Identification of a Novel Glial Cell Line-derived Neurotrophic Factor-inducible Gene Required for Renal Branching Morphogenesis

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In the developing kidney, activation of the rearrangement during transfection (RET) signaling system is required for renal branching morphogenesis. In this report, we describe a novel GDNF-inducible gene with a BTB/POZ domain and zinc finger motifs that are highly expressed in the ureteric bud of the metanephric kidney. The GDNF/RET signaling system is essential for normal development of the kidney.

The mammalian metanephric kidney develops from the metanephric mesenchyme, which appears in the region of the posterior intermediate mesoderm. It is well known that kidney development is induced by reciprocal signaling between the ureteric bud epithelium and the metanephric mesenchyme. On day 11 of mouse embryogenesis, the ureteric bud emerges near the caudal end of the Wolffian ducts and invades the metanephrogenic mesenchyme. Once the bud meets the mesenchyme, it begins to grow and branch repeatedly, thus generating the renal collecting duct system. At the same time, induction of the mesenchyme by signals from the bud initiates the condensation of the mesenchyme that differentiates to form the nephrons. In recent years, many important molecules that cooperatively function in the branching morphogenesis of the epithelial ureteric bud have been identified by in vitro and in vivo studies. These include transcription factors, secreted peptides, cell surface receptors, and extracellular matrices, which are expressed sequentially at specific sites of the developing kidney (2–4). Among them, glial cell line-derived neurotrophic factor (GDNF) and RET (rearrangement during transfection) have been recognized as critical regulators of ureteric bud branching. GDNF activates RET via its binding to glycosylphosphatidylinositol-linked cell surface protein, GFRα1 (GDNF family receptor α1) (5–7). The gene ablation studies revealed that GDNF, GFRα1, and RET are required for the development of the kidney and the enteric nervous system. Gdnf−/−, Gfra1−/−, or Ret−/− mice showed kidney aplasia or severe hypodysplasia and lacked enteric neurons in the whole intestinal tract, resulting in their death soon after birth (8–13). GDNF is secreted from the metanephric mesenchyme, and RET and GFRα1 are expressed on the cell surface of the branching ureteric bud epithelium (11, 14–16). Thus, it turned out that the interaction between the ureteric bud epithelium and the metanephric mesenchyme that leads to activation of the GDNF/RET signaling system is essential for normal development of the kidney.

Based on these findings, much attention has been paid to the genes that regulate the expression of GDNF and RET. It was suggested that transcription factors Pax2 and Eya-1 are responsible for Gdnf gene expression and that Emx2 is required to maintain the expression of both Gdnf and Ret in mouse metanephric kidney (2–4). In addition, Batourina et al. (17) reported that vitamin A signaling from stromal mesenchyme is necessary for Ret expression. However, it remains unknown which gene expression downstream of the GDNF/RET signaling is critical for renal development. In this report, we describe a novel GDNF-inducible gene with a BTB/POZ domain and zinc finger motifs that are highly expressed in the ureteric bud of the metanephric kidney. Analysis by the metanephric organ culture suggested that this gene plays a crucial role in the ureteric bud branching.

EXPERIMENTAL PROCEDURES

**Cell Lines**—TGW and NB39 human neuroblastoma cells, Neuro2a mouse neuroblastoma cells, and HEK293T human embryonic kidney cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB100265 and AB100266.

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1 The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; RET, rearrangement during transfection; GFRα1, GDNF family receptor α1; GZF1, GDNF-inducible zinc finger gene 1; GALABD, GAL4 DNA-binding domain; ODN, oligodeoxynucleotide; TK, thymidine kinase; EGFP, enhanced green fluorescent protein; BTB/POZ, broad complex, tramtrack, and bric-a-brac/poxvirus and zinc finger.
Identification of a New GDNF-inducible Gene

**Metaneprhic Organ Culture**—Metaneprhic organ culture was performed as described by Kanwar et al. (21). In brief, metanephric explants were isolated from E11.5 ICR mouse embryos and cultured in Transwell-Clear (Costar) for 4 days in a humidified incubator with 95% air and 5% CO2 at 37 °C. The culture medium consisted of equal volumes of Dulbecco’s modified Eagle’s medium and Ham’s nutrient mixture F-12 (Sigma) supplemented with transferrin (50 µg/ml), penicillin (100 µg/ml), and streptomycin (100 µg/ml). The medium was devoid of serum or any other growth factor. Sense, antisense, or scramble ONs of mouse GZF1 were added to the medium at a concentration of 0.5–2.0 µM. The metaneprhic explants were photographed directly by stereomicroscope and then stained with anti-pan cytokeratin antibody to highlight ureteric bud derivatives. The rudiments in metaneprhic organ culture were fixed in 100% methanol, washed three times in phosphate-buff ered saline, and permeabilized for 15 min in phosphate-buffered saline containing 0.2% Triton X-100. The rudiments were blocked with 3% bovine serum albumin in phosphate-buffered saline. The metaneprhic explants were incubated with anti-pan cytokeratin antibody diluted 1:50 at 4 °C overnight and then with Cy3-conjugated donkey anti-mouse IgG diluted 1:100 at 4 °C overnight. After immunostaining, slides were mounted in PermaFluor (Shandon) and observed by a fluorescent microscope with a charge-coupled device camera (BX50, DP70, Olympus).

