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# Congenital Insensitivity to Pain with Anhidrosis: Novel Mutations in the *TRKA* (*NTRK1*) Gene Encoding A High-Affinity Receptor for Nerve Growth Factor

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## Summary

**Congenital insensitivity to pain with anhidrosis (CIPA) is characterized by recurrent episodes of unexplained fever, anhidrosis (inability to sweat), absence of reaction to noxious stimuli, self-mutilating behavior, and mental retardation. Human *TRKA* encodes a high-affinity tyrosine kinase receptor for nerve growth factor (NGF), a member of the neurotrophin family that induces neurite outgrowth and promotes survival of embryonic sensory and sympathetic neurons. We have recently demonstrated that *TRKA* is responsible for CIPA by identifying three mutations in a region encoding the intracellular tyrosine kinase domain of *TRKA* in one Ecuadorian and three Japanese families. We have developed a comprehensive strategy to screen for *TRKA* mutations, on the basis of the gene's structure and organization. Here we report 11 novel mutations, in seven affected families. These are six missense mutations, two frameshift mutations, one nonsense mutation, and two splice-site mutations. Mendelian inheritance of the mutations is confirmed in six families for which parent samples are available. Two mutations are linked, on the same chromosome, to Arg85Ser and to His598Tyr;Gly607Val, hence, they probably represent double and triple mutations. The mutations are distributed in an extracellular domain, involved in NGF binding, as well as the intracellular signal-transduction domain. These data suggest that *TRKA* defects cause CIPA in various ethnic groups.**

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## Introduction

Congenital insensitivity to pain with anhidrosis (CIPA; MIM 256800) is a rare autosomal recessive disorder characterized by recurrent episodes of unexplained fever, anhidrosis (inability to sweat), absence of reaction to noxious stimuli, self-mutilating behavior, and mental retardation (Swanson 1963; Dyck 1984; McKusick 1994). CIPA is also known as "congenital sensory neuropathy with anhidrosis," "hereditary sensory and autonomic neuropathy type IV," and "familial dysautonomia type II." In CIPA, there is a lack of pain sensation, most likely the result of the absence of the dorsal root ganglia responsible for pain sensation. A feature of CIPA is the absence of small-diameter afferent neurons, which are activated by tissue-damaging stimuli (Swanson et al. 1965; Rafel et al. 1980). A loss of innervation of eccrine sweat glands, by sympathetic neurons, explains the phenomenon of anhidrosis (Langer et al. 1981; Ismail et al. 1998). Previously, the diagnosis of CIPA was established on the basis of clinical findings, pharmacological tests, and peripheral-nerve biopsy.

Nerve growth factor (NGF) is the first growth factor to be identified and characterized (Levi-Montalcini 1987). Cell survival, growth, and differentiation in the nervous system are mediated by numerous growth factors, including neurotrophic factors (neurotrophins). NGF supports the survival of sympathetic ganglion neurons and nociceptive sensory neurons in dorsal root ganglia, derived from the neural crest, and ascending cholinergic neurons of the basal forebrain (Levi-Montalcini 1987; Thoenen and Barde 1980). *TRKA* (also named *NTRK1*) was isolated from a colon carcinoma as a potential new member of the tyrosine kinase gene family (Martin-Zanca et al. 1986) and was later found to be expressed in the nervous system (Martin-Zanca et al. 1990). TRKA is a receptor tyrosine kinase that is phosphorylated in response to NGF (Kaplan et al. 1991;

Klein et al. 1991). A single transmembrane domain divides TRKA into an extracellular domain, important for NGF binding, and an intracellular tyrosine kinase domain, important for signal transduction.

Mice lacking the orthologous gene of human *TRKA* share dramatic phenotypic features of CIPA, including loss of responses to painful stimuli, although anhidrosis is not apparent in these animals (Smeyne et al. 1994; Snider 1994). In addition, all dorsal root ganglia neurons associated with nociceptive functions are lost in the deficient mice. They also have extensive neural cell loss in sympathetic ganglia and a decrease in cholinergic neurons of the basal forebrain. We therefore considered human *TRKA* as a candidate gene for CIPA. We identified the genetic basis for this disorder by showing that mutations in *TRKA* are associated with the absence of functional high-affinity NGF receptors in four patients (Indo et al. 1996). We focused on the region encoding the intracellular domain, since the whole structure of human *TRKA* was not known. CIPA is the first human genetic disorder implicated in the neurotrophin-signal–transduction system. The anomalous pain and temperature sensation in CIPA is probably because of a dramatic loss of unmyelinated C-fibers and small-diameter myelinated A-fibers in the afferent neurons. The NGF-TRKA system has a crucial role in the development and function of nociception, as well as the establishment of thermoregulation via sweating in humans.

Human *TRKA* maps to chromosome 1q (Miozzo et al. 1990; Morris et al. 1991). Recently, we and others independently determined the structure and organization of human *TRKA* (Greco et al. 1996; Indo et al. 1997; GenBank). We have established a method to amplify and analyze all coding exons from genomic DNA. This study describes the analysis of *TRKA* mutations in seven families from various ethnic groups, including nine CIPA patients, by direct sequencing of all exons and reports the detection of 11 novel mutations. The mutations responsible for CIPA are distributed in the extracellular domain, important for NGF binding, as well as in the intracellular domain, important for signal transduction. These data suggest that *TRKA* mutations cause CIPA in various ethnic groups and give insight into the structure and function relationship of the neurotrophin receptor tyrosine kinase family.

## Patients and Methods

### *Patients*

Seven affected families, including nine patients with CIPA, were studied: two each from Kuwait (KI-103 and KI-105) and Italy (KI-106 and KI-107); and one each from United Arab Emirates (KI-102), Spain (KI-104), and Canada (KI-108). All patients had characteristic features of CIPA, insensitivity to pain and anhidrosis. Lack of eccrine sweat-gland innervation was confirmed in the KI-103 patient (Ismail et al. 1998). Parents were consanguineous in families KI-102, -103, -104, -105, and -106. Affected individuals were diagnosed with CIPA on the basis of clinical findings or by pharmacological and pathological analyses. We also studied parents (obligate carriers) and sibling(s) in all families, except one. DNA samples of parents from family KI-107 were not available for testing. At least 50 unaffected Japanese individuals were screened to verify that the mutations found in patients with CIPA were absent in the normal population. DNA samples for all patients with CIPA, their family members, and normal individuals were obtained from peripheral blood leukocytes by standard methods. Blood was collected from patients with CIPA and family members, who were referred to the investigators, after giving informed consent.

