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Glial cell line-derived neurotrophic factor activates the receptor tyrosine kinase RET and promotes kidney morphogenesis

(neurotrophin/kidney development)

QUINN C. VEGA*, CAROLYN A. WORBY*, MARK S. LECHNER†‡, JACK E. DIXON*, AND GREGORY R. DRESSLER†‡

Departments of *Biological Chemistry and †Pathology and ‡Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI 48109

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ABSTRACT **The receptor tyrosine kinase RET functions during the development of the kidney and the enteric nervous system, yet no ligand has been identified to date. This report demonstrates that the glial cell line-derived neurotrophic factor (GDNF) activates RET, as measured by tyrosine phosphorylation of the intracellular catalytic domain. GDNF also binds RET with a dissociation constant of 8 nM, and ^{125}I-labeled GDNF can be coimmunoprecipitated with anti-RET antibodies. In addition, exogenous GDNF stimulates both branching and proliferation of embryonic kidneys in organ culture, whereas neutralizing antibodies against GDNF inhibit branching morphogenesis. These data indicate that RET and GDNF are components of a common signaling pathway and point to a role for GDNF in kidney development.**

The c-*ret* protooncogene (1) encodes a member of the receptor tyrosine kinase superfamily that is characterized by an extracellular ligand binding domain, a transmembrane domain, and an intracellular catalytic tyrosine kinase domain (2). In general, binding of ligand induces dimerization of receptor tyrosine kinases followed by autophosphorylation, which is required for kinase activation and signal transduction. The RET protein is expressed in a variety of peripheral and central nervous system tissues during development, including migrating cephalic neural crest cells, cranial ganglia, dorsal root ganglia, the myenteric ganglia of the gut, motor neurons, and the neuroretina (3, 4). RET is required for the migration of enteric neuronal precursor cells to the gastrointestinal tract and for the proliferation and branching of the ureteric bud epithelium during kidney development (5). In humans, RET mutations that inactivate the receptor result in Hirschsprung disease (6), a loss of parasympathetic innervation of the lower intestine, whereas constitutively active RET mutations lead to multiple endocrine neoplasia types 2A (7) and 2B (8) and familial medullary thyroid carcinoma (9). In the developing kidney, RET is expressed at the tips of the ureteric bud (3, 4) and is required for growth and branching morphogenesis but not for kidney mesenchyme differentiation (5). Thus, RET may receive inductive signals from the kidney mesenchyme that regulate local proliferation and directional growth.

The neurotrophic factor, glial cell line-derived neurotrophic factor (GDNF), was first identified by its ability to promote survival of midbrain dopaminergic neurons in culture (10). Recent data demonstrate that GDNF can lead to regeneration of dopamine nerve terminals in the adult that could have a profound influence on Parkinson disease (11). In addition, GDNF also has neurotrophic effects on neonatal and adult facial motor neurons (12, 13); these investigators suggest that GDNF may be useful in the treatment of motor neuron disorders like amyotrophic lateral sclerosis. The GDNF gene encodes a 211 amino acid precursor protein that is processed and secreted as a glycosylated, disulfide linked homodimer (14). GDNF shows 20% sequence identity to members of the transforming growth factor β superfamily. Structurally, GDNF contains a cystine knot motif that is also present in nerve growth factor, platelet-derived growth factor, and type β transforming growth factor (15). This motif consists of six cystines that form three intramolecular disulfide linkages between parallel β-sheets. The expression pattern of GDNF during murine embryogenesis correlates with RET expression and suggests a possible receptor–ligand relationship (16). GDNF is highly expressed in the mouse gut beginning at embryonic day 9 (E9) before migration of the neural crest derived enteric neurons. The expression of GDNF in the kidney is restricted to the undifferentiated mesenchymal cells along the periphery of the developing kidney as early as E11.5.

In this report, a series of experiments examine the function of GDNF in kidney development and show that GDNF can activate RET. Our results demonstrate that GDNF can activate RET in a dose-dependent manner and that GDNF coprecipitates with the RET receptor. GDNF binds RET with a dissociation constant of ≈8 nM. Furthermore, GDNF can stimulate kidney development *in vitro*, a process known to be RET-dependent. The data demonstrate that GDNF and RET are elements of a common signal transduction pathway that regulates important developmental processes in the kidney, enteric nervous system, and endocrine tissues.

