

Selective expression of TLQP-21 and other VGF peptides in gastric neuroendocrine cells and modulation by feeding

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Abstract

Although *vgf* gene knockout mice are hypermetabolic, administration of the VGF peptide TLQP-21 itself increased energy consumption. Agonist-antagonist roles are thus suggested for different VGF peptides, and the definition of their tissue heterogeneity is mandatory. We studied the rat stomach using antisera to C- or N-terminal sequences of known or predicted VGF peptides in immunohistochemistry and ELISA. TLQP (rat VGF_{556–565}) peptide/s were most abundant (162 ± 11 pmol/g, mean \pm S.E.M.) and were brightly immunostained in enterochromaffin-like (ECL) cells and somatostatin cells. A peptide co-eluting with TLQP-21 was revealed in HPLC of gastric and hypothalamic extracts, while the extended TLQP-62 form was restricted to the hypothalamus. Novel PGH (rat VGF_{422–430}) peptide/s were revealed in ghrelin cells, mostly corresponding to low MW forms (0.8–1.5 kDa), while VGF C-terminus peptides were

confined to neurons. VGF mRNA was present in the above gastric endocrine cell types, and was prominent in chief cells, in parallel with low-intensity staining for further cleaved products from the C-terminal region of VGF (HVLL peptides: VGF_{605–614}). In swine stomach, a comparable profile of VGF peptides was revealed by immunohistochemistry. When fed and fasted rats were studied, a clear-cut, selective decrease on fasting was observed for TLQP peptides only (162 ± 11 vs 74 ± 5.3 pmol/g, fed versus fasted rats, mean \pm S.E.M., $P < 0.00001$). In conclusion, specific VGF peptides appear to be widely represented in different gastric endocrine and other mucosal cell populations. The selective modulation of TLQP peptides suggests their involvement in peripheral neuro-endocrine mechanisms related to feeding responses and/or ECL cell regulation.

Journal of Endocrinology (2010) **207**, 329–341

Introduction

The *vgf* gene (non-acronymic) and its peptide products are proposed neuroendocrine regulators affecting food intake and/or energy balance (Salton *et al.* 2000, Levi *et al.* 2004), as well as other functions including water balance (Yamaguchi *et al.* 2007).

The *vgf* gene and its derived peptides are expressed in many tissues, including the central and peripheral nervous system, the endocrine system and the gut, suggesting that multiple sites of VGF peptide production, as well as actions are likely (Salton *et al.* 2000). Deletion of the mouse *vgf* gene (knockout) resulted in thin, small, hyperactive and hypermetabolic animals (Hahm *et al.* 1999), with derangement of hypothalamic outflow pathways regulating peripheral metabolic tissues and energy homeostasis (Hahm *et al.* 2002). More recently, an increased sympathetic drive in *Vgf*^{-/-} mice appeared to induce increased lipolysis, decreased lipogenesis and brown adipocyte differentiation in white adipose tissue (Watson *et al.* 2009). Surprisingly, i.c.v. injection of the naturally occurring VGF-derived TLQP-21

peptide increased energy expenditure and body temperature, and prevented diet-induced obesity in mice (Bartolomucci *et al.* 2006), while reducing food intake in Siberian hamsters (Jethwa *et al.* 2007). It was proposed that further end products of the VGF precursor (yet to be characterised) could be responsible for inhibiting energy expenditure, thus explaining the relevant aspects of the knockout phenotype (Bartolomucci *et al.* 2007, Jethwa & Ebling 2008).

Briefly, the *vgf* gene encodes a VGF polypeptide of 617 or 615 amino acids (in rat/mouse and human respectively, Salton *et al.* 2000), with more than 12 fully conserved stretches of basic amino acid residues representing potential or demonstrated processing sites for neuroendocrine prohormone convertases, such as PC1/3 and PC2 (Trani *et al.* 2002). In cells and tissues, proteolytic processing yields diverse peptides, including some of low MW, which were preferentially released by various stimuli (Possenti *et al.* 1989, 1999, Trani *et al.* 1995). These include peptides encompassing the VGF C-terminal portion, TLQP-21 and TLQP-62 peptides, and the recently identified peptides NERP-1 and -2 (Trani *et al.* 2002, Bartolomucci *et al.* 2007, Yamaguchi *et al.* 2007,

Bozdagi *et al.* 2008). While *vgf* gene expression is distinctly modulated in multiple models and conditions (Levi *et al.* 2004), differential processing and modulation of VGF peptides have been shown so far in the pituitary (Brancia *et al.* 2005), adrenal glands (D'Amato *et al.* 2008) and pancreas (Cocco *et al.* 2007).

The stomach is a complex organ involved in the regulation of food intake and in the response to feeding, and contains heterogeneous populations of endocrine cells including gastrin (G), enterochromaffin (EC), enterochromaffin-like (ECL) and somatostatin (D) cells (Solcia *et al.* 2000). The circulating hormone leptin is present and modulated not only in adipocytes, but also in gastric principal cells (Bado *et al.* 1998). The hormone ghrelin, acting on both food intake and GH secretion, is mainly released from gastric endocrine cells identified as P/D1 type in human and X/A-like type in rat (Rindi *et al.* 2002). A further novel hormone, apelin, is expressed in multiple gastric mucosal cells, including parietal cells, where from it participates in the regulation of acid secretion via ECL cells (Lambrecht *et al.* 2006). Most recently, the hypothalamic nesfatin-1 peptide was revealed in gastric ghrelin- and somatostatin-containing cells, with changes in connection with the nutritional status (Stengel *et al.* 2009).

We address the localisation and heterogeneity of several, including some novel, VGF peptides using antisera to short sequences corresponding to N- and C-terminal regions of known or predicted cleaved peptides. VGF peptide modulation was studied in response to the major physiological stimulus of feeding versus fasting.

Materials and Methods

Animals and tissue samples

Sprague–Dawley rats (250–350 g, Harlan, Milan, Italy) were kept at 20 °C with free access to water throughout. In a first set of experiments, rats ($n=8$, of either sex, fed *ad libitum*) were kept under standard light–darkness cycle (dark: 1900–0700 h), deeply anaesthetised with diethyl ether vapour and were exsanguinated. The stomach was opened, pinned on cork and was immersion fixed in paraformaldehyde (40 g/l, in 0.1 mol/l PO₄ buffer for 3 h at 0–4 °C). Further rats (three per cage) were kept under reversed light–darkness cycle (dark: 0900–2100 h, >4 weeks before killing), and received standard rat chow (20 g/rat) once per day (at 0900 h) for 2 weeks. These were allocated to either A) fasted group ($n=18$), deprived of chow 2 days before the killing, or B) fed group ($n=18$), fed as above until the killing. Rats were moved to the killing room one cage at a time (between 1200 and 1300 h), deeply anaesthetised with diethyl ether vapour, hence, either exsanguinated (for tissue extraction: male, $n=12$ per group) or perfused transcardially with paraformaldehyde, while the same fixative (~3–5 ml) was rapidly injected into the stomach (for immunohistochemistry: $n=4$ males + 2 females per group). From the latter animals, the

