**FUNCTIONAL ANALYSIS OF ZEBRAFISH GDNF**

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We have identified zebrafish orthologues of glial cell line-derived neurotrophic factor (GDNF) and the ligand-binding component of its receptor GFRα1. We examined the mRNA expression pattern of these genes in the developing spinal cord primary motor neurons (PMN), kidney, and enteric nervous systems (ENS) and have identified areas of correlated expression of the ligand and the receptor that suggest functional significance. Many aspects of zebrafish GDNF expression appear conserved with those reported in mouse, rat, and avian systems. In the zebrafish PMN, GFRα1 is only expressed in the CαP motor neuron while GDNF is expressed in the ventral somitic muscle that it innervates. To test the functional significance of this correlated expression pattern, we ectopically overexpressed GDNF in somitic muscle during the period of motor axon outgrowth and found specific perturbations in the pattern of CαP axon growth. We also depleted GDNF protein in zebrafish embryos using morpholino antisense oligos and found that GDNF protein is critical for the development of the zebrafish ENS but appears dispensable for the development of the kidney and PMN.

**Key Words:** zebrafish; GDNF; GFRα1; c-ret; kidney; ENS; primary motor neuron; morpholino.

**INTRODUCTION**

Glial cell line-derived neurotrophic factor (GDNF) has multiple roles during development. GDNF was originally identified as a potent survival factor for midbrain dopaminergic neurons in vitro (Lin et al., 1993) and subsequently has been shown to support the survival of several neuronal populations including motor neurons (Henderson et al., 1994), central noradrenergic neurons ( Arenas et al., 1995), cerebellar Purkinje neurons ( Mount et al., 1995), and autonomic neurons in peripheral ganglia (Ebalddal et al., 1995; Trupp et al., 1995). GDNF is the founding member of a family of structurally related molecules, of which there are currently four members: GDNF, neurturin (Koczbaure et al., 1996), persephin (Milbrandt et al., 1998), and artemin (Balo, et al., 1998b). Together, these factors form a distinct subgroup of the TGF-β superfamily.

The receptor for GDNF is composed of a multicomponent complex consisting of a GPI-linked, ligand binding component and a transmembrane signaling component, the tyrosine kinase c-ret (RET) (Jing et al., 1996; Treanor et al., 1996). Receptor specificity is provided by a set of different ligand binding subunits, designated GDNF family receptor alpha (GFRα). Currently there are four identified GFRα subunits that each preferentially binds to a different GDNF family member (Balo, et al., 1998a,b, 1997; Buj-Bello et al., 1997; Jing et al., 1996, 1997; Klein et al., 1997; Masure et al., 2000, 1998; Navelhan et al., 1998; Thompson et al., 1998; Trupp et al., 1998; Worby et al., 1998). GDNF preferentially binds to the GFRα1 subunit (Jing et al., 1996; Treanor et al., 1996), but can also bind and signal through GFRα2 (Balo et al., 1997; Creeden et al., 1997; Sanicola et al., 1997). Although RET acts as common receptor signaling component for all GFRα subunits, there is some evidence, from detailed comparative mRNA expression pattern studies (Golden et al., 1998, 1999; Homma et al., 2000; Nosrat et al., 1997; Trupp et al., 1997; Yu et al., 1998) and in vitro biochemical studies (Trupp et al., 1999), that the GFRα subunits may also signal through RET-independent pathways.

Although GDNF was originally identified as a neurotrophic factor, the embryonic expression pattern of GDNF and its receptors suggest multiple roles in regulating proliferation, differentiation, and inductive interactions during early development (Golden et al., 1999; Helmich et al., 1996; Homma et al., 2000; Luukko et al., 1997; Nosrat et al., 1997, 1996; Pachnis et al., 1993; Schuchardt et al., 1995; Suvanto et al., 1996; Wright and Snider, 1996). Functional studies support some of these additional functions of GDNF. Mice that have targeted deletions of either GDNF...
spinal cord motor neurons (Cacalano et al., 1998; Enomoto et al., 1998) or RET (Schuchardt et al., 1994) genes lack enteric neurons and metanephric kidneys. GDNF promotes in vitro the survival, proliferation, and differentiation of enteric neural crest precursors isolated from avian and rat embryonic gut (Chalazonitis et al., 1998; Hearn et al., 1998; Heuckereth et al., 1998). GDNF can also induce outgrowth and branching of the ureteric bud in culture (Pepicelli et al., 1997; Sainio et al., 1997). In addition GDNF appears to play an important role in the development of motor neurons. Mice that have had GDNF or GFRα1 genes deleted lack distinct groups of spinal cord motor neurons (Cacalano et al., 1998; Enomoto et al., 1998; Garces et al., 2000; M ore et al., 1996; Oppenheim et al., 2000; Pichel et al., 1996; Sanchez et al., 1996) and mice that overexpress GDNF in muscle develop hyperinnervation of neuromuscular junctions (Nguyen et al., 1998). We have isolated the zebrafish orthologues of GDNF and GFRα1, in order to further investigate GDNF's in vivo function. We find many aspects of zebrafish GDNF expression and function conserved with other vertebrates. However, while GDNF is critical for the development of the zebrafish enteric nervous system, it appears dispensable for the development of the kidney. We have also identified a new role for GDNF in the axonal patterning of specifically identified motor neurons but find that GDNF is not critical for their trophic support.