MATERIALS AND METHODS

Kidney Organ Cultures and Whole Mount Cytokeratin Staining. Kidneys were cultured on nucleopore filters (1.0 μm) that were suspended above the medium by wire grids as described (17). Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum was used in all assays. B49 (18) conditioned medium was harvested from near confluent dishes and frozen in aliquots. rhGDNF was purchased from Promega. Kidneys were fixed in cold methanol for 10 min, washed in PBST (10 mM sodium phosphate, pH 7.4/150 mM NaCl/0.1% Tween 20), and incubated for 2 h with a pan-cytokeratin monoclonal antibody mixture (Sigma). Kidneys were washed 3 times with PBST and incubated with a fluorescein conjugated anti-mouse secondary antibody. The anti-GDNF neutralizing antibody was a mouse monoclonal IgG (R & D Systems) and did not contain sodium azide or thimerasol. Organ cultures were examined after 3 or 4 days and the number of ureter terminal buds were counted. Only embryos from the same litter were compared for each experiment to control for slight differences in gestational times. Statistical analysis was done by calculating the *t* values and degrees of freedom using the Student's *t* test for two independent variables with different variance.

Cell Culture and Transient Transfection. LA-N-5 cells were grown in an 8% CO_2/92% air environment using DMEM

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Abbreviations: GDNF, glial cell line-derived neurotrophic factor; hrGDNF, human recombinant GDNF; E, embryonic day; HA, hemagglutinin; EGF, epidermal growth factor.

containing 15% fetal bovine serum (FBS). COS cells were grown in a 5% CO_2/95% air environment using DMEM with 10% FBS. Subconfluent COS cells in 10-cm dishes were transfected with 8 μg of the pRET-HA construct (19) using LipofectAMINE reagent as described by the manufacturer (Life Technologies, Grand Island, NY).

Immunoprecipitation and Western Blot Analyses. The RET polyclonal antibody was raised in rabbits (Cocalico, Reamstown, PA) by expressing the cytoplasmic domain of human RET (amino acids 718-1114) fused to glutathione *S*-transferase. LA-N-5 cells were plated onto 10-cm plates; cells were treated with ligand for 1–2 min, washed with phosphate-buffered saline (PBS), and lysed in PBS, 1% Triton X-100, 100 μM sodium vanadate, and 0.1 mg/ml phenylmethylsulfonyl fluoride. Extracts were precleared with pansorbin A (Calbiochem), incubated with RET antibody (1:200) for 2 h on ice followed by incubation with protein A agarose (GIBCO/BRL). The protein A agarose beads were washed 4 times with lysis buffer and the beads were boiled in Laemmli sample buffer. Samples were run on a 7% polyacrylamide gel, transferred to Immobilon (Millipore), and immunoblotted with hemagglutinin (HA) antibody (1:5000) (Boehringer Mannheim), phosphotyrosine antibody (1:10,000) (Upstate Biotechnology, Lake Placid, NY) in Tris-buffered saline (TBS), 0.1% Tween 20, and 2% BSA or a commercial RET antibody (1:50) (Santa Cruz Biotechnology) in TBST and 5% nonfat dry milk. For both immunoblots, secondary antibody conjugated to horseradish peroxidase (GIBCO/BRL) was used at a 1:10,000 dilution and the signal visualized by Enhanced Chemiluminescense (Amersham) using recommended protocols.

Coimmunoprecipitation and Equilibrium-Binding Analyses. For GDNF and RET association studies, GDNF was iodinated by the chloramine-T method (20) to a specific activity of 8×10^7 cpm/μg. For analysis of direct binding, LA-N-5 cells were incubated for 5 min in the presence or absence of 200 nM unlabeled GDNF followed by a 30-min incubation with 2 nM labeled GDNF at room temperature. RET was immunoprecipitated and the samples were run on an SDS/15% polyacrylamide gel. For the equilibrium-binding analysis, LA-N-5 cells were split into 12-well plates at least 24 h before the binding studies were performed. ^{125}I-labeled GDNF (8×10^4 cpm) was added to the cells in the presence of increasing amounts of unlabeled GDNF (0.25–50 nM) in DMEM containing 15% fetal calf serum in an 8% CO_2/92% air room temperature incubator. After 30 min, cells were washed four times with ice-cold PBS and 0.5 ml of 0.5 M NaOH added to the wells. The resulting solution was counted in a gamma counter. Data represent the average of triplicate points ± SD and are represented as percent specific binding where total binding was 23.9% of input counts and nonspecific binding was 9.3% of input counts. Nonspecific binding was determined by the addition of 500-fold molar excess of unlabeled GDNF to ^{125}I-labeled GDNF.

