

Congenital insensitivity to pain with anhidrosis (CIPA): effect of *TRKA* (*NTRK1*) missense mutations on autophosphorylation of the receptor tyrosine kinase for nerve growth factor

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Human *TRKA* (*NTRK1*) encodes the receptor tyrosine kinases (RTK) for nerve growth factor (NGF) and is the gene responsible for congenital insensitivity to pain with anhidrosis (CIPA), an autosomal recessive disorder characterized by a lack of pain sensation and anhidrosis. We reported 11 putative missense mutations in 31 CIPA families from various ethnic groups. Here we have introduced the corresponding mutations into the *TRKA* cDNA and examined NGF-stimulated autophosphorylation. We find that wild-type *TRKA* precursor proteins in a neuronal cell line and a non-neuronal cell line were differentially processed and phosphorylated in an NGF-dependent and -independent manner, respectively. Two mutants (L93P and L213P) in the extracellular domain were aberrantly processed and showed diminished autophosphorylation in neuronal cells. Five mutants (G516R, G571R, R643W, R648C and G708S) in the tyrosine kinase domain were processed as wild-type *TRKA* but showed significantly diminished autophosphorylation in both neuronal and non-neuronal cells. In contrast, R85S and (H598Y; G607V), detected previously as double and triple mutations, are probably polymorphisms in a particular ethnic background. The other putative mutant D668Y might be a rare polymorphism or might impair the function of *TRKA* without compromising autophosphorylation. Mutated residues in the tyrosine kinase domain are conserved in various RTKs and probably contribute to critical function of these proteins. Thus, naturally occurring *TRKA* missense mutations with loss of function provide considerable insight into the structure-function relationship in the RTK family. Our data may aid in developing a drug which targets the clinically devastating ‘complex regional pain syndrome’.

INTRODUCTION

Nerve growth factor (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 (1, 2). Human *TRKA* (also named *NTRK1*) was isolated from a colon carcinoma as a potential new member of the tyrosine kinase gene family (3) and expression of *TRKA(trk)* was later found in the nervous system (4). *TRKA* is a receptor tyrosine kinase which is phosphorylated in response to NGF (5, 6). The binding of NGF to *TRKA* stimulates homodimer formation and activation of tyrosine kinase activity. Phosphorylated tyrosine residues in the *TRKA* cytoplasmic domain serve as anchors for binding downstream signaling molecules (7, 8). Proteins known to become phosphorylated and activated in response to NGF include phospholipase C γ -1 (PLC γ -1), phosphatidylinositol 3-kinase (PI3K), the adapter protein Shc and the Src1-associated neurotrophic factor target (SNT, also called FRS2) (9). SNT/FRS2 was first identified as a specific target of differentiation factor-induced tyrosine kinase activity in neuronal cells (10) and then cloned as a lipid-anchored docking protein to link fibroblast growth factor and NGF receptor activation with signaling pathways essential for cell differentiation (11, 12). This protein is also phosphorylated in response to NGF (11, 13, 14).

The human *TRKA* gene, located on chromosome 1q21-q22 (15) is divided into 17 exons and 16 introns. The entire sequence was estimated to span > 23 kb region, coding for a protein of 790 or 796 amino acid residues (16, 17). Six amino acid residues encoded by exon 9 are in the extracellular domain of the neuronal-specific *TRKA* receptor (18). A single transmembrane domain divides *TRKA* protein into an extracellular domain and an intracellular domain (7, 8, 19). 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, including a juxtamembrane region, a tyrosine kinase domain and a very short carboxy-terminal tail, is phosphorylated in response to NGF and is critical for intracellular signaling. *In vitro* assessment has identified *TRKA* tyrosine residues - 490, 670, 674, 675 and 785 as autophosphorylation sites (20). The juxtamembrane domain contains an IXNPXpY motif, where p indicates phosphorylation at the -490Tyr residue of the activated *TRKA* (21). This motif is recognized by an Shc- adapter protein, required for activation of the Ras-MAPK pathway (20, 22). The Shc-association site is also important for PI3K activation. SNT/FRS2 also binds to the phosphorylated -490Tyr residue of *TRKA* (14). Tyrosines - 670,

674 and 675, which lie within the kinase activation loop, are involved in the regulation of the TRKA kinase activity and transphosphorylation (23). A consensus sequence motif YXXM, which interacts with PI3K, is located at the end of the kinase catalytic domain (-751Tyr in TRKA) (24, 25). There is another report indicating that PI3K and TRKA do not associate directly (26), and growth-associated binder-1 (Gab1)–docking protein is probably required for PI3K activation (27, 28). The short C-terminal tail of 15 amino acids includes a conserved tyrosine residue (-785Tyr in TRKA) which is responsible for binding of PLC γ -1 (29, 30).

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 (31, 32). We earlier 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 (33). CIPA is the first human genetic disorder implicated in the neurotrophin signal transduction system. We developed a comprehensive strategy to screen for *TRKA* mutations on the basis of the gene's structure and organization (34), and we characterized intragenic polymorphic sites and described the haplotypic associations of mutant alleles (35). We detected 25 mutations, including 6 frameshift mutations, 4 nonsense mutations, 4 splice-site mutations and 11 putative missense mutations in 31 CIPA families from various ethnic groups (33-35). Mendelian inheritance of the mutations has been confirmed in families from which samples from parents were available. Loss of TRKA function is self-evident in the frame-shift, splice-site or nonsense mutations. In contrast, putative missense mutations, including 'double' and 'triple mutations' (34), require an expression study for confirmation, as a cause of CIPA. Such studies provide an opportunity to consider the structure-function relationship of TRKA protein as well as findings in receptor tyrosine kinase (RTK) family members.

We have now examined 11 putative *TRKA* missense mutations, distributed in the extracellular domain as well as the intracellular signal transduction domain, by expressing them in two different cell lines and the ligand-induced autophosphorylation was scrutinized.

