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TrnR2, a Novel Receptor That Mediates Neurturin and GDNF Signaling through Ret

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Summary

Glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) comprise a family of TGF-β-related neurotrophic factors (TRNs), which have trophic influences on a variety of neuronal populations. A receptor complex comprised of TrnR1 (GDNFRα) and Ret was recently identified and found to be capable of mediating both GDNF and NTN signaling. We have identified a novel receptor based on homology to TrnR1, called TrnR2, that is 48% identical to TrnR1, and is located on the short arm of chromosome 8. TrnR2 is attached to the cell surface via a GPI-linkage, and can mediate both NTN and GDNF signaling through Ret in vitro. Fibroblasts expressing TrnR2 and Ret are ~30-fold more sensitive to NTN than to GDNF treatment, whereas those expressing TrnR1 and Ret respond equivalently to both factors, suggesting the TrnR2–Ret complex acts preferentially as a receptor for NTN. TrnR2 and Ret are expressed in neurons of the superior cervical and dorsal root ganglia, and in the adult brain. Comparative analysis of TrnR1, TrnR2, and Ret expression indicates that multiple receptor complexes, capable of mediating GDNF and NTN signaling, exist in vivo.

Introduction

The recent discovery of neurturin (NTN) (Kotzbauer et al., 1996), a neurotrophic factor related to glial cell line-derived neurotrophic factor (GDNF), has established the existence of a new family of neurotrophic factors that are structurally similar to TGF-β, for which we suggest the name TGF-β-related neurotrophic factors (TRNs). The mature NTN protein is 42% similar to mature GDNF, and was initially discovered and purified based on its ability to maintain the survival of rat superior cervical ganglion (SCG) neurons in culture (Kotzbauer et al., 1996). The full range of NTNs trophic activities is not yet known, but initial neuronal survival assays have indicated that NTN, like GDNF, supports the survival of rat superior cervical, nodose, and dorsal root ganglion neurons in vitro (Kotzbauer et al., 1996). With the recent demonstration that both GDNF and NTN can activate the Ret receptor tyrosine kinase, it is expected that more overlapping activities will be identified (Durbec et al., 1996; Jing et al., 1996, Treanor et al., 1996; Trupp et al., 1996; Creedon et al., in press).

GDNF was originally isolated based on its ability to maintain the survival of embryonic ventral midbrain dopaminergic neurons in vitro (Lin et al., 1993). Further studies in vivo have supported the role of GDNF as a target-derived trophic factor for embryonic and adult dopaminergic midbrain neurons (Stromberg et al., 1993; Hudson et al., 1995; Tomac et al., 1995), and exogenous GDNF treatment has shown protective effects in several animal models of Parkinson's disease, a disease in which midbrain dopaminergic neurons degenerate (reviewed by Lindsay, 1995; Gash et al., 1996; Choi-Lundberg et al., 1997). GDNF is now known to promote the survival of a wide variety of neuronal populations in both the central and peripheral nervous systems (CNS and PNS). In the PNS, GDNF can promote the survival of several autonomic and sensory neuron populations, cultured from either chicken or rat embryos, although with a much lower potency than its dopaminotrophic effects (Buj-Bello et al., 1995; Ebendal et al., 1995; Trupp et al., 1995; Kotzbauer et al., 1996).

Using an expression cloning strategy to identify high affinity binding proteins for GDNF, the first GDNF receptor, called GDNF receptor α (GDNFRα), was recently cloned by two groups (Jing et al., 1996; Treanor et al., 1996). GDNFRα has no transmembrane domain, and is attached to the cell surface via a glycosyl-phosphatidylinositol (GPI) linkage. In a surprising convergence, initiated by similarities in the phenotypes of Ret and GDNF deficient mice, the signaling component of the GDNF receptor was concurrently identified as the protein encoded by the proto-oncogene *c-ret*, a receptor tyrosine kinase (Durbec et al., 1996; Jing et al., 1996; Treanor et al., 1996 Trupp et al., 1996). GDNFRα and Ret form a functional GDNF receptor complex, and are expressed together in the developing nephron, midbrain, and motor neurons, all known targets of GDNF action (Treanor et al., 1996).

Given the redundancy of NTN and GDNF trophic activity in all survival assays performed thus far, we recently examined whether NTN can use the same receptor complex as GDNF, and found that NTN can stimulate Ret activation in SCG neurons, and that GDNFRα can function as a coreceptor for both NTN and GDNF in vitro (Creedon et al., in press). Here, we describe the cloning and initial characterization of a second coreceptor for both NTN and GDNF. To name the receptors appropriately for their ability to function in both GDNF and NTN signaling, we will refer to them as TGF-β- related neurotrophic factor receptors 1 and 2 (TrnR1 and TrnR2), where TrnR1 corresponds to GDNFRα. As described below, TrnR2 is GPI-linked to the cell surface, and is capable of mediating both NTN and GDNF signaling through Ret when expressed in fibroblasts. TrnR2–Ret expressing fibroblasts are 10- to 30-fold more sensitive to NTN than to GDNF treatment, whereas TrnR1–Ret

expressing fibroblasts respond similarly to both GDNF and NTN. TrnR2 and TrnR1 are expressed in both distinct and overlapping regions of the developing embryo and the adult. Comparative expression of TrnR1, TrnR2, and Ret indicates that the actions of NTN and GDNF can be mediated through multiple receptor complexes in vivo, and that a physiological pairing of ligands and receptors may exist for the TRNs as it does for the neurotrophins.

Results

TrnR2 is a TrnR1 Homolog Identified by Searching the EST Database

TrnR2 was identified by performing a BLAST (Basic local alignment search tool; Altschul et al., 1990) search of the dbEST database, using the full-length rat TrnR1 (GDNFRα) protein sequence as a query (Jing et al., 1996; Treanor et al., 1996) (accession number U59486). Three human ESTs were identified, which showed only partial but significant homology to rat TrnR1 (H12981, R02135, W73681). These ESTs were obtained from the WashU-Merck EST project and sequenced in their entirety. Alignment of these sequences indicated that all three ESTs encoded partial cDNAs of an identical transcript. The 5′ end of the cDNA was obtained by rapid amplification of cDNA ends (RACE) by polymerase chain reaction (PCR), using human brain and placenta Marathon RACE libraries (Clontech) as templates. Two alternatively spliced forms were identified in both brain and placenta; the shorter form is missing 399 nucleotides of the coding sequence from the 5′ end of the cDNA. The corresponding mouse cDNAs for both the full-length and splice variant were also obtained by PCR, using a brain cDNA template. The full-length murine cDNA contains a single long open reading frame (ORF) encoding a protein of 463 amino acids; the splice variant identified has an ORF encoding 330 amino acids (Figure 1A).