RESULTS

The ability of GDNF to affect ureteric bud branching was assayed by excising E11.5 day kidney rudiments and culturing them for 3 and 4 days with exogenous GDNF. At E11.5, the ureteric bud has induced the metanephric mesenchyme and exhibits a single bifurcation with mesenchymal cells condensed around both tips of the T-shaped ureteric bud. Initial experiments used conditioned media from B49 cells (18), a source of secreted GDNF. Development of the kidney cultures was examined by staining whole rudiments with a pan-cytokeratin antibody mixture to clearly visualize the ureteric bud and its branch points. The total numbers of ureteric bud tips were counted after 3 or 4 days in culture (Table 1). Control kidneys cultured in medium alone averaged 33 tips (33.2 ± 6.7) (Fig. 1*A*), whereas the kidneys cultured in B49 conditioned media were significantly larger ($P < 0.01$; Fig. 1*B*), with a 44% and 60% increase in the number of ureter bud tips for experiments 1 and 2, respectively. In B49-treated cultures, new buds were observed throughout the tissue, not just on the terminal ends of the major branches. Addition of 20 μg/ml of mouse monoclonal anti-GDNF antibody to the B49-conditioned media reduced the growth stimulatory effect by 50% (Table 1, experiment 2). Note that for experiment 2 a different batch of B49 media was used and the embryos were slightly older by ≈4 h. Subsequent experiments used human recombinant (hr) GDNF in DMEM or 10 μg/ml of the neutralizing anti-GDNF antibody in DMEM. Neutralizing antibodies have been used extensively to define the roles of other growth factors in kidney organ cultures (21–23). Kidneys cultured in the presence of anti-GDNF antibody were significantly smaller relative to controls with ≈33% fewer ureter bud tips after 3 days ($P < 0.01$; Table 1 and Fig. 1*C*). Kidneys cultured with 50 ng/ml rhGDNF for 3 days were larger relative to controls with ≈20% more ureter bud tips ($P < 0.08$; Table 1 and Fig. 1*D*). However, the hrGDNF-treated kidneys did not branch as extensively as the kidneys treated with B49-conditioned media. After 4 days in culture, hrGDNF significantly ($P < 0.05$) increased the ureteric terminal bud number by 20%, whereas the neutralizing antibody reduced growth by 25% ($P < 0.05$). These data suggest that GDNF can mediate growth of the embryonic kidney in culture.

A close comparison of the expression patterns of GDNF and RET demonstrate that they are expressed in a coordinate fashion in the developing kidney. In addition, the RET knockout mouse displays extensive problems in kidney development (5). These observations in conjunction with our results mentioned above suggested to us that GDNF and RET may function as partners in a specific signaling paradigm.

To test the ability of GDNF to interact with and activate RET, an antibody capable of immunoprecipitating RET was generated. Antibodies raised against the RET kinase domain or to a HA epitope tag, but not to the preimmune serum, were able to precipitate an epitope-tagged RET from transfected COS cells (Fig. 2*a*). Specificity of the antibody for RET was also confirmed by immunoblotting plasma membranes isolated from RET-HA-transfected or mock-transfected COS cells. Immunoblots of plasma membranes showed that the RET antibody recognized a 170-kDa band in RET-HA-transfected cells, whereas no signal was evident in the untransfected cells (data not shown). Efforts to demonstrate that GDNF induced

Table 1. Extent of ureteric bud branching in embryonic kidney cultures

Experiment (h)	DMEM	B49 (*P* value)	rhGDNF (*P* value)	α-GDNF (*P* value)
1 (72)	33.2 ± 6.7	47.7 ± 8.4 (<0.01)		
2 (72)	34.5 ± 4.2	55.8 ± 14.7 (<0.05)		45.3 ± 9.2 (<0.02)
3 (72)	28.2 ± 3.3		36.2 ± 9.6* (<0.1)	
4 (72)	30.8 ± 5.2		35.6 ± 3.0† (<0.08)	21.0 ± 3.0 (<0.01)
5 (96)	41.7 ± 3.2		49.0 ± 6.0† (<0.05)	33.0 ± 2.9 (<0.05)

Averages ± one SD, $n = 6$.
*10 ng/ml.
†50 ng/ml.