### *PCR Amplification of* TRKA *Exons and Sequencing*

*TRKA* exons were amplified from genomic DNA by use of specific primers, derived from the 5′ and 3′ intronic or exonic sequences (table 1), and AmpliTaq (PE Applied Biosystems) or Expand Long Template PCR (Boehringer-Mannheim). The annealing temperature for each pair of primers was 54°C–62°C. A microsatellite (D1S506) is located in intron 12. Two or three consecutive exons (i.e., 2 and 3; 5 and 6; 9, 10, and 11; and 13 and 14) were amplified simultaneously. The corresponding PCR products were purified by Microcon 100 (Millipore) or by agarose gel electrophoresis and QIAEX II Gel Extraction kit (QIAGEN). Direct sequencing of PCR products was performed with dRhodamine Terminator Cycle Sequencing Kit (PE Applied Biosystems). Sequences were resolved and analyzed on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Amplified segments were sequenced directly or were subcloned into the T-vector, in the case of a compound heterozygote in which there were two different mutations. The plasmid vector, pBluescript KS(+) (Stratagene), was digested with *Eco*RV and treated with *Taq* polymerase (Marchuk et al. 1991). Sequence was determined as described above. Detected mutations were described according to a nomenclature system for human gene mutations (Antonarakis 1998).

### *Restriction-Digestion Analysis*

For analysis of family KI-102, genomic DNA was amplified with a set of primers, gTRKA(5+6)F and TRKA-L600. PCR products were digested with *Mva*I and electrophoresed. Conditions for PCR are described above. For analysis of family KI-104, PCR products, amplified with a set of primers for exon 15, were digested with *Msp*I. The products were incubated with each restriction

**Table 1**

**Primer Sequence Used for the *TRKA* Gene**

| Analysis and Primer Name | Exons | Primer Sequence (5′→3′) | | PCR Product (bp) |
|---|---|---|---|---|
| | | Forward | Reverse | |
| PCR direct sequence: | | | | |
| gTRKA(1)F/R | 1 | AGCTGGGAGCGCACAGACGG | CAGCAAACAAGCAGCGAGTC | 323 |
| gTRKA(2+3)F/R | 2+3 | TGTGACTCCCATCCGCTCTC | TGTCCTCCCCAACTCCCACT | 516 |
| gTRKA(4)F/R | 4 | GCTCCTCCCCTCCCTCATTC | GTCATCCAACAGACCCAAGT | 224 |
| gTRKA(5+6)F/R | 5+6 | CTCCCTCTTATCCCCTGTGA | CTTTGTCCCCTGTGCTCTGC | 716 |
| gTRKA(7)F/R | 7 | CCTCCTCTCCTTTCCATCTG | CACCCTACATCCTCTTTTGA | 312 |
| gTRKA(8)F/R | 8 | TTCGGGTTCTACTCGCTTTG | TGGGGGCAGGGTTCAGGATG | 456 |
| gTRKA(9+10+11)F/R | 9+10+11 | CCAGCAGCCCACCTCCATCC | TACCCCAAACACACTCACAC | 895 |
| gTRKA(12)F/R | 12 | AGTTACAAGGTGGGGGTGAC | GGGCCTATGGCTGGAA | 388[a] |
| gTRKA(13+14)F/R | 13+14 | CAACCCCAGCCCTCCCAAGA | CTATGTTCACACATCTGCCT | 724 |
| gTRKA(15)F/R | 15 | CCAGTCTCCTCTCCCATCAC | TGGGATCCAGGGTGTCTACA | 406 |
| gTRKA(16)F/R | 16 | TAATGATGGGGCTGGGGTAG | AGGCCAGCAAGGGGTGACCA | 276 |
| gTRKA(17)F/R | 17 | GCTTTCTCCTCTGTCTCTCC | ATTCCGGCTAACCACTCCCA | 254 |
| Restriction enzyme: | | | | |
| gTRKA(5+6)F/TRKA-L600 | 5 (partial) | CTCCCTCTTATCCCCTGTGA | CATGACACTGCAGCTTCTGT | 176 |
| Exon trap: | | | | |
| gTRKA(4)F/gTRKA(5)R | 4+5 | GCTCCTCCCCTCCCTCATTC | TTTCAGTGCTTGCTCAGTGCC | ~1,400 |
| gTRKA(7)F/gTRKA(8)R | 7+8 | CCTCCTCTCCTTTCCATCTG | TGGGGGCAGGGTTCAGGATG | ~2,600 |
| SD6/TRKA-L600 | 5 (partial) | ATTTAGGTGACACTATAG | CATGACACTGCAGCTTCTGT | 268 |
| TRKA-U842/TRKA-L1144 | 7+8 (partial) | ATGTCACCAGTGACCTCAAC | GTGTAGTTGCCGTTGTTGAC | 323 |

[a] Number in which gt repeats of microsatellite D1S506 = 20.

enzyme and electrophoresed onto a 4% NuSieve agarose gel (FMC BioProducts).

### *Exon Trapping System*

An Exon Trapping System (Gibco BRL) was used to confirm putative splice-site mutations in vitro, according to the manufacturer's protocol. For a mutation on the splice-acceptor site of intron 4, a 1.4-kb fragment, containing exons 4 and 5, was amplified with a set of primers—forward, gTRKA(4)F and reverse, gTRKA(5)R—each from the patient or control. For a mutation on the splice-donor site of intron 7, a 2.6-kb fragment, containing exons 7 and 8, was amplified with a set of primers—forward, gTRKA(7)F and reverse, gTRKA(8)R—each from the patient or control. Amplified products were cloned into the T-vector and sequenced as described above. Subsequently, a *Xho*I-*Bam*I fragment, containing the insert from each clone, was subcloned into an exon trapping vector, pSPL3 (Gibco BRL). The pSPL3 derivatives were propagated in *Escherichia coli,* and the DNA was then isolated and transfected into COS-1 cells by Lipofectamin (Gibco BRL). The following day, cells were harvested and RNA isolated. To characterize a DNA segment trapped as an exon, mRNAs were amplified by RT-PCR, by use of random hexamer (for reverse transcription) and a set of primers, to locate corresponding exons of *TRKA*. Primer set SD6, in the pSPL3-vector sequence, and TRKA-L600, in exon 5, were used for detection of exon 5, whereas primer set TRKA-U842, in exon 7, and TRKA-L1144, in exon 8, were used for detection of exon 7. We also performed RT-PCR analysis of the *E2* gene of the branched-chain $\alpha$-keto-acid–dehydrogenase complex, as a positive control, to demonstrate the integrity of the mRNA and reverse transcription. A set of primers, E2-4/E2-7, for *E2,* was described elsewhere (Tsuruta et al. 1998).