RESULTS

Eleven putative *TRKA* missense mutations examined in the present study

We detected 11 putative missense mutations in 31 CIPA families from various ethnic groups (Table 1) (33-35). Arg85Ser and (His598Tyr; Gly607Val) were linked, on the same chromosome, to a splice-site mutation in intron 4 (IVS-1G>C) and a nonsense mutation in exon 1 (Gln9X). These were assumed to be ‘double’ and ‘triple’ mutations, respectively, since they were detected by screening the entire coding region of *TRKA* in consanguineous homozygous patients from United Arab Emirates and Italy, respectively (34). Positions of each putative mutation in the *TRKA* gene and its protein product were based on the domain structure (7, 17, 19, 36) and structure-based amino acid alignment of the insulin receptor (37, 38).

Transfection of the wild-type *TRKA* cDNA into two cell lines: SH-SY5Y and COS-1

We subcloned the full-length wild-type *TRKA* cDNA into a mammalian expression vector pCAGGS and transfected it into a neuronal cell line, SH-SY5Y. The *TRKA* precursor protein was detected and processed to a 140 and a 110 kDa form (Fig.1A). The primary translated product is the 110 kDa glycoprotein which becomes immediately glycosylated, presumably during its translocation into the endoplasmic reticulum, and this molecule is further glycosylated to yield the mature 140 kDa form (36). A faint band of 80 kDa is probably the common unglycosylated polypeptidic backbone of these proteins. The *TRKA*-transfected cells were exposed to NGF in order to study receptor activation, the result being autophosphorylation of tyrosine residues. Signal of the 140 kDa form was intense and phosphorylated in response to NGF. The signal detected by anti-phosphorylated -674/675Tyr antibody was weaker than that detected by anti-phosphorylated -490Tyr antibody. Thus, the *TRKA* precursor protein is processed to two 140 and 110 kDa glycosylated forms and these are phosphorylated in response to NGF in SH-SY5Y cells; however, phosphorylation of the latter is less than that of the former.

In the non-neuronal cell line COS-1, the *TRKA* precursor protein was processed mainly to the 110 kDa form, although the 140 and 80 kDa forms were also faintly visible (Fig. 1B). All forms were phosphorylated irrespective of the presence or absence of NGF.

Autophosphorylation of all these was slightly enhanced by NGF. In this experiments, we

cultured COS-1 cells in 0.5 % fetal calf serum to reduce the basal activation induced by a putative activator or ligand included in calf serum. We used a commercially available cell extract of PC-12 cells, with or without NGF stimulation, as a reference in order to compare the size of TRKA forms (bands) in each cell line. The 140 kDa form was the main TRKA protein and was phosphorylated in response to NGF in the PC-12 cell extract (data not shown). But the 110 kDa form was the main TRKA protein in COS-1 cells and was phosphorylated without NGF stimulation. We also examined an effect of NGF concentration on autophosphorylation of the TRKA protein expressed in COS-1 cells. TRKA protein was phosphorylated without adding NGF and the state of phosphorylation was not affected by increasing the amount of NGF (data not shown). Taken together, autophosphorylation apparently can occur without NGF stimulation of COS-1 cells. NGF-independent constitutive autophosphorylation of the TRKA protein has also been observed in other cell lines overexpressing this gene (5).

Transfection of putative mutant *TRKA* cDNAs into SH-SY5Y cells

We then transfected *TRKA* cDNAs harboring 11 putative missense mutations into SH-SY5Y cells and observed autophosphorylation of the intracellular tyrosine residues. Three putative mutations, R85S, L93P and L213P, were located in the extracellular domain. Both R85S and L93P mutations substitute amino acids in the first leucine-rich motif (Table 1). The former was detected as one of ‘double mutation’ in the same patient and the protein product was processed in cells and showed NGF-stimulated autophosphorylation as the wild-type *TRKA* (Fig. 2, box 1). In contrast, the product of the latter L93P mutation was processed only to the 110 kDa form in cells and showed a diminished response to NGF (Fig. 2, box 2). The third L213P mutation was in the first immunoglobulin-like domain and the product was also expressed and processed only to the 110 kDa form in cells. But there was significantly diminished NGF-stimulated autophosphorylation (Fig. 2, box 3). Thus, two mutations that substituted Pro for Leu in the same extracellular but in different domain seemed to impair the post-translational processing and NGF-stimulated autophosphorylation in SH-SY5Y cells.

Eight putative missense mutations, G516R, G571, H598Y, G607V, R643W, R648C, D668Y and G708S, were located in exons 13-16, each encoding part of the intracellular tyrosine kinase domain (Table 1). When transfected into SH-SY5Y cells, all these *TRKA* mutants were expressed and their protein products were processed as the wild-type to the 140 and 110 kDa form (Fig. 2, boxes 4-11). Five mutant proteins translated from G516R, G571R,

R643W, R648C and G708S showed a significantly diminished autophosphorylation (Fig. 2, boxes 4, 5, 8, 9 and 11). But the other three putative mutant proteins from H598Y, G607V and D668Y showed phosphorylation equivalent to that of the wild-type protein (Fig. 2, boxes 6, 7 and 10). These data indicate that eight putative missense mutations substituting amino acids in the intracellular tyrosine kinase domain were expressed, that their products were processed to the 140 kDa form in SH-SY5Y cells and that five of them showed severely impaired NGF-stimulated catalytic activity.

Transfection of putative mutant *TRKA* cDNAs into COS-1 cells

In COS-1 cells, the wild-type *TRKA* protein was processed mainly to the 110 kDa glycosylated and 80 kDa unglycosylated forms as well as to some degree to the 140 kDa form. All three forms were phosphorylated, irrespective of the presence or absence of NGF. The signal detected by anti-phosphorylated -674/675Tyr antibody was more intense in COS-1 cells than in SH-SY5Y cells and increased slightly in response to NGF (data not shown).