METHODS

Isolation of Zebrafish GDNF and GFRα1 Genes

Degenerate oligonucleotide primers for GDNF and GFRα1 were designed using CODEHOP (Consensus-Degenerate Hybrid oligonucleotide Primers; Rose et al., 1998). All known vertebrate sequences were aligned using CLUSTAL W, and blocks of conserved amino acid sequence were identified using Block Maker (Henikoff et al., 1995). Candidate primers were then identified from these blocks using the CODEHOP program. The sequences of the GDNF degenerate primers were as follows: Forward, 5′-CGAGAACAAAAGGTGATNTT(C/T)A/GNTA(C/T)TT-3′; Reverse, 5′-CCAGGAAGGACCTCGTCTCNGC-3′. The sequences of the two pairs of GFRα1 degenerate primers were as follows: Forward 1, 5′-CCTGTACAGCTCGGCGTC(A/T)AA(A/G)-TGA(G)-CNGG-3′; Reverse 1, 5′-TGCCGAGGGCCTTTAAGTGA(A/G)-GA(C/T)TTT-3′; Forward 2, 5′-AAGGCCCGCTGCGCA(A/G)TTT(C/T)TTT-3′; Reverse 2, 5′-GTTGACCGCTTGCGCAANGC(C/T)TG(A/G)TJTT-3′. The primers were used in RT-PCR with first-strand cDNA prepared from 24 hpf zebrafish embryos. The PCR conditions used were 1 cycle 94°C 3 min, 60°C 1 min, 72°C 1 min; 35 cycles 94°C 1 min, 55°C 1 min, 72°C 1 min; 1 cycle 94°C 1 min, 55°C 1 min, 72°C 1 min, 5 cycles 94°C 1 min, 55°C 1 min, 72°C 1 min; 39 cycles 94°C 1 min, 55°C 1 min, 72°C 1 min. Sequence analysis was carried out using Sequencher and MacVector. Sequence data has been submitted to GenBank (Accession Numbers AF329853, AF329854, AF329855).

Mapping

Genomic mapping of GDNF, GFRα1a, and GFRα1b was carried out using the LN54 radiation hybrid panel as previously described (Hukriede et al., 1999). Primers used were as follows: GDNF forward, 5′-GCAAGCCAAGCTGCAATTTGC-3′; GDNF reverse, 5′-TCTTGTGCTATGCTTCTCCGC-3′; GFRα1a forward, 5′-GGCCAGCGCTTGCAGAAGAG-3′; GFRα1a reverse, 5′-CAGAACCGCTGTAGTTGTC-3′; GFRα1b forward, 5′-GAAAGCGCTTCCTCAGGAC-3′; GFRα1b reverse, 5′-CGGAACGCGCAGATGC-3′.

Embryos

Zebrafish embryos were collected from a laboratory breeding colony kept at 28.5°C as previously described (Westerfield, 1993). Embryos were staged according to hours postfertilization (hpf) and morphological criteria (Kimmel et al., 1995).

Whole-Mount In Situ Hybridization

Embryos were processed for whole-mount in situ hybridization as previously described (Thiése et al., 1993). Digoxigenin-labeled riboprobes were synthesized from templates linearized with NotI using Sp6 RNA polymerase for GDNF, GFRα1a, and GFRα1b and T7 RNA polymerase for RET. Fluorescein-labeled riboprobe for GFR was synthesized using T7 RNA polymerase from a template linearized with BamHI. Autoradiography with NBT/BCIP was used to visualize the GDNF, GFRα1a, GFRα1b, and RET probes. Fast red (Roche) was used to visualize the GFP probe. Cross sections of whole-mount in situ hybridized embryos were made using a Reichart Jung cryostat. Embryos were equilibrated in 20% sucrose PBS solution and frozen in OCT. Sections (50 μm) were collected on Fisher superfrost plus rehydrated in PBS and mounted in 50% glycerol.

PMN Single-Cell Labeling

Individual motor neurons were labeled with lissamine fluoroscein dextran (3 × 106 MW; Molecular Probes) as described (Eisen et al., 1989). Embryos were then fixed and processed for in situ hybridization using the GFRα1b digoxigenin-labeled riboprobe. The hybridization signal was visualized with NBT/BCIP. Subsequently the embryos were processed immunohistochemically to detect the injected dye with alkaline phosphatase-conjugated anti-fluorescein antibodies and then visualized with Fast red.

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GDNF mRNA Injections

GDNF mRNA was synthesized using the mMessage mMachine kit (Ambion) from a pCS2-GDNF vector linearized with NotI and transcribed by SP6, ethanol precipitated, and resuspended in sterile distilled water for injection at a concentration of 50 ng/µl. GDNF mRNA was coinjected with GFP mRNA, also at a concentration of 50 ng/µl, to allow assessment of mRNA expression in injected embryos. Embryos for injection were obtained from natural matings. Approximately 1 nl of diluted mRNA was injected into 1- to 2-cell embryos using a gas-driven microinjection apparatus.

Embryos injected with mRNA were fixed at 24, 72, and 96 hpf and processed for immunocytochemistry as described in Beattie et al. (2000) using the following antibodies: 24 hpf embryos were stained with ZNP-1 (University of Oregon), a monoclonal antibody (mAb) that recognizes primary and secondary motor axons (Melancon et al., 1997; Trevorrow et al., 1990); 72 hpf embryos were stained with α6F (Developmental Studies Hybridoma Bank), an mAb that recognizes an anti-Na+/K+ ATPase that is specifically expressed in the epithelia of the developing zebrafish pronephric kidney (Drummond et al., 1998; Takeyasu et al., 1988); 96 hpf were stained with anti-Hu mAb 16A11 (University of Oregon) that labels differentiated neurons (Marusich et al., 1994). All mAbs were visualized using an Alexa Fluor 568 anti-mouse IgG antibody (Molecular Probes). Embryos were examined and scored for aberrant PMN pathfinding at 24 hpf, defective pronephric kidney development at 72 hpf, and the number of differentiated neurons in the intestine at 96 hpf.

Muscle Actin Promoter GDNF ORF Plasmid Construction, Embryo Injections, and Analysis of PMN Axon Projection

A BamHI-CIal fragment containing the complete GDNF ORF including the stop codon was inserted into a pCS2-GFP vector to create pCS2-GDNF(GFP), encoding the full ORF of GDNF and GFP. This plasmid was cut with HindIII, filled in with Klenow, and then cut with NotI. The GDNF(GFP) SV40 poly(A) fragment released was gel purified and inserted into the ϕlGFP plasmid (Higashijima et al., 1997) that had had the EGFP poly(A) sequence excised by digestion with Agel, filled in with Klenow, then cut with NotI. The resulting fusion gave rise to ϕlGDNF(GFP) plasmid.