brains were removed, the stomach was opened and emptied of contents and then both were washed and stored in PBS–sucrose (0.01 mol/l PO₄, pH 7.2–7.4, containing 0.15 mol/l NaCl, 70 g/l sucrose and 0.2 g/l NaN₃). For the rat stomach, the glandular portion was sampled, including the oxyntic and antro-pyloric regions. Additional samples were taken from pigs (females and castrated males, 7–9 months, $n=10$, from a local abattoir). According to routine butchery procedures, pigs received a limited amount of food on the day preceding slaughtery. Samples of oxyntic, cardiac and pyloric mucosa (~4×4 cm) were deprived of muscularis, stretched onto filter paper (mucosal side up), immersion fixed in paraformaldehyde (40 g/l, in 0.1 mol/l PO₄ buffer for 3 h at 0 °C) and stored in PBS–sucrose. For cryosectioning, tissue samples were oriented in aluminium foil moulds in cryoembedding medium (Cocco *et al.* 2003) and were frozen in the melting freon (cooled with liquid nitrogen). Sections (5–6 µm) were cut using a cold-blade cryomicrotome (Microm HM-560, Walldorf, Germany) and were collected on slides coated with either poly-L-lysine (for immunocytochemistry) or 3-aminopropyltriethoxysilane (TESPA, Sigma, for *in situ* hybridisation, 20 ml/l in acetone, followed by overnight heating at 180 °C), air dried, wrapped in aluminium foil and stored in a liquid nitrogen tank (vapour phase) until used. For peptide extraction, the whole stomach from each rat was emptied of contents, rinsed in cold saline, blotted dry, weighed and coarsely minced with a scalpel. A portion of the brain encompassing the hypothalamus was taken in parallel. The above tissues were immediately homogenised in PBS (~10 ml/g tissue, 3–5 min, on ice) containing protease inhibitor cocktail (P8340, Sigma, 5 µl/ml) using an Ultra-Turrax homogeniser (Ika-Werke, Staufen, Germany), heated in a vigorously boiling water bath (10–15 min) and centrifuged (3000 g, 10–15 min). Supernatants were kept frozen until used (–20 °C or lower).

Experimental design and procedures were approved by the relevant ethical committee at the University of Cagliari and the national controlling body (Italian Ministry of Health) and were performed in accordance with the care and use of animals approved by the American Physiological Society and EEC Council Directive of 24 November 1986 (86/609) throughout.

VGF peptide antibodies

Antisera (Table 1) were raised against peptide sequences at the C-terminus of the VGF precursor protein or adjacent to the putative or demonstrated cleavage sites (Fig. 1) and were conjugated with either bovine thyroglobulin or keyhole limpet haemocyanin (KLH). At the C-terminal end of rat and mouse VGF, a His₆₁₅–Arg₆₁₆–Pro₆₁₇ sequence is found, as opposed to Arg₆₁₃–Arg₆₁₄–Pro₆₁₅ in human (Salton *et al.* 2000), hence antisera were raised against the corresponding nonapeptides conjugated at their N-terminus (Ferri *et al.* 1995). A peptide corresponding to human VGF_{603–612} (identical to rat VGF_{605–614}), and immediately preceding

Table 1 VGF peptide antisera

Antigen	Short name	Use	Species	References
Rat VGF ₆₀₉₋₆₁₇	Rat VGF C-term.*	I, E	Rabbit	Ferri <i>et al.</i> (1995) and Trani <i>et al.</i> (1995, 2002)
Human VGF ₆₀₇₋₆₁₅	Hum VGF C-term.*	I	Rabbit	Brancia <i>et al.</i> (2005) and Cocco <i>et al.</i> (2007)
Rat VGF ₆₀₅₋₆₁₄ **	HVLL	I, E	Rabbit	D'Amato <i>et al.</i> (2008)
Rat VGF ₅₅₆₋₅₆₅	TLQP	I, E	G-pig	Brancia <i>et al.</i> (2005)
Rat VGF ₄₂₂₋₄₃₀	Rat PGH	I, E	Rabbit	Rindi <i>et al.</i> (2007) and D'Amato <i>et al.</i> (2008)
Rat VGF ₄₄₃₋₅₈₈	b-gal-VGF ₄₄₃₋₅₈₈	I	Rabbit	Ferri <i>et al.</i> (1992)

*C-terminus; ** identical to human VGF₆₀₃₋₆₁₂. b-gal, β-galactosidase; use: I, immunohistochemistry; E, ELISA; G-pig, guinea-pig.

the Arg₆₁₃–Arg₆₁₄ stretch found in human VGF, was conjugated at its N-terminus (HVLL peptide, corresponding to the C-terminus of the VGF precursor deprived of its last three amino acids). TLQP peptides were isolated from the rat brain and were proved to be cleaved from VGF at the rat VGF₅₅₃₋₅₅₅ (Arg₅₅₃–Pro₅₅₄–Arg₅₅₅) processing site (Trani *et al.* 2002), at least their N-terminal five amino acids (Thr-Leu-Gln-Pro-Pro) being identical in human and rat VGF. Hence, the rat VGF₅₅₆₋₅₆₅ peptide was synthesised and conjugated at its C-terminus (Brancia *et al.* 2005). The rat VGF₄₂₂₋₄₃₀ 'PGH' peptide, immediately preceding a putative Arg-Arg-Lys cleavage site, was conjugated via an N-terminal tyrosine (Ferri *et al.* 1995, D'Amato *et al.* 2008). The VGF₄₄₃₋₅₈₈ fusion protein antiserum has been described previously in detail (Ferri *et al.* 1992).

Immunohistochemistry and in situ hybridisation

Sections were soaked in Triton X-100 (1 g/l in PBS for 1 h; Merck), incubated with one, two or three primary antiserum/a (raised in different species, overnight at room temperature), hence with the relevant species-specific secondary antibody/ies (from donkey, conjugated with either AMCA, Cy2 or Cy3; Jackson Immunoresearch Laboratories, West Grove, PA, USA). Endocrine cell populations were characterised as follows: i) ECL cells by combined immunostaining for vesicular monoamine transporter 2 (VMAT-2; Chemicon, Temecula, CA, USA) and ghrelin, such cell type expressing VMAT-2 but not ghrelin (Rindi *et al.* 2002); ii) principal cells by immunostaining for pepsinogen II (Abcam, Cambridge, UK); iii) ghrelin, gastrin, somatostatin and serotonin-containing (EC) cells by

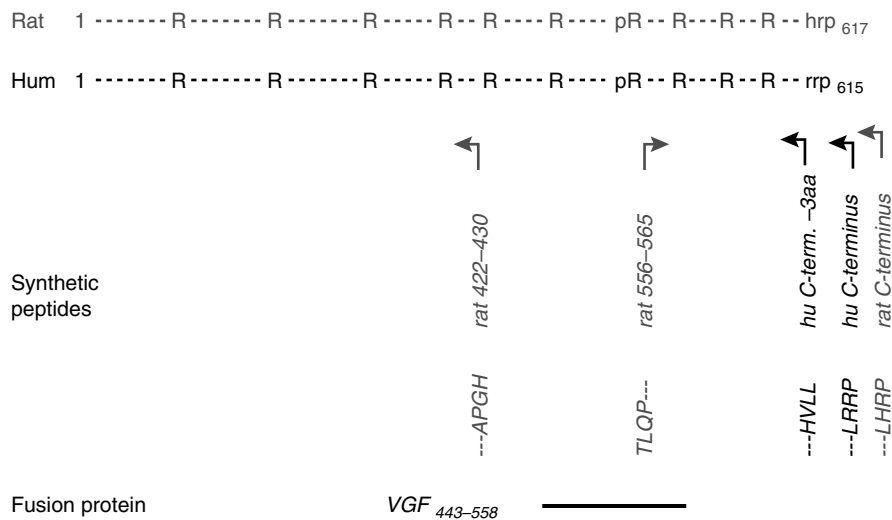


Figure 1 Scheme of the VGF precursor protein (rat and human), and peptides/fusion protein used to raise the antisera. Immunisations were aimed to obtain antibodies specific for sequences at the VGF C-terminus, or immediately adjacent to the demonstrated/putative cleavage sites (N-terminus of the TLQP peptide/s, C-terminus of HVLL and PGH peptides), hence, a cysteine residue was added at their other extreme and used for conjugation. Arrows indicate expected reactivity of antibodies, which would be selective for a free/cleaved N-terminus (for TLQP peptides), or C-terminus (HVLL, PGH and C-terminus peptides), whichever length or modification native peptides may show at their other extreme. The far longer fusion protein was intended to raise antibodies to multiple portions of its included VGF sequence, expectedly cross-reacting with the VGF precursor. 'rat'/hum': schemes of rat and human VGF (precursor) protein respectively; 'R': putative/demonstrated cleavage sites, with two or more Arg/Lys basic residues; 'pR': rat Arg₅₅₃–Pro₅₅₄–Arg₅₅₅ processing site giving raise to TLQP peptides (Trani *et al.* 2002); 'rrp'/'hrp': last three C-terminal amino acids of rat/human VGF (Arg-Arg-Pro and His-Arg-Pro respectively).