**RESULTS AND DISCUSSION**

To identify GDNF-inducible genes, we performed differential display analysis using RNA from a human neuroblastoma cell line, TGW, expressing RET and GFRα1. We detected 124 cDNA bands in which the intensity increased after GDNF stimulation. After isolating and sequencing these cDNA bands, 73 independent cDNA clones were identified and used for Northern blotting to confirm their increased expression after GDNF stimulation. As a consequence, we found the increased expression of 14 genes by GDNF, although the time course of the induction was different depending on the genes (data not shown). These included 10 known genes such as c-FOS, CREM, and cell division cycle-like kinase genes and four unpublished sequences. In this study, we focused on a new gene with a BTB/POZ domain and C2H2-type zinc finger motifs that was named GZF1 (GDNF-inducible zinc finger gene 1).

We isolated its full-length cDNA from the library constructed from RNA of GDNF-treated TGW human neuroblastoma cells. The cDNA sequence revealed that it encodes a protein of 711 amino acids containing the BTB/POZ domain at the amino-terminal region and 10 C2H2-type zinc fingers (Fig. 1A). In addition, the GZF1 sequence contained a nuclear localization signal between the BTB/POZ domain and the zinc finger motifs. We also isolated its mouse ortholog cDNA from a mouse testis cDNA library with an amino acid sequence (706 amino acids) showing 84% identity with the human GZF1 sequence (Fig. 1A). The human and mouse GZF1 genes were located on chromosomes 20 and 2, respectively. When the GZF1 expression was examined in various human tissues, a single 4.8-kb mRNA was detected in brain, heart, skeletal muscle, kidney, and liver tissues (Fig. 1B). In addition, it was expressed at relatively high levels in brain and kidney tissue among human fetal tissues examined (Fig. 1B). The GZF1 gene expression was also detected in several mouse tissues including kidney and in different stages of mouse embryos (7–17-day embryos) (Fig. 1C).

The induction of the GZF1 gene in TGW cells showed two peaks at 1 h and at 24–48 h after GDNF stimulation by Northern blotting (Fig. 2A). This pattern of the GZF1 gene induction by GDNF was confirmed by reverse transcriptase-PCR analysis (data not shown). To further investigate the GZF1 protein expression, we developed a rabbit polyclonal antibody against the 19 carboxy-terminal amino acids of hu man GZF1. The antibody specifically detected 95- and 115-kDa proteins in TGW cells and a 115-kDa protein in Neuro2a mouse neuroblastoma cells (Fig. 2B). Because we were not able to detect either N-linked or O-linked glycosylation in these pro-
proteins (data not shown), the difference between expected and apparent molecular masses of GZF1 (80 kDa as expected molecular mass and 95 and 115 kDa as apparent molecular masses) may be caused by their peculiar protein conformations or other modifications. When the GZF1 expression was examined in GDNF-stimulated TGW and NB39 human neuroblastoma cells in which both RET and GFRα1 are expressed, its expression significantly increased 24–72 h after stimulation (Fig. 2C). The early induction of the GZF1 protein was not observed, probably because of transient up-regulation of GZF1.

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Fig. 1. Deduced amino acid sequences and expression of human and mouse GZF1. A, alignment of human and mouse GZF1 amino acid sequences. A BTB/POZ domain, a nuclear localization signal (NLS), and 10 C2H2-type zinc finger motifs are indicated above the sequences. The identical amino acids between human and mouse sequences are shaded. The sequences of human and mouse GZF1 cDNAs and proteins were deposited under GenBank™/EMBL/DDBJ accession numbers AB100265 and AB100266, respectively. B and C, Northern blot analyses of the expression of human (B) and mouse (C) GZF1. A single 4.8-kb transcript was detected in several human and mouse tissues (upper panels). The same membranes were probed with β-actin cDNA as a control (lower panels). The membranes were purchased from Clontech.
mRNA for a short period at an early phase (1–2 h after GDNF stimulation).