## Results

### *Physical Characteristics of a Patient with CIPA*

We present a patient, to describe the physical characteristics associated with this disorder (fig. 1). The patient is a 5-year-old white girl of mixed Czech and English-German ancestry, born to nonconsanguineous parents. There are no abnormalities of the hair, eyebrows, or eyelashes (fig. 1*A*). She presents with recurrent unexplained fevers and anhidrosis by history. She developed palmoplantar hyperkeratosis, with significant fissuring of the plantar skin (fig. 1*B, C*). Since a very young age, she has demonstrated self-mutilating behavior (avulsion of teeth, biting of distal extremities), and she has had several deep heel ulcers that take a long time to heal, yet are painless (fig. 1*C*). The mutations in this patient are described below (family KI-108).

### *Mutations in* TRKA *in CIPA Families*

Seven unrelated CIPA families were screened for mutations in the *TRKA* gene. These include nine patients, four normal siblings, and 12 obligate carriers. All 17

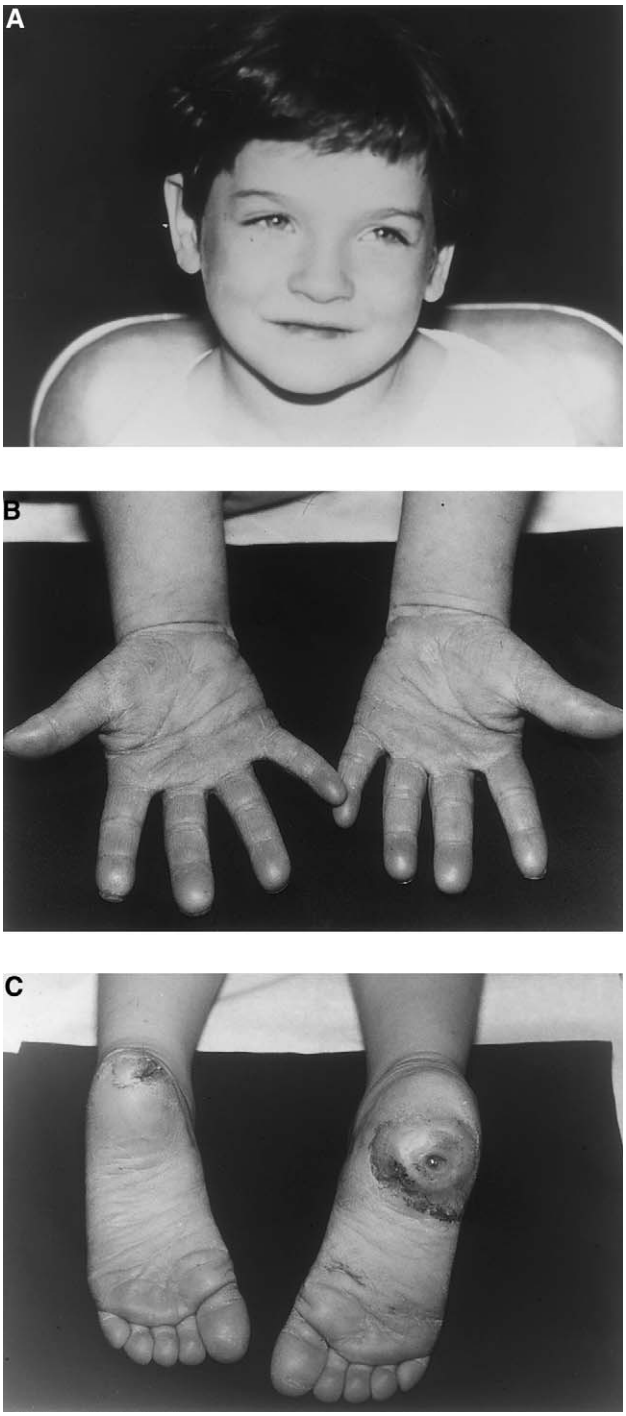


**Figure 1** Physical characteristics of a patient with CIPA, a 5-year-old white girl born to nonconsanguineous parents. *A*,There are no abnormalities of the hair, eyebrows, or eyelashes. *B*, Palmar skin is dry and hyperkeratotic. *C*, Plantar skin is dry and hyperkeratotic, with significant fissuring. Deep heel ulcers are painless and slow to heal. Informed consent for publication of photographs was obtained from her parents.

exons of the *TRKA* gene, including their flanking intronic sequences, were amplified from the genomic DNA of each patient with CIPA by use of the PCR primers specified in table 1. All exons and intron-exon boundaries were sequenced. We detected putative mutation(s) in the proband. For missense mutations, we compared DNA sequences from 100 nonaffected chromosomes to reduce the likelihood that the described change represented a polymorphism.

In a male patient with CIPA, of family KI-102, sequencing of genomic DNA revealed that a 3′ splice site contained a G→C transversion in the first position of intron 4 (IVS4−1G→C; fig. 2*A* and table 2). The substitution destroys a restriction enzyme site for *Mva*I. Digestion analysis with this enzyme also demonstrated that the proband and parents were homozygous and heterozygous, respectively (fig. 2*A*). In addition, we detected a C→A transversion at nucleotide 337 in exon 2, which causes an Arg→Ser substitution at amino acid 85 (Arg85Ser; table 2). Both parents were heterozygous for this substitution (data not shown).

In family KI-103 a single base A at nucleotide 284 was deleted, in exon 1 of the two female patients (fig. 2*B* and table 2). The deletion (~284delA) causes a frameshift and premature termination codon after amino acid Asn 67 (Asn67fr). The patients and parents were homozygous and heterozygous, respectively. One of three male siblings with normal phenotype was wild type, and the other two were heterozygous for this deletion. Thus, ~284delA is apparently the cause of CIPA in this family.

In family KI-104 we found a C→T transition, at nucleotide 2011 in exon 15 (~2011C→T), which causes an Arg→Trp substitution at amino acid 643 (Arg643Trp) (fig. 2*C* and table 2). After amplification of exon 15 from parental genomic DNA, we detected mutant and wild-type alleles. The substitution destroys a restriction-enzyme site for *Msp*I. Digestion analysis with this enzyme showed that the patient and parents were homozygous and heterozygous, respectively (fig. 2*C*).

In two female patients with CIPA, from family KI-105, a 5′ splice site of intron 7 contained a G→A substitution in the first position (IVS7+1G→A) (fig. 2*D* and table 2). The proband and parents were homozygous and heterozygous for IVS7+1G→A, respectively. A female sibling with normal phenotype was heterozygous.