We then transfected *TRKA* cDNA harboring 11 putative missense mutations into COS-1 cells and observed autophosphorylation of the intracellular tyrosine residues. The R85S mutation was expressed in the cells and its product was processed and phosphorylated as the wild-type *TRKA* (Fig. 3, box 1). The two missense mutations L93P and L213P were expressed and their products were processed to the 110 kDa form, but in some degree to the smaller 80 kDa form (Fig. 3, boxes 2 and 3). Phosphorylation of the L93P protein was equivalent to that of the wild-type *TRKA* protein in COS-1 cells. This was unexpected because autophosphorylation of the L93P was reduced in SH-SY5Y cells compared with the wild-type protein, as described above. In contrast, the L213P mutant showed a significantly diminished autophosphorylation, irrespective of the presence or absence of NGF (Fig. 3, box 3). Thus, the processing of two mutant proteins substituting Pro for Leu in the same extracellular domain but in different domains seemed to be similar in COS-1 cells, albeit with a differential pattern of phosphorylation.

Eight putative missense *TRKA* mutations in the tyrosine kinase domain were expressed and their products were processed to the 110 and/or 80 kDa forms, as the wild-type *TRKA* in COS-1 cells (Fig. 3, boxes 4-11). Five mutant proteins translated from G516R, G571R, R643W, R648C and G708S showed a significantly diminished autophosphorylation (Fig. 3, boxes 4, 5, 8, 9 and 11). But the other three putative mutant proteins from H598Y, G607V

and D668Y showed phosphorylation equivalent to that of the wild-type protein, irrespective of NGF stimulation (Fig. 3, boxes 6, 7 and 10). These data indicate that all eight putative mutations in the tyrosine kinase domain were expressed and their products were processed to the 110 kDa form in COS-1 cells and that five of them showed severely impaired catalytic activity for autophosphorylation of -490Tyr and -674/675Tyr residues.

Alignment of the mutated residues in the TRKA protein with various RTKs

Crystallographic structures of the RTK family, together with extensive biochemical studies, led to a better understanding of the structure and function of their mechanisms (37-39). We made an alignment of eight mutated TRKA residues in the tyrosine kinase domain, using various RTKs (Fig. 4). Five were invariant amino acids perfectly conserved among at least 15 RTKs while the other three were not conserved, although one (-668Asp) was relatively well conserved among 12 of 15 RTKs. G512R and G571R substituted for the last invariant Gly of the glycine-rich nucleotide-binding loop (TRKA residues 511-516) and the invariant Gly of the β 4-strand (569-573), respectively. Either H598Y or G607V substitutes an amino acid in the kinase-insert region that showed significant variance among various RTKs. Both R643W and R648C substitute the invariant Arg residues of the catalytic loop (residues 642-649), in which all residues (except for Thr) are well conserved. D668Y substitutes an amino acid in the activation loop (residues 661-682) that includes -674/675Tyr and is important for activation of tyrosine kinase. G708S substitutes for the central residue of α F helix (700-716).

DISCUSSION

We transfected the wild-type *TRKA* and 11 putative missense mutants into two cell lines, SH-SY5Y and COS-1, using as a vector the mammalian expression plasmid, pCAGGS. These two cell lines showed a low-level expression of the endogenous *TRKA* gene *in vitro*. Transfection of SH-SY5Y cells with the exogenous wild-type *TRKA* restored NGF responsiveness in terms of TRKA autophosphorylation that can be detected by immunoblotting, using two specific antibodies to recognize phosphorylated -490Tyr and -674/675Tyr of the TRKA protein. The wild-type *TRKA* was expressed and its product was processed to the 140 and 110 kDa forms. Both forms were phosphorylated in response to NGF, the latter showed a reduced response compared with the former. A previous study indicated that the mature 140 kDa form crosses the plasma membrane and is exposed to the cell exterior, but both forms possess *in vitro* kinase activity specific for tyrosine residues (36). In addition, our study suggested that a small proportion of the 110 kDa proteins could become exposed to the cell exterior since autophosphorylation was enhanced slightly by NGF stimulation. TRKA protein precursors in SH-SY5Y and COS-1 cells were differentially processed and phosphorylated in an NGF-dependent and NGF-independent manner, respectively, under the conditions used in this study. In COS-1 cells, the wild-type *TRKA* was strongly expressed and its product was processed mainly to the 110 kDa form that was phosphorylated, irrespective of the presence or absence of NGF. This strong expression might in part be due to the origin of DNA replication derived from SV40 in the pCAGGS plasmid, making way for the plasmid to replicate in COS-1 cells that produce SV40 T antigen (40). The observation that overexpression of *TRKA* results in NGF-independent activation has been described before (5, 20). The difference in the apparent size of the TRKA protein in two cell lines probably reflects the difference in post-translational modification, mainly glycosylation of the extracellular domain (36). Critical elements for TRKA processing may vary from cell to cell, hence studying this receptor in a neuronal context is important.

We examined 11 putative mutations in the *TRKA* gene that we detected in 31 CIPA affected families of various ethnic origins (33-35). Three putative extracellular mutations substituted amino acids in the extracellular domain. Two mutations, L93P and L213P, were expressed in SH-SY5Y cells but their products were aberrantly processed. These two mutations probably impair post-translational processing and transport of receptor protein through the endoplasmic reticulum and Golgi apparatus to the plasma membrane.

Presumably, these do not fold normally, therefore are not transported efficiently, as demonstrated in several mutants of the human insulin receptor (41) and RET (42-45). Both mutant proteins showed diminished NGF-stimulated autophosphorylation in SH-SY5Y cells. However, the L93P protein was phosphorylated in COS-1 cells, irrespective of the presence or absence of NGF, suggesting that overexpression of this mutant protein could cause NGF-independent autophosphorylation of the intracellular domain. In contrast, the L213P protein showed severely impaired autophosphorylation in COS-1 cells, suggesting that it also lacked the tyrosine kinase catalytic activity in addition to a defect of post-translational processing. A previous study also stressed the importance of expression levels play in assessing the consequence of receptor mutations (23). Thus, a comparison of these two mutations indicates that the expression level as well as use of a suitable cell line are important in order to demonstrate specific effect(s) of mutation when mutated RTKs are being functionally evaluated in *in vitro* studies. The other putative mutation in the extracellular domain, R85S, was detected as one of the homozygous ‘double’ mutations in a consanguineous CIPA family from the United Arab Emirates. The proband of this family also has a splice site mutation on the same chromosome, which causes an aberrant splicing of exon 5, encoding the second cysteine clusters in the extracellular domain (34). The R85S was expressed and its product was processed and autophosphorylated as the wild-type *TRKA*, in both cell lines. It is likely that R85S is a polymorphism in a particular ethnic background, although we have not yet studied normal populations of the same ethnic origin.

