Gliol cell line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins


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ABSTRACT Glial cell line-derived neurotrophic factor (GDNF)-dependent activation of the tyrosine kinase receptor RET is necessary for kidney and enteric neuron development, and mutations in RET are associated with human diseases. Activation of RET by GDNF has been shown to require an accessory component, GDNFR-α (RETL1). We report the isolation and characterization of rat and human cDNAs for a novel cell-surface associated accessory protein, RETL2, that shares 49% identity with RETL1. Both RETL1 and RETL2 can mediate GDNF dependent phosphorylation of RET, but they exhibit different patterns of expression in fetal and adult tissues. The most striking differences in expression observed were in the adult central and peripheral nervous systems. In addition, the mechanisms by which the two accessory proteins facilitate the activation of RET by GDNF are quite distinct. In vitro binding experiments with soluble forms of RET, RETL1 and RETL2 demonstrate that while RETL1 binds GDNF tightly to form a membrane-associated complex which can then interact with RET, RETL2 only forms a high affinity complex with GDNF in the presence of RET. This strong RET dependence of the binding of RETL2 to GDNF was confirmed by FACS analysis on RETL1 and RETL2 expressing cells. Together with the recent discovery of a GDNF related protein, neurturin, these data raise the possibility that RETL1 and RETL2 have distinctive roles during development and in the nervous system of the adult. RETL1 and RETL2 represent new candidate susceptibility genes and/or modifier loci for RET-associated diseases.

The RET protooncogene encodes a receptor tyrosine kinase that is expressed in a variety of tissues during development including the peripheral and central nervous systems and the kidney. Analysis of ret null mice has defined ret as critical for the migration and innervation of enteric neurons to the hindgut and for proliferation and branching of the ureteric bud epithelium during kidney development (1). In humans, mutations in RET can engender at least four different disease phenotypes (2–4). Somatic rearrangements of RET which result in receptor activation are associated with papillary thyroid carcinoma and germline activating mutations of RET are linked to the cancer syndromes multiple endocrine neoplasia type 2A and 2B ( MEN2A and MEN2B), Familial Hirschsprung disease (HSCR), which is characterized by a lack of enteric nerve innervation to the hindgut, can arise from mutations in the endothelin pathway or in RET.

The search for a key component of the RET signaling pathway, the RET ligand, has been an area of intensive research. Recently, it has been shown that mice null for the gene encoding glial cell line-derived neurotrophic factor (GDNF) manifest a phenotype similar to that of mice null for ret (5–7). Both GDNF and ret null mice exhibit renal agenesis or severe dysgenesis and lack enteric neurons. The latter phenotype is reminiscent of human HSCR (1, 5–7). The similarity in the mutant phenotypes implied that GDNF and ret act in the same pathway. Initial experiments confirmed that GDNF could activate RET on cells, but a direct physical interaction between GDNF and RET was not demonstrated (8–10). Recent studies have shown that GDNF-dependent RET signaling requires a cell-surface-associated accessory protein, GDNFR-α. GDNFR-α binds GDNF to form a stable complex that can activate RET (11, 12). No binding of GDNFR-α to RET was seen in the absence of GDNF.

Using a direct expression cloning strategy, we have isolated a cDNA for GDNFR-α (RETL1) by its ability to interact with the extracellular domain of RET, demonstrating for the first time that a direct interaction between GDNF-α and RET can be observed. We also report the identification of a novel cell surface protein, RETL2, which can mediate GDNF-dependent phosphorylation of RET. Human RETL2 shares 49% identity with human RETL1 but functions in a mechanistically distinct way. In contrast to RETL1, RETL2 can only bind GDNF with high affinity in the presence of RET. RETL1 and RETL2 display different expression patterns that could account for some of the tissue-specific phenotypic differences observed in human disease patients carrying RET mutations. RETL1 and RETL2 may represent new candidate susceptibility or modifying genes for RET-associated diseases.