Embryos for injection were obtained from natural matings. Approximately 1 nl of supercoiled plasmid DNA diluted to a concentration of 50 ng/µl was injected in 1- to 2-cell embryos using a gas-driven microinjection apparatus. Any dead or dying embryos were removed between 3- 6 h. Embryos were fixed at 24 hpf and processed for in situ hybridization using a GFP antisense riboprobe. After developing the Fast red color reaction product, embryos were washed in PBS and processed for immunocytochemistry with an anti-acetylated tubulin antibody (Sigma) as described (Beattie et al., 2000). An Alexa Fluor488 anti-mouse IgG antibody (Molecular Probes) was used to visualize the acetylated tubulin antibody. Embryos were examined using a confocal microscope and scored for any perturbation in the normal pattern of CaP PMN axon projection into ventral somites in segments 10-22.

GDNF Antisense Oligo Injections

The antisense oligonucleotide used was a 25-mer morpholino oligo (Gene Tools LLC) with the base composition 5'-TGT-CCCATAACTTCATTTTAGACTC-3'. The oligo was resuspended in sterile filtered water and diluted to working concentrations in a range between 1 and 5 µg/µl. Approximately 1 nl of diluted morpholino was injected into 1- to 2-cell embryos using a gas-driven microinjection apparatus.

Injected and uninjected control embryos were fixed at 24, 72, and 96 hpf. Embryos were processed for immunocytochemistry, as described previously, with ZNP-1, α6F, and anti-Hu mAbs to determine its effect on PMN axon pathfinding at 24 hpf; pronephric kidney development at 72 hpf, and the number of differentiated neurons in the intestine at 96 hpf.

GDNF morpholino-injected embryos and control uninjected embryos were also fixed at 18 hpf and processed for whole-mount in situ hybridization with antisense digoxigenin-labeled riboprobes for Crestin (Rubinstein et al., 2000) and Dlx-2 (Akimenko et al., 1994) as described previously (Thisue et al., 1993). After NBT/BCIP color reaction development, the specification and pattern of vagal neural crest migration was assessed.

Other GDNF morpholino-injected and control-uninjected embryos were fixed at 24, 48, 72, and 96 hpf and the number of apoptotic cells were identified in whole-mount embryos by TUNEL histochemistry using Roche in situ cell death detection kit AP as described in Abdellah et al. (1996). The number of apoptotic profiles was determined in the region posterior to the otic vesicle up to the second somite of 24 and 48 hpf embryos and in the intestine of 72 and 96 hpf embryos.

In morpholino rescue experiments GDNF morpholino, at a concentration of 1-5 µg/µl, was coinjected with GDNF mRNA, at a concentration of 50 ng/µl, into 1-2 cell stage embryos. These embryos were allowed to develop until 96 hpf fixed and processed for immunocytochemistry with anti-Hu mAb as described above. Embryos were examined and scored for the number of differentiated neurons in the intestine at 96 hpf.

Photography

Whole-mount fixed embryos were mounted in 50% glycerol between bridged coverslips and photographed using a Zeiss Axioskop. A cetylated tubulin mAb stained embryos were imaged using a Zeiss Pascal confocal microscope. Images were scanned and adjusted in Adobe Photoshop for brightness/contrast and color balancing.

RESULTS

The Cloning and Molecular Analysis of Zebrafish GDNF and GFRα1 Orthologues

We have isolated clones representing the full open reading frame of zebrafish GDNF. We isolated partial cDNAs of zebrafish GDNF by RT-PCR using CODEHOP designed degenerate primers (Rose et al., 1998). Subsequently we used 5' and 3' RACE to identify flanking coding regions. PCR using primers designed to amplify the full-length open reading frame identified a single product. The zebrafish GDNF open reading frame is 708 bases in length and encodes for a putative protein of 235 amino acid residues with a predicted molecular weight of 27 kD (Fig. 1A). Sequence comparison by BLAST and using PHYLIP revealed that the zebrafish gene is most similar to other GDNF orthologues.
FIG. 1. Cloning of zebrafish GDNF and GFRα1 genes. (A) Alignment of the predicted amino acid sequence zebrafish GDNF with those of chick, mouse, and human GDNFs. Amino acid identities with the zebrafish sequence are indicated by dashes, and gaps are indicated by periods. At the amino acid level zebrafish GDNF has 46.9, 47.3, and 49.0% identity with chick, mouse, and human GDNF, respectively. Box I indicates a potential secretion signal. Box II indicates the consensus sequence for proteolytic processing in the constitutive secretion pathway. The predicted amino acid sequence of mature GDNF is underlined; the N-linked glycosylation sites are indicated by D, and asterisks mark the conserved cysteine residues. (B) Alignment of the predicted amino acid sequence of zebrafish GFRα1a and GFRα1b fragments with each other and with the corresponding amino acid sequence of chick, mouse, and human GFRα1s. Comparison of the amino acid sequence alignment of these 600 base pair fragments to each other and to the corresponding regions of other previously identified GFRα1s show that the zebrafish GFRα1 genes have 87.7% identity to each other. Zebrafish GFRα1a has 81.2, 79.4, and 78.5% identity, and zebrafish GFRα1b has 80.8, 78.0, and 78.0% identity with the chick, mouse, and human GFRα1 genes, respectively. Asterisks mark the conserved cysteine residues.

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Amino acid sequence analysis reveals that all functionally identified motifs that are found within other vertebrate GDNF family members are present in the zebrafish sequence. All eight conserved cysteines and two putative conserved N-linked glycosylation sites are present. As with other vertebrate GDNFs, zebrafish GDNF appears to be synthesized as a precursor protein that is then processed and secreted as a mature protein. A potential secretion signal is present after the initial Met and is predicted to be cleaved after Thr19 (von Heijne, 1986). In addition, there is a consensus sequence for proteolytic processing in the constitutive secretion pathway predicted to release the mature GDNF after cleaving at Arg19 (Hosaka et al., 1991).