Eight putative missense mutations were distributed in the intracellular tyrosine kinase domain important for signal transduction. We aligned the mutated amino acid residues in the tyrosine kinase domain with various RTKs (Fig. 4). We demonstrated that five mutant *TRKA* genes (G516R, G571R, R643W, R648C and G708S) were expressed and their products were processed as the wild-type *TRKA* in both SH-SY5Y and COS-1 cell lines; however, they showed a significantly diminished autophosphorylation. In contrast, the other three mutants (H598Y, G607V and D668Y) were expressed and their products were processed in SH-SY5Y cells; they showed the NGF-stimulated autophosphorylation seen in the wild-type *TRKA*. Catalytic activity for autophosphorylation in the COS-1 cells was evident. The G516R mutant substituted the highly conserved glycine residue in a glycine-rich loop predicted to be a nucleotide-binding site. A Gly→Val mutation at the equivalent position was also noted in the insulin receptor (37, 41). Because ATP is the phosphate donor in the reaction catalyzed by the RTK, a mutation in the binding site would inactivate the kinase activity. We first detected the G571R mutation in a Japanese family (33). -571Gly in the

insulin receptor is predicted to be in a β -sheet conformation. The functional significance of the region is not yet apparent. Among the RTK family members, -571Gly is invariant suggesting that it probably plays an important role in tyrosine kinase activity. Recently, this mutation has been characterized and was found to exert a loss-of-function effect (46). Equivalent residues of both R643W and R648C mutations were located in the catalytic loop in the insulin receptor (37, 38). R643W mutation substitutes Trp for -643Arg, a residue immediately preceding the putative catalytic base, -644Asp, that is highly conserved among the RTK family members. -643Arg is one of the charge pair partners for phosphorylated tyrosines in the activation loop (47). Interestingly, a mutation substituting Gln for Arg at the equivalent position was also noted in the insulin receptor (37). R648C mutation substitutes Cys for -648Arg, whose equivalent carbonyl oxygen in the insulin receptor is predicted to be hydrogen-bonded via a water molecule to one of the ribose hydroxyl group of ATP (38). Thus, it is likely that both R643W and R648C mutations affect catalytic activity of the TRKA protein. The G708S mutation substitutes Ser for -708Gly that is invariant in the RTK family and the equivalent residue in the insulin receptor is predicted in an α -helical conformation. Again, it is likely that G708S mutation affects catalytic activity of the TRKA protein, although the functional significance of this α -helical region is not yet apparent.

Both H598Y and G607V were detected as one of the ‘triple’ mutations in a consanguineous family from Italy. The patient also has a nonsense mutation (Q9X) in exon 1 on the same chromosome (34). Interestingly, sporadic human medullary thyroid carcinomas were examined for a putative mutation in the *TRKA* gene (48). The group detected both H598Y and G607V as polymorphisms, on the same chromosome and in a race-matched control population, although they did not analyze exon 1 in *TRKA* and their numbering system differs from ours. In addition, G607V has also been detected in homozygous healthy Bedouin individuals (49). Our expression study and mutation searches by other investigators strongly indicate that these two amino acid substitutions are polymorphisms in a particular ethnic background, not mutations.

We detected D668Y as one allele in a compound heterozygote in four Japanese families with CIPA (35). D668Y was not detected in race-matched control chromosomes; this was linked to the same *TRKA* haplotype in four families. We searched all coding exons and flanking exon-intron junctions in the *TRKA* in these families. The other allele in each patient was a nonsense, frameshift, splice-site or missense mutation. The missense mutation was R648C, as described above. These findings strongly suggest that D668Y is a missense

mutation responsible for CIPA. D668Y mutant protein appeared at least capable of autophosphorylating -490Tyr and -674/675Tyr. We repeated the expression study with respect to D668Y, starting at the *in vitro* mutagenesis step and obtained similar results. One possible interpretation is that the kinase activity in this mutant is sufficient to autophosphorylate these sites but is impaired with respect to phosphorylation of other tyrosine residues on TRKA protein or its various protein substrates. In this context, it is interesting to note that mutation of any one of the activation loop tyrosines (-670Tyr, 674Tyr or 675Tyr) results in selective impairment of NGF-dependent PLC γ -1 phosphorylation (23). TRKA-mediated PLC γ -1 phosphorylation is nearly completely compromised by mutations of single activation loop tyrosines, whereas Shc phosphorylation can be rescued substantially by overexpression of the same mutants (23). Alternatively an additional tyrosine residue in the activation loop introduced by the D668Y mutation might exert a deleterious effect on the function of TRKA protein through other mechanisms rather than defects of autophosphorylation. We noted that the D668Y mutation is likely to be activating when introduced into other tyrosine kinase molecules, as deduced from the structural homology among protein kinases. Murine KIT receptor kinase (50) and human MET receptor kinase (51) were seen to be activated by amino acid changes at the Asp residue corresponding to position of this TRKA mutant. The former is interesting since it substitutes Tyr for Asp, as observed in our D668Y mutation. It is possible that the activating mutation may be incompatible with survival of neuronal cells when introduced homozygously into the embryo. Of course one cannot completely rule out the possibility that D668Y might be a rare polymorphism linked to a putative mutation that could not be detected using our present method. Experiments are in progress to address these questions by examining the downstream signal transduction of the TRKA protein.

In the present study, we identified at least five loss-of-function mutations in the intracellular tyrosine kinase domain of the TRKA protein. We aligned these residues as well as putative polymorphisms with various RTKs, as shown in Figure 4. All five mutations are perfectly conserved among these receptors, strongly suggesting that these five amino acid residues play a critical role in the autophosphorylation induced by each corresponding ligand binding. Thus, naturally occurring missense mutations in the human *TRKA* gene provide considerable insight into structure-function relationships in the RTK family.