In family KI-106 we found a C→T transition at nucleotide 109 (109C→T) in exon 1, which changes a Gln to a termination codon at amino acid 9 (Gln9X) in the proband (fig. 2*E* [*upper*] and table 2). In addition, we detected two further base changes, both in exon 15: a C→T transition at nucleotide 1876 (~1876C→T) and a G→T transversion at nucleotide 1904 (~1904G→T) that cause a His→Tyr substitution at amino acid 598 (His598Tyr) and a Gly→Val substitution at amino acid 607 (Gly607Val), respectively (data not shown). The

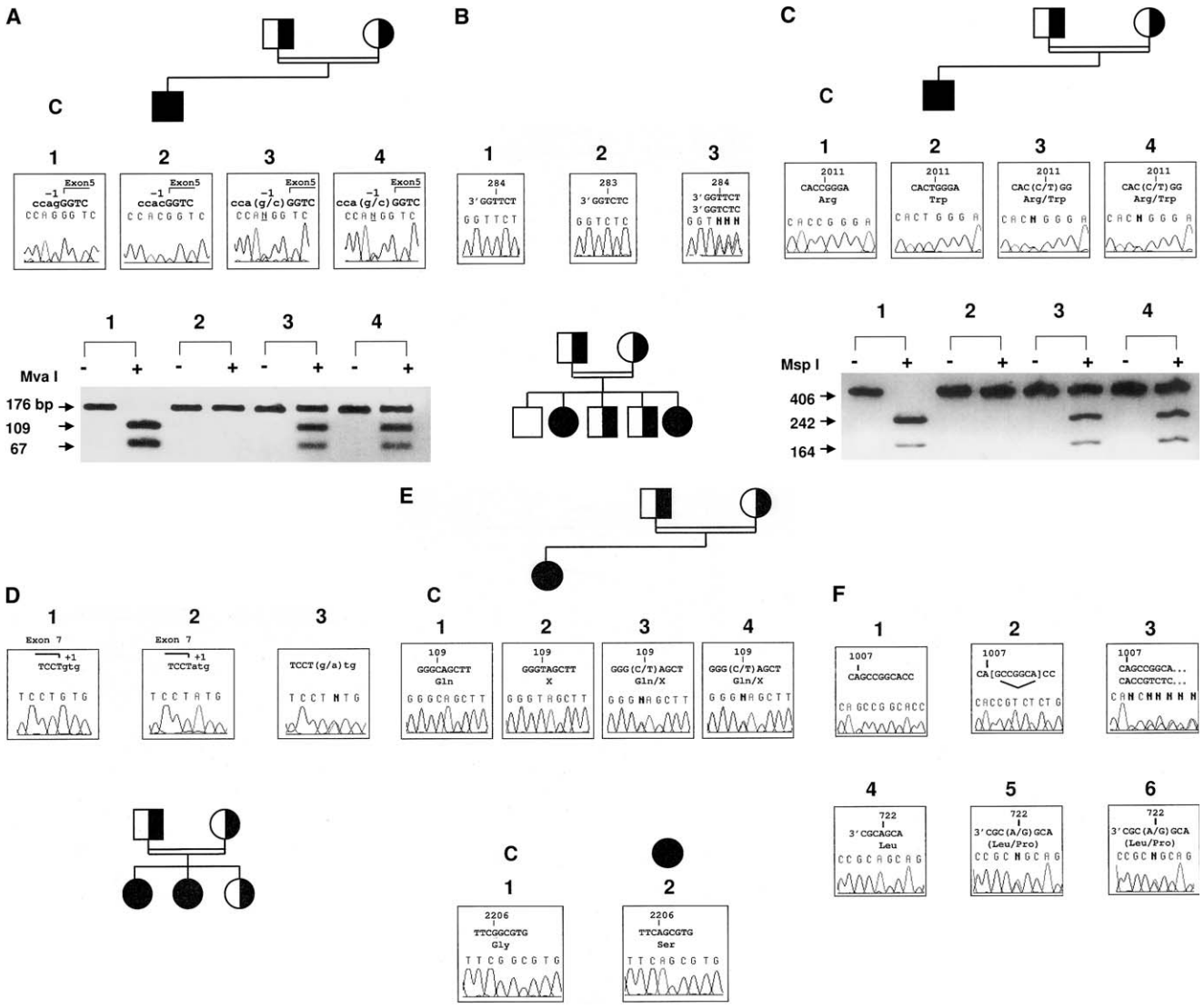


**Figure 2** Mutation analysis of human *TRKA,* in seven CIPA families. *A,* In family KI-02 a G→C transversion at the first position of the 3′ splice site in intron 4 (IVS4−1G→C) (*upper*) and restriction-digestion analysis with *Mva*I (*lower*) are shown. Shown are a normal control (*lane 1*), the patient (*lane 2*), and the patient's father and mother (*lanes 3 and 4*). *B,* A single base deletion at nucleotide 284 of exon 1, in family KI-103. Reverse sequences are shown. Shown are a normal control (*box 1*); the proband, as a representative patient in this family (*box 2*); and the father, as a representative heterozygote (*box 3*). The proband and her younger sister with CIPA are homozygous for the mutation. An older brother is normal. Two younger brothers with normal phenotype and both parents are heterozygous. *C,* In family KI-104 a C→T transition at nucleotide 2011 in exon 15 (*upper*) and restriction-digestion analysis with *Msp*I (*lower*) are shown. The transition causes an Arg→Trp substitution at amino acid 643. Shown are a normal control (*lane 1*); the patient (*lane 2*); and the patient's father and mother (*lanes 3 and 4*). *D,* A G→A transition at the first position of the 5′ splice site in intron 7, in family KI-105. Shown are a normal control (*box 1*); the proband, as a representative patient in this family (*box 2*); and the father, as a representative heterozygote (*box 3*). The proband and her younger sister with CIPA are homozygous for the mutation. Both parents and her younger sister with normal phenotype are heterozygous. *E,* In family KI-106, a C→T transition at nucleotide 109 in exon 1 causes a Gln codon to become a termination codon at position 9, as shown in the upper part. Shown are a normal control (*box 1*), the patient (*box 2*), and the patient's father and mother (*boxes 3 and 4*). In family KI-107, a G→A transition at nucleotide 2206 in exon 16 causes a Gly→Ser substitution at amino acid 708, as shown in the lower part. DNA samples from parents were not available for testing in this family. *F,* Two different mutations at the *TRKA* locus, in family KI-108. A 7-bp deletion, from nucleotide 1008 to 1014 in exon 8 (~1008–1014delGCCGGCA), causes a frameshift after amino acid Gln308 and premature termination codon downstream, as shown for control and subcloned mutant sequences, respectively (*boxes 1 and 2*). This mutation is derived from her father (*box 3*). The other, a T→C transition at nucleotide 722 in exon 6, causes a Leu→Pro substitution at amino acid 213; results of the PCR direct sequence are shown in reverse orientation for control and mutant sequences, respectively (*boxes 4 and 5*). The latter mutation is derived from her mother (*box 6*).