We have also identified two zebrafish genes coding for GFRα1, the ligand-binding component of the GDNF receptor complex. We isolated partial clones of zebrafish GFRα1 using degenerate PCR and RACE. Sequence comparison by BLAST revealed that both clones appear to be orthologues of GFRα1 rather than other members of the GFRα family. Both partial fragments of the zebrafish GFRα1 genes contain the 19 conserved cysteines found in the corresponding regions of all other vertebrate GFRα1 genes. We suspect that the two zebrafish GFRα1 genes, GFRα1a and GFRα1b, arose as a result of the proposed ancient genome duplication. BLAST revealed that both clones appear to be orthologues of GFRα1 rather than other members of the GFRα family.Both partial fragments of the zebrafish GFRα1 genes contain the 19 conserved cysteines found in the corresponding regions of all other vertebrate GFRα1 genes. We suspect that the two zebrafish GFRα1 genes, GFRα1a and GFRα1b, arose as a result of the proposed ancient genome duplication.

We mapped zebrafish GDNF, GFRα1a, and GFRα1b genes on the LN54 radiation-hybrid mapping panel to determine their respective genomic locations (Hukriede et al., 1999). GDNF maps to linkage group 10, 9.10 cR from Z6410. GFRα1a maps to linkage group 13, 19.72 cR from Z15438, and GFRα1b maps to linkage group 12, 25.36 cR from Z4188. None of these map positions correspond to any published mutants. The finding that GFRα1a and GFRα1b map to different linkage groups demonstrates that they are indeed different genes rather than splice variants.

Expression of GDNF and Its Receptor Components in the Developing Zebrafish Embryo

We examined the embryonic expression of zebrafish GDNF, GFRα1a, GFRα1b, and RET by in situ hybridization with antisense riboprobes. We focused on the expression pattern of these genes in the developing primary motor neuron system, kidney, and enteric nervous system. We hypothesized that GDNF might play an important role in the development of these systems in zebrafish given the results of previous studies in other model organisms.

Expression in the primary motor system. In zebrafish, each muscle segment is innervated by three primary motor neurons (PMNs) that can be individually identified by the positions of their cell bodies in the spinal cord and the regions of the muscle they innervate (Eisen, 1999). The three are designated CaP, MiP, and RoP for caudal, middle, and rostral primary motor neuron, respectively. A fourth PMN, VaP, is variably present and has equivalent developmental potential to CaP (Eisen, 1992; Eisen et al., 1990; Pike et al., 1992). Smaller secondary motor neurons arise later as clusters of cells within each hemisegment (Pike et al., 1992). CaP, MiP, and RoP innervate different territories: CaP innervates ventral myotome, MiP innervates dorsal myotome, and RoP innervates a central territory in between.

To determine whether GDNF could be involved in the development of the primary motor neurons, we examined GFRα1 expression in the ventral spinal cord. GFRα1a is not expressed at any time examined. In contrast, GFRα1b expression is first detected at 14 hpf in one or two bilateral pairs of cells at the level of somite 2 (not shown). Subsequently the number of GFRα1b positive cells increases, as cells are added in a rostral to caudal direction following a segmentally arranged pattern corresponding to the overlying somite (Fig. 2A). By 20 hpf the number of GFRα1b expressing cells increases to two or three bilateral pairs of cells per overlying muscle segment. Expression of GFRα1b decreases after 48 hpf. The segmental arrangement and location of these GFRα1b expressing cells is consistent with some of these cells being primary motor neurons. Other GFRα1b expressing cells in the ventral cord are likely to be interneurons. We do not see any GFRα1b expression in secondary motor neurons.

Expression of GFRα1b coincides with localization of mRNA for RET tyrosine kinase. We confirmed the previously reported pattern of RET expression in the spinal cord (Bisgrove et al., 1997; Marcos-Gutierrez et al., 1997) and compared it to the expression of GFRα1. RET expression precedes that of GFRα1b by 2-3 h. RET is first detectable around 11 hpf in one or two bilateral pairs of cells at the level of the second somite (not shown). Subsequently RET is expressed in two or three pairs of bilateral cells per segment by 15 hpf persisting through 24 hpf (Fig. 2B). By 30 hpf there are more RET expressing cells per segment in the spinal cord than GFRα1b expressing cells (data not shown). This difference is probably due to RET being expressed in secondary motor neurons (Bisgrove et al., 1997). We find RET expression persists through 48 hpf, longer than previously reported (Bisgrove et al., 1997), but becomes undetectable by 72 hpf.

To determine whether specific primary motor neurons express GFRα1b, we compared its mRNA distribution to cells phenotypically identified by dye labeling. We identified CaP, MiP, and RoP somata by their characteristic positions in live embryos at 22 hpf and injected them with lysinated fluorescein dextran. The characteristic axonal trajectories of injected cells confirmed their identities. Fluorescein and GFRα1b expressing cells were detected by immunohistochemistry and in situ hybridization, respectively. Only CaP motor neurons express GFRα1b at this stage (Fig. 2C). In addition GFRα1b is expressed in an unidentified interneuron. MiP (Fig. 2D) and RoP (not shown) do not express GFRα1b. Previously it had been shown that RET is expressed in both CaP, MiP, and RoP (Bisgrove et al., 1997). These results suggest that GFRα1b is expressed in only a subset of RET-expressing cells, leading
FIG. 2. Expression of zebrafish GDNF, GFRα1b, and RET genes in the trunk of 24 hpf embryos (A and B). Low power lateral views of the trunks of 24 hpf embryos hybridized with riboprobes for GFRα1b (A) and RET (B). (C, D) High power lateral views of whole-mount 24 hpf embryos hybridized with GFRα1b riboprobe (blue reaction product) that had been injected with lysinated fluorescence dextran (red reaction product) to identify CaP (C) and MiP (D) PMN prior to in situ hybridization. Low power lateral view of the trunk of 24 hpf embryos hybridized with riboprobes for GDNF (E). Transverse section taken through a 24 hpf embryo hybridized with probe for GDNF at the level of somite 14. SC, spinal cord; N, notochord; VS, ventral somite; Y, yolk. Arrow in (B) indicates pronephric duct. Arrows in (C) and (D) indicate the identified PMN. In (A–E) rostral is to the left. Scale bars: (A), (B), (E), and (F), 50 μm; (C) and (F), 25 μm.
to the possibility that other GFRα family members may be expressed in MIP or RoP. Since ligand specificity is conferred by the GFRα subunit, these results also suggest that the primary motor neurons are differentially responsive to various GDNF family members.