The protein encoded by the cDNAs, which we have termed TrnR2, shows significant homology with TrnR1 (Figure 1B). The predicted protein for TrnR2 contains a putative signal peptide at the amino terminus (von Heinje, 1986), three potential N-linked glycosylation sites, and has a stretch of 16 carboxyl-terminal hydrophobic amino acids. The presence of the N- and C-terminal hydrophobic regions indicates that TrnR2 is potentially a GPI-linked protein (Udenfriend and Kodukula, 1995), as has been demonstrated for the closely related TrnR1 (Jing et al., 1996; Treanor et al., 1996). Human TrnR2 is 48% identical to human TrnR1 at the amino acid level, and shares 30 of 31 cysteine residues with nearly identical spacing, indicating a conserved cysteine backbone structure. All features of TrnR2 indicate that it is closely related to TrnR1, suggesting that it may also interact with the Ret receptor tyrosine kinase to promote signaling of NTN and/or GDNF.

We isolated a human P1 artificial chromosome (PAC) genomic clone for TrnR2, and used fluorescence in situ hybridization (FISH) analysis to determine its chromosomal location (Figures 1C and 1D). Hybridization of biotin or digoxygenin-labeled TrnR2 probes to normal human chromosomes revealed a symmetrical fluorescent signal on the short arm of chromosome 8, in region p12–21. Because TrnR2 is a receptor for the neurotrophic factors NTN and GDNF, we searched for neurological diseases which have been genetically mapped to this locus. A search of the database revealed only one such disease, SPG5A, an autosomal recessive form of spastic paraplegia, localized to the paracentric region of chromosome 8 (Hentati et al., 1994). Also, an amplification event on 8p12 has been observed in some cases of breast and ovarian cancer (Imbert et al., 1996).

TrnR2 and RET Comprise a Second Functional Receptor Complex for Both NTN and GDNF

To examine the possibility that TrnR2 can form a functional receptor complex with Ret for NTN and/or GDNF, we generated NIH3T3 fibroblasts (subclone MG87; Zhan et al., 1987), which stably express either Ret alone or both Ret and TrnR2. The cells were treated with recombinant GDNF or NTN, and then lysed. The lysates were immunoprecipitated with an anti-phosphotyrosine antibody and analyzed by Western blot using an anti-Ret antibody (Figure 2A). Fibroblasts expressing only the Ret tyrosine kinase showed no Ret phosphorylation after treatment with either NTN or GDNF at doses ranging from 50–3000 ng/ml (Figure 2A; data not shown). However, cells which expressed both Ret and TrnR2 showed a strong band of approximately 170 kDa upon stimulation with either NTN or GDNF at 50 ng/ml, indicating that Ret was being phosphorylated on tyrosine residues. Furthermore, pretreatment of the Ret–TrnR2 fibroblasts with phosphatidylinositol-specific phospholipase C (PI-PLC), which specifically cleaves GPI-linked proteins from the cell surface, significantly depleted the NTN- or GDNF-induced phosphorylation of Ret (Figure 2B). These data indicate that NTN and GDNF are able to stimulate Ret phosphorylation only if TrnR2 is present to function as a coreceptor. This is analogous to the previously described requirement of TrnR1 as a coreceptor with Ret for GDNF (Jing et al., 1996; Treanor et al., 1996) and NTN (Creedon et al., in press) signaling.

The level of Ret phosphorylation in the presence of the coreceptor TrnR2 was dependent upon the concentration of NTN or GDNF applied (Figure 2C). In the TrnR2–Ret expressing fibroblasts, Ret phosphorylation was clearly observed in response to NTN treatment at 0.3 ng/ml; an equivalent response to GDNF was observed at 10 ng/ml. Similar results were obtained with multiple batches of recombinant GDNF and NTN, and with another stable TrnR2–Ret transfectant (data not shown). To further examine the differential dose responsiveness to GDNF versus NTN, we compared the dose dependency of the TrnR2–Ret fibroblasts with fibroblasts expressing Ret and TrnR1 treated with identical recombinant NTN or GDNF preparations. We have shown previously that TrnR1–Ret expressing fibroblasts respond to both GDNF and NTN stimulation by Ret phosphorylation (Creedon et al., in press). When Ret–TrnR1-expressing fibroblasts were treated with NTN or GDNF, the extent of Ret phosphorylation in response to each factor was equivalent at all doses tested. The observed difference in the dose-response curves of the Ret–TrnR1 and Ret–TrnR2 fibroblasts to NTN and GDNF suggests that there is a difference in the functional affinity of the ligands for the two receptor complexes, TrnR1–Ret and