immunostaining for their respective hormone (anti-ghrelin, raised in sheep against a human octanoylated ghrelin 1–10 peptide conjugated to KLH; anti-gastrin, anti-somatostatin and anti-serotonin; Biomol, Exeter, UK). Washings were carried out in PBS, and antibodies were diluted in PBS containing 30 ml/l normal serum of the secondary donor species (donkey), and 30 ml/l normal serum (from a pool) of the same species being immunostained (either rat or pig). Routine controls included substitution of each layer, in turn, with PBS or pre-immune serum. In absorption controls, each primary antiserum was pre-incubated overnight with its own antigen in a range of concentrations (0.03–100 µmol/l, at 0–4 °C). Absorption with ~10 µmol/l concentrations of antigen, or higher, resulted in virtually complete prevention of the corresponding labelling in each case.

As described below (see section Results), distinct localisation patterns were shown with VGF_{443–588}, TLQP and PGH peptide antibodies, hence, these were used for quantitation of the relevant co-localisation profiles versus hormones and/or other markers. Single- and double-labelled cells showing a visible (unlabelled) nuclear profile were counted in four rats per group (fed versus fasted), using three non-adjacent sections, two areas each (encompassing the whole mucosa, from muscularis mucosa to lumen).

For *in situ* hybridisation, ~600 bp fragment of rat VGF cDNA (BamHI–XhoI 235–854 bp from the ATG of the coding sequence) was cloned in both orientations, downstream of the T7 polymerase promoter in pGEM-4Z plasmid (Promega Corporation). Labelled antisense and sense probes were obtained by *in vitro* transcription of the linearised plasmids with T7 polymerase, using Megascript labelling kits (Ambion, Austin, TX, USA) and digoxigenin-11-UTP (Roche, Monza, Italy). Sections were digested with proteinase K (1 µg/ml in PBS containing 1 mmol/l EDTA for 3 min; ICN, Milan, Italy), treated with glycine (25 mmol/l in PBS for 5 min) and post fixed in paraformaldehyde (40 g/l in PBS for 5 min). After a pre-incubation step

with the hybridisation buffer (at 70 °C for 1 h), preparations were hybridised overnight with the relevant probe (at 70 °C, as described in Moonman *et al.* 2001). After RNase digestion and stringency washes (at 70 °C), sections were incubated with alkaline phosphatase-labelled anti-digoxigenin Fab fragment antibodies (Roche), using a 4-nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indolyl-phosphate substrate kit (Vector, Burlingame, CA, USA). Controls included the use of the corresponding sense probe, substitution of the probe with the hybridisation buffer alone, as well as pre-digestion with RNase, and confirmed the specificity of the staining. Preparations were observed with an Olympus BX60 fluorescence microscope equipped with Fuji FinePix S2 Pro and S3 Pro digital cameras (Fujifilm, Milan, Italy).

ELISA

Assays used are summarised in Table 2. Multiwell plates (Nunc, Milan, Italy) were coated with the corresponding synthetic peptide (5–50 nmol/l, in carbonate-bicarbonate buffer, pH 9.6, either for 4 h at 37 °C or for 16 h at 0–4 °C), hence were treated with normal serum from the secondary antibody donor species (90 ml/l in PBS, PBS-donkey). Primary incubations were carried out in duplicate, including relevant standards (0.005–500 nmol/l), or serially diluted samples (100 µl/well incubation volume, in PBS-donkey for 4 h at room temperature under constant agitation). After the incubation (1 h) with the relevant, biotinylated secondary antibody (Jackson) and streptavidin-peroxidase conjugate (Biospa, Milan, Italy), wells were filled with tetramethylbenzidine substrate (100 µl/well; Kem-En-Tec Diagnostics, Taastруп, Denmark). Upon development, the reaction was stopped with HCl (1 mol/l, 100 µl/well), and optical density was measured at 450 nm using a multilabel plate reader (Chameleon; Hidex, Turku, Finland). Synthetic peptides were used for assay characterisation.

Table 2 ELISA characterisation

Assay	Peptide	IC ₅₀ (pmol) ^a	Percentage of cross-reactivity
VGF C-term. (rat)	Rat VGF _{609–617}	0.02	100
	Hum VGF _{607–615}	> 200	< 0.01
	Hum VGF _{603–612} (HVLL)	> 200	< 0.01
TLQP peptides	Rat VGF _{556–565}	1.1	100
	Rat VGF _{556–576} (TLQP-21)	0.6	183
	Rat VGF _{556–567} (TLQP-11)	0.9	122
HVLL peptides	Hum VGF _{603–612} (HVLL) ^b	0.3	100
	Hum VGF _{607–615}	28	1.0
	Rat VGF _{609–617}	8.7	3.4
PGH peptides (rat)	Rat VGF _{422–430} (APGH)	0.3	100
	Rat VGF _{422–431} (APGH-R) ^c	> 2000	< 0.015
	Hum VGF _{419–427} (TPGH)	8.5	3.5
	Hum VGF _{419–428} (TPGH-R) ^c	> 2000	< 0.015

^apmol per assay well, 100 µl incubation volume.

^bIdentical to rat VGF_{605–614}.

^cFor PGH peptide assays, cross-reaction with C-terminally extended forms, or the VGF precursor itself was addressed using peptides with an additional Arg residue at their C-terminus.