MATERIALS AND METHODS

RET Fusion Proteins. A cDNA encoding the extracellular domain of rat c-ret was isolated using the reverse transcription–PCR method. Poly(A) selected RNA from the day 14 embryonic rat kidney was converted to cDNA using avian myeloblastosis virus reverse transcriptase and amplified using Taq polymerase in a standard PCR with oligomers kid-013 (nucleotides 150–169 of GenBank sequence X15262; human c-ret) and kid-015 (complement of nucleotides 1894–1914 of GenBank sequence X67812; murine c-ret). The resulting PCR fragment was cloned and sequenced and found to encode the extracellular domain of rat RET, which exhibits 92% identity to murine RET (data not shown). To generate a rat RET–Ig fusion protein, a DNA fragment encoding amino acids 1–657

Abbreviations: GDNF, glial cell line-derived neurotrophic factor; GDNFR-α, GDNF receptor-α; HSCR, Hirschsprung disease; MEN2A and -2B, multiple endocrine neoplasia type 2A and 2B; AP, alkaline phosphatase; HRP, horseradish peroxidase; EBNA, Epstein–Barr virus-encoded nuclear antigen; FACS, fluorescence-activated cell sorter, PI, phosphatidylinositol.

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of rat RET was ligated to a fragment containing the Fc domain of human IgG1 and cloned into the Biogen expression vector pMDR901 to generate plasmid pJC022. Plasmid pJC022 was transfected into Chinese hamster ovary cells to generate a stable cell line producing the fusion protein. Clarified conditioned medium from the cell line was loaded by gravity directly onto Protein A Sepharose (Pharmacia). The column was washed with five column volumes each of PBS, PBS containing 0.5 M NaCl, and 25 mM sodium phosphate, 100 mM NaCl (pH 5.0). The bound protein was eluted with 25 mM Na2HPO4, 100 mM NaCl (pH 2.8) and immediately neutralized with 1/10 volume fraction of 0.5 M Na2HPO4 (pH 8.6). A plasmid encoding a fusion protein between the extracellular domain of rat RET and placental alkaline phosphatase (AP) was constructed as described (13) in the CH269 expression vector (described below) and expressed in 293-Epstein–Barr virus-encoded nuclear antigen (EBNA) cells. Conditioned medium was produced and used without further purification. SDS/PAGE analysis of the rat RET–AP fusion protein indicated a size consistent with its predicted molecular weight and gel filtration analysis indicated that it was produced as a dimer.

Expression Cloning. A cDNA library was prepared from Wistar rat day 18 embryonic kidney mRNA in vector CH269 (derived from the Invitrogen vector, pCEP4, by excising the EBNA-1 gene), containing approximately 1 × 10⁸ clones with an average insert size of 1.5 kb. Pools of 5000 colonies from the library were generated; part of the culture was used to make DNA. DNA was purified using Qiagen (Chatsworth, CA) Qiafilter cartridges and Qiagen plasmid midi kits. Used to make DNA. DNA was purified using Qiagen (Chatsworth, CA) Qiafilter cartridges and Qiagen plasmid midi kits.

Screening of the library was performed essentially as described by Cheng and Flanagan (13), except for modifications described below. DNAs from 256 pools were individually transfected into 293-EBNA cells (8 × 10⁶ cells on a 60-mm Biocoat plate from Collaborative Biomedical Products, Bedford, MA) using lipofectamine (Becton Dickinson, San Jose, CA) Qiafilter cartridges and Qiagen plasmid midi kits. After 48 hr, the cells were washed with 0.5 mg/ml BSA, 20 mM Hepes (pH 7.0) and 0.1% NaCl and incubated with 20 µg/ml rat RET–Ig in Tris-buffered saline plus 1 mM MgCl₂ and CaCl₂ for 60–90 min at room temperature. Following this incubation, the cells were washed four times with 0.5 mg/ml BSA, 20 mM Hepes (pH 7.0), and 0.1% NaCl and then fixed with 60% acetone/3% formaldehyde/20 mM Hepes (pH 7.0) for 30 sec. Following two washes with HBS buffer (150 mM NaCl/20 mM Hepes, pH 7.0), the cells were incubated with an AP-coupled secondary antibody [goat F(ab’2) anti-human IgG (Jackson ImmunoResearch)]. Signals were visualized with biotinylated horseradish peroxidase (HRP) and avidin (Pierce) and the enhanced chemiluminescence detection system (Amersham).