To confirm that the bands detected by the antibody represent the GZF1 protein, we constructed the EGFP-GZF1 fusion gene and transfected it into HEK293T cells. Although the fusion gene encodes a protein of 976 amino acids in which the expected molecular mass is 107 kDa, Western blotting with anti-GZF1 and anti-GFP antibodies revealed that the fusion protein was detected mainly as a 140-kDa band (Fig. 2D). The difference (33 kDa) between expected and apparent molecular masses of the EGFP-GZF1 fusion protein was almost the same as that between expected and apparent molecular masses of the endogenous GZF1 protein (80 and 115 kDa), indicating that the bands recognized by anti-GZF1 antibody represent the GZF1 protein. The 95-kDa protein detected in human cells may be translated from another methionine (e.g. codon 192) that is
present only in the human GZF1 gene or may be influenced by differences in modification or processing, although further investigation is necessary.

Cell fractionation experiments revealed that the GZF1 protein was detected in both nuclear and cytosolic fractions (Fig. 2E), although the nuclear expression was predominant. Because it is known that the BTB/POZ domain is involved in transcriptional repression (22), we next investigated the transcriptional activity of GZF1 by the luciferase reporter gene assay. The human GZF1 cDNA was fused to the GAL4 DNA-binding domain (Fig. 3A, GAL4BD) in the expression plasmid containing the SR/H9251 promoter, and the resulting construct was transfected into HEK293T cells together with the luciferase reporter plasmid containing five tandem repeats of the GAL4-binding sequence, a binding site for the serum response element (SRE) and the thymidine kinase minimal (TKm) promoter (Fig. 3A) (20). As a consequence, it turned out that expression of GZF1 reduced the luciferase activity by ∼80% as compared with that by expression of the GAL4BD only (Fig. 3C).

It was reported that two conserved charged residues within the BTB/POZ domains of PLZF and Bcl-6 proteins are important for their transcriptionally repressive activity (22). Thus, we replaced the corresponding amino acids (aspartic acid 32 (D32) and lysine 50 (K50)) are indicated by arrowheads. D32 in GZF1 corresponds to D35 in PLZF and D33 in Bcl-6, and K50 in GZF1 corresponds to R49 in PLZF and K47 in Bcl-6 (22). C, transcriptional repressive activity of GZF1. 293T cells were cotransfected with wild-type or mutant GZF1 fused to GAL4BD, pGL3 luciferase reporter plasmid, and pRL-TK. Luciferase activity in cells transfected with the plasmid containing GAL4BD alone was set at 100%, and luciferase activities of cells transfected with the designated effector plasmids were expressed as percentages of control value ± S.E. Each value represents a result of at least three independent experiments.

FIG. 3. Transcriptional repressive activity of GZF1. A, physical map of each effector and reporter construct. The effector plasmid was constructed by ligating GAL4BD in-frame with full-length human GZF1 cDNA. The reporter plasmid contains five tandem repeats of GAL4-binding sites followed by the serum response element (SRE) as an enhancer and a herpes simplex virus thymidine kinase minimal (TKm) promoter. B, sequence alignment of the pocket region in the BTB domains of GZF1, PLZF, and Bcl-6. Two conserved charged residues (aspartic acid 32 (D32) and lysine 50 (K50)) are indicated by arrowheads. D32 in GZF1 corresponds to D35 in PLZF and D33 in Bcl-6, and K50 in GZF1 corresponds to R49 in PLZF and K47 in Bcl-6 (22). C, transcriptional repressive activity of GZF1. 293T cells were cotransfected with wild-type or mutant GZF1 fused to GAL4BD, pGL3 luciferase reporter plasmid, and pRL-TK. Luciferase activity in cells transfected with the plasmid containing GAL4BD alone was set at 100%, and luciferase activities of cells transfected with the designated effector plasmids were expressed as percentages of control value ± S.E. Each value represents a result of at least three independent experiments.

FIG. 4. Immunohistochemical analysis of GZF1 expression in mouse embryonic kidneys. Metanephric kidneys from 13.5–14.5-day embryos were stained with anti-RET or anti-GZF1 antibody. A and C, RET expression in branching ureteric buds of 13.5- and 14.5-day embryonic metanephiroi. B and D, GZF1 expression in 13.5- and 14.5-day metanephiroi. GZF1 was expressed at high levels in some branching ureteric buds of 13.5–14.5-day metanephiroi. A along with C along with D are serial sections of the same metanephiroi. RET and GZF1 expression was detected in the same ureteric buds of metanephiroi. When the staining was performed with protein A-purified normal rabbit IgG instead of the anti-GZF1 antibody as a negative control, no positive staining was observed in each section (data not shown). Bars indicate 100 μm.
charged amino acids for transcriptional repressive activity of GZF1.