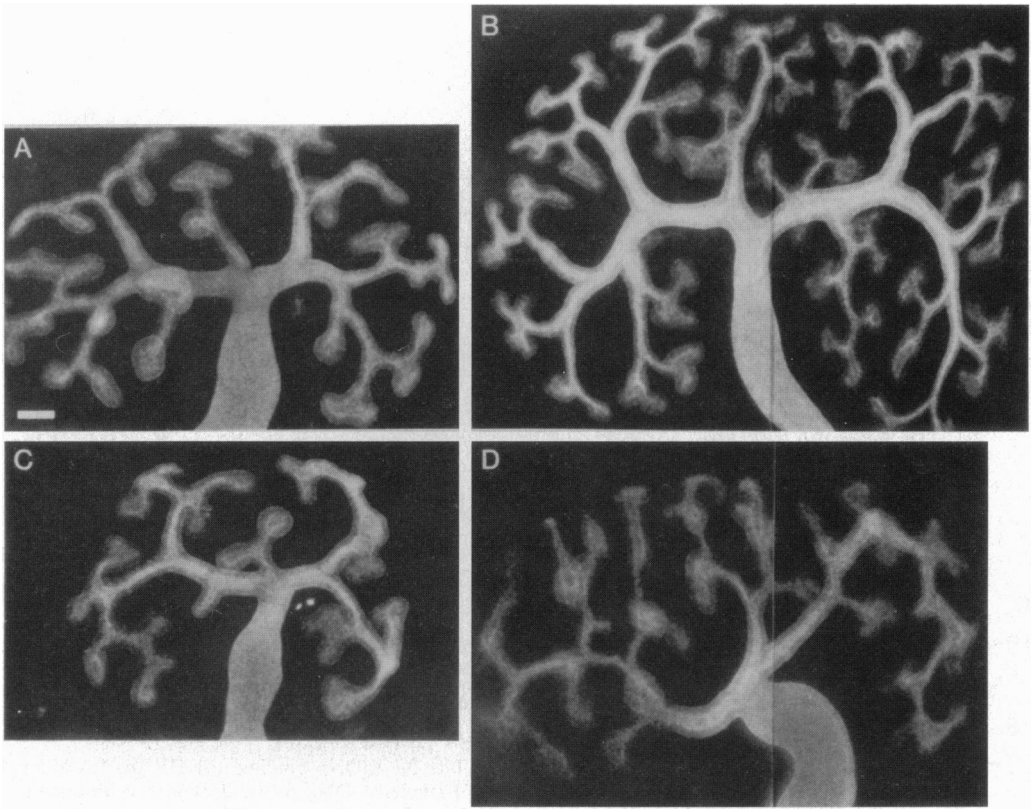

FIG. 1. Whole mount cytokeratin staining of embryonic kidney organ cultures. E11.5 day mouse kidney rudiments were cultured for 3 days in control media (*A*), B49 conditioned media (*B*), 10 μg/ml of anti-GDNF neutralizing antibody (*C*), and 50 ng/ml of rhGDNF (Promega) (*D*). For each experiment, kidneys from a single litter were compared with control for slight variations in gestation times. Representative micrographs are shown for each culture condition. (Bar = 100 μm for all panels.)

phosphorylation of the RET receptor in COS cells were hampered by the high levels of autophosphorylation of the receptor in the absence of ligand.