**Table 2**

**Mutation Analysis of CIPA Families**

| Family | Consanguinity | Status | Nucleotide Change (Location) | Mutation Type (Predicted Consequence) | Country of Origin |
|---|---|---|---|---|---|
| KI-102 | Yes | Homozygote | IVS4−1G→C; ~337C→A (intron 4; exon 2) | Splice site; missense (aberrant splicing of exon 5; Arg85Ser) | UAE |
| KI-103 | Yes | Homozygote | ~284delA (exon 1) | Frameshift (truncation after Asn67) | Kuwait |
| KI-104 | Yes | Homozygote | ~2011C→T (exon 15) | Missense (Arg643Trp) | Spain |
| KI-105 | Yes | Homozygote | IVS7+1G→A (intron 7) | Splice site (aberrant splicing of exon 7) | Kuwait |
| KI-106 | Yes | Homozygote | ~109C→T; ~1876C→T; ~1904G→T (exon 1; exon 15 ; exon 15) | Nonsense; missense; missense (Gln9X; His598Tyr; Gly607Val) | Italy |
| KI-107 | No | Homozygote | ~2206G→A (exon 16) | Missense (Gly708Ser) | Italy |
| KI-108 | No | Compound heterozygote | ~722T→C (exon 6); c.1008^1014delGCCGGCA (exon 8) | Missense (Leu213Pro); frameshift (truncation after Gln308) | Canada[a] |

NOTE.—Positions of nucleotide change are from the transcription start site, as described by Martin-Zanca et al. (1989). The ATG initiation codon is located at nucleotide position c85. The structure and organization of human *TRKA* were described by Indo et al. (1997).

[a] Family from Canada is of mixed Czech and English-German ancestry.

proband and both parents were homozygous and heterozygous for all base substitutions, respectively.

In family KI-107 a homozygous G→A transition at nucleotide 2206 (~2206G→A), in exon 16, was predicted to cause a Gly→Ser substitution at amino acid 708 (Gly708Ser) (fig. 2*E* [*lower*] and table 2). DNA samples from parents were not available for testing.

In family KI-108 the proband was a compound heterozygote, having two different mutations at the *TRKA* locus on separate chromosomes (fig. 2*F* and table 2). Each mutant allele was subcloned into a plasmid vector and sequenced. One contained a 7-bp deletion, from nucleotide 1008 to 1014 (~1008–1014delGCCGGCA) in exon 8, which is predicted to cause a frameshift after amino acid Gln308 and create of a downstream premature termination codon (Gn308fr). The other was a T→C transition at nucleotide 722 (~722T→C) in exon 6, which causes a Leu→Pro substitution at amino acid 213 (Leu213Pro). Sequence analysis of the parents revealed that (~1008–1014delGCCGGCA) and (~722T→C)
were derived from the father and mother, respectively.

### *Exon Trap Analysis of Splice-Site Mutations*

We characterized the IVS4−1G→C mutation in family KI-102 and the IVS7+1G→A mutation in family KI-05 by using exon trap analysis, since mRNA preparations from the patients were not available for testing. A 1.4-kb fragment containing exons 4 and 5, or a 2.6-kb fragment containing exons 7 and 8, as well as part of the flanking introns, was amplified from proband and control samples. Each was subcloned into the exon trap vector, pSPL3, to prepare a minigene construct. After transfection of the minigene and incubation of cells, we observed whether exon 5 or exon 7 was incorporated into a transcript. RT-PCR analysis demonstrated that the corresponding exon was incorporated in the control but not in the patient (fig. 3*A, B*). A faint, large DNA fragment was observed in the patient sample from family KI-02 (fig. 3*A*). Sequence analysis of the fragment showed that it was derived from an alternative splicing event, which occurred at a cryptic splice-acceptor site in intron 4 (IVS4-41; data not shown).

Thus, 11 novel mutations have been detected in seven CIPA families from five countries. None of these mutations were detected in 100 chromosomes from unrelated controls. Further, all appear to be "private mutations," restricted to each individual family.

## Discussion

We reported elsewhere that *TRKA* is responsible for CIPA (Indo et al. 1996). Given the lack of information on the structure of the entire human *TRKA* gene, we restricted our analysis to the region encoding the intracellular tyrosine kinase domain. We detected three mutations, R548fs, G571R, and IVS15+3A→C, in three consanguineous Japanese CIPA families and in one consanguineous Ecuadorian CIPA family. These mutations are located in exon 14 and intron 15 (fig. 4). It has not been confirmed whether all patients with CIPA have mutation(s) in *TRKA* or whether there is genetic heterogeneity among various ethnic groups. Complete analysis of patients with CIPA was hampered by the lack of a full-length gene sequence; hence we report on the structure and organization of the whole human *TRKA,* to facilitate mutation analysis (Indo et al. 1997).

Human *TRKA* is divided into 17 exons and 16 introns. The entire sequence was estimated to span ⩾23 kb, coding for a protein of 790 or 796 amino acid res-