RETL1 clone, 86–17. The human RETL2 clone, DSW240, was identified. DNA sequence analysis of both clones revealed the same open reading frame encoding a protein of 468 amino acids (referred to as RETL1). A human RETL1 clone, GJ107, was isolated from a human embryonic kidney library obtained from Clontech, using a probe from the rat

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generated. To assess the binding of RET–Ig to these cells, the cells were washed with PBS and removed from the flask with 5.0 mM EDTA in PBS. Cells (2 × 10^5) were incubated with rat RET–Ig (final concentration of 20.0 μg/ml) in 0.1 ml FAC buffer (PBS/BSA/0.1% NaN₃) supplemented with 1 mM CaCl₂ and 1 mM MgCl₂ for 1 hr at room temperature, with or without 0.25 μg/ml recombinant human GDNF (R & D Systems). The cells were washed once with FAC buffer, resuspended in 0.2 ml FAC buffer, and read in the FACSCAN. The binding of GDNF to these cells was assessed using essentially the same procedure but in the absence of RET–Ig. GDNF was detected using a murine mAb to GDNF (Promega; final concentration 20.0 μg/ml) for 30 min at room temperature, followed by cleavage with PI-specific phospholipase C, confirmed that RETL1 can be tethered to the cell via a PI linkage (data not shown) (11, 12). A DNA clone, GJ107, encoding the human homolog of RETL1, was isolated from a human embryonic kidney library. The human protein is 93.3% identical to that of the rat (Fig. 1). Subsequent to our identification of RETL1, two groups reported the isolation of rat cDNAs encoding RETL1 that they obtained as a result of their efforts to clone a receptor for the neurotrophic factor GDNF (11, 12). They named the protein GDNFR-a because it binds to GDNF with high affinity. Jing et al. (11) also report the sequence of the human protein, which differs from our human sequence by two single amino acids and a five-amino acid insertion.

The peptide sequence of rat RETL1 was used to search the GenBank database with the program BLAST (19) to identify related proteins. Two significant matches were obtained. One was with GenBank accession no. R02249, a 229-bp expressed sequence tag (EST) from a combined human fetal liver and spleen cDNA library, and the other was with GenBank accession no. H12981, a 521-bp EST from a human infant brain

RESULTS

Cloning of cDNAs for RETL1 and RETL2. An expression cloning strategy was used to identify proteins that interact with the extracellular domain of RET. This was accomplished using a soluble form of rat RET containing the RET extracellular domain fused to the hinge and CH2 and CH3 regions of the human IgG1 heavy chain. The purified rat RET–Ig fusion protein was active in a fetal kidney organ culture assay (14, 15), where RET is essential for the growth and branching of the ureteric bud epithelium that gives rise to the collecting ducts. A clear reduction in the overall size and the degree of branching of the collecting ducts was seen in RET–Ig treated kidneys (data not shown), indicating that the fusion protein blocked RET signaling. The RET–Ig fusion protein was next used in an expression cloning approach to screen a rat day 18 embryonic kidney cDNA library for potential RET binding proteins (see Materials and Methods). A single gene product of 468 amino acids was identified that we refer to as RETL1 because of its ability to bind RET. Subsequent testing revealed that an essential part of the expression cloning protocol was a fixing step. Without the fixing step, no binding of RET–Ig to the RETL1 transfected cells could be observed, suggesting that the interaction between RET–Ig and RETL1 was of relatively low affinity.