Since there are no cell lines corresponding to the proliferating ureteric bud, activation experiments used a neuroblastoma cell line (LA-N-5) that expresses high endogenous levels of RET mRNA (24). Treatment with increasing levels of GDNF resulted in a dose-dependent increase in RET tyrosine phosphorylation (Fig. 2*b*). There was no apparent change in RET tyrosine phosphorylation in response to treatment with 1 or 5 ng/ml GDNF but tyrosine phosphorylation increased significantly with 10 ng/ml GDNF treatment. Treatment with higher concentrations of GDNF also lead to a dramatic increase in RET tyrosine phosphorylation. The induction of RET tyrosine phosphorylation was specific for GDNF since no increase in RET tyrosine phosphorylation was observed with activin, nerve growth factor, or epidermal growth factor (EGF) treatment (Fig. 2*c*).

GDNF-dependent RET tyrosine phosphorylation could occur by either direct or indirect activating mechanisms. To show a direct association, RET was immunoprecipitated from LA-N-5 cells that had been incubated with ^{125}I-labeled GDNF (Fig. 3*a*). Labeled GDNF coimmunoprecipitated with RET in the absence of cold competitor. However, no ^{125}I-labeled GDNF was precipitated from LA-N-5 cells incubated with an excess of unlabeled GDNF or when preimmune serum was used for the immunoprecipitation. After demonstrating an association between RET and GDNF, binding affinity of GDNF was examined in LA-N-5 cells. LA-N-5 cells were incubated either in the presence of increasing amounts of ^{125}I-labeled GDNF or in the presence of a constant amount of ^{125}I-labeled GDNF and increasing unlabeled competitor. Scatchard analysis of LA-N-5 cells treated with increasing amounts of ^{125}I-labeled GDNF shows that LA-N-5 cells bind GDNF with a dissociation constant (K_d) of 8.2 ± 1.9 nM and that there are approximately 100,000 receptors per cell (data not shown). Competition studies revealed that addition of increasing levels of cold GDNF competed with ^{125}I-labeled GDNF binding to LA-N-5 cells at a K_d of ≈7 nM (Fig. 3*b*). This dissociation constant is similar to the K_d determined by Trupp *et al.* (25) in neuronal cells. GDNF competition was specific since EGF, at equivalent concentrations, did not compete with ^{125}I-labeled GDNF for binding.

DISCUSSION

The data indicate that GDNF activates the RET receptor as measured by tyrosine phosphorylation. GDNF stimulates tyrosine phosphorylation of the RET protein in neuroblastoma cells, whereas other related growth factors cannot. This is particularly striking since GDNF is normally placed in the transforming growth factor β superfamily. Members of this family do not signal through receptor tyrosine kinases, but appear to signal via a family of receptor serine kinases that are heterodimers with high-affinity binding requiring both a type I and type II subunit (26).

We have demonstrated that unlabeled GDNF but not EGF can compete with labeled GDNF for binding to LA-N-5 cells and that labeled GDNF can coimmunoprecipitate with RET. A binding constant of 8 nM is higher than similar constants noted for the various TRK receptors and their corresponding neurotrophins (27, 28). In addition, although our coimmunoprecipitation experiments demonstrate that there is a direct interaction between RET and GDNF, they do not exclude the possibility that an accessory protein could be an essential component of the RET/GDNF complex. The presence of an additional protein in the RET/GDNF complex could also mediate high-affinity binding. Other receptor subclasses such