Our study also has clinical implications to treat acquired 'complex regional pain syndrome or sympathetically maintained pain', including causalgia and reflex sympathetic dystrophy.

Detailed mechanisms of these conditions remain to be elucidated. Pain in these conditions is often associated with abnormal skin color, temperature change, abnormal sudomotor activity, or edema (52). According to our previous study on CIPA, the NGF-TRKA system has a crucial role in the development and function of the nociceptive reception as well as establishment of thermoregulation via sweating in humans (33). The sudomotor system is innervated and regulated by the sympathetic nervous system and nociceptive and sympathetic neural pathways are supported and maintained by the neurotrophic action of NGF (53-55). Availability of a viable therapy could specifically inhibit these pathways. Drugs designed to target the TRKA receptor would be one attractive candidate. Indeed, a synthetic TRKA-IgG fusion molecule was studied to sequester endogenous NGF in the treatment of experimental inflammatory pain states in laboratory animals (56). Their data suggest that peripherally produced NGF normally functions to maintain sensitivity of nociceptive sensory neurons and that in some inflammatory states, an up-regulation of NGF is responsible for alterations in pain-related behavior. Thus, the present study provides some clues to develop a specific drug that targets the NGF-TRKA signal transduction, based on a structure-based design.

MATERIALS AND METHODS

Cell lines

SH-SY5Y, a cell line derived from human neuroblastoma (57), and COS-1, an SV40-transformed simian cell line (58), were cultured in RPMI1640 containing 10 % fetal calf serum.

Construction of expression plasmid for wild-type *TRKA* cDNA

The plasmid pLM6, which carries the full-length 2.7 kb cDNA for the human TRKA receptor (36), was a generous gift from Dr. L. F. Parada. A mammalian expression vector (pCAGGS) used for this study was a generous gift of Dr. J. Miyazaki. The pCAGGS vector has a strong CAG promoter based on a modified chicken β -actin promoter with cytomegalovirus immediate early enhancer, a rabbit β -globin gene sequence including a polyadenylation signal, and an SV40 origin of DNA replication (40, 59). The pLM6 plasmid was digested with the restriction enzyme *EcoRI* and subjected to an agarose gel electrophoresis. The full-length *TRKA* cDNA fragment was recovered from the gel and subcloned into the *EcoRI* site of pCAGGS vector. The junction between the pCAGGS vector and cDNA was sequenced to verify orientation.

***In vitro* site-directed mutagenesis**

To construct *TRKA* mutants, the GeneEditor *In Vitro* Site-Directed Mutagenesis System (Promega, Madison, WI) was used according to the manufacturer's instruction and with the pCAGGS expression vector containing the wild-type *TRKA* cDNA as template. In brief, a selection oligonucleotide provided in the system and a specific mutagenesis oligonucleotide described below were annealed to the plasmid DNA template. A mutant strand was synthesized with T4 DNA polymerase and T4 DNA ligase. The heteroduplex DNA was then transformed into the repair-minus *Escherichia coli* strain BMH 71-18 *mutS* and the cells were grown in selective media to select clones containing the mutant plasmid. Plasmids resistant to the antibiotic selection mix were then isolated and transformed into the final host strain, JM 109, using the same selection conditions.

Mutations were verified by sequencing DNA in the region of interest, as described (33). The specific mutagenesis oligonucleotides were 5'-phosphorylated and complementary to the *TRKA* cDNA, except for a region of mismatch near the center. Sequences of the oligonucleotides are as follows. Each underline indicates the position of the desired mutation.

- 5'-pCTGGAGCTCAGTGATCTGAG-3' for R85S;
- 5'-pGGCCTGGGGGAGCCGAGAAACCTCACC-3' for L93P;
- 5'-pGACGTGCTGCCCGCGGTGCCAG-3' for L213P;
- 5'-pGGGCGCCTTTAGGAAGGTCTTC-3' for G516R;
- 5'-pGCGCTTCTTCCGCGTCTGCAC-3' for G571R;
- 5'-pCCTCCGATCCTATGGACCCG-3' for H598Y;
- 5'-pCTGCTGGCTGTTGGGGAGGATG-3' for G607V;
- 5'-pCATTTTGTGCACTTGGGACCTG-3' for R643W;
- 5'-pCCTGGCCACATGCAACTGTC-3' for R648C;
- 5'-pCATGAGCAGGTATATCTACAG-3' for D668Y;
- 5'-pGTGGAGCTTCAGCGTGGTGCTC-3' for G708S.

Sequencing of DNA

The plasmid DNA was sequenced using a dRhodamine Terminator Cycle Sequencing kit (PE Biosystems). The sequences were resolved and analyzed on an ABI PRISM 310 Genetic Analyzer (PE Biosystems).

Transfection of expression plasmids

The expression plasmid DNA was prepared using a QIAGEN Plasmid Maxi Kit 10. Transfections were performed using the Lipofectamine Reagent (Life Technologies), according to the manufacturer's protocol. In brief, nearly 50-80% confluent cells in a six-well tissue culture plate were washed three times with serum-free medium RPMI 1640. One microgram of the plasmid DNA was diluted and mixed with the Lipofectamine reagent in a sterile polystyrene tube. After diluting with serum-free medium, the DNA-Lipofectamine complex was overlaid onto the rinsed cells. The cells with the complex were incubated for 16-24 hr at 37°C in a CO₂ incubator. The

DNA-Lipofectamine complex was removed by aspiration. The cells were further incubated after adding RPMI 1640 medium containing fetal calf serum.