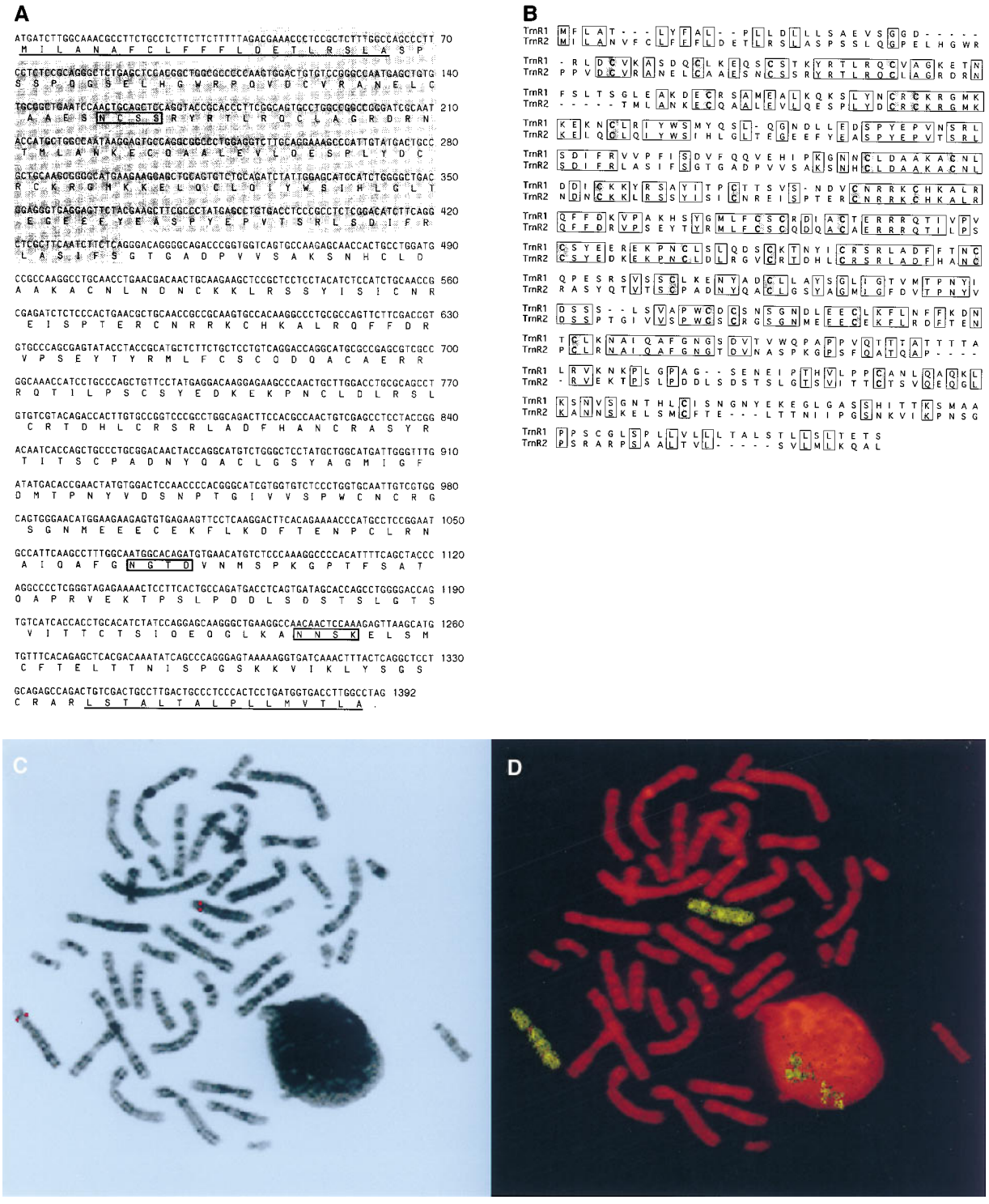

Figure 1. Sequence Analysis and Chromosomal Localization of TrnR2

(A) The nucleotide sequence and amino acid translation of mouse TrnR2. The N-terminal signal sequence and C-terminal hydrophobic domain are underlined, and potential N-linked glycosylation sites are boxed. The region absent in the splice variant is shaded.

(B) Alignment of the human TrnR1 and TrnR2 amino acid sequences. Identical residues are boxed, and shared cysteine residues are shaded.

(C) The chromosomal location of human TrnR2. Biotin and digoxygenin-labeled human TrnR2 probes showed symmetrical labeling of chromosome 8p12–21. The human TrnR2 PAC clone used as a probe was isolated and labeled as described in the Experimental Procedures.

(D) The same metaphase as in (C) showing painting after rehybridization with a chromosome 8 specific probe.

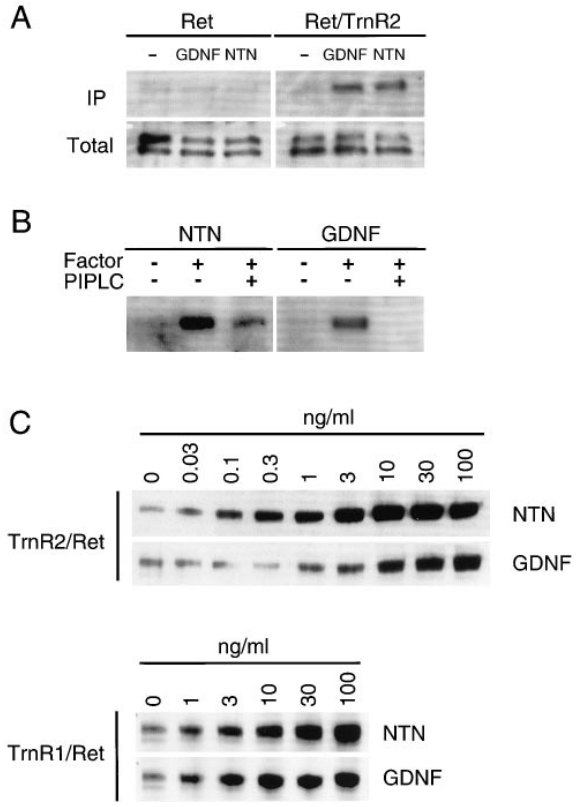

Figure 2. TrnR2 and Ret Comprise a Functional NTN and GDNF Receptor Complex

(A) Fibroblasts stably transfected with Ret alone (Ret), or both Ret and TrnR2 (Ret–TrnR2), were assayed for Ret phosphorylation by immunoprecipitation with anti-phosphotyrosine antibody and immunoblotting with an anti-Ret antibody (IP). Cells were treated for 10 min with 50 ng/ml NTN or GDNF or left untreated (-). Anti-Ret immunoblot analysis of the corresponding total lysates below illustrates equal Ret expression (Total). Two bands are visible in the total lysate corresponding to both the immature (150 kDa) intracellular protein and the glycosylated mature protein (170 kDa). Only the mature form is immunoprecipitated by the phosphotyrosine antibody. Cells expressing both TrnR2 and Ret, but not Ret alone, responded to NTN and GDNF treatment.

(B) Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment of Ret–TrnR2 fibroblasts depletes Ret phosphorylation induced by NTN and GDNF. Ret–TrnR2 fibroblasts were pre-treated with 1 U/ml PI-PLC for 45 min, washed twice, and processed as in (A).

(C) Dose-response of NTN and GDNF treatment on Ret phosphorylation in fibroblasts stably expressing both Ret and TrnR2 (TrnR2–Ret) or Ret and TrnR1 (TrnR1–Ret). Cells were stimulated with the indicated amount of factor for 6 min, immunoprecipitated, and immunoblotted as in (A).

TrnR2–Ret. The TrnR2–Ret complex may function preferentially as a NTN receptor, whereas the TrnR1–Ret complex responds equivalently to either factor.