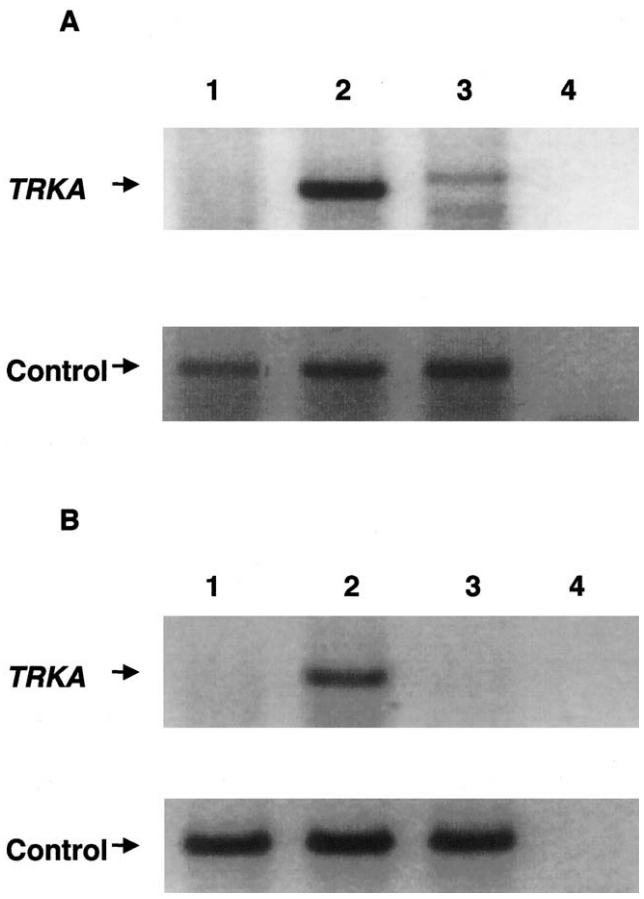


**Figure 3** Exon-trap analysis of splice-site mutations in *TRKA*. *A,* Characterization of the IVS4−1G→C mutation detected in family KI-102. A fragment containing exons 4 and 5, as well as parts of flanking introns, was subcloned into the exon trap vector, pSPL3, and transfected into COS-1 cells. RT-PCR analysis was done on the mRNA from such cells transfected with each vector construct. Shown are a vector alone (*lane 1*), a vector with a normal *TRKA* gene (*lane 2*), a vector with a mutant *TRKA* gene (*lane 3*), and PCR reaction without a product by reverse transcription (*lane 4*). Exon 5 is incorporated in the control but not in the patient. A 268-bp fragment was amplified from the normal construct (*lane 2*). Faint bands in lane 3 are products of alternative splicing, as described in the text. A housekeeping gene was analyzed, as a control for integrity of the mRNA and of the RT-PCR reaction. *B,* Characterization of the IVS7+1G→A mutation in family KI-05. A fragment containing exons 7 and 8, as well as parts of flanking introns, was used in a similar way, as described above. Exon 7 is incorporated in the control but not in the patient. A 323-bp fragment was amplified from the normal construct (*lane 3*).

idues. A single transmembrane domain divides the TRKA protein into an extracellular and an intracellular domain (Schneider and Schweiger 1991; Barbacid 1995; Bothwell 1995). The extracellular domain is important for specific NGF binding and includes a signal peptide; three tandem leucine-rich motifs, flanked by two cysteine clusters; and two immunoglobulin-like domains (or motifs). The intracellular domain includes a juxtamembrane region, a tyrosine kinase domain, and a very short carboxyl-terminal tail. There is a general correlation between the genomic organization of *TRKA* and the functional organization of the TRKA protein product (fig. 4) (Indo et al. 1997). Exon 1 contains the signal peptide and the first cysteine cluster. Three leucine-rich motifs are encoded by exons 2, 3, and 4. Exon 5 contains the second cysteine cluster. The first immunoglobulin-like motif is encoded by exons 6 and 7, whereas the second immunoglobulin-like motif is encoded by the single exon 8. Thus, the splice sites of *TRKA,* encoding the extracellular domain, separate the functional domains, so that each is encoded by separate exons. Exon 9 is small (18 bp) and incorporated into mRNA by alternative splicing. Six amino acid residues encoded by this exon are present in the extracellular domain of the neuronal-specific TRKA receptor (Barker et al. 1993). The transmembrane domain is encoded by exons 10 and 11, and the intracellular domain of TRKA is encoded by exons 11–17. The juxtamembrane domain is encoded by exons 11 and 12. The domain contains an IXNPXpY motif, where "p" indicates phosphorylation at the Tyr-490 residue of the activated TRKA (Dikic et al. 1995). This motif is encoded by exon 12 and is recognized by an Shc-adapter protein, required for activation of the Ras-MAPK pathway (Obermeier et al. 1994; Stephens et al. 1994). The tyrosine kinase domain that is phosphorylated in response to NGF—and is critical for the intracellular signaling—is encoded by exons 13–17. A consensus–sequence motif YXXM, which interacts with phosphatidylinositol-3′ kinase, is located at the end of the kinase catalytic domain (Tyr-751 residue in TRKA) (Obermeier et al. 1993*b*; Soltoff et al. 1992) and encoded by exon 17. The short carboxyl-terminal tail of 15 amino acids is also encoded by exon 17 and includes a conserved Tyr residue (Tyr-785 in TRKA) that is responsible for binding of phospholipase Cγ (Obermeier et al. 1993*a*; Loeb et al. 1994).

In this report, we present the methodology to amplify and analyze all coding exons from genomic DNA by PCR direct sequencing. A total of 11 novel mutations are described, in seven families from various ethnic groups (fig. 4). CIPA mutations are spread throughout the signal peptide, extracellular domain, and intracellular domain. There are six missense mutations (Arg85Ser, Leu213Pro, His598Tyr, Gly607Val, Arg643Trp, and Gly708Ser), two frameshift mutations (Asn67fs and Gln308fs), one nonsense mutation (Gln9X), and two splice mutations (IVS4−1G→C and IVS7+1G→A).

Three missense mutations, Leu213Pro, Arg643Trp, and Gly708Ser, are probably responsible for CIPA. They have not been observed in 100 normal chromosomes. The Leu213Pro mutation is detected in a state of compound heterozygosity in the patient who has a 7-bp de-