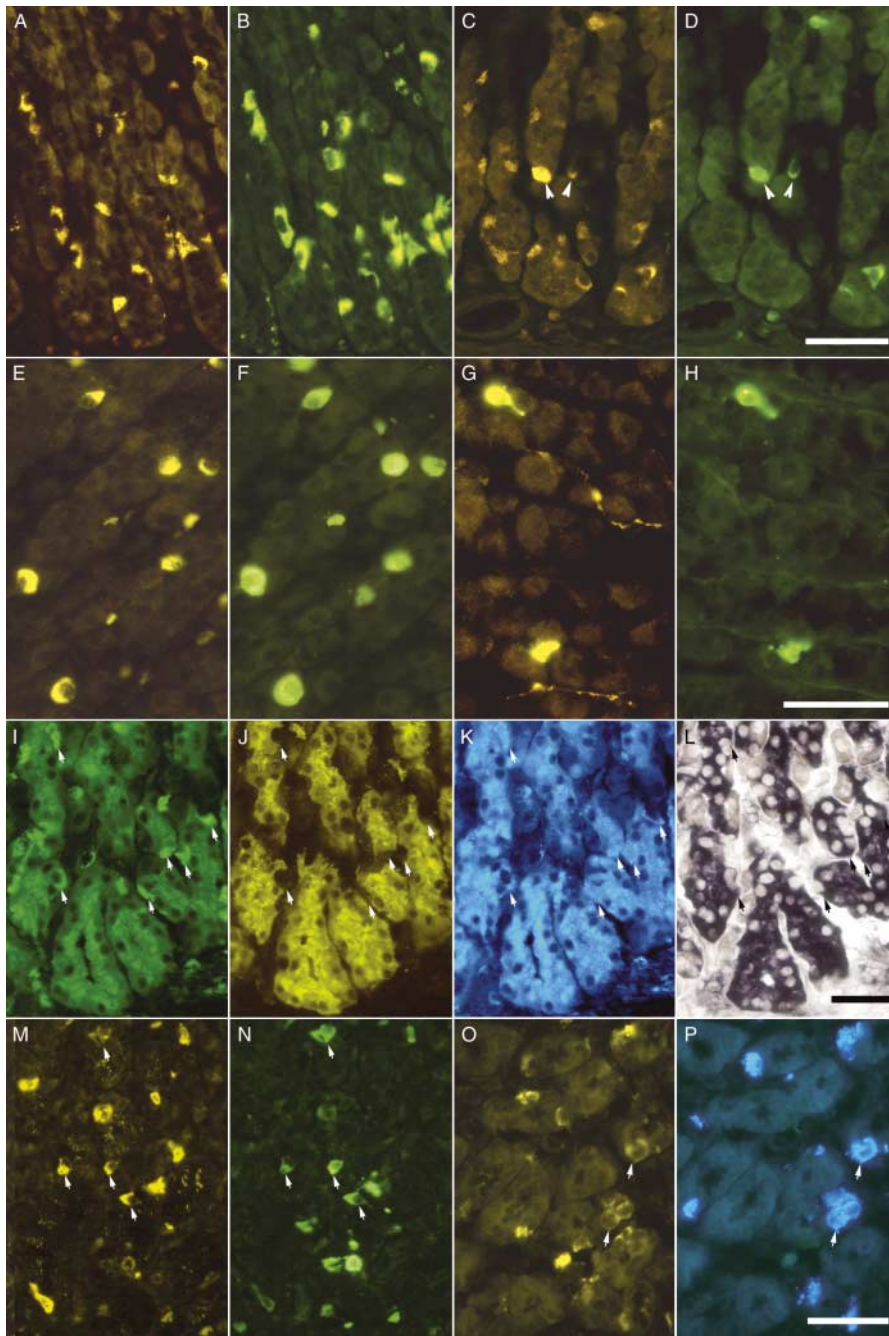


Figure 2 Differential distribution of VGF peptides in gastric endocrine cells. TLQP peptides were abundant and brightly labelled in VMAT-2-immunoreactive ECL cells (A: TLQP; B: VMAT-2), and in somatostatin cells (arrowheads in C: TLQP; D: somatostatin), irrespective of the animal's fed/fast condition. Somatostatin cells, with the addition of a few mucosal nerve axons, were also labelled by the VGF₄₄₃₋₅₈₈ antibody (G: VGF₄₄₃₋₅₈₈; H: somatostatin). PGH peptides were selectively shown in closed-type endocrine cells, recognised as ghrelin-containing cells in double immunofluorescence (E: PGH; F: ghrelin). At a distinctly lower intensity, TLQP immunoreactivity was revealed in a subpopulation of EC and G cells (M and O: TLQP; N: serotonin; P: gastrin), and in chief cells (about half of the animals, I: TLQP; J: pepsinogen), irrespective of the animal's feeding state. Diffuse low-intensity labelling for HVLL peptides was found in chief cells, in association with a prominent expression of VGF mRNA (I: TLQP; J: pepsinogen; K: HVLL peptides, all in the same section; L: VGF mRNA, in an immediately adjacent serial section). Single-cell profiles (arrows in panels I–K) can be seen to be negative for pepsinogen (K), while being labelled for the TLQP in the same section (I). In the adjacent serial section (L), corresponding cell profiles are seen to contain VGF mRNA. These represent ECL and/or somatostatin cells, few in number compared to chief cells. (A–L) Rat stomach, oxyntic region; (M–P) swine stomach, pyloric mucosa; scale bars: 50 μ m. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0189>.

Chromatography

Gastric extracts (~2 ml, $n=4+4$) from rats of the fed and fasted groups respectively were individually loaded onto a Sephadex G-50S column (Sigma, $2\text{ cm}^2 \times 1\text{ m}$), equilibrated with 50 mM ammonium bicarbonate and eluted with the same buffer. A MW marker kit (MWGF70; Sigma) was used for column calibration. Fractions (3 ml) were reduced in volume using a Vacufuge Concentrator (Eppendorf, Milan, Italy) and were assayed. Recovery after the chromatography ranged between 80 and 120% for all the peptides tested.

The relevant gel chromatography fractions (rat stomach, $n=3$; rat hypothalamus, $n=2$) were dried and redissolved in 200 μl distilled water. HPLC was carried out using a Waters 1525 system, equipped with a $0.46 \times 15\text{ cm}$ ODS ultra sphere C18 column (Beckman Coulter, Brea, CA, USA), and a variable wavelength u.v. detector. The mobile phase was made with two solvents: A and B. Solvent A was 0.5 g/l trifluoroacetic acid (TFA) in water and solvent B was 0.5 g/l TFA in acetonitrile/water (80/20, vol/vol). A linear gradient was used, from 5 to 30% solvent B in 40 min, then to 50% solvent B during the next 15 min, at a 1 ml/min flow rate, at room temperature. Rat synthetic TLQP peptides (TLQP-11, TLQP-21, TLQP-30 and TLQP-62, all containing the TLQP sequence at their N-terminal end; Primm, Milan, Italy) were used as standards, and their elution times were measured by absorbance at 210 nm. Twenty-five HPLC fractions (2 ml) were collected, dried with a Vacufuge Concentrator, redissolved in PBS and tested in ELISA.

Results

Immunohistochemistry and in situ hybridisation

Most of the VGF antibodies used labelled endocrine cells in the rat gastric mucosa (Fig. 2), and their overall differential localisation is summarised in Table 3. As mentioned (see section Materials and Methods), absorption controls

resulted in virtually complete prevention of immunostaining in each case (Fig. 3A and B). TLQP peptides were abundant and intensely labelled in cells of varied morphology, numerous in the oxyntic region (Fig. 2A and C). These were identified as VMAT-2-labelled, ghrelin-negative ECL cells (Fig. 2A and B), with the addition of somatostatin cells (Fig. 2C and D). Most ECL cells showed bright immunostaining for TLQP peptides ($94.5 \pm 1.4\%$, mean \pm S.E.M., $n=4$), while almost all somatostatin cells were intensely labelled with both TLQP and VGF₄₄₃₋₅₈₈ antibodies (96.9 ± 1.1 and $99.6 \pm 0.4\%$ respectively; Fig. 2C, D, G and H). Immunoreactivity for the novel PGH peptide/s was revealed in cells showing the typical morphology of closed-type endocrine cells, and mostly abundant in the oxyntic mucosa (Fig. 2E). These proved to be ghrelin-containing cells (Fig. 2E and F), with a virtually complete co-localisation profile (>98%). Though few, antral ghrelin cells also contained PGH immunoreactivity.