The expression of GDNF mRNA is consistent with the idea that the primary motor neurons respond differentially to ligand. GDNF message is first detectable at 14 hpf in the ventral half of anterior somites (data not shown). This ventral expression persists and extends into more posterior somites over the next 12 h (Fig. 2E). By 30 hpf the somitic expression ceases. Cross-sections through embryos at 24 hpf show that GDNF message is found throughout the ventral somitic/mesodermal tissue (Fig. 2F).

The patterns of GDNF expression in the ventral somite before and during the period of PMN axonogenesis, and the expression of GDNF receptor components in the ventrally projecting CaP PMNs, raise the possibility that GDNF might function as a chemotropic axon guidance cue for CaP helping distinguish its ventral projection from the more dorsal projections of MIP and RoP.

Expression in the developing kidney. The zebrafish larval excretory system is a relatively simple pronephric kidney consisting of two nephrons with fused glomeruli and paired pronephric tubules and ducts (Drummond et al., 1998). The pronephric ducts arise from a primordia within the intermediate mesoderm soon after the onset of somitogenesis and are well-defined by 16 hpf (Kimmel et al., 1995). The nephron arises from a primordium ventral to the third somite beginning around 24 hpf (Drummond et al., 1998). Development of the nephric glomeruli and tubules is completed between 40–48 hpf when glomerular filtration begins.

Zebrafish GDNF expression is consistent with it playing a role in kidney development. As described above, GDNF message is first detectable at 14 hpf in the ventral half of anterior somites and the intermediate mesoderm where the pronephric duct condenses. The pattern of GDNF expression persists and extends posteriorly continuing through 24 hpf (Figs. 2E, 2F, and 3A) but ceases by 30 hpf.

Expression of GDNF receptor components is also consistent with GDNF having a role in the development of the zebrafish kidney. GFRα1a is only very transiently expressed in the developing pronephric ducts between 18–20 hpf (Fig. 3B). A high level of expression is restricted to the most posterior part of the ducts near the anus where the two pronephric ducts fuse. Weak expression is seen in the ducts further anteriorly extending as far as somite 11. No expression of GFRα1a is seen in more anterior parts of the duct at any stage examined. The expression of GFRα1a in the pronephric duct ceases by 22 hpf. GFRα1b is only very weakly expressed in the posterior pronephric duct from somite 12 to the anus between 18–20 hpf (Fig. 3C). This expression is also transient, ceasing by 22 hpf.

We find that RET is initially expressed along the whole length of the pronephric duct at 16 hpf, as previously reported. Expression becomes discontinuous by 20 hpf. Posteriorly, RET expression extends from somite 11 to the anus (Figs. 2B and 3D). Anteriorly, RET is expressed in the putative primordium of the pronephric kidney in the lateral mesoderm deep to somites 2–3 (Bisgrove et al., 1997; Marcos-Gutierrez et al., 1997, not shown). This pattern of RET expression ceases by 48 hpf. As in the PMN system, RET expression precedes that of GFRα1 gene expression. In addition GFRα1 gene expression is only in a subset of the pronephric duct cells that express RET. Interestingly, GDNF mRNA is expressed throughout the tissue where the pronephric duct forms.

Expression in the intestine and enteric nervous system. The development of the enteric nervous systems (ENS) is intimately related to the normal development of the intestine. The zebrafish intestine can be initially identified as a cord of radially aligned cells at 36 hpf, though the anus and esophagus have not developed at this age. By 52 hpf the lumen of the gut develops rostrally but remains unconnected to the pharynx. By 72 hpf the lumen extends the full length of the gut, the esophagus having formed connecting the proximal intestine to the pharynx. During this same period cellular polarization and differentiation begin in the intestinal epithelium. To date little is known about the development of the zebrafish ENS. Currently the earliest known marker for what is believed to be ENS precursors is RET (Bisgrove et al., 1997). ENS neuronal differentiation within the intestine occurs in a rostral to caudal gradient (Kelsh and Eisen, 2000). Differentiated neurons are clearly identifiable in rostral intestine at 72 hpf and are found along the whole length of the gut by 96 hpf.

Zebrafish GDNF is expressed in positions consistent with a role in ENS development. As described above, GDNF is expressed throughout the ventral trunk mesoderm and endoderm of the zebrafish at 24 hpf (Figs. 2E and 2F). By 36 hpf GDNF expression becomes restricted to the endodermal cells forming the gut. At 72 hpf GDNF message can be seen to be expressed along the whole length of the early developing gut tube (Fig. 4A). This pattern of expression continues through 96 hpf, the latest developmental age we examined.

Expression of GDNF receptor components is also consistent with a role in ENS development. GFRα1a and 1b messages are expressed within what we believe to be migrating enteric precursors from 36 hpf (data not shown). Cells expressing these messages are initially seen at this age in the rostral-most part of the gut endoderm. Over the next 36 h, cells expressing these messages are found progressively in more caudal parts of the gut. By 72 hpf GFRα1a and 1b positive cells are found along the whole length of the gut (Figs. 4B and 4C). This pattern of expression persists through 96 hpf but the level GFRα1a and 1b messages decreases significantly. At all ages examined the levels of GFRα1a expression were significantly lower than that of GFRα1b message. Currently we are unable to determine whether all ENS precursors express both the 1a and 1b
messages or whether different precursors express either one or the other GFRα1 messages.

The pattern of RET expression in the ENS we observe is the same as that previously reported (Bisgrove et al., 1997; Marcos-Gutierrez et al., 1997). As with the GFRα1 subunits, RET expression is found in cells throughout the length of the intestine by 72 hpf (Fig. 4D). We find, however, that in contrast to previous reports RET expression persists in the ENS through 96 hpf. As in other embryonic structures, RET message expression precedes that of GFRα1a and 1b. Currently we cannot determine whether all the RET-expressing ENS precursors express one or both of the GFRα1 messages.