Expression of TrnR2 Is Observed in the Brain and Testis of the Adult Mouse, and Shows Partial Overlap with TrnR1

Northern analysis of TrnR2 message in adult mouse tissues revealed expression in brain and testis (Figure 3A). Two messages were observed in brain, differing only slightly in size (Figures 3A and 6). These two bands likely correspond to the two splice forms found in brain while performing RACE PCR to amplify the 5′ end of the TrnR2 cDNA, the shorter of which is missing 399 nucleotides from the coding region (see Figure 1A). Two different bands were also observed in testis, which were significantly smaller (~1.5–1.8 kb) than either of the transcripts detected in the brain (~4 kb). One of the smaller TrnR2 messages in testis may be analogous to a small TrnR1 mRNA reported which encodes a truncated protein of 158 amino acids (Treanor et al., 1996). Low level expression may also be present in the spleen and in the adrenal. These results indicate that the tissue distribution of TrnR2 is more limited than that of TrnR1, which has been detected in liver, kidney, and brain of adult rat and mouse (Jing et al., 1996).

We focused further expression analysis on known sites of GDNF and/or NTN action (Figure 4). In situ hybridization analysis showed only low level expression of TrnR2 in the substantia nigra in the adult mouse, and in the ventral mesencephalon of an E14 mouse, in contrast to high level expression of TrnR1 and Ret (Figure 4A; data not shown). Motor neurons in the ventral horn of the adult spinal cord also express TrnR1 and Ret, but not TrnR2 (Figure 4B). TrnR2 is highly expressed in the developing and adult dorsal root ganglia (DRG), along with Ret and TrnR1 (Figure 4C; data not shown). In the developing kidney and gut, there is high level expression of TrnR1 and Ret, but not TrnR2 (Figure 4C). Finally, we observed significant expression of both TrnR2 and Ret in the rat SCG, with only low level, diffuse staining of TrnR1 (Figure 4D). These data indicate a partially overlapping expression pattern for TrnR1 and TrnR2 in embryonic and adult central and peripheral nervous tissue. In several areas of known GDNF action, including nigral and motor neurons, high levels of TrnR1 and Ret are expressed, with only low or undetectable levels of TrnR2 expression. Based on this initial survey, TrnR2 expression is largely limited to neuronal tissue in both embryo and adult, with highest levels of expression in sensory and sympathetic neuronal populations.

TrnR2 Is Expressed in Newborn Rat SCG Neurons, a Population Responsive to Both NTN and GDNF

Both NTN and GDNF promote the survival of newborn rat SCG neurons in culture, presumably through their activation of the Ret signaling pathway (Trupp et al., 1995; Kotzbauer et al., 1996; Creedon et al., in press). As shown in Figure 4D, in situ hybridization analysis indicates that TrnR2 and Ret are expressed at high levels in rat SCG neurons, whereas TrnR1 is expressed diffusely and does not appear to be localized to neurons. To further assess the cellular localization of TrnR1, TrnR2, and Ret mRNAs in this ganglion, we analyzed their expression in primary SCG cultures by reverse transcription polymerase chain reaction (RT-PCR). Because the primary cultures contain a small contaminating population of nonneuronal cells (Schwann cells and fibroblasts), neuronal specific messages were identified by inducing apoptosis in the neuronal population. Removal of nerve growth factor (NGF) from the culture medium results in near complete death of the neuronal population within 48 hours (Martin et al., 1988; Deckwerth and

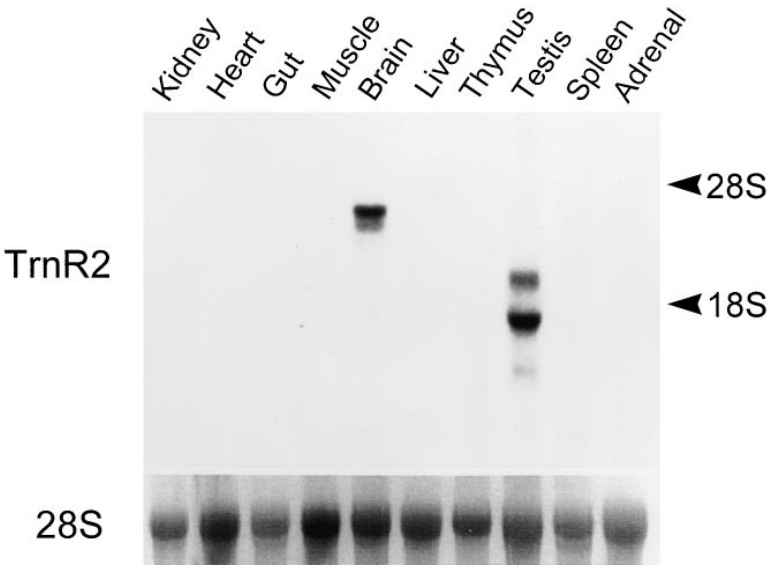

Figure 3. RNA Blot Analysis of TrnR2 Expression in Adult Mouse Tissues

Samples of total RNA (25 mg) isolated from the indicated tissues were loaded into each lane. A ^{32}P-labeled TrnR2 probe was generated and hybridized to the blot as described in the Experimental Procedures. Two TrnR2 mRNA species are visible in the brain. The 28S ribosomal RNA band visualized with ethidium bromide is shown below to demonstrate equal loading.

Johnson, 1993; Edwards and Tolkovsky, 1994). During this period, neuronal messages decrease whereas messages from nonneuronal cells remain constant (Estus et al., 1994; Freeman et al., 1994). Reverse transcribed cDNA libraries were prepared from cultures at multiple time points between 0 and 36 hr, and PCR was performed to assess the expression of Ret, TrnR1, and TrnR2 (Figure 5). Ret and TrnR2 messages decreased as the neurons died, in a manner similar to neuron-specific enolase (NSE). In contrast, TrnR1 levels remained constant, similar to the Schwann cell marker S-100. These data indicate that Ret and TrnR2 expression is largely limited to neurons in neonatal rat SCG cultures, and likely mediates the functional response of these neurons to NTN and GDNF. This is consistent with in situ hybridization analysis (Figure 4D), which also indicates that the expressed receptor complex in SCG neurons consists of TrnR2 and Ret. Interestingly, NTN is more potent in promoting the survival of SCG neurons than GDNF (Kotzbauer et al., 1996), which is consistent with the higher sensitivity of the TrnR2–Ret receptor complex to NTN treatment (Figure 2C). Although some low level neuronal expression of TrnR1 cannot be excluded by this assay, these data indicate that it is predominantly expressed in the nonneuronal population, consistent with its previously observed expression in Schwann cells (Treanor et al., 1996; Figure 6).