RETL1 contains a signal sequence, 31 cysteines, two potential N-linked glycosylation sites, and a hydrophobic C-terminal, indicating that it may be linked to the cell via a phosphatidylinositol (PI) linkage (Fig. 1). Transient expression of the RETL1 cDNA clone, 86–17, in 293-EBNA cells, followed by cleavage with PI-specific phospholipase C, confirmed that RETL1 can be tethered to the cell via a PI linkage (data not shown) (11, 12). A DNA clone, GJ107, encoding the human homolog of RETL1, was isolated from a human embryonic kidney library. The human protein is 93.3% identical to that of the rat (Fig. 1). Subsequent to our identification of RETL1, two groups reported the isolation of rat cDNAs encoding RETL1 that they obtained as a result of their efforts to clone a receptor for the neurotrophic factor GDNF (11, 12). They named the protein GDNFR-a because it binds to GDNF with high affinity. Jing et al. (11) also report the sequence of the human protein, which differs from our human sequence by two single amino acids and a five-amino acid insertion.

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![Fig. 1](image-url) Comparative analysis of RETL1 and RETL2 protein sequences. Alignment of rat and human RETL1 and RETL2 predicted protein sequences. Residues conserved in all four sequences are boxed. Sequences were aligned using the CLUSTAL method (16).
cDNA library. The two ESTs share 99% identity in a region of overlap, indicating that they are from the same cDNA. A cDNA clone corresponding to these EST sequences was isolated from a human fetal liver cDNA library and found to contain an ORF encoding a protein of 464 amino acids, which we refer to as RETL2 (Fig. 1). The human RETL2 protein is 49.1% identical to human RETL1. It shares in common with human RETL1 a hydrophobic N terminus indicative of a signal sequence, and a hydrophobic C terminus indicative of a phophatidylinositol glycan linkage motif. In addition, 30 cysteines out of the 31 that are present in each protein are conserved. We have also isolated a cDNA for rat RETL2; the protein is 94.6% identical to human RETL2 (Fig. 1).

**Functional Analysis of RETL1 and RETL2.** To evaluate whether RETL1 and RETL2 can facilitate GDNF dependent phosphorylation of RET, NB41A3 cells were transfected with RETL1 and RETL2, exposed to GDNF, and analyzed for tyrosine phosphorylation of RET. As shown in Fig. 2, treatment of vector transfected cells with GDNF results in only a very slight increase in RET phosphorylation. In contrast, cells transfected with either RETL1 or RETL2 show a significant increase in the level of RET tyrosine phosphorylation after treatment with GDNF. Jing et al. (11) and Treanor et al. (12) have provided similar results for RETL1 expressed in a different cell line, Neuro2A. The results shown in Fig. 2 provide the first evidence that, like RETL1, RETL2 can also facilitate GDNF dependent phosphorylation of RET.

Direct binding of RETL1 and RETL2 to GDNF and RET were evaluated using an ELISA type format in which 96-well plates were coated with GDNF and incubated with soluble forms of RET, RETL1, and RETL2. The soluble RET protein used in this experiment was a fusion of the RET extracellular domain with AP (RET–AP); both soluble RETL1 and RETL2 proteins were fusions with the Fe portion of human IgG1 (RETL1–Ig and RETL2–Ig). Fig. 3a shows that RETL1–Ig (detected with an anti-human Fe antibody) binds to GDNF coated plates with an apparent 

$$K_d = 5 \text{ nM}$$

whereas very little binding of RETL2–Ig is observed under the same conditions. Fig. 3b shows that, in the presence of RET–AP (90 ng/ml), RETL2–Ig binds tightly to GDNF with an apparent 

$$K_d = 1 \text{ nM}$$

the same concentration of RET–AP had no significant effect on the binding of RETL1–Ig. Detection of binding via the enzymatic activity of the RET–AP fusion rather than by the anti-human Fe antibody showed that RET–AP is a component in the complexes of both RETL1–Ig and RETL2–Ig with GDNF (Fig. 3c). In additional experiments the binding of RETL1–Ig was shown to be insensitive to the density of GDNF coating on the plate, whereas the extent of RET–AP-

**Expression of RETL1 and RETL2.** Northern blot analyses of various embryonic and adult rat tissues were performed to compare the expression profiles of RETL1 and RETL2 (Fig. 5). Three transcripts of 3.1, 4.0, and 8.8 kb were observed with the RETL1 analysis, while two transcripts of 3.1 and 4.0 kb were observed for RETL2. Both RETL1 and RETL2 are expressed in embryonic brain, lung, kidney, and intestine, with higher independent binding of RETL2–Ig was sensitive to variations in GDNF density (data not shown). Under all conditions tested the binding of RETL2–Ig to GDNF was very weak in the absence of RET–AP, and was strongly enhanced by its presence.