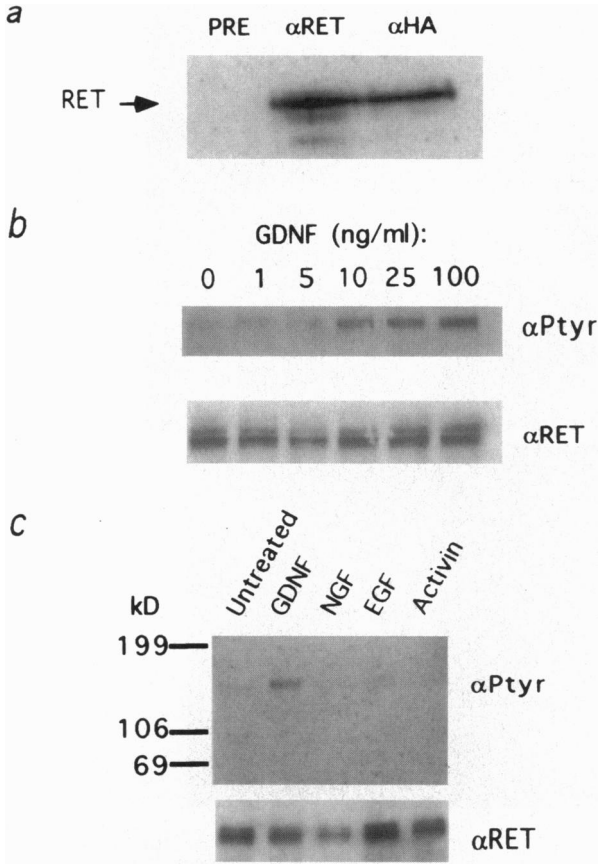

FIG. 2. RET tyrosine phosphorylation in response to GDNF. (*a*) HA immunoblot of RET-HA transfected COS cells immunoprecipitated with rabbit pre-immune serum, anti-GST-RET, or an antibody specific for HA. (*b*) LA-N-5 cells incubated with increasing amounts of GDNF, immunoprecipitated with anti-RET antibody, and immunoblotted with either phosphotyrosine (*Upper*) or an anti-RET specific antibody (*Lower*). (*c*) Same as *b* except that cells were treated with 50 ng/ml EGF, nerve growth factor, GDNF, or activin.

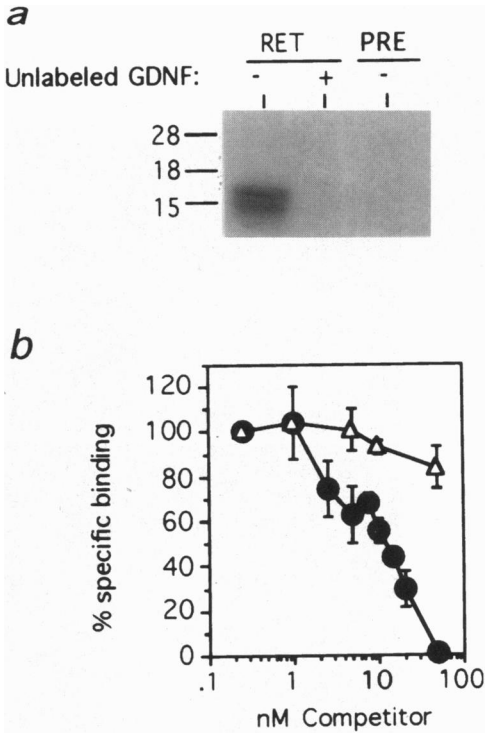

FIG. 3. GDNF binding to RET. (*a*) LA-N-5 cells incubated with ^{125}I-labeled GDNF in the presence or absence of unlabeled GDNF followed by immunoprecipitation with RET specific antibodies or preimmune serum. (*b*) Competition of ^{125}I-labeled GDNF binding to LA-N-5 cells with increasing amounts of unlabeled GDNF (●) or unlabeled EGF (△).

as the interleukin 6-type cytokine receptors have been shown to associate with an additional membrane protein, such as gp130 (29).

The effect of GDNF on ureteric bud branching *in vitro* is consistant with RET activation and with classical experiments demonstrating that mesenchyme provides signals for epithelial branching and growth (30, 31). GDNF-treated kidneys exhibit more pronounced branching morphogenesis, with more end points and longer branches. Conditioned media from B49 cells were more effective than recombinant human GDNF. Upon the addition of GDNF-neutralizing antibodies to B49 media, stimulation was reduced by 50% (data not shown), suggesting that much of the growth promoting effect present in B49 is due to GDNF. The decreased biological activity of hrGDNF may reflect the lack of glycosylation (14), resulting in decreased affinity and/or activity in the organ culture assay. Alternatively, there may be additional factors present in the B49 media that augment the activity of GDNF or stimulate branching independently. Other growth factors, such as hepatocyte growth factor, insulin-like growth factor I, and insulin-like growth factor II (21–23), are thought to regulate kidney development based on *in vitro* organ culture experiments. However, mutations in their respective receptors, c-met (32) and IGFr (33), do not exhibit specific kidney defects. The GDNF/RET signaling pathway is clearly required for ureteric bud growth, as both RET (5) and GDNF (34–36) mutations have similar renal agenesis phenotypes.