TrnR1, but Not TrnR2, Is Up-Regulated in Distal Sciatic Nerve after Nerve Injury

GDNF is a well-characterized trophic factor for both embryonic and adult motor neurons (Henderson et al., 1994; Li et al, 1995; Oppenheim et al., 1995; Yan et al., 1995). In the adult animal, GDNF expression is up-regulated in the distal segment of the sciatic nerve after transection and in denervated muscle (Springer et al., 1995; Trupp et al., 1995). This is similar to observations regarding NGF and the p75 low affinity neurotrophin receptor (p75NTR), which are both upregulated by Schwann cells in the distal segment of the sciatic nerve after transection (Taniuchi et al., 1986; Heumann et al., 1987). Because GDNF is up-regulated after injury, and because TrnR1 is expressed by Schwann cells (Treanor et al., 1996), we hypothesized that one or both of the TRN coreceptors might also be up-regulated after transection, in a manner analogous to the up-regulation of p75. We examined the expression of TrnR1 and TrnR2 in the distal segment of the rat sciatic nerve before and after transection, using RNA blot analysis (Figure 6). Seven days after nerve transection, the distal portion of the sciatic nerve showed a dramatic increase in the level of TrnR1 mRNA. In contrast, TrnR2 mRNA was not detected in the nerve either before or after transection. Consistent with the observed differential expression of TrnR1 and TrnR2 in Schwann cells, RT-PCR analysis of the JS-1 Schwann cell line also showed expression of TrnR1, but not TrnR2 (data not shown). These results indicate that TrnR2 is unlikely to play a major role in Schwann cell mediated peripheral trophic support of the regenerating nerve. However TrnR1, in conjunction with GDNF produced by the distal sciatic nerve and muscle, could potentially provide a potent trophic substrate for growth of the regenerating nerve.

Discussion

We have identified, isolated and begun to characterize a novel receptor for the TGF-β related neurotrophic factor (TRN) family of ligands, which we have called TrnR2. Like TrnR1 (GDNFRα), TrnR2 is GPI-linked to the cell surface, and is able to mediate both NTN and GDNF signaling through the Ret tyrosine kinase receptor. Presumably a complex of Ret, TrnR2, and ligand is formed which initiates Ret dimerization and autophosphorylation, although further experiments will be necessary to determine both the exact kinetics and the stoichiometry of this complex. Our data concur with those of others, which indicate that Ret alone is insufficient for NTN and GDNF signaling, and that a coreceptor is necessary (Jing et al., 1996; Treanor et al., 1996; Creedon et al., in press). Because NTN and GDNF are able to signal through both coreceptors, we believe that the TrnR nomenclature is

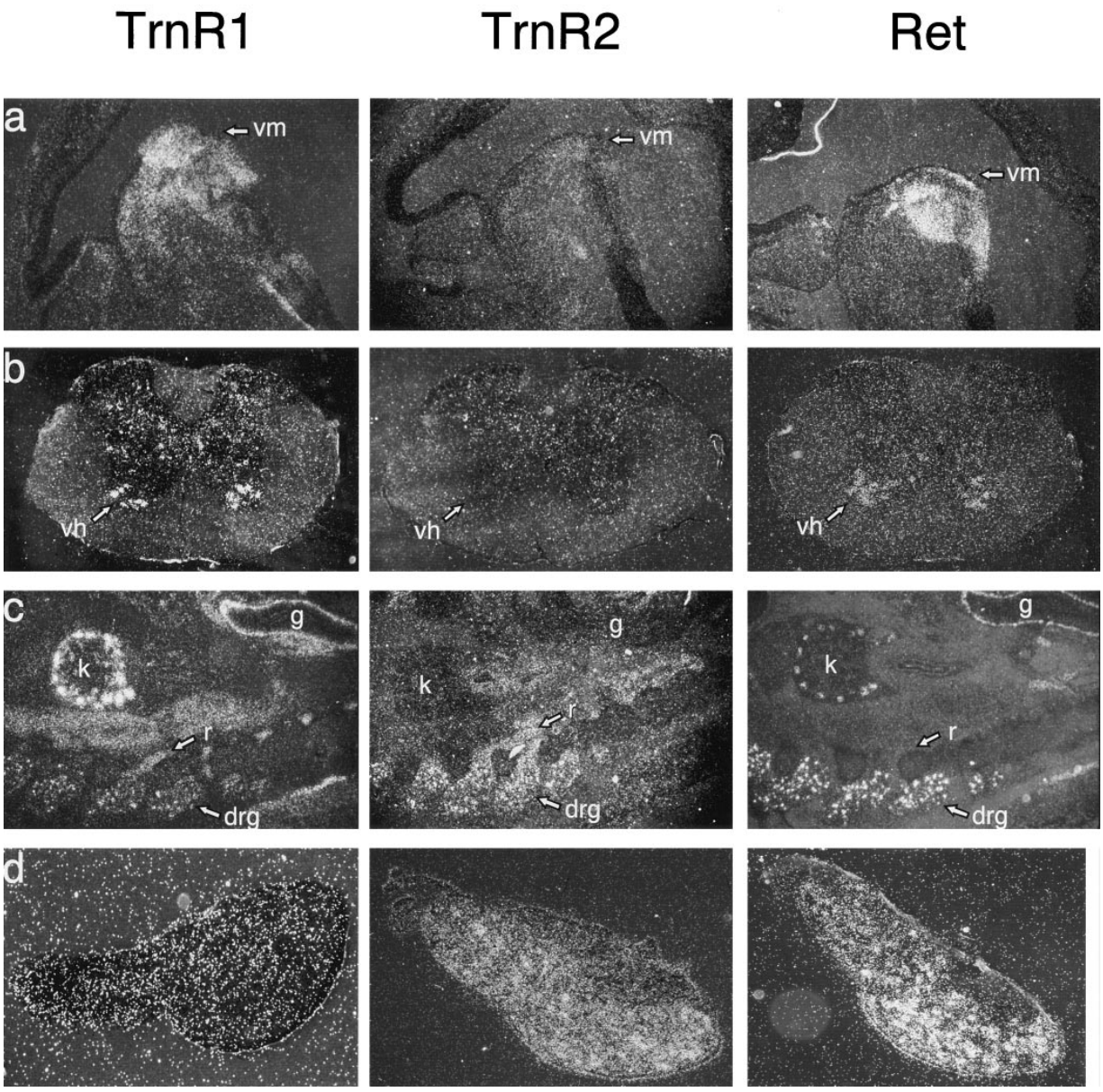

Figure 4. In Situ Hybridization Analysis of TrnR1, TrnR2, and Ret in Targets of GDNF and NTN Action

(A) E14 mouse ventral mesencephalon (vm). Strong staining of TrnR1 and Ret are observed in the developing ventral mesencephalon, with low levels of TrnR2 transcripts detected.

