Expression of VGF mRNA in developing neuroendocrine and endocrine tissues

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Abstract

Analysis of knockout mice suggests that the neurotrophin-inducible secreted polypeptide VGF (non-acronymic) plays an important role in the regulation of energy balance. VGF is synthesized by neurons in the central and peripheral nervous systems (CNS, PNS), as well as in the adult pituitary, adrenal medulla, endocrine cells of the stomach and pancreatic beta cells. Thus VGF, like cholecystokinin, leptin, ghrelin and other peptide hormones that have been shown to regulate feeding and energy expenditure, is synthesized in both the gut and the brain. Although detailed developmental studies of VGF localization in the

CNS and PNS have been completed, little is known about the ontogeny of VGF expression in endocrine and neuroendocrine tissues. Here, we report that VGF mRNA is detectable as early as embryonic day 15.5 in the developing rat gastrointestinal and esophageal lumen, pancreas, adrenal, and pituitary, and we further demonstrate that VGF mRNA is synthesized in the gravid rat uterus, together supporting possible functional roles for this polypeptide outside the nervous system and in the enteric plexus.

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Introduction

VGF was originally identified as a rapidly regulated gene product in nerve growth factor (NGF)-treated PC12 cells (Levi *et al.* 1985, Cho *et al.* 1989, Salton *et al.* 1991). Of particular interest, the VGF polypeptide is highly conserved among several mammalian species, and is stored in large dense core vesicles where it is differentially processed and released from the regulated secretory pathway of both neuronal and endocrine cells (Possenti *et al.* 1989, 1999, Benson & Salton 1996). Recent studies of VGF-deficient mice that were generated by gene targeting indicate that VGF plays a crucial role in the control over energy balance (Hahm *et al.* 1999, 2002). VGF mutant mice are lean, hypermetabolic and hyperactive, and analysis of hypothalamic gene expression indicates that these mice have elevated levels of neuropeptide Y (NPY) and agouti-related peptide mRNAs, encoding two powerful orexigenic neuropeptides, and decreased pro-opiomelanocortin mRNA that encodes the satiety-inducing peptide alpha-melanocyte stimulating hormone. Consistent with this phenotype, VGF mRNA levels are regulated in the

hypothalamic arcuate nucleus in response to fasting (Hahm *et al.* 1999, 2002), and in the dorsal motor nucleus of the vagus and nucleus tractus solitarius in response to gastrointestinal manipulation (Kanemasa *et al.* 1995a,b, Hahm *et al.* 1999, 2002). Since the abnormalities noted in VGF mutant mice extend to low circulating insulin and glucose levels, and VGF is stored and secreted from pancreatic beta cells, it remains possible that central nervous system-, peripheral nervous system- and/or peripherally-synthesized VGF peptides may each function to control energy homeostasis. Recent studies of VGF mutant mice further suggest a role for VGF in the regulation of energy storage in peripheral metabolic tissues that are innervated by the autonomic nervous system. Little, however, is known about VGF expression in extra-neuronal sites in the developing rodent. We therefore examined the ontogeny and distribution of VGF mRNA in non-neural tissues during rat development. We report that VGF is expressed as early as embryonic day 15.5 in several neuroendocrine and endocrine tissues, consistent with a possible functional role for this secreted polypeptide outside the nervous system.

Materials and methods

Postnatal or timed-pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA; Zivic-Miller Laboratories, Allison Park, PA, USA) were killed by CO₂ narcosis, and rapidly dissected on ice. The day following the evening of conception was considered embryonic day 0 (E0), and the day of birth postnatal day 0 (P0). Brains, heads, or whole embryos were then embedded in OCT compound (Miles, Elkhart, IN, USA), frozen in 2-methyl-butane on dry ice powder, and stored at -20 °C before sectioning. Frozen sections (10 µm) were cut on a Bright-Hacker cryostat (Huntingdon, Cambs, UK), thaw-mounted onto Superfrost/Plus slides (Fisher Scientific, Fairlawn, NJ, USA), and stored at -20 °C or -80 °C.

