest that VGF20 results from cleavage within this motive that conforms to a consensus recognition site for prohormone convertases. We have been up to now unable to locate the prohormone convertase target site responsible for the production of VGF18.

Discussion

As mentioned in the introduction, the vgf gene is specifically expressed in neuroendocrine and neuronal cells. Depending on cell type, the primary gene product (proVGF) may be processed to a varied range of VGF-peptides, including 80, 20 and 10 kDa forms (VGF80, VGF20 and VGF10, respectively). Of these, VGF20 and VGF10 are produced in a tissue-specific manner and are secreted by neurons and by pancreatic islet cells in response to external stimuli (Trani *et al*. 1995; Possenti *et al*. 1999). In order to address VGF processing, we studied the main prohormone convertases expressed in neuroendocrine cells and neurons, i.e. PC1/3 and PC2, while focusing on VGF peptides retaining the C-terminal portion of proVGF.

The relationship between the action of PC1/3 and the conversion of proVGF to VGF20 and VGF10 was firstly supposed following the observation that PC1/3 is inducted in parallel with VGF peptides biosynthesis during neuronal maturation of cerebellar granule cells. Mature cerebellar granules (8 days *in vitro*) do contain PC1/3, in the absence of PC2, though not in high quantities (Fig. 1). PC2 was also undetectable in AtT20, in the presence of an elevated content of mature PC1/3, where we found VGF10 but no VGF20. Conversely, prevailing presence of PC2 convertase, as in the glucagon-producing alpha TC1-6 cells (Rouille *et al*. 1994), resulted in VGF20 being the most abundant species of VGF. These observations suggest that varying proportions of PC1/3 and PC2 are likely to result in varied spectra of VGF peptides in different cell types. On the other hand the presence of VGF80 in GH3 cells, in the absence of PC1/3 and PC2, suggests that other proprotein convertases (such as furin or PC5, which are both expressed in GH3) can produce VGF80. Other than in GH3 cells, this larger VGF product appeared to accumulate in PC12 cells and in adrenal medulla (Trani *et al*. 1995) in which further processing to smaller VGF peptides is not found. It is possible that this high molecular weight form has a function by itself beside being the precursor of the low molecular mass species. A precedent for this hypothesis is provided by the chromogranin/secretogranins, which are hormone precursors, and, their non-processed form, promote the sorting of peptide precursors from the *trans*-Golgi network to secretory granules (Natory and Huttner 1996).

The repertoire of VGF peptides observed following ectopic expression of PC1/3 and PC2 convertases in stable tranfectants of GH3 cells is in accord with the hypothesis that VGF10 is produced specifically by PC1/3. We, as opposed to others, did not detect either of PC1/3 or PC2 convertase in GH3 cells (Fig. 1, lane 7). Strain variations are likely, as some researchers found PC2 protein in their GH3 cells (Friedman *et al*. 1996), while others did not (reviewed in Seidah *et al*. 1994). In any event this allowed us to express the two convertases separately in a cell line which contains high level of proVGF. Importantly GH3 cells also synthesize 7B2, a protein which plays the double function of promoting correct maturation of PC2, as well as preventing its activity in early compartments of the secretory pathway (Benjannet *et al*. 1995; Muller and Lindberg 1999; Westphal *et al*. 1999). The presence of 7B2 guarantees that ectopically expressed PC2 is fully functional. In stably transfected GH3 PC2 activity lead to the production of VGF20 in the absence of detectable VGF10. Upon transfection with PC1/3, forms corresponding to VGF20 and VGF10 were found enriched in the culture medium (Fig. 2). Production of both forms of VGF peptides distinguish GH3 ectopically expressing PC1/3 from AtT20. We suppose that different level of convertase expression and/or different time of retention of secretory granules within the cells may account for such a cell type-specific pattern of VGF peptides production. In this respect it should be noted that as our antibodies recognize the most C-terminal portion of VGF only VGF10 would be detected following complete cleavage at the site that generate VGF10 within VGF20 sequence.