(B) Adult mouse spinal cord. TrnR1 and Ret are expressed in large motor neurons of the ventral horn (vh), whereas TrnR2 is not. Ret is localized predominately to motor neurons, whereas TrnR1 shows additional staining in the intermediate and dorsal horns of the cord.

(C) E14 mouse kidney, gut, and dorsal root ganglia. Strong expression of TrnR1 and Ret, but not TrnR2, is visible in the developing kidney (k) and gut (g). In both kidney and gut, TrnR1 staining is stronger, and more diffuse than Ret. TrnR1, TrnR2, and Ret are all expressed in the dorsal root ganglia (drg). In addition, the exiting nerve root (r) shows strong expression of TrnR1 and TrnR2 with no expression of Ret.

(D) TrnR2 and Ret are highly expressed in the adult rat superior cervical ganglion (SCG). Diffuse, low level TrnR1 expression was also observed.

more appropriate than the previous GDNFR nomenclature and allows the addition of future members of the receptor family, without an inappropriately restrictive designation based on GDNF or NTN alone.

The TrnR2–Ret receptor complex responded to stimulation with ligand in a dose-dependent manner. In TrnR2–Ret expressing fibroblasts, NTN stimulated Ret phosphorylation at an approximately 30-fold lower dosage than GDNF. TrnR1–Ret expressing cells responded equivalently to stimulation with either ligand at all doses tested. Together these results indicate redundancy exists in the signaling ability of both GDNF and NTN through TrnR1 and TrnR2, and that NTN may preferentially signal through the TrnR2–Ret receptor complex. We chose a fibroblast-based model system as opposed to a Ret expressing neuroblastoma (Jing et al., 1996) because the MG87 fibroblasts do not contain endogenous TrnR1 or TrnR2 mRNA as assessed by RT-PCR

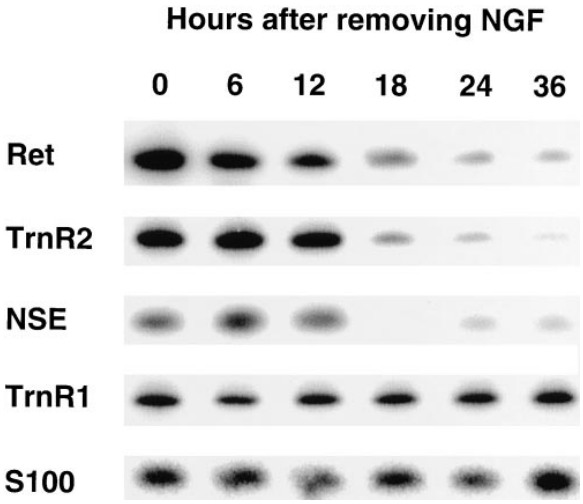

Figure 5. RT-PCR Analysis of Ret, TrnR1, and TrnR2 Expression in E21 Rat SCG Cultures

SCG neurons from E21 rats were deprived of NGF after 5 days in culture. mRNA isolated at 6, 12, 18, 24, and 36 hours after NGF removal was analyzed by RT-PCR for Ret, TrnR1, and TrnR2. RT-PCR was performed in the linear range of amplification for all transcripts. Each cDNA preparation was normalized to the level of actin message (not shown). Neuron-specific enolase (NSE) and S-100 are shown as examples of neuronal and Schwann cell specific markers, respectively.

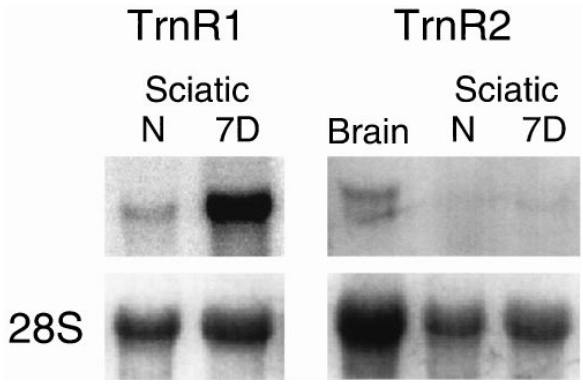

Figure 6. Up-Regulation of TrnR1, but Not TrnR2, in the Distal Segment of the Sciatic Nerve after Nerve Transection

RNA blot analysis of TrnRs demonstrating expression before and after nerve injury. Total RNA was prepared from normal rat sciatic nerve (N), and the distal segment of the sciatic nerve 7 days posttransection (7D). Brain is included as a positive control for detection of TrnR2 message. ^{32}P-labeled TrnR1 and TrnR2 probes were generated and hybridized to blots as described in the Experimental Procedures. The 28S ribosomal RNA band, visualized using ethidium bromide, is shown below to demonstrate equal loading.

analysis (data not shown); therefore, the properties of defined receptor complexes could be ascertained. We have found that neuroblastoma lines may express either TrnR1, TrnR2, or both, and that despite this heterogeneity those that respond to GDNF or NTN always respond to both factors (unpublished data). It is notable that in several ligand–receptor systems in which multiple family members have been identified, there is evidence of cross-talk between different ligands and receptors, particularly in fibroblast-based model systems. The neurotrophins BDNF, NT-3, and NT-4/5 are all able to stimulate phosphorylation of the TrkB receptor expressed in fibroblasts, with an equivalent dose-response relationship (Ip et al., 1993). However, in PC12 cells, BDNF and NT-4/5 stimulate TrkB phosphorylation at 100-fold lower dosages than NT-3. It is believed that the p75NTR may play a role in the restriction of NT-3 binding to TrkB in PC12 cells and neurons (Ip et al., 1993). Although the efficiency of the interaction is disputed, there is also evidence that NT-3 can signal through TrkA (Cordon-Cardo et al., 1991; Ip et al., 1993), and, furthermore, that different splice forms of TrkA interact differentially with NT-3 but identically with NGF (Clary and Reichardt, 1994). Therefore, although in vitro model systems are useful, they obviously cannot recreate the neuronal environment in vivo, which may include additional accessory factors and multiple forms of the existing receptor components, which can alter specificity. We cannot exclude the possibility that other accessory factors, and splice variants of both TrnR1 and TrnR2, may confer differential specificity for GDNF or NTN. Indeed, the splice variant form of TrnR2 we have found has a large deletion in the coding region, which may alter ligand binding properties or interaction with Ret. Of course, to fully understand the interactions between ligand and receptor in vivo, detailed expression analysis and assessment of multiple biological responses, in the context of both normal mice and those with null mutations for ligands and receptors, will be required.