The RET signaling pathway is clearly required for development of the enteric nervous system in the intestinal tract and the kidney (5). Aberrant RET signaling can also lead to a variety of neoplasms in endocrine tissues (7–9). The expression patterns in the developing kidney and the mutant phenotypes of the knockout mice suggest that RET and GDNF are elements of a common signaling pathway that is active during branching morphogenesis of the ureteric bud. During kidney development, the ureteric bud must invade the mesenchyme to induce nephrogenesis and must then continue to grow in a radial manner such that new nephrons are induced along the periphery of the organ. In the absence of surrounding mesenchyme, the ureteric bud epithelium quickly degenerates *in vitro*. The epithelium can be rescued by recombination with metanephric mesenchyme but not with heterologous mesenchyme. To precisely regulate this growth, RET is expressed first along the entire ureteric bud and finally on the tips of the growing ureter buds (3, 4), whereas GDNF is restricted to the most distal mesenchyme (16). Repression of GDNF after epithelial conversion may thus limit the amount of ureter bud branching in the more proximal regions of the organ such that the radial patterning is preserved. This report also demonstrates an essential function for GDNF in branching morphogenesis through RET signaling. We can now examine the signaling mechanism alluded to in classical tissue recombination studies using modern molecular tools. In addition, the role of GDNF in diseases previously associated with RET can also be addressed.

Note Added in Proof. Since this paper was submitted, Trupp *et al.* (37) and Jing *et al.* (38) have also shown RET activation induced by GDNF.