Stimulation of cells with NGF

Transfections were performed as described above. The cells were cultured in 2 ml of RPMI 1640 medium containing 10% and 0.5% fetal calf serum for SH-SY5Y and for COS-1 cells, respectively. Cells without transfection and cells transfected with the plasmid pCAGGS vector without the *TRKA* cDNA were used as a negative control and as a mock-treated control, respectively. The mutant or the wild-type *TRKA*-transfected cells and mock-transfected cells as well as non-transfected cells were treated under the same conditions in a six-well tissue culture plate. The plate was incubated in humidified air containing 5% CO₂ for 48 hr. The cells were washed three times with serum-free medium and incubated for 2 hr in a serum-free medium. After the medium was aspirated, 1 ml of fresh serum-free medium containing 100 ng of a 2.5 S murine NGF (Promega) and 100 µg of bovine serum albumin was added, followed by incubation at 37°C for 5 min. The cells were then immediately washed three times with phosphate-buffered saline containing 2 mM of a phosphatase inhibitor, sodium orthovanadate (Na₃VO₄; Wako). After adding 1 ml of the same buffer, the cells were harvested by scraping, transferred into a 1.5 ml tube and collected by centrifuging at 12 000 g for 5 min in a microfuge.

Detection of phosphorylated TRKA protein by immunoblotting

Lysis of cell pellets was done by adding 100 µl of lysis buffer (20mM HEPES, pH 7.4, 100mM NaCl; 0.5% NP-40; 10% glycerol), containing 10 µg/ml aprotinin (Boehringer Mannheim), 10 mM EDTA and 2 mM Na₃VO₄. The tube content was vortexed and incubated on ice for 10 min. The tube was centrifuged at 12 000 g for 5 min at 4°C. Supernatant fluid containing total cell lysate proteins was transferred to a fresh microfuge tube. The protein concentration was estimated by using the BCA Protein Assay kit (PIERCE). Then 20 µg of protein per lane was resolved by 6% sodium dodecyl sulfate - polyacrylamide gel electrophoresis. Kaleidoscope Prestained Standards (Bio-Rad) were run as a molecular weight marker for protein. Proteins were transferred from the gel to a membrane (Immobilon PVDF transfer membranes; Millipore), using an electroblotting

apparatus. Primary antibodies used were as follows: a rabbit polyclonal antibody, Trk (C-14; Santa Cruz Biotechnology) and two phospho-specific antibodies, [TrkA(Tyr490) and TrkA(Tyr674/675); New England BioLabs]. The former was raised against a peptide corresponding to amino acids 777-790, the C-terminus of TRKA protein, and was used to directly detect total TRKA protein. The latter two phospho-specific antibodies were prepared to detect phosphorylated -490Tyr and -674/675Tyr residues. The membrane was incubated with the primary antibody according to the manufacturer's protocol. Next the membrane was incubated at room temperature for 1 hr with a secondary anti-rabbit antibody conjugated to horseradish peroxidase (DAKO). For detection we used ECL western blotting detection reagents (Amersham Pharmacia Biotech) and exposure to X-ray film.

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FIGURES

Figure 1. Expression of the wild-type *TRKA* cDNA and processing and autophosphorylation of its product in two different cell lines: SH-SY5Y and COS-1.

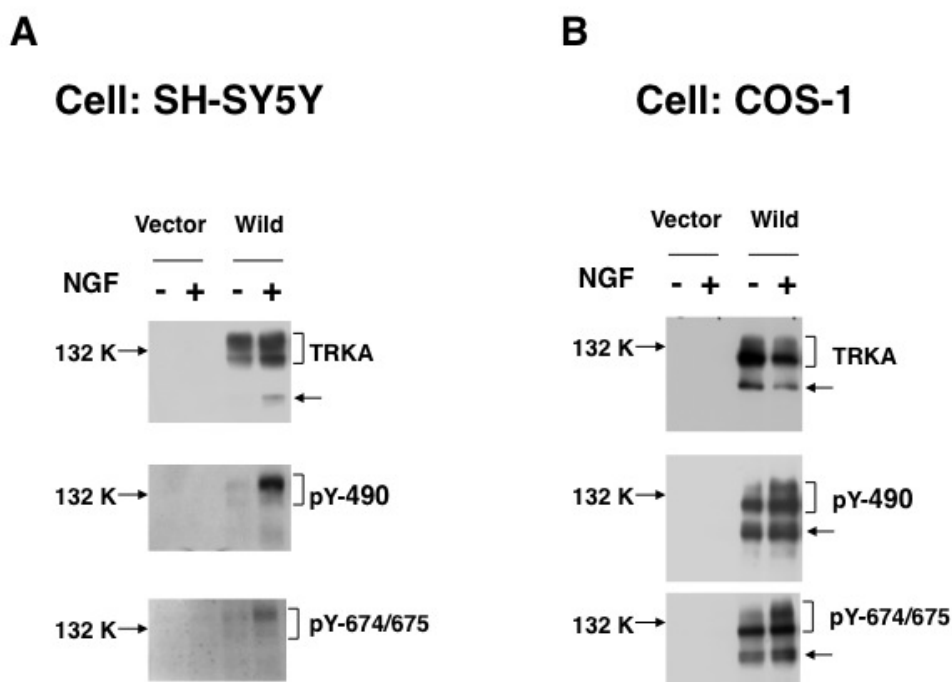


Figure 1. Expression of the wild-type *TRKA* cDNA and processing and autophosphorylation of its product in two different cell lines: SH-SY5Y and COS-1. The full-length cDNA for human *TRKA* receptor (36) was subcloned into a mammalian expression vector (pCAGGS). The *TRKA* expression plasmid was transfected into SH-SY5Y cells (A) or COS-1 cells (B). Each cell transfected with the plasmid pCAGGS vector, without the *TRKA* insert, was used as a mock-treated control. Cells were incubated in the presence (+) or absence (-) of nerve growth factor (NGF) (100ng/ml) for 5 min and were harvested for detection of the total or phosphorylated *TRKA* proteins by immunoblotting. An antibody recognizes the C-terminal *TRKA* protein (*TRKA*, top panel). Two phospho-specific antibodies recognize the phosphorylated -490Tyr residue (pY-490, middle panel) and -674/675Tyr residues (pY-674/675, bottom panel) of *TRKA* protein. An arrow on the left side of each blot shows

the position of a prestained molecular weight marker. A bracket and an arrow on the right side indicate the position of glycosylated and unglycosylated TRKA proteins, respectively. (A) In SH-SY5Y cells, the TRKA precursor protein was detected and processed to 140 and 110 kDa forms. An 80 kDa form (arrow) is probably the unglycosylated polypeptidic backbone of these proteins. The 140 kDa form was strongly phosphorylated in response to NGF. (B) In COS-1 cells, the TRKA precursor protein was mainly processed to the 110 kDa form, although 140 and 80 kDa forms were also visible. The 110 kDa form as well as 140 and 80 kDa forms were phosphorylated, irrespective of the presence or absence of NGF stimulation.