TrnR1 and TrnR2 are expressed in a partially overlapping manner in the adult brain and in the embryo. In regions where they are coexpressed, such as the DRG and the brain, more detailed study will be required to determine whether TrnR1 and TrnR2 are expressed in distinct cell populations or are coexpressed in individual cells. Furthermore, we observed regions that express TrnR1 and TrnR2 but do not contain detectable levels of Ret (data not shown), indicating that these receptors may be able to interact with other signal-transducing molecules, may act in a soluble form with Ret, or perhaps may have additional functions independent of signal transduction. When TrnR1 was first identified in a screen for high affinity GDNF binding proteins, it was noted that Ret and TrnR1 are expressed in most targets of known GDNF action, including embryonic and adult nigra, motor neurons, gut, and kidney (Treanor et al., 1996). Our data confirm these observations, and further indicate that TrnR2 is expressed at low levels or not at all in these regions, in both the E14 embryo and adult mouse. We have also shown that TrnR1 appears to be up-regulated by Schwann cells in response to nerve injury in conjunction with GDNF, whereas TrnR2 is not. In contrast, TrnR2 and Ret appear to comprise the expressed receptor complex in SCG neurons, whereas low levels of TrnR1 mRNA are present in the nonneuronal cells of the ganglion.

NTN was discovered based upon its ability to support the survival of neonatal SCG neurons (Kotzbauer et al., 1996). GDNF has shown variable survival promotion of cultured SCG neurons from multiple species, with a generally lower potency than that of its actions on midbrain

dopaminergic neurons (Henderson et al., 1994; Buj-Bello et al., 1995; Ebendal et al., 1995; Trupp et al., 1995). The only study that directly compared the actions of NTN and GDNF on SCG neurons found that NTN was more potent than GDNF at promoting SCG survival (Kotzbauer et al., 1996). We believe the differential dose-response relationship, and the accumulating data on GDNF, NTN, TrnR1, TrnR2, and Ret expression, together suggest that a physiological pairing of ligand and receptor may exist for the TrnRs as it does for the Trks. It must be noted that if such a physiological pairing does exist, there is already potential evidence of cross-talk between ligands and receptors (Figure 2C). Mice lacking GDNF show a ~30% loss of SCG neurons (Moore et al., 1996; Pinchel et al., 1996; Sanchez et al., 1996), indicating that at some point in development a subpopulation of SCG neurons are singularly dependent on GDNF trophic support for survival. This may represent signaling of GDNF through TrnR2, temporally restricted expression of TrnR1, or the existence of an as yet unidentified receptor in these neurons.

An emerging theme in the expression of TrnR1 is its presence in Schwann cells in the absence of Ret. Expression of TrnR1 without Ret was originally observed by RT-PCR in a Schwann cell line (Treanor et al., 1996). We have found the expression of TrnR1, but not TrnR2, in the JS-1 schwannoma line and in adult sciatic nerve. Also, no Ret was detected in the spinal nerve root of the E14 embryo, whereas both TrnR1 and TrnR2 were detected. Furthermore TrnR1 likely has functional significance in peripheral nerve injury in the adult, in that it is clearly up-regulated in distal sciatic nerve after transection. TrnR1 may be functioning to concentrate GDNF and present it to the regrowing nerve, analogous to the proposed role of the p75NTR in the same injury paradigm (Taniuchi et al., 1986). Presumably the regenerating motor, sympathetic, and sensory axons express receptor complexes for GDNF, as they are present in the ventral horn (TrnR1–Ret), DRG (TrnR1–R2–Ret), and sympathetic neurons (TrnR2–Ret in the SCG). This may represent an efficient mechanism for trophic support of the regenerating nerve, with the potent neurotrophic factor GDNF concentrated and presented to the regenerating nerve, which expresses multiple signaling receptors.

In summary, we have characterized a second coreceptor for the TGF-β-related neurotrophic factors, GDNF and NTN. Initial in vitro functional data and expression analysis together suggest that NTN is the preferred ligand for TrnR2. The preferred ligand status of members of the neurotrophin family (NGF for TrkA, BDNF and NT-4/5 for TrkB, and NT-3 for TrkC) has been determined by the synthesis of multiple experimental analyses in the context of both normal and null-mutant mice. A similar synthesis of multiple experimental approaches will be necessary to determine if GDNF and NTN have a preferred receptor in vivo, and if significant cross-talk exists.

Experimental Procedures

Sequence Analysis, PCR Techniques, Cloning

All sequencing was performed on an Applied Biosystems 373 DNA sequencer using Taq DyeDeoxy Terminator cycle sequencing kits (Applied Biosystems). Sequence editing, mapping, alignment, and contig production were performed using the DNAstar software package. ESTs were obtained from the WashU-Merck EST project and sequenced completely. RACE was performed using Klentaq LA PCR (Barnes, 1994) and human brain and placenta cDNA libraries, per manufacturers instructions (Clontech). Products were cloned into the EcoRV site of pBluescript KS (Stratagene, La Jolla, CA) and sequenced, and a contig was generated of the full-length human cDNA.

For the TrnR2 expression construct, the coding region of the long form of human TrnR2 cDNA was amplified by Klentaq LA PCR from the same Marathon RACE human brain library, using primers 5′-GCGGTACCATGATCTTGGCAAACGTCTGC-3′ and 5′-GCTCTA GAGTCAGGCGGCTGTTCTTGTCTGCG-3′. The product was cloned into pCMV-neo (Brewer, 1994), and the insert was confirmed by sequencing. Human and mouse TrnR2 genomic clones were obtained by screening P1 (mouse) and PAC (human) genomic libraries with a PCR assay generated from the TrnR2 coding region (Genome Systems, St. Louis, MO). Mouse genomic sequence was used to design primers to amplify the full-length mouse TrnR2 cDNA by PCR. This fragment was cloned into pBluescript, and the nucleotide sequence was determined.