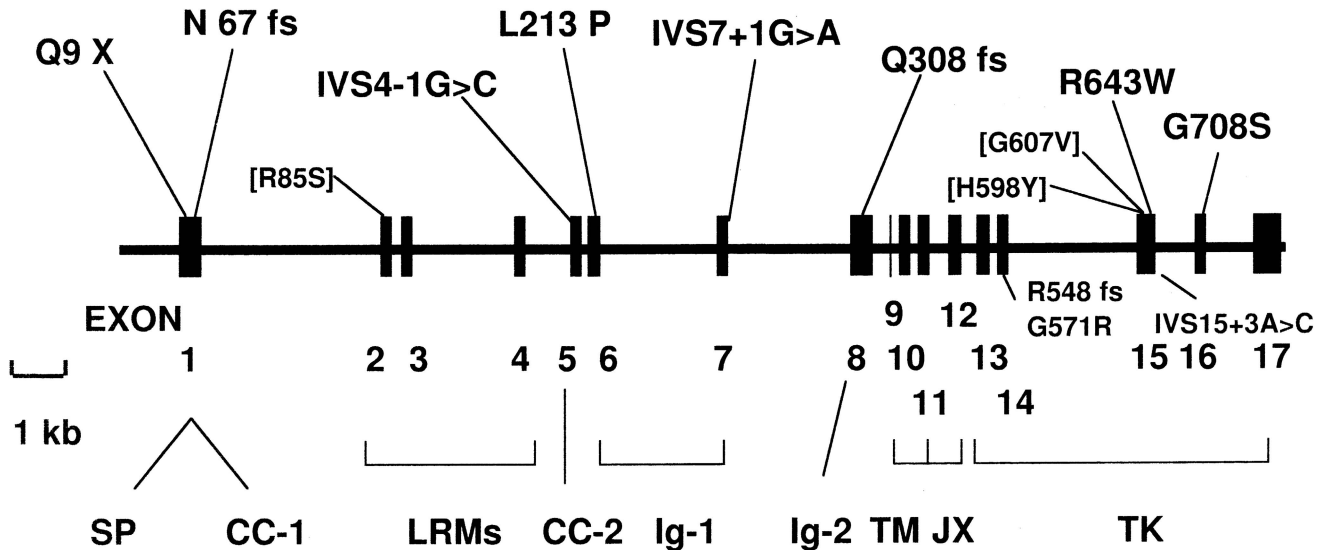


**Figure 4** Disease-associated mutations in *TRKA*. Amino acid numbering of the TRKA protein and structure of the *TRKA* gene are according to Martin-Zanca et al. (1989) and Indo et al. (1997), respectively. Mutations reported in this study are listed above the *TRKA* diagram, and three mutations, previously reported, are listed below the diagram. A nomenclature system for human gene mutations is used, according to the recommendations of Antonarakis (1998). Mutations in brackets are detected as double or triple mutations, as described in the text. Abbreviations listed in the lower part show the domain structures encoded by the corresponding exon(s) (Schneider and Schweiger 1991; Indo et al. 1997). SP = signal peptide; CC-1 and CC-2 = the first and second cysteine clusters, respectively; LRMs = leucine-rich motifs; Ig-1 and Ig-2 = the first and second immunoglobulin-like motifs, respectively; TM = transmembrane; JX = juxtamembrane; and TK = tyrosine kinase.

letion on a separate chromosome. It is located in the first immunoglobulin-like motif in the extracellular domain, which is important for NGF binding, and is conserved between TRKA and TRKB. Both the Arg643Trp and Gly708Ser mutations are located in the intracellular tyrosine kinase domain and conserved among ⩾14 receptor tyrosine kinases, including human TRKB and TRKC (Martin-Zanca et al. 1989; Nakagawara et al. 1995). This suggests that they are important for enzyme activity. Arg85Ser and (His598Tyr and Gly607Val) are detected as a double and triple mutation, respectively, and are described below. The His598Tyr mutation is located in the intracellular tyrosine kinase domain and conserved in three TRK families. However, Arg85Ser (located in the leucine-rich motif of the extracellular domain) and Gly607Val (in the intracellular tyrosine kinase domain) are not conserved. The effects of all these missense mutations on the function of the TRKA protein are currently unknown, although experiments are in progress to address this question.

Two frameshift mutations, Asn67fs and Gln308fs, are located in the first cysteine cluster and the second immunoglobulin-like motifs, respectively. These should result in truncation of the TRKA polypeptide in the extracelluar domain. Thus, these two mutations almost certainly cause defects in NGF signal transduction.

The nonsense mutation, Gln9X, is located on the signal peptide and is associated with two missense mutations (His598Tyr and Gly607Val) on the same chromosome. The parents in this family are consanguineous, and both are carriers of these three mutations. The patient is homozygous for all three. Thus, they probably represent a triple mutation, although Gln9X is the most likely cause of CIPA in this family. Triple mutation is rare and has been reported in Gaucher disease (Hong et al. 1990). In contrast, double mutations have been reported in several clinically relevant genes, such as the gene encoding the $\alpha$-subunit of hexosaminidase A (Ainsworth and Coulter-Mackie 1992), the low-density lipoprotein receptor (Jensen et al. 1997), and so on. Without screening the entire coding region of *TRKA* in the consanguineous homozygous patient, these CIPA mutations would not have been detected. The triple mutation awaits further study.

Two splice-site mutations (IVS4−1G→C and IVS7+1C→A) cause skipping of exon 5 and exon 7, respectively, in vitro. We predict that IVS4−1G→C and IVS7+1G→A cause a skip of exon 5 or alternative splicing, and a skip of exon 7, respectively, in vivo. These exons are located in a region encoding the extracellular domain of TRKA. These changes would result in a deletion of the second cysteine cluster or of a part of the first immunoglobulin-like motifs. Further, these two changes would result in a frameshift and cause a trun-

cation of the TRKA protein. This structural change in the extracellular domain would affect the function of TRKA in NGF binding, and hence these splice mutations are probably responsible for CIPA. In addition, the splice IVS4−1G→C mutation is associated with Arg85Ser on the same chromosome. The patient and both parents are homozygous and heterozygous for these two substitutions in this consanguineous family. Thus, these two probably represent a double mutation. The IVS4−1G→C is likely the main cause of CIPA in this family, considering the effect of exon skipping or alternative splicing.

The data presented in this article represent the first extensive mutation analysis of the whole *TRKA* gene in CIPA patients from various ethnic groups. Mendelian inheritance of the mutations was confirmed by sequence analysis of the relevant amplified DNA fragment in family members. These mutations do not seem to represent frequent polymorphisms because they are not detected in unaffected control individuals. We cannot completely rule out the possibility that they are rare polymorphisms. The three amino acid substitutions detected as double or triple mutations might be rare polymorphisms in a particular ethnic background.

Mutations responsible for CIPA are located in the extracellular domain as well as in the intracellular domain of TRKA.They provide insight into the structure and function, for neurotrophin members, of the TRK receptor family. We did not observe any genotype-phenotype correlation in the patients with CIPA we analyzed in this study. All patients have typical features: insensitivity to pain and anhidrosis. There seems to be no genetic heterogeneity in CIPA, although we cannot rule out the possibility that mutation(s) in other gene(s) are responsible for clinical phenotypes similar to CIPA.

CIPA is a painless but severe genetic disorder associated with devastating complications, often leading to crippling or fatal consequences. This study will facilitate analyses of CIPA mutations in *TRKA* and provide an invaluable aid for the diagnosis of this disorder and for genetic counseling.

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## Electronic-Database Information

Accession numbers and URLs for data in this article are as follow:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for CIPA [MIM 256800]).