Preparation of cRNA probes and *in situ* hybridization

The VGF cRNA and sense strand control probes were transcribed using ³⁵S-labeled UTP (New England Nuclear, Boston, MA, USA), Riboprobe System II transcription buffers (Promega, Madison, WI, USA), either T3 or T7 RNA polymerase (Stratagene, La Jolla, CA, USA) and a linearized plasmid construct that was previously described and contains a 304 base pair cDNA fragment corresponding to bases 1592–1896 of the 3' end of the VGF coding region (Salton *et al.* 1991). Labeled cRNA probes were purified by Sephadex G100 (Pharmacia, Piscataway, NJ, USA) column chromatography, and stored at -80 °C in 100 mM dithiothreitol until use.

In situ hybridization was carried out essentially as previously described by Snyder *et al.* (1998). Briefly, slides were fixed in RNase-free 4% paraformaldehyde, 50 mM NaCl, 100 mM sodium phosphate (pH 7.2) fixative for 2–15 min, rinsed and dehydrated through a graded ethanol series, then rehydrated and incubated in a 0.05 M triethanolamine, 0.026 M acetic acid solution for 2 min. Slides were then acetylated for 10 min, rinsed, and dehydrated. Sections were prehybridized for 1–2 h, hybridized overnight at 50 °C, washed for 30 min twice, treated with ribonuclease A, then washed several times to a final stringency of 0.2 × SSC at 65–70 °C for 2 h, as previously detailed (Snyder *et al.* 1998). In each experiment, the appropriate VGF sense-strand cRNA probe was hybridized to adjacent sections in parallel with antisense probe. After dehydration through a graded ethanol series containing 300 mM ammonium acetate, slides were air dried and exposed to Hyperfilm-βmax film (Amersham, Arlington Heights, IL, USA) alongside [¹⁴C]micro-scales (Amersham) for 2 days to 2 weeks. Films were developed and slides were then dipped in a 1:1 mixture of Kodak NTB2 emulsion and HPLC-grade dH₂O at 42 °C, exposed for 1–4 weeks, then developed. Slides were counterstained with cresyl violet (Eastman Kodak, Rochester, NY, USA), dehydrated, cleared in two

changes of Polyclear (D-Limonene; Florida Chemical, Winter Haven, FL, USA), and coverslipped with DPX mountant (Fluka Chemicals, Ronkonkoma, NY, USA).

Image preparation

Emulsion-dipped sections were visualized on a Stemi SV8 dissecting microscope (Zeiss, Germany) and a Microphot FX microscope (Nikon, Japan), and bright field and dark field photomicrographs were taken using Kodak Ektachrome 160T slide film. Ektachrome slides and film autoradiographs were scanned into a Power Macintosh computer (Apple, Cupertino, CA, USA) using an ARCUS II flatbed scanner and Fotolook software (Agfa, Wilmington, MA, USA). Composite figures were made using Photoshop (Adobe Systems, Mountain View, CA, USA) on a Power Macintosh computer with minimal adjustments to brightness, contrast, and sharpness.

Results

To characterize the ontogenic expression pattern of the *vgf* gene, the regional and cellular localization of VGF mRNA was studied using *in situ* hybridization in rats from embryonic day 11.5 (E11.5) to postnatal day 10 (P10). Specific hybridization was first observed at midgestation in cranial, sympathetic and sensory ganglia (Snyder *et al.* 1998), after which the distribution broadened throughout the nervous system. Expression in non-neural tissues was first noted at E11.5, not in the embryo proper but rather in the gravid uterus where strong VGF mRNA hybridization in a subpopulation of cells in the uterine wall was noted. At this age, hybridization was also seen between the trophoblast and the maternal decidua (Fig. 1A and B), while at E13.5, VGF mRNA was expressed in a monolayer of cells (Fig. 1C and D), a pattern that might be expected for intramural postganglionic parasympathetic neurons.

Gastrointestinal, pancreatic and adrenal expression of VGF

Within the embryo, VGF mRNA was initially detected outside peripheral and central nervous tissues at E15.5, in the primordial myenteric plexus of the stomach (Fig. 2A and B). By E17.5, VGF mRNA expression in the developing stomach and surrounding bowel was more robust (Fig. 2C). A parasagittal section of the E19.5 embryo demonstrated a strong VGF hybridization signal in the esophageal mucosa and developing pancreas, and discrete labeling of the developing myenteric plexus and enteric ganglia in both the esophagus and stomach (Fig. 2D and E). In the developing stomach, VGF hybridization in cells of the non-glandular (non-GI) mucosa was detected (Fig. 2F). In addition, VGF mRNA expression in the adrenal gland was also noted as early as E15.5 (Fig. 2G and H). Previous reports demonstrated relatively early expression