To elucidate the importance of GZF1 on kidney development, we stained the mouse embryonic kidney with anti-GZF1 antibody. Interestingly, the ureteric bud epithelia of 13.5–14.5-day embryonic metanephroi were strongly stained (Fig. 4, B and D). Using serial sections, we detected the expression of GZF1 and RET in the same ureteric buds of 13.5–14.5-day metanephroi (Fig. 4, A–D). Both nuclear and cytoplasmic staining of GZF1 were observed in the ureteric buds (Fig. 4, B and D). Like RET, the GZF1 expression significantly decreased in the kidney after birth (data not shown). When immunostaining was performed with protein A-purified rabbit IgG instead of the anti-GZF1 antibody as a negative control, no staining was observed in these tissues (data not shown).

Finally, we investigated whether antisense phosphorothioated ODNs of the GZF1 gene impair the ureteric bud branching in the metanephric organ culture. We designed six antisense ODNs (18–20 mer) were designed for the indicated sequences of mouse GZF1 (AS-1, -2, -3, -4, -5, and -6). B, inhibition of GZF1 expression in Neuro2a mouse neuroblastoma cells. Neuro2a cells were transfected with each of six antisense ODNs (200 nM) using OligofectAMINE reagent. Similarly, sense (SS-4) and scramble (SC-4) ODNs corresponding to the AS-4 sequence were synthesized and transfected into Neuro2a cells. The cells were incubated for 48 h after transfection, and the resulting cell lysates were subjected to Western blotting with anti-GZF1 antibody or anti-α-tubulin antibody; 115-kDa GZF1 and 55-kDa α-tubulin. C, inhibition of ureteric bud branching in the metanephric organ culture. Whole E11.5 mouse kidney explants from ICR mice were cultured for 4 days in the presence of 1.5 μM SS-4, AS-4, or SC-4. In the upper panels, stereomicroscopic findings showed normal renal organogenesis (ureteric bud branching and mesenchymal condensing) in metanephroi treated with SS-4 or SC-4, but marked dysmorphogenesis was observed in metanephroi treated with AS-4. In the lower panels, the ureteric buds were visualized by staining with mouse anti-pan cytokeratin antibody followed by incubation with Cy3-conjugated donkey anti-mouse IgG. Bars indicate 500 μm.

In the past 10 years, a variety of factors including transcription factors and secreted peptides have been reported to be involved in normal development of metanephric kidney (2–4). Genetic evidence has established a crucial role for the GDNF/RET signaling in the ureteric bud development and branching. GDNF that is secreted from the metanephric mesenchyme is essential for correct outgrowth and branching of the ureteric bud in which RET and GFRα1 are expressed (8–13). It was demonstrated that several transcription factors including Pax2, Pax8, Eya-1, and Emx-2 are expressed in nephric duct
and metanephric mesenchyme in spatial and temporal fashions and regulate the GDNF and/or RET expression in the developing kidney (23–26). In addition, vitamin A signaling to the stromal mesenchyme (a third renal cell type) that activates the retinoic acid receptors, Rara and Rarβ2, was required for RET expression in the ureteric bud (17), indicating that there are kidney-specific regulatory mechanisms for the activation of the GDNF/RET signaling system. Moreover, the forkhead/winged helix transcription factor, Foxd1 (27), and the basic helix-loopp helix transcription factor, Pod1 (28), appeared to control the spatially restricted RET expression during renal development. Foxd1−/− and Pod−/− mice showed a transition in RET expression from the normal restricted pattern at tips of the branching ureteric buds to ectopic expression throughout the collecting duct system, resulting in decreased ureteric bud branching and renal hypoplasia. However, what gene expression (as regulated by the GDNF/RET signaling) is required for normal ureteric bud branching has not been elucidated so far. In this study, we identified for the first time a GDNF-inducible gene, GZF1, involved in ureteric bud branching. The facts that RET and GZF1 are highly expressed in the same ureteric buds and that antisense ODNs of GZF1 markedly impaired their branching in the metanephric organ culture strongly supported the view that GZF1 is an important branching factor that functions downstream of the GDNF/RET signaling pathway.

The GZF1 gene contained the BTB/POZ domain as well as zinc finger motifs. It was reported that the BTB/POZ domains were found in 0.6 and 0.8% of total genes in the human and mouse genome, respectively (29). BTB/POZ domain-containing proteins comprise a large and diverse family of factors involved in multiple cellular processes including development and cell growth (30–33), and the BTB/POZ domains appear to be required for dimerization and/or transcription repression of the proteins (22, 34). Using a luciferase reporter gene assay, we further investigated GZF1 functions and identification of the genes in which expression is regulated by GZF1 would promote our understanding of mechanisms of renal branching morphogenesis.

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