The strong dependence of the binding of RETL2 to GDNF on the presence of RET was confirmed by FACS experiments in which we measured the ability of GDNF to bind to cells stably expressing RETL1 or RETL2. In Fig. 4, a–c show cells evaluated for binding GDNF, while Fig. 4 d–f show cells evaluated for binding RET–Ig in the presence or absence of GDNF. Although cells transfected with the vector CH269 show some background binding of GDNF (Fig. 4a), cells expressing RETL1 show a significant shift (Fig. 4b) compared with the vector control, indicating that GDNF can bind to RETL1 expressing cells. In contrast, the majority of cells expressing RETL2 show only a minor shift (Fig. 4c) relative to the vector control, indicating that GDNF binds relatively poorly to RETL2 expressing cells. The shoulder to the right of this peak in Fig. 4c may represent a subpopulation of cells that express a high level of RETL2. Fig. 4e and f show that, in the presence of GDNF, cells transfected with either RETL1 or RETL2 bind RET–Ig equally well indicating that, when RET is present, RETL2 is as effective as RETL1 at supporting the formation of the three-component complex.
levels of RETL1 observed in intestine and kidney and higher levels of RETL2 observed in lung. The 4.0 kb RETL2 transcript is expressed in all the adult tissues analyzed; the highest level of expression of RETL2 is in the lung and placenta. The 4.0 kb RETL1 transcript can be detected in all the adult tissues analyzed, while the 8.8-kb RETL1 transcript is expressed in kidney, heart, and placenta.

To further define the expression profiles of RETL1 and RETL2, a Northern analysis of human tissues within the central and peripheral nervous systems was performed (Fig. 6). One predominant transcript of 9.6 kb and two minor transcripts of 3.8 and 1.9 kb were observed with the indicated tissue. (a–c) The ability of these cell lines to bind RET-Ig was evaluated using a mAb to GDNF as described in the experimental methods. In each of these panels, curves on the left show curves incubated in the absence of GDNF. (d–f) The ability of these cell lines to bind RET-Ig was evaluated using an antibody against the human Fc region as described in the experimental methods. Curves labeled RET–Ig show cells incubated with RET-Ig in the presence of GDNF; curves labeled RET–Ig + GDNF show cells incubated with RET–Ig in the presence of GDNF. In each of these panels, control curves (which fall on top of the curves labeled RET–Ig) show cells incubated in the absence of both RET–Ig and GDNF.

The highest level of expression of RETL2 is observed in the occipital, frontal, and temporal lobes and the cerebral cortex. Overall, the Northern analysis shows that while RETL1 and RETL2 are sometimes expressed in the same tissues, their expression profiles differ significantly, implying that they may perform distinct roles in the embryo and in the adult.

**DISCUSSION**

We report the isolation and characterization of rat and human cDNAs for RETL1 and RETL2. RETL1 and RETL2 encode two cell surface proteins that function as accessory molecules for RET. They are widely expressed throughout development but differ in their tissue distribution. The human RETL1 and RETL2 proteins share 49% identity, while the rat and human homologs share 93.3% (RETL1) and 94.6% (RETL2) identity. Both RETL1 and RETL2 can mediate GDNF-dependent phosphorylation of RET. However, our studies indicate that RETL1 and RETL2 differ in their ability to bind GDNF in a RET independent versus RET dependent manner. The differences in mechanisms and in the expression profiles of RETL1 and RETL2 could translate into functional differences in RET signaling in vivo.