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank/index.html (for the complete nucleotide sequences for human *TRKA* and its transcript [AB019480-AB019488 and M23102])

## References

Ainsworth PJ, Coulter-Mackie MB (1992) A double mutation in exon 6 of the $\beta$-hexosaminidase $\alpha$ subunit in a patient with the B1 variant of Tay-Sachs disease. Am J Hum Genet 51:802–809

Antonarakis SE (1998) Recommendations for a nomenclature system for human gene mutations. Nomenclature Working Group. Hum Mut 11:1–3

Barbacid M (1995) Structural and functional properties of the TRK family of neurotrophin receptors. Ann NY Acad Sci 766:442–458

Barker PA, Lomen HC, Gensch EM, Meakin SO, Glass DJ, Shooter EM (1993) Tissue-specific alternative splicing generates two isoforms of the trkA receptor. J Biol Chem 268: 15150–15157

Bothwell M (1995) Functional interactions of neurotrophins and neurotrophin receptors. Ann Rev Neurosci 18:223–253

Dikic I, Batzer AG, Blaikie P, Obermeier A, Ullrich A, Schlessinger J, Margolis B (1995) Shc binding to nerve growth factor receptor is mediated by the phosphotyrosine interaction domain. J Biol Chem 270:15125–15129

Dyck PJ (1984) Neuronal atrophy and degeneration predominantly affecting sensory and autonomic neurons. In: Dyck PJ, Thomas PK, Lambert EH, Bunge R (eds) Peripheral neuropathy. W. B. Saunders Company, Philadelphia, pp 1557–1599

Greco A, Villa R, Pierotti MA (1996) Genomic organization of the human NTRK1 gene. Oncogene 13:2463–2466

Hong CM, Ohashi T, Yu XJ, Weiler S, Barranger JA (1990) Sequence of two alleles responsible for Gaucher disease. DNA Cell Biol 9:233–241

Indo Y, Mardy S, Tsuruta M, Karim MA, Matsuda I (1997) Structure and organization of the human TRKA gene encoding a high affinity receptor for nerve growth factor. Jpn J Human Genet 42:343–351

Indo Y, Tsuruta M, Hayashida Y, Karim MA, Ohta K, Kawano T, Mitsubuchi H, et al (1996) Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. Nat Genet 13:485–488

Ismail EA, Al-Shammari N, Anim JT, Moosa A (1998) Congenital insensitivity to pain with anhidrosis: lack of eccrine sweat gland innervation confirmed. J Child Neurol 13: 243–246

Jensen HK, Jensen TG, Faergeman O, Jensen LG, Andresen BS, Corydon MJ, Andreasen PH, et al (1997) Two mutations in the same low-density lipoprotein receptor allele act in synergy to reduce receptor function in heterozygous familial hypercholesterolemia. Hum Mut 9:437–444

Kaplan DR, Hempstead BL, Martin ZD, Chao MV, Parada LF (1991) The trk proto-oncogene product: a signal trans-

ducing receptor for nerve growth factor. Science 252: 554–558

Klein R, Jing SQ, Nanduri V, O'Rourke E, Barbacid M (1991) The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 65:189–197

Langer J, Goebel HH, Veit S (1981) Eccrine sweat glands are not innervated in hereditary sensory neuropathy type IV: an electron-microscopic study. Acta Neuropathol Berl 54: 199–202

Levi-Montalcini R (1987) The nerve growth factor: thirty-five years later. EMBO J 6:1145–1154

Loeb DM, Stephens RM, Copeland T, Kaplan DR, Greene LA (1994) A Trk nerve growth factor (NGF) receptor point mutation affecting interaction with phospholipase C-$\gamma$ 1 abolishes NGF-promoted peripherin induction but not neurite outgrowth. J Biol Chem 269:8901–8910

Marchuk D, Drumm M, Saulino A, Collins FS (1991) Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nuclic Acids Res 19: 1154

Martin-Zanca D, Barbacid M, Parada LF (1990) Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. Genes Dev 4:683–694

Martin-Zanca D, Hughes SH, Barbacid M (1986) A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. Nature 319:743–748

Martin-Zanca D, Oskam R, Mitra G, Copeland T, Barbacid M (1989) Molecular and biochemical characterization of the human trk proto-oncogene. Mol Cell Biol 9:24–33

McKusick VA (1994) Mendelian inheritance in man. The Johns Hopkins University Press, Baltimore, pp 2073

Miozzo M, Pierotti MA, Sozzi G, Radice P, Bongarzone I, Spurr NK, Della PG (1990) Human TRK proto-oncogene maps to chromosome 1q32-q41. Oncogene 5:1411–1414

Morris CM, Hao QL, Heisterkamp N, Fitzgerald PH, Groffen J (1991) Localization of the TRK proto-oncogene to human chromosome bands 1q23-1q24. Oncogene 6:1093–1095

Nakagawara A, Liu XG, Ikegaki N, White PS, Yamashiro DJ, Nycum LM, Biegel JA, et al (1995) Cloning and chromosomal localization of the human TRK-B tyrosine kinase receptor gene (NTRK2). Genomics 25:538–546

Obermeier A, Bradshaw RA, Seedorf K, Choidas A, Schlessinger J, Ullrich A (1994) Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLC $\gamma$. EMBO J 13:1585–1590

Obermeier A, Halfter H, Wiesmuller KH, Jung G, Schlessinger J, Ullrich A (1993*a*) Tyrosine 785 is a major determinant of Trk–substrate interaction. EMBO J 12:933–941

Obermeier A, Lammers R, Wiesmuller KH, Jung G, Schlessinger J, Ullrich A (1993*b*) Identification of Trk binding sites for SHC and phosphatidylinositol 3′-kinase and formation of a multimeric signaling complex. J Biol Chem 268: 22963–22966

Rafel E, Alberca R, Bautista J, Navarrete M, Lazo J (1980) Congenital insensitivity to pain with anhidrosis. Muscle Nerve 3:216–220

Schneider R, Schweiger M (1991) A novel modular mosaic of cell adhesion motifs in the extracellular domains of the neurogenic trk and trkB tyrosine kinase receptors. Oncogene 6: 1807–1811

Smeyne RJ, Klein R, Schnapp A, Long LK, Bryant S, Lewin A, Lira SA, et al (1994) Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature 368:246–249

Snider WD (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell 77:627–638

Soltoff SP, Rabin SL, Cantley LC, Kaplan DR (1992) Nerve growth factor promotes the activation of phosphatidylinositol 3-kinase and its association with the trk tyrosine kinase. J Biol Chem 267:17472–17477

Stephens RM, Loeb DM, Copeland TD, Pawson T, Greene LA, Kaplan DR (1994) Trk receptors use redundant signal transduction pathways involving SHC and PLC-$\gamma$ 1 to mediate NGF responses. Neuron 12:691–705

Swanson AG (1963) Congenital insensitivity to pain with anhidrosis. Arch Neurol 8:299–306

Swanson AG, Buchan GC, Alvord EC (1965) Anatomic changes in congenital insensitivity to pain. Arch Neurol 12: 12–18

Thoenen H, Barde Y-A (1980) Physiology of nerve growth factor. Physiol Rev 60:1284–1335

Tsuruta M, Mitsubuchi H, Mardy S, Miura Y, Hayashida Y, Kinugasa A, Ishitsu T, et al (1998) Molecular basis of intermittent maple syrup urine disease: novel mutations in the E2 gene of the branched-chain $\alpha$-keto acid dehydrogenase complex. J Hum Genet 43:91–100