Figure 2. Expression of mutant *TRKA* cDNAs and autophosphorylation of their products in SH-SY5Y cells.

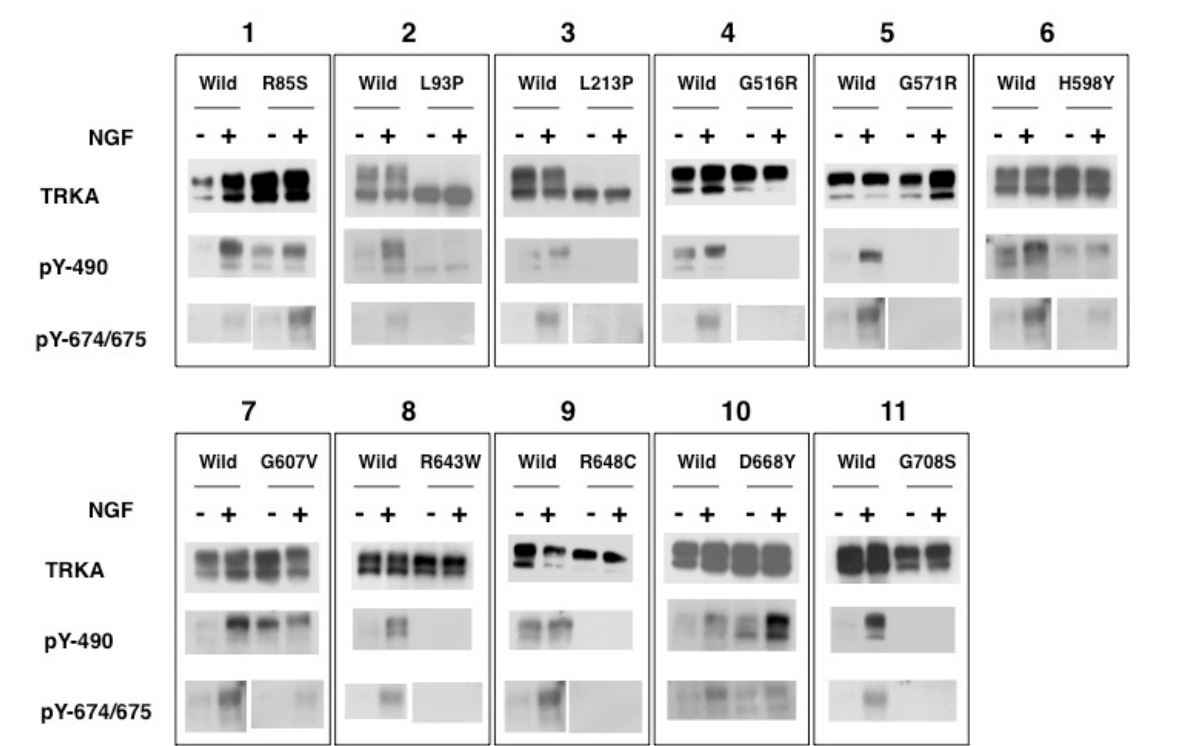


Figure 2. Expression of mutant *TRKA* cDNAs and autophosphorylation of their products in SH-SY5Y cells.

TRKA cDNAs harboring 11 putative missense mutations were prepared, using *in vitro* site-directed mutagenesis. Each specific mutagenesis oligonucleotide was complementary to the wild-type *TRKA* cDNA, except for a region of mismatch. The mutant and the wild-type *TRKA* cDNAs were transfected into SH-SY5Y cells, under the same conditions. Autophosphorylation of the *TRKA* protein was detected after stimulation with NGF.

Figure 3. Expression of mutant *TRKA* cDNAs and autophosphorylation of their products in COS-1 cells.