**Ectopic Misexpression of GDNF**

To further investigate GDNFs in vivo function in the developing zebrafish embryo, synthetic GDNF mRNA was injected at the 1–2 cell stage. The development of the PMN projections, pronephric kidney, and ENS was assessed, as these tissues have correlated expression of ligand and receptor components. The patterns of PMN axon projections were analyzed at 24 hpf by staining injected embryos with the ZNP-1 antibody (Melancon et al., 1997; Trevarrow et al., 1990). The development of the pronephric kidney was analyzed by staining 72 hpf injected embryos with an anti-Na+/K+ ATPase antibody, α6F (Drummond et al., 1998; Takeyasu et al., 1988). The development of the ENS was analyzed by assessing the presence of differentiated neurons in the intestine at 96 hpf by staining embryos with the neuron-specific anti-Hu antibody (Kelsh and Eisen, 2000). Subsequently the number of Hu-positive neurons in a 4-somite-length segment of the intestine was counted. GFP mRNA was coinjected with GDNF mRNA to determine indirectly which cells were misexpressing GDNF. No difference in PMN axon projections was seen in embryos that had been injected with GDNF/GFP mRNA compared to those in control GFP mRNA injected embryos (data not shown).
Pronephric kidney development in GDNF-injected embryos was also unaffected (data not shown). There was, however, a small (17%) but statistically significant increase in the number of Hu-positive neurons in the intestine of 96 hpf experimental embryos as compared to control embryos (Fig. 6C).

**Transient Misexpression of GDNF in Somitic Muscle**

To address whether more discrete localized sources of GDNF could effect PMN axon projections, we used a muscle-specific actin promoter to overexpress GDNF in somitic muscle. In control experiments, the muscle-specific actin promoter directed expression of green-fluorescence protein (GFP) in a mosaic fashion beginning around 18 hpf. GFP expression continued in somitic muscle throughout the period of PMN axogenesis. To determine if there were any perturbations in PMN axon projections, embryos were fixed at 24 hpf and stained with an anti-acteylated tubulin antibody. No significant perturbation was seen in the pattern of PMN projection when compared to uninjected embryos (Table 1).

In contrast, mosaic expression of GDNF had profound effects on CaP motor neuron projections. One-cell stage embryos were injected with the muscle-specific promoter GDNF construct, allowed to develop until 24 hpf, fixed, and processed for in situ hybridization. A probe that was directed to a heterologous 3’ UTR tag in the GDNF construct was used to determine the pattern of the construct’s expression. Embryos were then immunohistochemically stained to determine the patterns of PMN axon projection. When

**FIG. 4.** Enteric nervous system and gut endoderm mRNA expression of zebrafish GDNF, GFRα1, and RET at 72 hpf. (A–D) Lateral view of the trunk of 72 hpf embryos from somites 9–13 that have been hybridized with GDNF (A), GFRα1a (B), GFRα1b (C), and RET (D) riboprobes. Y, yolk sac extension. Arrows in (A) indicate gut endoderm. Arrows in (B–D) indicate enteric neuron processors in the gut endoderm. Rostral is to the left. Scale bar: 50 μm.
GDNF was overexpressed in a discontinuous/focal pattern (1–2 misexpressing cells per somite), more than half of the embryos had CaP motor axon projection errors (Table 1). These errors included inappropriate branching toward overexpressing cells, and in the most severe cases, failure of the CaP axon to project beyond overexpressing cells into ventral somite (Fig. 5B). In contrast, where GDNF was uniformly overexpressed (three or more misexpressing cells per somite in two or more consecutive somites), no errors were seen in the pattern of motor axon projections (Fig. 5C; Table 1). No errors in the projection of MiP axons were ever observed (not shown). These results suggest that changing the local distribution of GDNF is sufficient to specifically alter the projection patterns of CaP motor axons.

**Table 1**

<table>
<thead>
<tr>
<th>Construct Injected</th>
<th>Pattern of Misexpression</th>
<th>Total No. of Embryos</th>
<th>No. of Embryos with CaP Errors</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>N/A</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>Mus:GFP Uniform</td>
<td></td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>Mus:GFP Focal</td>
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<td>27</td>
<td>1</td>
</tr>
<tr>
<td>Mus:GDNF Uniform</td>
<td></td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Mus:GDNF Focal</td>
<td></td>
<td>74</td>
<td>43</td>
</tr>
</tbody>
</table>

Scoring of CaP motor projection errors was determined in acetylated tubulin stained 24 hpf embryos between somites 10–22. Mus, muscle actin promoter.

**Morpholino Antisense Injections**

To determine whether GDNF was necessary for zebrafish embryonic development, we used a “morpholino” antisense approach to deplete embryos of GDNF protein. Morpholino oligonucleotides block mRNA translation when designed as complementary to the sequence around the translation initiation site (Summerton and Weller, 1997). Morpholino oligos have recently been shown to be highly effective gene specific translational inhibitors in zebrafish (Nasevicius and Ekker, 2000). One-cell stage embryos were injected with a morpholino oligo complementary to the initiation site of GDNF mRNA. Injected embryos were analyzed immunohistochemically for defects in PMN axon projection pattern, early development of the pronephric kidney, and development of the enteric nervous system.

At all concentrations of GDNF morpholino tested (1–5 μg/μl), injected embryos were morphologically indistinguishable from control embryos. In addition, no defects were observed in the patterns of the PMN axon projections at 24 hpf or in the development of the pronephric kidney at 72 hpf (data not shown). In contrast, the number of enteric neurons was drastically reduced in 95% of morpholino-injected embryos as compared to control embryos (Fig. 6A and 6B). When cells were counted in a 4-somite-wide

**Fig. 5.** Focal overexpression of GDNF in zebrafish somitic muscle cells causes PMN axon pathfinding errors. (A–C) Lateral view of the trunks of 24 hpf antiacetylated tubulin antibody stained control (A), focal GDNF overexpressing embryos (B), and uniform GDNF overexpressing embryos (C). Red cells in (B) and (C) express the muscle actin promoter; GDNF DNA construct as revealed by whole-mount in situ hybridization with a riboprobe against a 3' UTR tag contained in the injected DNA construct. Arrow in (B) indicates a stalled PMN axon projection associated with a GDNF overexpressing cell. Scale bar: 50 μm.
segment of the intestine at 96 hpf, injected embryos showed a 90% reduction compared to controls (Fig. 6C). To control for the specificity of the antisense oligo, we attempted to rescue the morpholino phenotype by coinjecting the morpholino with a GDNF mRNA that has a different sequence at the initiation site. In embryos injected with GDNF mRNA alone, cell counts revealed a small but significant increase in the number of Hu-positive neurons (Fig. 6C). In morpholino mRNA coinjected embryos the synthetic GDNF mRNA partially rescued the decrease in ENS neurons (Fig. 6C). Other morpholino oligonucleotides injected at similar concentrations to the GDNF morpholino have no effect on ENS neuron number (data not shown).