Figure 1 VGF mRNA expression in the gravid rat uterus at E11.5 (A and B) and E13.5 (C and D). Note strong VGF hybridization in a subpopulation of cells in the uterine wall (arrows, A and B) at E11.5, and the development of a monolayer of labeled cells (arrowheads, C and D) in this region between the trophoblast layer and the maternal component of the decidual reaction at E13.5. Scale bars: A, C and D, 1 mm; B, 80 μ m.

of VGF mRNA in developing sympathetic and sensory neurons that innervate some of these peripheral targets (Snyder *et al.* 1998). In the current study, we further noted that cholinergic ganglia that are located in the submandibular gland, adjacent to ducts (I_1) and acini (I_2) were also strongly labeled with VGF probes at E19.5 (Fig. 2I).

Pituitary expression of VGF

The ontogeny of VGF mRNA expression in the pituitary from E15.5 to birth is illustrated in Fig. 3. VGF mRNA was first seen in the developing pars tuberalis (A and A'). By E17.5 (B and B'), scattered cells in the pars distalis also displayed strong VGF labeling. This pattern was maintained at E19.5 (C and C'). By P0 (D and D'), however, expression levels in the pars tuberalis had declined some-

what and a low level of labeling was now detectable in the pars intermedia. Coronal sections through the P1 and adult pituitary revealed continued expression of VGF mRNA in the anterior pituitary, and low levels in the intermediate lobe (Fig. 4). No VGF-specific labeling was seen in the pars nervosa at any stage of development studied (Figs 3 and 4).

Discussion

VGF is expressed in a number of endocrine and neuro-endocrine tissues, and is first detectable in the pituitary, adrenal and developing myenteric plexus at E15.5. Later in embryogenesis, expression expands to the pancreas and mucosal lining of the esophagus, stomach and intestine. Consistent with a possible functional role in the gastrointestinal (GI) tract, VGF expression in cholinergic ganglia

Figure 2 VGF mRNA expression in the developing GI tract and adrenal of the E15.5, E17.5 and E19.5 rat embryo. In A and B, emulsion autoradiographs of the developing E15.5 stomach (St) and surrounding regions are shown in bright and dark field. Arrowheads highlight VGF mRNA hybridization in the developing myenteric plexus. In C, dark field emulsion autoradiography of the developing stomach and surrounding bowel in an E17.5 embryo is shown. In D and E, abdominal organs of an E19.5 embryo are shown in bright and dark field. Note intense hybridization over cells of the esophageal (Es) lining and the pancreas (Pa). In F, mucosal hybridization in the non-glandular (non-GI) portion of the stomach of an E19.5 embryo is shown. Arrowheads (E-F) indicate the labeled enteric ganglia. In G and H, the developing kidney (K) and adrenal gland (Ad) and surrounding region are shown in bright and dark field at E15.5. In I, labeling of parasympathetic ganglia adjacent to the submaxillary gland (SubM) duct (boxed region in I corresponds to dark field autoradiograph in I₁) is shown. Longer exposure of ganglia adjacent to the SubM acini of another E19.5 embryo is shown in I₂. L, liver; Sml, small intestine. Scale bars: B, 200 µm; C, 250 µm; E and F, 500 µm; H, 200 µm; I, 200 µm; I₂ 50 µm.

Figure 3 Ontogeny of VGF mRNA expression in parasagittal sections through the rat pituitary gland and surrounding tissue is shown in bright and dark field. (A and A') E15.5; (B and B') E17.5; (C and C') E19.5; (D and D') P0. H, hypothalamus; InR, infundibular recess; pd, pars distalis; pi, pars intermedia; pn, pars nervosa; pt, pars tuberalis; Pon, pontine nucleus; Rp, Rathke's pouch; 3 V, third ventricle. Scale bars, 250 μ m.