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1. Takahashi, M. & Cooper, G. M. (1987) *Mol. Cell. Biol.* **7,** 1378–1385.
2. Ullrich, A. & Schlessinger, J. (1990) *Cell* **61,** 203–212.
3. Avantaggiato, V., Dathan, N. A., Grieco, M., Fabien, N., Lazzaro, D., Fusco, A., Simeone, A. & Santoro, M. (1994) *Cell Growth Differ.* **5,** 305–311.
4. Pachnis, V., Mankoo, B. & Constantini, F. (1993) *Development (Cambridge, U.K.)* **119,** 1005–1017.
5. Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Constantini, F. & Pachnis, V. (1994) *Nature (London)* **367,** 380–383.
6. Romeo, G., Ronchetto, P., Luo, Y., Barone, V., Seri, M., Ceccherini, I., Pasini, B., Bocciardi, R., Lerone, M., Kaariainen, H. & Martucciello, G. (1994) *Nature (London)* **367,** 377–378.
7. Mulligan, L. M., Kwok, J. B. J., Healey, C. S., Elsdon, M. J., Eng, C., Gardner, E., Love, D. R., Mole, S. E. M., Moore, J. K., Papi, L., Ponder, M. A., Telenius, H., Tunnacliffe, A. & Ponder, B. A. J. (1993) *Nature (London)* **363,** 458–460.
8. Hofstra, R., Landsrater, R. M., Ceccherini, I., Stulp, R. P., Stelwagen, T., Luo, Y., Pasini, B., Hoppener, J. W. M., Ploos van Amstel, H. K., Romeo, G., Lips, C. J. M. & Buys, C. H. C. M. (1994) *Nature (London)* **367,** 375–376.
9. Mulligan, L. M., Eng, C., Healey, C. S., Clayton, D., Kwok, J. B. J., Gardner, E., Ponder, M. A., Frilling, A., Jackson, C. E., Lehnert, H., Neumann, H. P. H., Thibodeau, S. N. & Ponder, B. A. J. (1994) *Nat. Genet.* **6,** 70–74.
10. Lin, L. F., Doherty, D. E. H., Lile, J. D., Bektes, S. & Collins, F. (1993) *Science* **260,** 1130–1132.
11. Tomac, A., Lindquist, E., Lin, L., Ogren, S., Young, D., Hoffer, B. & Olson, L. (1995) *Nature (London)* **373,** 335–339.
12. Oppenheim, R., Hovenou, L., Johnson, J., Lin, L., Li, L., Lo, A., Newsome, A., Prevette, D. & Wang, S. (1995) *Nature (London)* **373,** 344–346.
13. Yan, Q., Matheson, C. & Lopez, O. T. (1995) *Nature (London)* **373,** 341–344.
14. Lin, L.-F. H., Zhang, T. J., Collins, F. & Armes, L. G. (1994) *J. Neurochem.* **63,** 758–768.
15. McDonald, N. Q. & Hendrickson, W. A. (1993) *Cell* **73,** 421–424.
16. Hellmich, H. L., Kos, L., Cho, E. S., Mahon, K. A. & Zimmer, A. (1996) *Mech. Dev.* **54,** 95–106.
17. Rothenpieler, U. W. & Dressler, G. R. (1993) *Development (Cambridge, U.K.)* **119,** 711–720.
18. Schubert, D. A., Heinemann, S., Carlisle, W., Tarikas, H., Kimes, B., Patrick, J., Steinboch, J. H., Culp, W. & Brandt, B. L. (1974) *Nature (London)* **249,** 224–227.
19. Liu, X., Vega, Q. C., Decker, R. A., Pandey, A., Worby, C. A. & Dixon, J. E. (1996) *J. Biol. Chem.* **271,** 5309–5312.
20. Kuo-Jang, K. & Ramirez, V. D. (1978) *J. Endocrinol. Invest.* **1,** 233–238.
21. Woolf, A. S., Kolatsi-Joannou, M., Hardman, P., Anderwarchen, E., Moorby, C., Fine, L. G., Jat, P. S., Noble, M. D. & Gherardi, E. (1995) *J. Cell Biol.* **128,** 171–184.
22. Rogers, S. A., Ryan, G. & Hammerman, M. R. (1991) *J. Cell Biol.* **113,** 1447–1453.
23. Rogers, S. A., Ryan, G., Purchio, A. F. & Hammerman, M. R. (1993) *Am. J. Physiol.* **264,** F996–F1002.
24. Bunone, G., Borrello, M. G., Picetti, R., Bongarzone, I., Peverali, F. A., de Franciscis, V., Della Valle, G. & Pierotti, M. A. (1995) *Exp. Cell. Res.* **217,** 92–99.
25. Trupp, M., Ryden, M., Jornvall, H., Funakoshi, H., Timmusk, T., Arenas, E. & Ibanez, C. F. (1995) *J. Cell Biol.* **130,** 137–148.
26. Mathews, L. S. (1994) *Endocr. Rev.* **15,** 310–325.
27. Klein, R., Jing, S., Nanduri, V., O'Rourke, E. & Barbacid, M. (1991) *Cell* **65,** 189–197.
28. Lamballe, F., Klein, R. & Barbacid, M. (1991) *Cell* **66,** 967–979.
29. Baumann, H., Symes, A. J., Comeau, M. R., Morella, K. K., Wang, Y., Friend, D., Ziegler, S. F., Fink, J. S. & Gearing, D. P. (1994) *Mol. Cell. Biol.* **14,** 138–146.
30. Grobstein, C. (1953) *Science* **118,** 52–55.
31. Grobstein, C. (1953) *J. Exp. Zool.* **130,** 319–339.
32. Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A. & Birchmeier, C. (1995) *Nature (London)* **376,** 768–771.
33. Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J. & Efstratiadis, A. (1993) *Cell* **75,** 59–72.
34. Sanchez, M. P., Silos-Santiago, I., Frisen, J., He, B., Lira, S. A. & Barbacid, M. (1996) *Nature (London)* **382,** 70–73.
35. Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A. C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J., Sariola, H. & Westphal, H. (1996) *Nature (London)* **382,** 73–76.
36. Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver-Moore, K. & Rosenthal, A. (1996) *Nature (London)* **382,** 76–79.
37. Trupp, M., Arenas, E., Fainzilber, M., Nilsson, A.-S., Sieber, B.-A., Grigoriou, M., Kilkenny, C., Salazar-Grueso, E., Pachnis, V., Arumae, U., Sariola, H., Saarma, M. & Ibancz, C. F. (1996) *Nature (London)* **381,** 785–789.
38. Jing, S., Wen, D., Yu, Y., Hoist, P. L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., Louis, J.-C., Hu, S., Attrock, B. W. & Fox, G. M. (1996) *Cell* **85,** 1113–1124.