To investigate when GDNF morpholinos affects ENS development, we examined the specification and initial migration pattern of vagal neural crest using two different markers, Crestin (Rubinstein et al., 2000) and Dlx-2 (Akiemenko et al., 1994). No difference in the specification and pattern of vagal neural crest migration was seen after GDNF morpholinos injection (data not shown). To address whether GDNF morpholinos cause cell death of either a subpopulation of vagal neural crest cells that gives rise to the ENS during its initial migration, or whether morpholinos cause cell death of ENS precursors while the precursors are migrating along the gut endoderm, we used TUNEL histochemistry to determine the extent of apoptosis in morpholino-injected embryos as compared to control embryos. TUNEL histochemistry was carried out on 24, 48, 72, and 96 hpf morpholino-injected and control embryos. No difference in the number of apoptotic cells was observed in morpholino-injected embryos as compared to uninjected controls in either the migrating vagal crest population at 24 or 48 hpf or in the gut endoderm at 72 and 96 hpf (data not shown).

**DISCUSSION**

In the present study we report the identification of zebrafish orthologues of GDNF and GFRα1. We describe the mRNA expression patterns of these genes in the developing zebrafish PMN, kidney, and enteric nervous systems and have identified areas of correlated expression of receptor and ligand that suggest possible functional significance. Comparison of the zebrafish GDNF and GFRα1 mRNA expression patterns with those reported previously in the developing mouse and chick show that there is conservation of expression in the early developing renal and enteric nervous systems (Golden et al., 1999; Homma et al., 2000). In all three species GDNF is expressed in the pronephric-intermediate mesoderm while GFRα1 is expressed to varying degrees in the pronephric duct epithelia. In the enteric nervous system, GDNF is expressed in the avian, mouse, and zebrafish intestinal endoderm, while GFRα1 is expressed in the ENS precursors and neurons of zebrafish and mice. The avian GFRα1 mRNA expression pattern in ENS precursors and neurons has not been reported.

**FIG. 6.** GDNF antisense morpholino injections cause a loss of enteric neurons in zebrafish embryos. Lateral view of the trunks of 96 hpf embryos whole-mount stained with anti-Hu antibody. (A) Control embryo. (B) GDNF morpholino-injected embryo. (C) A bar graph showing the number of enteric neurons in control; GDNF RNA injected; GDNF morpholino injected; and GDNF morpholino plus GDNF RNA injected embryos as a percentage of control. The error bars in (C) indicate 95% confidence interval. Arrows in (A) and (B) indicate gut endoderm. Rostral is to the left in (A) and (B). Scale bars: 100 μm.
There are, however, a number of marked differences in the pattern of GDNF and GFRα1 mRNA expression in motor neurons and somitic tissue. There are no published reports of GDNF being expressed in mouse or chick ventral somitic mesoderm during the initial period of motor axon outgrowth as seen in zebrafish. In addition, avian and mouse GFRα1 is expressed in dorsolateral somitic tissue (Hellmich et al., 1996; Homma et al., 2000), which is not the case in zebrafish. This difference in expression could be due to the organizational and functional differences in the mouse and avian somite compared to the zebrafish somite (Morin-Kensicki and Eisen, 1997). In the avian, mouse, and zebrafish spinal cord GFRα1 is expressed in a subset of motor neurons at later stages of development (Garces et al., 2000; Soler et al., 1999). However, at earlier stages of motor neuron development there are distinct differences in the pattern of GFRα1 expression. In mice GFRα1 is expressed in all motor neurons (Golden et al., 1999), while avian GFRα1 is not expressed in any motor neurons (Homma et al., 2000). In contrast, zebrafish GFRα1 is always specifically expressed only in CaP motor neurons. The difference in these early patterns of expression may indicate functional differences for GDNF in the development of motor projections in zebrafish as compared to its role in other vertebrates.

We have investigated the functional significance of the correlated expression of GDNF in ventral somite and its receptor expression in ventrally projecting CaP primary motor neurons. While global overexpression of GDNF in zebrafish embryos resulted in no change in the pattern of PMN axon projections, we found that localized GDNF overexpression can alter CaP's normal axon projection pattern. Our results could be interpreted as evidence that GDNF acts as a chemoattractive guidance cue, guiding the CaP axon to ventral somite. The idea that GDNF could act as such a chemoattractive cue is not without precedent. In vitro experiments have shown that Xenopus spinal cord neuronal growth cones reorient toward a localized source of other neurotrophins such as NT-3 and BDNF (Ming et al., 1997). An alternative explanation is that GDNF does not act to guide axons, but instead stabilizes projections by providing trophic support for the CaP motor neuron. In this model, a CaP motor axon is guided to its target by multiple guidance cues but the axon stops extending when it encounters a cell expressing a high level of GDNF.

Our results, however, support the idea that CaP patterning is affected by local differences in GDNF expression. When GDNF was more uniformly overexpressed in zebrafish myotomes, by the αp-GDNF (GFP) construct or by the injection of GDNF mRNA at 1–2 cell stage, no effects on CaP axon projection were observed. Similarly, no differences in CaP axons were observed after morpholino-mediated GDNF depletion. These observations suggest that the absolute concentration of GDNF is not critical for CaP axon patterning or survival, and in the absence of GDNF other signals are used. In contrast, absolute levels of GDNF affect the development of motor neurons in mice. When GDNF was uniformly overexpressed in mouse muscle under control of the myogenin promoter, neuromuscular junctions were hyperinnervated by motor neurons, with the level of innervation correlated with the overall levels of GDNF expression (Nguyen et al., 1998). Observations in mice also suggest that GDNF acts trophically to promote motor neuron survival rather than affect axon outgrowth. In GDNF transgenic mice, more motor neurons are present in the spinal cord compared to controls, and in GDNF-deficient mice, a small but significant reduction in motor neurons was observed (Oppenheim et al., 2000). Our results suggest that GDNF does not have the same function in motor neuron development in zebrafish as compared to that in mice. GDNF does not appear to be an essential trophic survival factor for subsets of zebrafish motor neurons.