A far weaker labelling pattern, clearly distinct from the one described for ECL, somatostatin and ghrelin cells, was revealed with several VGF peptide antibodies in additional rat gastric mucosal cells. The HVLL peptide antiserum (Fig. 2K) yielded a consistent, low-intensity immunostaining in chief cells (Fig. 2J), while TLQP antibodies weakly labelled a fraction of gastrin and 5-HT cells (5.9 ± 1.2 and $15.7 \pm 3.8\%$ of gastrin and 5-HT cells, mean \pm S.E.M. respectively) as well as chief cells in about half of the animals (irrespective of their fed or fasted state; Fig. 2I–J). VGF C-terminus immunoreactivity was confined to neuronal structures, including myenteric perikarya and axons in all layers of the wall, with no labelling of endocrine or other mucosal cells, as reported previously (Severini *et al.* 2009). Nerve structures were also revealed by the VGF₄₄₃₋₅₈₈ antiserum (Fig. 2G), with the addition of a small number of weakly labelled axons depicted by the HVLL antibodies, while no nerve labelling was obtained in the stomach with any further VGF antibody.

Table 3 Gastric mucosal elements showing VGF peptide immunoreactivity. Summary of the localisation of VGF peptides studied in gastric mucosal cells, including various endocrine cell populations and chief cells, as well as in the gastric innervation

VGF peptide immunostained	Gastric cell type						Neuronal structures
	ECL	D	X/A like	EC	G	Chief cells	
TLQP	++++	++++	–	+ ^a	+ ^a	\pm ^b	–
APGH	–	–	++++	–	–	–	–
HVLL	–	–	–	–	–	+ ^c	+
VGF ₄₄₃₋₅₈₈	–	++++	–	–	–	–	+++
C-terminus	–	–	–	–	–	–	++++

Intense immunoreactivity, + + + +; low immunoreactivity, +; no immunoreactivity, –.

^aIn $15.7 \pm 3.8\%$ of EC cells and $5.9 \pm 1.2\%$ of G cells, fed rats, mean \pm S.E.M., $n=4$.

^bAbout half of the animals, irrespective of fed/fast state.

^cAll animals, immunostained neuronal structures included axons in the mucosal lamina propria, submucosa and muscle layers, and a fraction of perikarya in myenteric ganglia.

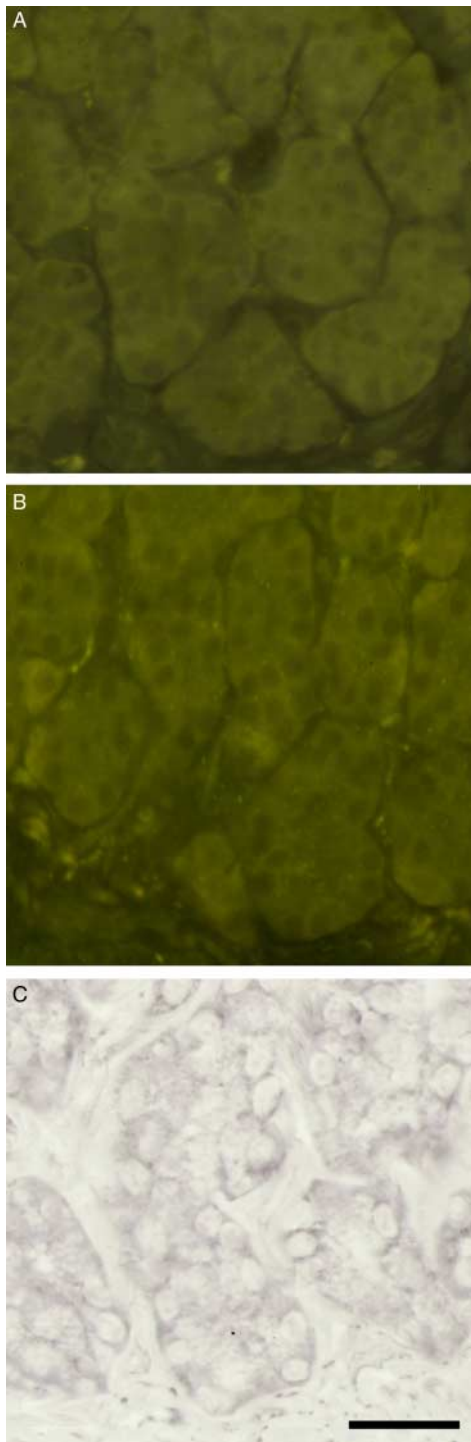


Figure 3 Negative control sections for immunohistochemistry and *in situ* hybridisation. Sections from rat oxyntic mucosa showed no labelling with TLQP and HVLL antisera (A and B respectively), when pre-absorbed with their respective peptides. Similarly, *in situ* hybridisation resulted in no labelling, when the sense probe was used (C). Scale bar: 50 μ m. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0189>.

While VGF mRNA was traced to each of the above VGF peptide-containing cell types in serial sections, the most prominent VGF mRNA-containing population was composed of pepsinogen-, as well as HVLL-immunoreactive chief cells in fundic glands (Fig. 1J–L). Corresponding sense probe controls were negative (Fig. 3C). Multiple myenteric perikarya expressed VGF mRNA in the various regions of the stomach (Fig. 4C), while submucous perikarya were seen only occasionally.

To gain some insight into another mammalian species, a few parallel experiments were carried out by immunocytochemistry on pig gastric mucosa, encompassing the cardiac, oxyntic and antral regions. The overall VGF peptide localisation in swine gastric endocrine cells proved comparable with the one seen in rat, namely in ECL, ghrelin and somatostatin cells, as well as in a subpopulation of antral serotonin (Fig. 2M and N) and gastrin cells (Fig. 2O and P). Neither HVLL nor TLQP labelling was seen in swine chief cells. Since porcine VGF has not been sequenced, the antisera against the human and rat VGF C-terminus peptides were both tested. The former proved most effective in labelling a few bright mucosal axons, but not endocrine cells (the muscularis externa was not included in the swine specimen studied).

Since TLQP peptides were originally identified and sequenced from the rat brain (Trani *et al.* 2002, Bartolomucci *et al.* 2006), we briefly tested their distribution in the rat brain, compared with VGF C-terminus peptides (Fig. 4A and B). While the latter were widely distributed, TLQP peptides were mostly found in the hypothalamic region, in densely labelled terminal fields in the external layer of the median eminence (Fig. 4B), with the addition of delicately labelled axons in the medial hypothalamus (especially in the arcuate, peri- and para-ventricular regions). While their analysis is beyond the scope of the present investigation, scattered VGF C-terminus-immunoreactive perikarya were seen in the medial hypothalamus and in other brain regions. VGF C-terminus and TLQP peptides appeared to co-distribute and co-localise in portions of the external median eminence (Fig. 4A and B).

Immunohistochemistry and molecular heterogeneity

In the stomach of *ad libitum* fed rats, TLQP peptides were most abundant (162 ± 11 pmol/g, mean \pm S.E.M., $n = 12$). Lower concentrations were found for PGH (24 ± 4.0 pmol/g), HVLL (12 ± 1.2 pmol/g) and VGF C-terminus peptides (7.4 ± 1.0 pmol/g).