Figure 4 Postnatal VGF expression in coronal sections through the isolated rat pituitary gland. Bright field (A, B) and dark field (C, D) images of adult (A, C) and P1 (B, D) pituitary glands are shown. PL, posterior lobe; IL, intermediate lobe; AL, anterior lobe.

was also noted. Sites of VGF synthesis in both the myenteric plexus of the gut and possibly the intramural postganglionic parasympathetic neurons in the uterus would be consistent with a proposed role for VGF and/or VGF-derived peptides in smooth muscle contraction (A Levi, personal communication). The pattern of expression of VGF mRNA in the decidualized endometrium of the uterus at E11.5 was similar to that previously noted for peptidylglycine α -amidating monooxygenase mRNA (Zhang *et al.* 1997), encoding an enzyme involved in the activation of peptides by amidation. The VGF polypeptide notably has greater than 10 paired basic amino acid clusters, several of which are cleaved to generate VGF-derived peptides, one of which is predicted to be amidated (Salton *et al.* 2000, Trani *et al.* 2002). Other studies of the adult rat uterus have localized secretoneurin, the 33 amino acid peptide that is cleaved from secretogranin II, in sensory fibers and neurons that are apposed to postganglionic parasympathetic neurons (Collins *et al.* 2000). Secretogranin II, like VGF and other members of the chromogranin/secretogranin family, is also found in and secreted from large dense core vesicles of neuronal, neuroendocrine and endocrine cells. Thus VGF is expressed in the uterus during pregnancy; it is also of interest to note that female homozygous VGF knockout mice are infertile, although it is unlikely that this is due solely to uterine and/or placental dysfunction (Hahm *et al.* 1999).

In the developing gut, a similar pattern of distribution to that of VGF in the developing myenteric plexus has been noted for the serotonin 5-HT_{2B} receptor (Fiorica-Howells *et al.* 2000) and for cholecystokinin (CCK) (Lay *et al.* 1999). Previous studies demonstrated that VGF mRNA was first detected in migrating neural crest cells and sympathetic and sensory ganglia (Snyder *et al.* 1998), consistent with our localization of VGF in the enteric innervation of the GI tract since the enteric nervous system is derived from neural crest cells that migrate into the gut. Relatively robust expression of VGF mRNA in the developing mucosal lining of the gut was also noted in

the current study. In the adult, several gastric endocrine cell types appear to contain VGF peptides (Ferri *et al.* 1992; G-L Ferri, personal communication), reminiscent of the distribution of the hormone leptin, a key peripheral regulator of energy balance. Thus, the distribution of VGF in peripheral tissues such as the developing stomach that we noted in this study, as well as in the brain and adult GI tract, is similar to a number of peptides that have been implicated in the control of feeding behavior, facilitation of nutrient uptake and regulation of gastric contractility, including ghrelin and CCK. Of interest, VGF is induced in NPY-containing neurons in the medial arcuate nucleus of the hypothalamus in response to fasting, and, furthermore, VGF mutant mice are lean, hypermetabolic and hyperactive (Hahm *et al.* 1999). This suggests that VGF may function to decrease metabolic rate, the normal response to starvation, and perhaps to centrally regulate food intake, as do a number of other similarly localized peptide hormones.

In the pituitary, the onset of VGF expression in the pars tuberalis seemed to occur simultaneously throughout, just at the time these cells are differentiating. By E15.5, the cells of the developing pars tuberalis have been shown to have become non-mitotic and to possess a differentiated secretory morphology, making this the earliest differentiating lobe of the adenohypophysis (Stoeckel *et al.* 1973, 1993). At this time, expression of pituitary cell-type terminal differentiation markers is first noted (reviewed in Dasen & Rosenfeld 2001). Throughout both the non-mitotic differentiation and proliferative periods, intense *in situ* hybridization for the glycoprotein hormone α -subunit has been described in the pars tuberalis and pars distalis, in a nearly identical pattern to that seen for VGF hybridization except that the α -subunit was shown to be expressed in Rathke's pouch as early as E12 (Stoeckel *et al.* 1993). Immunoreactivity for the adrenal, gonadal and thyroid stimulating hormones of the pars distalis has also been described by E15 in pars tuberalis, one day earlier than similar expression in the pars distalis (Nemeskeri *et al.*

Pitx-1 binding elements

(G/A)GATTA(G/T/C)

Putative Pitx-1 binding elements

Human VGF

-666 to -660

GGATTAC

-2302 to -2296

TGATTAA

Rat *vgf*

-658 to -652

TGATTAC

-836 to -829

GGATTAA

GATA binding elements

(A/T)GATA(A/G)