Results from the morpholino antisense experiments suggest that the only critical role for GDNF during zebrafish embryogenesis is in the development of the ENS. This result may not be surprising since GDNF-deficient mice lack nearly all ENS neurons (Moore et al., 1996; Piché et al., 1996; Sanchez et al., 1996). In vitro experiments have shown that GDNF is required for the proliferation, differentiation, and trophic support of neural crest precursors isolated from avian and rat intestines (Chalazonitis et al., 1998; Heem et al., 1998; Heuckeroth et al., 1998). The 10% of remaining ENS neurons seen in the 96 hpf morpholino-injected zebrafish embryos could be a subset of ENS neurons that are GDNF independent. In mouse, a subset of ENS neurons are dependent on another GDNF family ligand, neurturin (Heuckeroth et al., 1999; Rossi et al., 1999). It is also possible that if neurturin is present in zebrafish gut endoderm it could provide some trophic support for the GDNF-dependent enteric precursors. In vitro studies have shown that neurturin can bind to and signal through the GFRα1/RET receptor complex (Baloh et al., 1997; Creedon et al., 1997; Sanicola et al., 1997). Furthermore, other in vitro studies have shown that neurturin can trophically support and promote the differentiation of ENS precursors to the same extent as GDNF (Heuckeroth et al., 1998). An alternate explanation for the 10% remaining ENS neurons in the morpholino-injected embryos is that by 96 hpf, a small subset of late migrating, GDNF-dependent ENS precursors may have sufficient GDNF trophic support to survive and differentiate. This could occur if the GDNF morpholino is no longer at a high enough concentration in gut endoderm cells to block GDNF mRNA translation due to cellular degradation of the morpholino or due to dilution of the morpholino caused by cell division.

We have not been able to determine the developmental stage at which GDNF is critically needed for ENS precursor development. Our studies of vagal neural crest specification, migration pattern, and cell death in morpholino-injected embryos failed to identify a stage when there is a detectable difference between morpholino-injected embryos as compared to controls. These results suggest that GDNF does not play a role in determining the pattern of ENS precursor migration at 18 hpf. However as we do not know the number and precise migration pattern of the vagal...
neural crest cells that give rise to the ENS. It is possible that if there is only a small number of these cells any aberrant migration pattern might not be detectable using our current methods. Similarly if there is only a small number of vagal crest cells that give rise to the ENS precursors any increase in the number of apoptotic cells in morpholino-injected embryos may be easily missed. Ongoing studies investigating the cell fate of vagal neural crest cells, and other studies attempting to identify zebrafish ENS precursor specific markers, will help address the question of where and when GDNF is necessary for ENS development.

The lack of a detectable kidney phenotype in morpholino-injected zebrafish embryos may be surprising given that GDNF-deficient mice die from failure of kidney development (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). However, these results may simply reflect differences in overall kidney development in mice as compared to zebrafish. The mouse kidney progresses from pronephric through mesonephric to metanephric stages during embryonic development (Saxen, 1987), where as fish larvae retain a pronephric arrangement only developing a mesonephric kidney as juvenile fish mature into adults (Drummond et al., 1998). Mouse GDNF is critical during the early stages of metanephric development as a signal for ureteric bud outgrowth from the Wallerian duct into the metanephric mesenchyme (Pepicelli et al., 1997; Sainio et al., 1997). The development of the pro and mesonephric kidney in GDNF-deficient mice has not been reported; however, in GFRα1-deficient mice that have the same metanephric kidney phenotype as GDNF-deficient mice, there are no defects in the pro or mesonephric kidney (Cacalano et al., 1998). As zebrafish have no metanephric kidney, GDNF-specific morpholinos would not necessarily have any effect on the development of its renal system.

There are alternate explanations as to why GDNF morpholino injection does not affect motor neuron and kidney development. First, the GDNF morpholino may not persist at concentrations able to block protein translation during critical periods of PMN axonogenesis and pronephric duct and tubule formation. However, GDNF morpholinos dramatically affect the ENS, which develops much later than both the pronephric kidney and CaP axon outgrowth. A second plausible explanation is that other GDNF family ligands, or even a second unidentified GDNF orthologue that arose from the actinopterygian genome duplication (Postlethwait et al., 1998), may act redundantly with GDNF during these processes. We are currently trying to identify other members of the GDNF family to test this hypothesis. A third possible explanation is that ENS neurons may be much more sensitive to changes in GDNF protein concentration as compared to the pronephric kidney or PMN. A small reduction in GDNF concentration may be enough to cause the ENS phenotype in morpholino-injected embryos but this small change has no effect on PMN or pronephric kidney development. Currently we cannot determine what the in vivo GDNF protein concentration is in GDNF morpholino-injected embryos as compared to uninjected embryos. It is possible that the morpholino may only partly reduce the concentration of GDNF, though data from Nasenius and Ekker (2000) suggest that morpholinos can effectively block protein translation of a ubiquitously expressed gene in zebrafish embryos up to 28 hpf. We cannot rule out the possibility that a true GDNF null mutant zebrafish embryo might also have PMN and pronephric kidney defects.

Taking advantage of the cell biological and embryological strengths of the zebrafish, we are further investigating the function of GDNF in the developing zebrafish ENS. We intend to determine whether GDNF has a later role in the developing ENS after the precursors have migrated along the intestine and differentiated into neurons. We are interested in testing whether GDNF might help pattern the axon projections of subset enteric neurons in the myenteric and submucosal plexae, as it does for primary motor neurons.

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