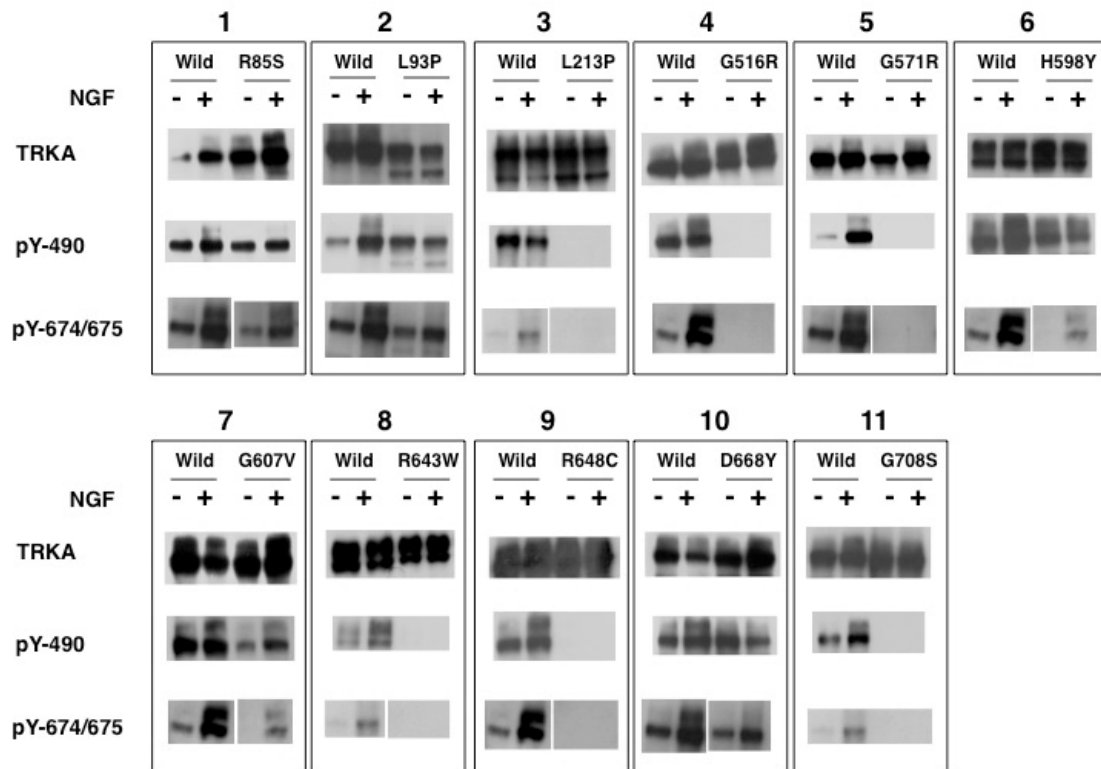


Figure 3. Expression of mutant *TRKA* cDNAs and autophosphorylation of their products in COS-1 cells. The mutant and the wild-type *TRKA* cDNAs harboring 11 putative missense mutations were transfected into COS-1 cells, under the same conditions, and phosphorylation of their products was observed.

Figure 4. Conservation of mutated residues in the TRKA protein in various receptor tyrosine kinases (RTKs).

	Nucleotide-binding loop		Kinase insert region		Catalytic loop		Activation loop	α F
	β 4							
	G516R (1008*)	G571R (1064*)	H598Y	G607V	R643W (1131*)	R648C (1136*)	D668Y (1156*)	G708S (1196*)
	●	●	○	○	●	●	⊙	●
TRKA	GEGAFG	FFGVVC	SHGPDAKLLAGGE		HRDLATRNL	DFGMSRDVY	SDVWSFGV	
TRKB	GEGAFG	FYGVVC	AHGPDVAVLMAEGN		HRDLATRNL	DFGMSRDVY	SDVWSLGV	
TRKC	GEGAFG	FYGVVC	AHGPNAMILVDGQ		HRDLATRNL	DFGMSRDVY	SDVWSFGV	
IR	GQGSFG	LLGVV	SLRPEA-----EN		HRDLAARN	DFGMTRDIY	SDMWSFGV	
IGF-IR	GQGSFG	LLGVV	SLRPEM-----EN		HRDLAARN	DFGMTRDIY	SDVWSFGV	
ROS	GSGAFG	QLGVC	KARMAT-----		HRDLAARN	DFGLARDIY	SDVWSFGI	
MET	GRGHFG	LLGIC	NETHNP-----		HRDLAARN	DFGLARDMY	SDVWSFGV	
EPH	GEGEFG	LEGVV	EREDQL-----		HRDLAARN	DFGLTRLLD	SDVWSFGI	
EGFR	GSGAFG	LLGIC	EHKDNI-----		HRDLAARN	DFGLAKLLG	SDVWSYGV	
ERBB2	GSGAFG	LLGIC	ENRGRL-----		HRDLAARN	DFGLARLLD	SDVWSYGV	
RET	GEGEFG	LYGAC	SRKVGP [16aa]		HRDLAARN	DFGLSRDVY	SDVWSFGV	
CSF-1R	GAGAFG	LLGAC	RKAEAM [62aa]		HRDVAARN	DFGLARDIM	SDVWSYGI	
PDGFR	GSGAFG	LLGAC	RNKHTF [92aa]		HRDLAARN	DFGLARDIM	SDVWSFGI	
KIT	GAGAFG	LLGAC	RKRDSF [69aa]		HRDLAARN	DFGLARDIK	SDVWSYGI	
FGFR1	GEGCFG	LLGAC	ARRPPG-----LE		HRDLAARN	DFGLARDIH	SDVWSFGV	

Figure 4. Conservation of mutated residues in the TRKA protein in various RTKs. Shown are regions in the tyrosine kinase domain flanking amino acid residues mutated in each patient. Missense mutations characterized in the present study are listed above the diagram, based on alignment of previous reports (36, 37, 60). Assignment of the nucleotide-binding loop, β4, kinase-insert region, catalytic loop, activation loop and αF structure was based on the three-dimensional structure of the human insulin receptor (37, 38). Parts of the kinase-insert region, the activation loop and the αF structure are shown. Each circle corresponds to the position of an amino acid substitution: closed and open circles indicate invariant and non-invariant amino acid residues, respectively, and the shaded circle indicates an amino acid residue conserved among 12 of 15 RTKs. Asterisks in parenthesis indicate the position in the human insulin receptor (37). A minimal number of gaps (-) was introduced in the kinase-insert region. aa, amino acid residues not included in the alignment.

Table 1. Putative missense mutations in human *TRKA* from 31 families with CIPA

Mutation ^a	Position in <i>TRKA</i> gene	Position in TRKA protein ^b	Reference
Arg85Ser ^c	Exon 2	Leucine rich motif 1	34
Leu93Pro	Exon 2	Leucine rich motif 1	35
Leu213Pro	Exon 6	Immunoglobulin-like domain 1	34
Gly516Arg	Exon 13	Tyrosine kinase (nucleotide-binding loop)	35
Gly571Arg	Exon 14	Tyrosine kinase (β 4)	33
His598Tyr ^c	Exon 15	Tyrosine kinase (kinase-insert region)	34
Gly607Val ^c	Exon 15	Tyrosine kinase (kinase-insert region)	34
Arg643Trp	Exon 15	Tyrosine kinase (catalytic loop)	34
Arg648Cys	Exon 15	Tyrosine kinase (catalytic loop)	35
Asp668Tyr	Exon 15	Tyrosine kinase (activation loop)	35
Gly708Ser	Exon 16	Tyrosine kinase (α F)	34

^a Based on the published sequence of TRKA cDNA (36) and TRKA gene (17).

^b Based on the domain structure (19) and structure-based sequence alignment of the insulin receptor (37, 38).

^c Arg85Ser and (His598Tyr; Gly607Ser) were linked, on the same chromosome, a splice acceptor site mutation in intron 4 and a nonsense mutation in exon 1, respectively (34).