Enzymes of the family of kexin/subtisilin-like serine proteinases like PC1/3 and PC2, cleave, generally, C-terminally at the motif: (Arg/Lys)-(X)n – Arg (where $n = 0, 2, 4$, or 6; Seidah and Chretien 1999). The KRKRKK sequence (amino acids 483–488 of rat proVGF), whose deletion prevents production of VGF20, conforms to the recognition motif for prohormone convertases. On the contrary the RPR^{555} sequence, which is the cleavage site utilized by PC1/3 for the production of VGF10, as demonstrated by sequencing of this peptide, is a novel, non-canonical recognition site for this prohormone convertase. Possibly, in the context of VGF sequence, PC1/3 recognises a proline-directed arginine processing site, similar to those described by Schwartz (Schwartz 1986), whereas PC2 has low affinity for such a motif.

Considering that VGF20 is produced by both PC1/3 and PC2 and that this convertase has a wider tissue distribution than PC1/3 (Schafer *et al*. 1993; Seidah and Chretien 1999) it is likely that VGF10 has a more restricted expression and possibly functional role.

A major finding of this work was the identification of novel C-terminal VGF peptides present *in vivo*. Beside a peptide of 6151 85 Da (VGF6), which correspond to a molecular mass detectable also by western blot of rat brain

extracts, smaller species which escaped such analysis were isolated from rat brain and analysed by the combined use of MALDI-Tof mass spectrometry and Edman sequencing. A 30 aminoacid VGF peptide appeared to be the rat equivalent (with a 2 aminoacid difference) of peptide V, previously isolated from bovine hypophysis (Liu *et al*. 1995) and corresponding to the human VGF sequence (Canu *et al*. 1997). Finally, smaller VGF fragments corresponded to $VGF_{598-617}$, $VGF_{599-617}$, $VGF_{601-617}$ and $VGF_{602-617}$, were collectively named VGF2.

Sequencing of VGF6 indicates that it is generated by endoproteolysis following the Arg^{564}-Arg^{566} site of proVGF which is a consensus motif for cleavage by prohormone convertases. On the contrary, the extended peptide V and the larger of VGF2 peptides ($VGF_{598-617}$) both showed an N-terminal Arg residue, probably resulting from cleavage within the relevant dibasic (Arg-Arg) site by endopeptidase/s other than PC1/3, or PC2. Potential candidates may include the aspartyl protease proopiomelanocortin-converting enzyme (PCE), or the multicatalytic cysteine protease called prohormone thiol-protease (PTP), responsible for maturation of proenkephalin (Hook *et al*. 1996). The mentioned N-terminal Arg residue were then probably removed by aminopeptidases. The two smallest VGF2 species, corresponding to further trimming of $VGF_{599-617}$, might be either bioactive VGF peptides, or degradation products generated in the extracellular compartment by exopeptidases (Turner and Tanzawa 1997).

In conclusion, VGF is a relatively large protein and peptide precursor. Out of approximately 15 potential processing sites, we have focused onto some close to the C-terminal region of proVGF. It is likely that further peptides are generated from other regions of proVGF, as shown for fragments derived from the N-terminal portion of VGF which were recently found in the human cerebrospinal fluid (Stark *et al*. 2001). Recent analysis of knockout mice has demonstrated that VGF plays a critical role in the control of energy homeostasis (Hahm *et al*. 1999; Salton *et al*. 2000). On the other hand, VGF gene has been shown to be highly responsive to a number of conditions and stimuli, including nerve injury, seizure and long-term potentiation (Lombardo *et al*. 1995; Hevroni *et al*. 1998; Snyder *et al*. 1998), suggesting it may contribute to neuronal plasticity. Future studies will help to understand how any single VGF peptide participates to the various function of this gene.

Acknowledgements

We gratefully acknowledge Dr Delio Mercanti for help in immunoaffinity purification of VGF peptides, for constant encouragement and many insightful discussions. We thank Maria Teresa Ciotti for technical assistance and Prof Donatella Barra for valuable discussion. Supported by Telethon research grant # E830 to A. L and MURST-COFIN research grants to RP and G-LF.

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