RETL1 and RETL2 are expressed in a wide variety of tissues in the embryo and adult. An examination of RETL1 and RETL2 expression in rat embryonic tissues indicates a high level of RETL1 in fetal intestine. This is consistent with the expression profiles of RET and GDNF and the observation that mice null for GDNF or RET exhibit hindgut defects reminiscent of HSCR (1, 5–7, 12). Clearly, it will be interesting to determine if any of the familial cases of HSCR can be attributed to genetic alterations in RETL1. Expression of RETL1 is also observed in the fetal kidney, again consistent with sites associated with GDNF and RET expression and with an organ known to be dysgenetic in GDNF and RET null mice. In adult rat tissues, RETL1 is most abundant in heart and kidney, while RETL2 shows strong expression in placenta and lung. The high level of RETL2 expression in lung is intriguing because there are a few reported cases of small cell lung carcinomas being associated with alterations in RET (20). RETL1 and RETL2 exhibit different patterns of expression in human adult central nervous system tissues. Most striking is the high level of expression of RETL1 in the substantia nigra and caudate nucleus. Recent studies (21) indicate that GDNF when injected into the substantia nigra can show efficacy in models of Parkinson disease suggesting that GDNF signaling through RET in these cells is mediated via RETL1. RETL2 in the human brain is more discrete and is expressed in the...
cerebral cortex, predominantly in the occipital and frontal lobes.

The generation of soluble forms of RETL1, RETL2 and RET allowed us to directly study the binding properties of the components in vitro. Although both RETL1 and RETL2 mediate GDNF-dependent RET phosphorylation through formation of a three component complex, the mechanisms through that the complexes form are quite distinct. While we and others have shown that RETL1 can bind GDNF with high affinity in a RET-independent manner, our in vitro data suggest that RETL2 can only form a high affinity complex with GDNF in the presence of RET. These mechanistic differences could result in functional consequences in RET signaling in vivo. The strong RET-dependence of the GDNF/RETL1 interaction suggests that dissociation of the RETL2, RET, GDNF complex would release GDNF from the cell surface while dissociation of the RETL1, RET, GDNF complex would leave GDNF tightly bound to RETL1, and thus free to reassociate with RET and reform the complex. These differences might cause RETL2 expressing cells to be more sensitive than RETL1 expressing cells to fluctuations in the local concentration of free GDNF, because retention of GDNF on the surface of RETL1 expressing cells would allow RET to remain activated even if the concentration of free GDNF had decreased.

A second implication of our data is that the affinity of RETL2 for RET may be higher than that of RETL1 for RET. This raises the possibility that RETL2 and RET might reassociate on the membrane even in the absence of GDNF. Such an interaction might serve to regulate the proportion of RET for RETL1 and RETL2. We have demonstrated that an interaction can be observed between RET-Ig and RETL1 (this paper) and RETL2 (data not shown) expressed on cells, in the absence of GDNF, provided that a fixing step is included. Experiments are currently in progress to evaluate the relative affinities of RET for RETL1 and RETL2.

The ability of GDNF, a transforming growth factor β family member, to activate RET, a receptor tyrosine kinase was a surprising result since most members of the transforming growth factor β superfamily bind serine-threonine kinase receptors (22, 23). RETL1 and RETL2 may have evolved as adaptor molecules that allowed a receptor tyrosine kinase family member to recognize a new class of ligands (i.e., a transforming growth factor β family member). These accessory molecules could also facilitate the interaction of multiple ligands with RET. In fact, the existence of a new GDNF-like molecule, neurturin, has just been reported (24). It will be interesting to determine if neurturin will bind RETL1 and RETL2 and activate RET.

RETL1 and RETL2 represent two new candidate susceptibility genes for RET associated human diseases. Families with heritable cases of MEN2A, -2B, and HSCR not attributable to RET or GDNF (25) should be screened for alterations in RETL1 or RETL2. The existence of RETL1 and RETL2 may already suggest an explanation for the low penetrance seen in patients with familial HSCR due to loss of function mutations. For example, many different wildtype alleles of RETL1 and RETL2 could be present in the population and some of these alleles may compensate for and/or suppress the RET loss of function mutations associated with HSCR. In a few families,activating RET mutations that result in MEN2A can cosegregate with a HSCR disease phenotype (2, 25–28). This observation is often postulated to be due to a difference in degree of penetrance in various tissues of the RET mutation or, alternatively, to a closely linked modifiers. It will be interesting to determine if RETL1 and RETL2 are linked to RET.

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