Generation of Fibroblasts Expressing Ret and TrnR2 or TrnR1

NIH3T3 fibroblasts (subclone MG87) expressing Ret, Ret–TrnR1, and Ret–TrnR2 were generated as described (Creedon et al., in press). Briefly, fibroblasts were transfected with a Ret-CMV-neo plasmid, selected in 1 mg/ml G418, and screened for Ret expression by Western blotting with an anti-Ret antibody (Santa Cruz). A clonal Ret expressing line was used as the parent to generate TrnR1–Ret and TrnR2–Ret expressing cells. The TrnR2-CMV-neo (or TrnR1-CMV-Neo) and SV2-HisD (gift from Richard Mulligan) plasmids were cotransfected into a Ret expressing clone, and selected in 2mM L-Histidinol (Sigma, St. Louis, MO). TrnR2–Ret and TrnR1–Ret expressing clones were confirmed by Western (Ret) and Northern (TrnR2/TrnR1) blotting.

Ret Phosphorylation Assays, PI-PLC Treatment

NIH3T3 cells were grown in DMEM plus 10% calf serum. Fibroblasts were seeded on 6-well plates, at 2.5×10^5/well, and grown to confluence. Cells were treated with recombinant NTN and GDNF for 10 min. Recombinant factors were prepared as described (Creedon et al., in press). Cells were then washed twice with cold PBS (Sigma) and lysed in 500 ml immunoprecipitation (IP) buffer (1 mM EDTA, 1mM EGTA, 0.2 mM NaOrthovanadate, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 0.2mM PMSF, and 10mM Tris [pH 7.4]). A portion of each sample was removed for assaying total lysates. The remaining lysates were incubated with agarose-conjugated anti-phosphotyrosine antibody (Calbiochem) for 1 hr at 4°C. The immunoprecipitate was washed three times with cold IP buffer and solubilized by boiling in SDS sample buffer for 5 min. Samples were run on 7.5% polyacrylamide gels. Gels were transferred to PVDF (Millipore, Bedford, MA) or nitrocellulose membranes and incubated in blocking solution (Tris Buffered Saline [TBS: 150 mM NaCl in 50 mM Tris, pH 7.4] with 0.1% Tween-20, 5% dry milk) for 1 hr at 25°C. Blots were then incubated overnight, at 4°C, in blocking solution containing a 1:300 dilution of anti-Ret antibody (Santa Cruz). Blots were washed (3×15 min) in blocking solution and then incubated with secondary antibody (1:1000 dilution of alkaline-phosphatase conjugated anti-rabbit antibody [New England Biolabs, Beverly, MA] in blocking solution) for 1 hr at 25°C. Blots were then washed in blocking solution (3×15 min); then in TBS plus 0.1% Tween-20 (2×10 min); and finally in 1mM $MgCl_2$, 10mM NaCl, and 10mM Tris-HCl (pH 9.5; 2×10 min). Blots were incubated in CDP-Star (Tropix, Bedford, MA) and exposed to X-ray film. For PI-PLC assays, cells were seeded as above and treated with 1 U/ml PI-PLC (Oxford Glycosystems) for 45–60 min at 37°C. The cells were then washed twice with warm media, treated with factors, and processed as above.

RT-PCR Expression Analysis

mRNA in neuronal cultures was assessed using semiquantitative RT-PCR as described in Freeman et al. (1994) and Estus et al. (1994)

and extensively detailed by Estus (1996). Five-day-old SCG cultures were switched to medium containing anti-NGF antibodies for various times. Polyadenylated RNA was isolated from the cultures using the QuickPrep Micro Kit (Pharmacia, Piscataway, NJ), according to the manufacturer's instructions. Half of the poly-A RNA was converted to cDNA by reverse transcription with Moloney murine leukemia virus reverse transcriptase, with random hexamers (16 μM) as primers. cDNA from approximately 150 cells was used in a 50 μl PCR reaction. After amplification, the PCR products were separated by electrophoresis on 10% polyacrylamide gels, visualized by autoradiography of the dried gels, and quantified with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). The following primer sets were used to generate the PCR products: mouse Ret forward 5′-TGGCACACCTCTGCTCTATG-3′ and reverse 5′-TGTTCCCAGGA ACTGTGGTC-3′; TrnR1 forward 5′-GCACAGCTACGGGATGCTCTT CTG-3′ and reverse 5′-GTAGTTGGGAGTCATGACTGTGCCAATC-3′; TrnR2 forward 5′-AGCCGACGGTGTGGCTCTGCTGG-3′ and reverse 5′-CCAGTGTCATCACCACCTGCACG-3′.

Fluorescence In Situ Hybridization Analysis (FISH)

A 120 kb genomic fragment cloned into a PAC vector labeled with biotin or digoxygenin (Bio-prime BRL, Gaithersburg, MD) was used for in situ hybridization of human chromosomes derived from methotrexate-synchronized normal peripheral lymphocyte cultures. The conditions of hybridization, the detection of hybridization signals, digital-image acquisition, processing and analysis, and direct fluorescent signal localization on banded chromosomes were performed as described (Zimonjic et al., 1995; Svaren et al., 1996). The majority of metaphases hybridized with the biotin or digoxigenin-labeled genomic TrnR2 probe had specific fluorescent signals at identical sites on both chromatids of the short arm of chromosome 8. The hybridization signal was localized in 100 metaphases with rhodamine signal on DAPI generated and enhanced G-like banding. Fifty metaphases were analyzed directly with both homologously labeled probes by imaging of DAPI generated and enhanced G-like banding at region 8p12–21. To confirm the identity of chromosomes with specific signal, the metaphases were rehybridized with a chromosome 8 probe. All labeled chromosomes were positive for painting.

Expression Analysis by Northern Blotting and In Situ Hybridization

Total RNA was prepared from mouse and rat tissues, and electrophoresis was performed on 1% agarose/formaldehyde gels and blotted onto nylon membranes (Zetaprobe) as previously described (Chomczynski and Sacchi, 1987). Fragments of the mouse TrnR2 and rat TrnR1 cDNAs were ^{32}P-labeled and used as hybridization probes. Surgical procedures for obtaining rat sciatic nerve samples were performed according to NIH guidelines for care and use of laboratory animals at Washington University. Sciatic nerves were transected and recovered as previously described (Araki and Milbrandt, 1996). In situ hybridization on fresh frozen tissue samples was performed as described (Wanaka et al., 1990). Sense and antisense ^{33}P-labeled RNA probes were transcribed from fragments of the TrnR1, TrnR2, and Ret cDNAs.

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GenBank Accession Numbers

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