Upon Sephadex chromatography (Fig. 5), several forms of TLQP peptides were shown in stomach extracts (Fig. 5A), including: (a) a major wide peak in the ~ 2.5 –6 kDa region and (b) a lower peak at ~ 8 kDa MW, broadly corresponding to a similar one seen for VGF C-terminus and HVLL peptides (Fig. 5B and D), with the addition of low amounts of immunoreactivity in the low MW and void volume fractions. Upon reverse-phase HPLC (Fig. 6, upper panel), fractions

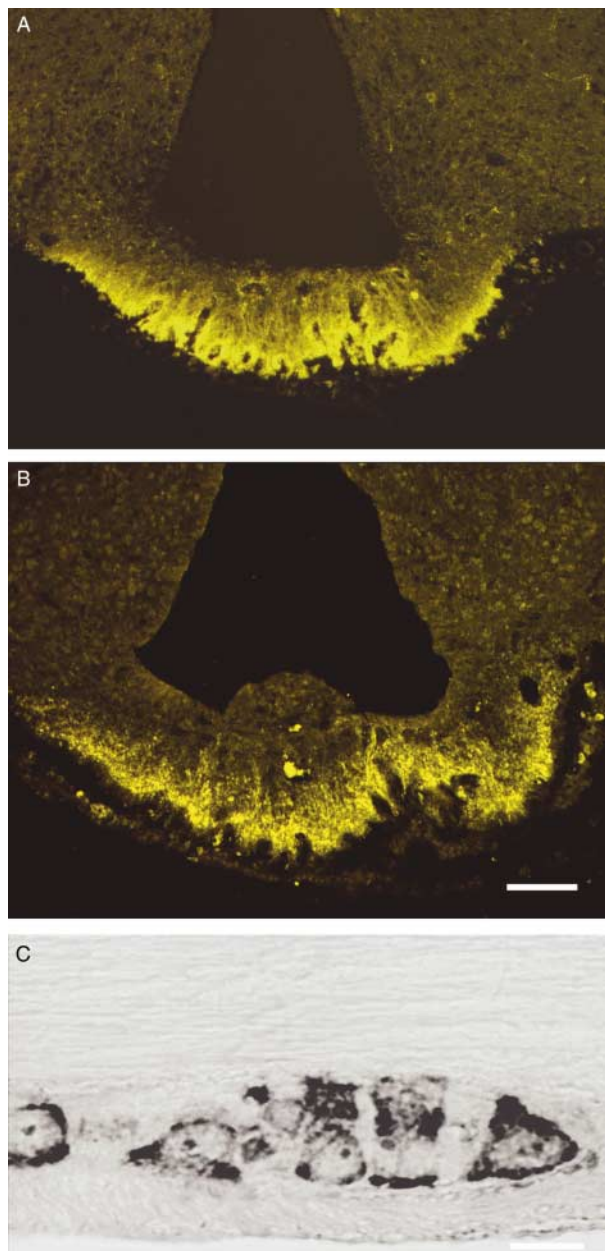


Figure 4 VGF peptides in rat hypothalamus, and VGF mRNA in myenteric neurons. VGF C-terminus peptides were most abundant in the median eminence (A), with the addition of thin axons in various hypothalamic regions, while TLQP peptides were mostly seen in the external layer of the median eminence (B). In the rat stomach, *in situ* hybridisation revealed a proportion of myenteric perikarya labelled for VGF mRNA (C). Scale bar in (A and B) 50 μ m; (C) 20 μ m. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0189>.

related to the (a) peak showed TLQP immunoreactivity at elution positions coinciding with synthetic TLQP-21 and TLQP-30 standards, with the addition of a larger molecular form eluting shortly after the latter. When fractions

corresponding to the (b) peak were similarly loaded, a small amount of reactivity was found at a position eluting earlier than the TLQP-62 standard. The latter fractions showed HVLL peptide, but no detectable VGF C-terminus immunoreactivity in ELISA (~ 0.5 and < 0.1 pmol/HPLC fraction respectively). When hypothalamic extracts were similarly analysed by Sephadex chromatography and HPLC, TLQP peptides co-eluting with the TLQP-21 and TLQP-62 standards were revealed (Fig. 6), with the addition of an unknown form apparently co-eluting with the one seen in the gastric extracts. In ELISA, hypothalamic HPLC fractions co-eluting with the TLQP-62 standard did show VGF C-terminus immunoreactivity (~ 1.13 pmol/fraction), in agreement with the presence of authentic TLQP-62. PGH peptides (Fig. 5C) mostly eluted as low MW forms in Sephadex chromatography, ~ 0.8 – 1.5 kDa in range, with the addition of a broad peak close to the void volume.

For the study of the VGF peptide response to feeding, as mentioned, the entire stomach from each rat was extracted for VGF peptide assays. Clear-cut, selective differences were found for TLQP peptides, which showed more than 50% decrease in fasted animals (Fig. 7; 162 ± 11 vs 74 ± 5.3 pmol/g, fed versus fasted rats, mean \pm s.e.m., $P < 0.00001$). Conversely, none of the other VGF peptide studies showed any significant change (Fig. 7). Immunohistochemistry of fed and fasted animal samples did not reveal distinct changes for either TLQP or other VGF peptides, neither in their immunostaining intensity nor in the abundance of labelled cells. Hence, co-localisation of VGF peptides versus the relevant cell marker/s, or hormone, was addressed quantitatively, and revealed no change in the relevant co-localisation profiles between the fed and fasted groups (Table 4). Comparable molecular profiles were revealed in the two groups in gel chromatography (data not shown).

Data were expressed as mean \pm s.e.m. throughout. Statistical analyses were carried out by one-way ANOVA followed by *t*-test as appropriate (Statistix software, www.statistix.com).

Discussion

On the basis of this study, VGF-derived peptide/s appear to be well represented in the stomach of rat and swine. The comparable distribution we observed in the two species, as shown previously for the pancreas and adrenal glands (Cocco *et al.* 2007, D'Amato *et al.* 2008), fits well with the largely conserved sequence of the VGF precursor from rat to human, including virtually all its internal cleavage sites (Salton *et al.* 2000). The far higher reactivity found in pig for human (compared with rat) VGF C-terminus antibodies suggests that swine VGF, which is yet to be sequenced, may be similar to the human form in its C-terminal region.

Major cleaved VGF products showed a distinct differential distribution in the stomach, TLQP peptides being expressed at high level in ECL and D cells, and PGH peptides in ghrelin

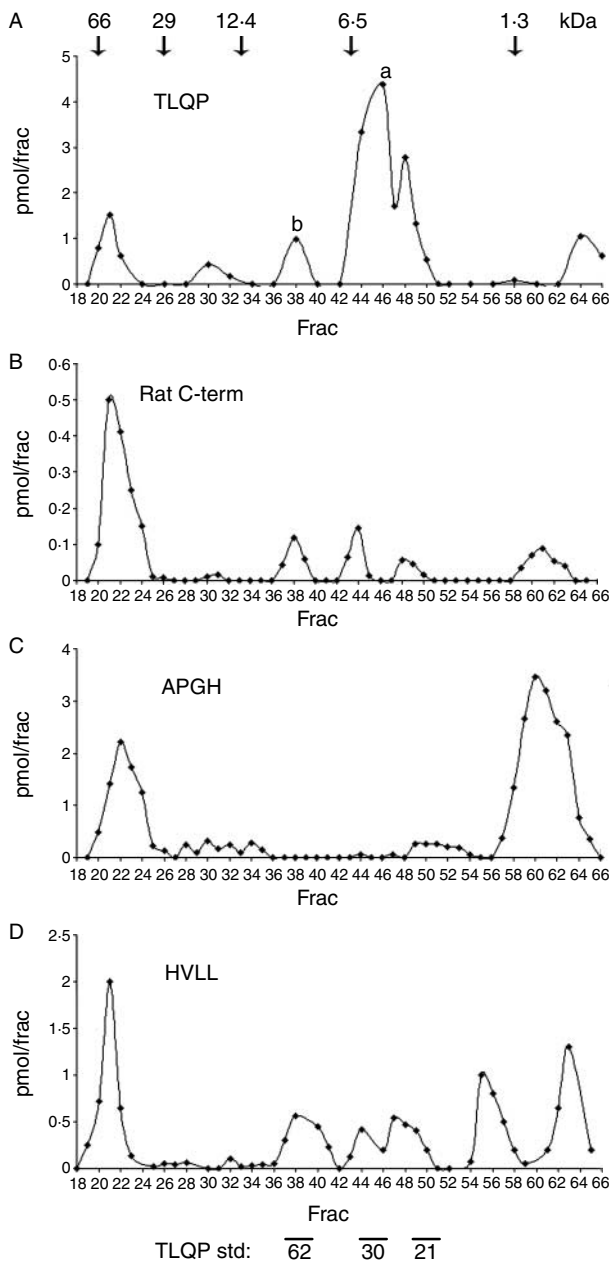


Figure 5 VGF peptides in Sephadex chromatography of gastric extracts. Several forms of TLQP peptides were shown (A), including a major wide peak in the ~ 2.5 – 6 kDa region (a), and a lower peak at ~ 8 kDa MW (b), broadly corresponding to a similar one seen for VGF C-terminus (B) and HVLL peptides (D), with the addition of low amounts of immunoreactivity in the low MW and void volume fractions. PGH peptides (C) mostly eluted as low MW forms in Sephadex chromatography, in an ~ 0.8 – 1.5 kDa range, with the addition of a broad peak close to the void volume. Representative chromatography profiles from the fed rat group. Molecular weight markers are shown in (A), while 'TLQP std' 62, 30 and 21 indicate the elution positions of the respective peptide standards (D).