Putative GATA binding elements

Human VGF

-1061 to -1056

AGATAC

-1027 to -1022

GTATCT

-2237 to -2232

CTATCT

Rat *vgf*

-1061 to -1056

AGATAC

-801 to -796

TTATCA

to

Steroidogenic Factor-1 (SF-1) binding elements

TGACCTTG(T/G)

CAGCCTTGG mouse 21-hydroxylase (-141 to -133)

TGGCCATGT human LH β (-129 to -121)

Putative Steroidogenic Factor-1 (Sf-1) binding element

Human VGF

-1454 to -1446

TGACCTTGG

Steroidogenic Factor-1 (Sf-1)-like binding elements conserved in rat and human *vgf*

Human VGF

-91 to -83

TCCCCATGA

-545 to -537

CGTCCTTGG

Rat *vgf*

-92 to -84

TCCCCATGA

-557 to -549

CGTCCTTGG

Figure 5 Elements in the rat and human *vgf* promoters that are similar to Pitx-1, GATA-2 and SF-1 binding sites. Putative *vgf* binding sites from the rat (Salton *et al.* 1991; chromosome 12 WGS supercontig Rn12_330) and human (Canu *et al.* 1997) sequences are compared with consensus elements for Pitx-1 (Melamed *et al.* 2002), GATA-2 (Steger *et al.* 1994), and SF-1 (Halvorson *et al.* 1996, Catalano *et al.* 2003).

1988, Japon *et al.* 1994). In addition, the melatonin receptor, MT1, has been shown to be expressed in the pars tuberalis by E14 (Johnston *et al.* 2003) as has the transcription factor, pituitary homeobox-1 (Pitx-1), which stimulates the promoter activity of many pituitary-specific genes (Lamonerie *et al.* 1996, Szeto *et al.* 1996, Tremblay *et al.* 1998, Lanctot *et al.* 1999). Pitx-1 expression is highest in the pars tuberalis and pars distalis of ventral origin, co-localizing with luteinizing hormone beta (LH- β) expression and with the distribution of VGF described here. As a consequence of these striking similarities in distribution, we re-examined the human and rat *vgf* promoter sequences for putative Pitx-1 binding sites. Two candidate sites resembling the consensus element in each

promoter were noted (see Fig. 5). Another lineage-specifying transcription factor that regulates gonadotrope development and function, Egr-1/NGFI-A, has been previously noted to bind the rat *vgf* promoter (D'Arcangelo *et al.* 1996) and to induce both LH- β (Tremblay & Drouin 1999) and VGF transcription (D'Arcangelo *et al.* 1996). Finally, consensus binding elements for factors that are involved in the transactivation of gonadotrope-associated genes, including GATA binding factor 2 and steroidogenic factor-1 (SF-1), were also found in the *vgf* promoter (see Fig. 5). This suggests the possibility that some of these proteins could form functional complexes with one another (see Dasen & Rosenfeld 2001), or with additional transcription factors including CREB that

are critical for VGF regulation in neurons (see Salton *et al.* 2000). Lastly, the primate pars tuberalis and other lobes of the adenohypophysis express neurotrophin and p75^{NTR} immunoreactivity during development and in the adult (Borson *et al.* 1994), providing a possible link between VGF expression and neurotrophin activity in the maturing pituitary.

From E17.5 onward, scattered cells in the pars distalis also displayed strong VGF mRNA expression, and at P0, hybridization could be seen in the pars intermedia. In postnatal and adult coronal sections, VGF mRNA expression was seen scattered throughout the anterior pituitary, while no signal was detected in the posterior pituitary. Immunohistochemical studies have found VGF immunostaining in occasional anterior pituitary lactotrope in adult males, and in gonadotropes and a few lactotropes in adult females, the levels of which vary with the estrous cycle (Ferri *et al.* 1995). In addition to anterior pituitary endocrine cells, VGF is also found in parvocellular and magnocellular hypothalamic neurons projecting to the median eminence and to the posterior pituitary respectively (Liu *et al.* 1994, Ferri *et al.* 1995, Hahm *et al.* 2002). Our studies further confirm that the C-terminal VGF peptide isolated from the posterior pituitary (Liu *et al.* 1994) is unlikely to have been synthesized in the posterior pituitary since no VGF mRNA was detected, but rather is most likely transported from the hypothalamus.

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