cells. Conversely, VGF C-terminus-containing peptides were confined to neuronal structures, including intrinsic gut neurons and probably axons of extrinsic origin. Co-localisation of the above VGF peptides with VGF mRNA, and the additional presence of VGF_{443–588} immunoreactivity in certain location/s (e.g. D cells) strongly argue for authentic VGF peptides being revealed. In addition, VGF mRNA is one of the mRNAs expressed in highly purified ECL cell preparations, while absent in the parietal cell fraction (Lambrecht *et al.* 2006).

ECL cells appeared to selectively express TLQP peptides, including authentic TLQP-21, as well as the following additional forms: a) a TLQP-30 peptide, related to cleavage at the rat VGF_{586–587} Arg-Arg sequence; b) an additional form that could be tentatively cleaved at a further Arg-Arg sequence (rat VGF_{597–598}); and c) a longer peptide extending towards the VGF C-terminus, but distinct from TLQP-62. A further point is that TLQP peptides (only) were selectively modulated by changes in the nutritional status, with a clear-cut reduction in tissue TLQP peptide/s content on fasting. ECL cells appeared to be the major cell population involved, with the possible addition of somatostatin cells, which also contained TLQP and some other peptides related to the VGF_{443–588} region. Previous studies using purified preparations of ECL cells showed a significant reduction in VGF mRNA on fasting ($\sim 49\%$ decrease, Lambrecht *et al.* 2007), thus indicating that the changes observed may be at least partly regulated at the transcriptional level. In our investigation, VGF mRNA was expressed in the oxyntic mucosa by numerous chief cells, largely obscuring any change one might observe in the comparatively few ECL and somatostatin cells. In addition, indirect immunofluorescence and the *in situ* hybridisation method used (Moorman *et al.* 2001) were set up aiming for the highest possible sensitivity, rather than to a proportional representation of the quantitative peptide or mRNA content in each cell. Hence, changes in both the techniques of the order mentioned above may result in minor differences in the overall detection signal (fluorochrome-labelled secondary antibody, or alkaline phosphatase reaction product respectively), when the change diffusely affects the cell population/s involved. Furthermore, the consistent pattern of TLQP peptide co-localisation in fed and fasted animals, as confirmed by quantitative analysis, further supports a general involvement of the ECL (and possibly somatostatin) cell populations.

In the fasting rat, ECL cells showed a decrease in both their L-histidine substrate uptake transporter SN2 and their VMAT-2 responsible for histamine uptake into the secretory vesicles (Dimaline *et al.* 1993b, Dimaline & Struthers 1996, Lambrecht *et al.* 2007). The reduction in histamine biosynthesis required under fasting conditions would thus be achieved by reducing substrate availability, as well as the histamine secretory pool, rather than inhibiting the biosynthetic enzyme histidine decarboxylase (Lambrecht *et al.* 2007). The localisation of TLQP peptides in both ECL and D cells is also intriguing, in view of their inhibitory

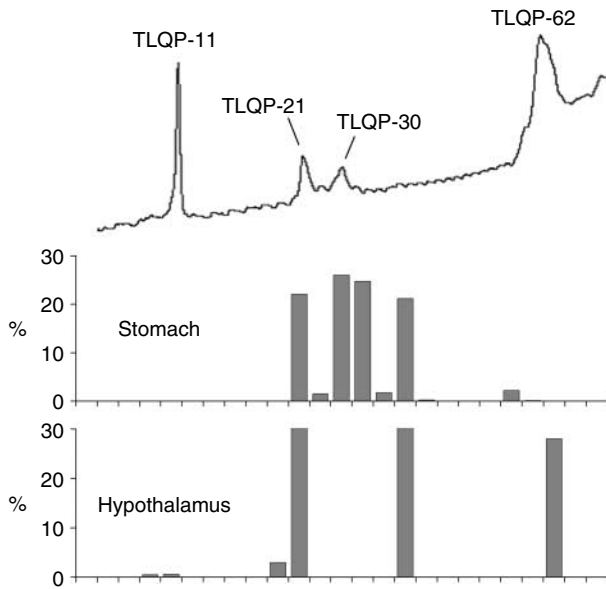


Figure 6 TLQP peptides in HPLC chromatography of gastric and hypothalamic extracts. In the extracts of rat stomach (middle panel), distinct peaks co-eluting with synthetic TLQP-21 and TLQP-30 were revealed, together with a possibly larger molecular form eluting shortly after the latter. A small amount of reactivity was found at an elution position earlier than the TLQP-62 standard. Conversely, in hypothalamic extracts, TLQP peptides co-eluting with the TLQP-21 and TLQP-62 standards were revealed (bottom panel), together with an unknown form apparently co-eluting with the one seen in gastric extracts. Representative HPLC chromatography from the fed rat group. Bars represent percentages of total TLQP immunoreactivity measured in all fractions from each chromatography.

connection in the fundic stomach, and the resulting effects upon ECL cell histamine and gastric acid secretion (Dimaline *et al.* 1993a,b, Lambrecht *et al.* 2006). On the whole, TLQP peptides could have a role in the regulatory machinery of the ECL cells or, upon release from ECL and/or somatostatin cells, could act locally in a paracrine manner or via neuro-endocrine pathways such as those involving the vagus nerve (Dockray 2009). So far, peripheral administration of TLQP-21 proved ineffective (Jethwa *et al.* 2007, Severini *et al.* 2009). In the brain, TLQP-62 and TLQP-21 were suggested to have an agonist–antagonist action upon hippocampal synaptic potentiation (Bozdagi *et al.* 2007), while the bioactivity of the additional forms we found remains to be studied.

Though unchanged in fasting/feeding conditions, the localisation of novel VGF-derived products, namely PGH peptides in ghrelin cells, warrants the precise identification of the molecular species involved. Of these, the high MW form we observed close to the void volume might correspond to a product cleaved at the rat VGF_{431–433} putative cleavage site (Arg-Arg-Lys, Salton *et al.* 2000) and encompassing an extensive domain towards the VGF N-terminus. In the adrenal gland, PGH peptides were also present in a

comparable profile of molecular forms; however, PGH immunoreactivity was broadly localised in the whole medulla, while TLQP peptides were confined to adrenalin cells (D'Amato *et al.* 2008). The same or similar peptides were also found in human, in ECL tumour cells (Rindi *et al.* 2007).

The localisation of VGF mRNA and the related peptide/s immunoreactivity in further gastric endocrine and mucosal cells is worth noting. The TLQP immunoreactivity found in a fraction of gastrin and serotonin cells, in both rat and swine, may indicate a low expression of the relevant peptide/s. Extended molecular forms, of low reactivity with the antibodies we used, could also be involved, as shown for certain endocrine cell types in sheep pituitary (Brancia *et al.* 2005). Most human G- and EC-cell tumours, be it of gastric, intestinal or pancreatic origin, appeared to display both C-terminal and PGH peptide immunoreactivity to an extent comparable to ECL tumours, while TLQP peptides were not investigated (Rindi *et al.* 2007). As for the chief cells, the intense mRNA signal, consistent immunostaining for HVLL peptides, and the occasional labelling with TLQP antibodies all converge to indicate local synthesis of the VGF precursor, at least in rat. Whether further cleavage products or the HVLL peptide/s themselves are part of the functional machinery of such cell type remains to be determined. Interestingly, gastric chief cells have been shown to produce and release peptide hormones including gastric leptin (Bado *et al.* 1998) and apelin (Susaki *et al.* 2005). On the other hand, the localisation of VGF peptides in the gastric chief cells suggests that they might be secreted into the gastric lumen from where they would travel and act onto different locations, as seen for leptin and apelin released into the gastric lumen and stimulating

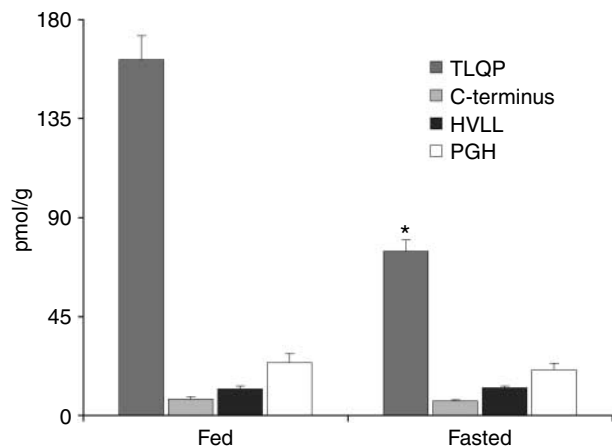


Figure 7 VGF peptide immunoreactivity in the whole stomach, the fed versus fasted rat groups. TLQP peptides were most abundant, followed by PGH peptides, hence the others studied. A clear-cut, selective decrease in TLQP peptides (only) was revealed in fasted rats compared with fed ones, while all other VGF peptides studied were unchanged. The whole stomach was extracted for the assay from each animal. Data are expressed as mean \pm S.E.M., $n = 12$ male rats/group. * $P < 0.00001$.

Table 4 VGF peptides in gastric endocrine cells: co-localisation profiles in fed versus fasted rats. Upper panel: the majority of enterochromaffin-like cells (ECL), D and X/A-type endocrine cells showed the relevant VGF peptide immunoreactivity in both fed and fasted animals, with no significant change in co-localisation profiles. Lower panel: TLQP peptide/s-containing cells were largely identified as ECL cells, with the addition of a lower number of D cells, and a small fraction of further uncharacterised cells. No significant shift in the contribution of either cell type to the overall TLQP-containing population was revealed between fed and fasted rats. Data are expressed as mean \pm S.E.M., $n=4$ rats/group. Endocrine cell types were characterised on the basis of their relevant markers, or hormone (see section Materials and Methods)

VGF peptide immunostained		Percentage of immunostained cells		P
		Fed group (%)	Fasted group (%)	
Endocrine cell type				
ECL	TLQP	97.9 \pm 0.9	94.0 \pm 4.8	0.4
D	TLQP	98.0 \pm 0.8	98.6 \pm 1.4	0.3
D	VGF ₄₄₃₋₅₈₈	98.0 \pm 1.9	93.4 \pm 3.9	0.3
X/A like	PGH	98.7 \pm 1.6	97.8 \pm 2.3	0.4
Percentage of TLQP immunostained cells				
Endocrine cell type		Fed group	Fasted group	P
VGF peptide immunostained				
TLQP	ECL	90.3 \pm 1.2	92.9 \pm 1.9	0.6
	D	6.3 \pm 0.9	6.5 \pm 1.7	0.6
	Not characterised	3.4 \pm 1.4	0.6 \pm 0.3	0.1

CCK secretion in the proximal small intestine (Sobhani *et al.* 2002, Susaki *et al.* 2005).

While the investigation of nerve pathways containing VGF peptides is beyond the scope of this paper, the selective localisation of VGF C-terminus peptides in myenteric neurons, and in mucosal nerves probably including at least some of extrinsic origin, should not be overlooked. In fact, VGF mRNA was rapidly induced in both sensory and efferent vagal neurons upon cysteamine ulceration of the duodenum (Kanemasa *et al.* 1995a,b), suggesting an involvement of yet-to-be-determined VGF peptides in the gastric response to injury or other stimuli, and possibly in mucosal defence mechanisms. The only VGF peptide studied so far, TLQP-21, induced gastric longitudinal muscle contraction *in vivo*, while inhibiting gastric emptying when given *i.c.v.* (Severini *et al.* 2009).

The heterogeneity of VGF peptides implies complex post-translational processing mechanisms. The action of prohormone convertases PC1/3 and PC2 on the VGF precursor has been studied, and showed that PC1/3 is responsible for cleavage at the N-terminus of TLQP and other peptides (Trani *et al.* 2002), in keeping with the accumulation of the C-terminal VGF-derived peptide V in mice lacking PC1/3 (Pan *et al.* 2005). Conversely, the enzyme/s affecting C-terminal cleavage of TLQP-21, as well as others involved in the production of multiple VGF peptides revealed in the human cerebro-spinal fluid (Selle *et al.* 2005) remain to be clarified. A parallel is often drawn between VGF and the chromogranin-secretogranin family of proteins, because of their selective localisation in secretory granules, as well as their role as precursors of multiple bioactive peptides (Helle 2004). Chromogranins, too, are differentially processed in gastric endocrine cells (Watkinson *et al.* 1991, Watkinson & Dockray 1992,

Portela-Gomes & Stridsberg 2002), although to a profile different from the one we observed for VGF peptides. In fact, G cells showed immunoreactivity to virtually all region-specific antibodies to chromogranin A (Portela-Gomes & Stridsberg 2002), as opposed to the limited reactivity of VGF peptides we could demonstrate in such cell type.

In conclusion, diverse VGF peptides appear to be selectively expressed in different gastric endocrine and other mucosal cells. Of these, TLQP peptides rapidly respond to feeding conditions. Their further investigation is warranted, with respect to their possible involvement in mechanisms regulating the response to feeding and starvation, as well as the neuro-endocrine integratory pathways connecting the gut and the brain.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was partly supported by research grants from MIUR FIRB (RBNE013XSJ_002 and RBNE01JKLF_002 to G L F) and Ministry of Health, Italy. C B and B N were supported by an RAS grant (Regione Autonoma Sardegna, PO FSE 2007-2013, L.R. 7/2007 for promotion of Scientific Research and Technological Innovation in Sardinia).

Author contribution statement

F D, B N and C B carried out the ELISA assays; F S and A A performed the HPLC; C B and C C performed the immunohistochemistry; C B and R P performed the *in situ* hybridisation; G L F raised the VGF antibodies and supervised the study; and C B, C C and G L F wrote the paper.

Acknowledgements

M Collu, B Tuveri and M Tuveri, as well as M C Mostallino at the Institute of Neuroscience, National Research Council (CNR), Cagliari are acknowledged for rat housing; G Boi, F Incollu and the COALBE Company for swine tissues; P Nicolussi for animal housing for antibody production.

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Received in final form 22 September 2010

Accepted 27 September 2010

Made available online as an Accepted Preprint
27 September 2010