Novel Pathogenic Mechanisms of Congenital Insensitivity to Pain with Anhidrosis Genetic Disorder Unveiled by Functional Analysis of Neurotrophic Tyrosine Receptor Kinase Type 1/Nerve Growth Factor Receptor Mutations*

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Congenital insensitivity to pain with anhidrosis (CIPA) is a rare genetic disease characterized by absence of reaction to noxious stimuli and anhidrosis. The genetic bases of CIPA have remained long unknown. A few years ago, point mutations affecting both coding and noncoding regions of the neurotrophic tyrosine receptor kinase type 1 (NTRK1)/nerve growth factor receptor gene have been detected in CIPA patients, demonstrating the implication of the nerve growth factor/NTRK1 pathway in the pathogenesis of the disease. We have previously shown that two CIPA mutations, the G571R and the R774P, inactivate the NTRK1 receptor by interfering with the autophosphorylation process. We have extended our functional analysis to seven additional NTRK1 mutations associated with CIPA recently reported by others. Through a combination of biochemical and biological assays, we have identified polymorphisms and pathogenic mutations. In addition to the identification of residues important for NTRK1 activity, our analysis suggests the existence of two novel pathogenic mechanisms in CIPA: one based on the NTRK1 receptor processing and the other acting through the reduction of the receptor activity.

The NTRK1 gene (also called TRKA) encodes one of the receptors for nerve growth factor (NGF) (1, 2) and consists of 17 exons distributed within a 25-kb region on chromosome 1q21-22 (3, 4). The NTRK1 protein comprises an extracellular portion, including Ig-like and cysteine-rich domains; a single transmembrane region; a juxtamembrane domain; a tyrosine kinase (TK) domain; and a C-terminal tail (Ref. 5; Fig. 1A).

Several studies have shown that the activity of the NTRK1 receptor requires the phosphorylation of five different tyrosine residues, located in the juxtamembrane domain (Tyr490), in the tyrosine kinase domain (Tyr785, Tyr867, Tyr874, and Tyr875), and in the C-terminal tail (Tyr1165, Tyr1167, and Tyr1174). Several studies have shown that the activity of the NTRK1 receptor requires the phosphorylation of five different tyrosine residues, located in the juxtamembrane domain (Tyr490), in the tyrosine kinase domain (Tyr785, Tyr867, Tyr874, and Tyr875), and in the C-terminal tail (Tyr1165, Tyr1167, and Tyr1174). Several studies have shown that the activity of the NTRK1 receptor requires the phosphorylation of five different tyrosine residues, located in the juxtamembrane domain (Tyr490), in the tyrosine kinase domain (Tyr785, Tyr867, Tyr874, and Tyr875), and in the C-terminal tail (Tyr1165, Tyr1167, and Tyr1174). Several studies have shown that the activity of the NTRK1 receptor requires the phosphorylation of five different tyrosine residues, located in the juxtamembrane domain (Tyr490), in the tyrosine kinase domain (Tyr785, Tyr867, Tyr874, and Tyr875), and in the C-terminal tail (Tyr1165, Tyr1167, and Tyr1174). Several studies have shown that the activity of the NTRK1 receptor requires the phosphorylation of five different tyrosine residues, located in the juxtamembrane domain (Tyr490), in the tyrosine kinase domain (Tyr785, Tyr867, Tyr874, and Tyr875), and in the C-terminal tail (Tyr1165, Tyr1167, and Tyr1174). Several studies have shown that the activity of the NTRK1 receptor requires the phosphorylation of five different tyrosine residues, located in the juxtamembrane domain (Tyr490), in the tyrosine kinase domain (Tyr785, Tyr867, Tyr874, and Tyr875), and in the C-terminal tail (Tyr1165, Tyr1167, and Tyr1174). Several studies have shown that the activity of the NTRK1 receptor requires the phosphorylation of five different tyrosine residues, located in the juxtamembrane domain (Tyr490), in the tyrosine kinase domain (Tyr785, Tyr867, Tyr874, and Tyr875), and in the C-terminal tail (Tyr1165, Tyr1167, and Tyr1174). Several studies have shown that the activity of the NTRK1 receptor requires the phosphorylation of five different tyrosine residues, located in the juxtamembrane domain (Tyr490), in the tyrosine kinase domain (Tyr785, Tyr867, Tyr874, and Tyr875), and in the C-terminal tail (Tyr1165, Tyr1167, and Tyr1174). Several studies have shown that the activity of the NTRK1 receptor requires the phosphorylation of five different tyrosine residues, located in the juxtamembrane domain (Tyr490), in the tyrosine kinase domain (Tyr785, Tyr867, Tyr874, and Tyr875), and in the C-terminal tail (Tyr1165, Tyr1167, and Tyr1174). Several studies have shown that the activity of the NTRK1 receptor requires the phosphorylation of five different tyrosine residues, located in the juxtamembrane domain (Tyr490), in the tyrosine kinase domain (Tyr785, Tyr867, Tyr874, and Tyr875), and in the C-terminal tail (Tyr1165, Tyr1167, and Tyr1174). Several studies have shown that the activity of the NTRK1 receptor requires the phosphorylation of five different tyrosine residues, located in the juxtamembrane domain (Tyr490), in the tyrosine kinase domain (Tyr785, Tyr867, Tyr874, and Tyr875), and in the C-terminal tail (Tyr1165, Tyr1167, and Tyr1174). Several studies have shown that the activity of the NTRK1 receptor requires the phosphorylation of five different tyrosine residues, located in the juxtamembrane domain (Tyr490), in the tyrosine kinase domain (Tyr785, Tyr867, Tyr874, and Tyr875), and in the C-terminal tail (Tyr1165, Tyr1167, and Tyr1174).
chemical and biological analysis. Our studies allowed the identification of polymorphisms and pathogenic mutations. With respect to the latter, in addition to the interference with auto-phosphorylation, two novel mechanisms of NTRK1 deregulation responsible for the CIPA phenotype were unveiled; one is based on processing alteration, and the other involves a reduction of the receptor activity.

EXPERIMENTAL PROCEDURES

In Vitro Site-directed Mutagenesis—The NTRK1 mutants were constructed by the GeneEditor™ in Vitro Site-directed Mutagenesis System (Promega). According to the manufacturer’s instructions, the expression vector template the NTRK1 cDNA cloned into the pRC/CMV expression vector (plasmid NTRK1wt) (24). The sequences of the oligonucleotides used are as follows, with the mutated nucleotides in boldface type: 5'-CTGGAGGCTAGCTATGAGG-3' for R553S, 5'-CTGCTGCTGCCCGTGGCC-A3' for L213P; 5'-CTCCGATCCTATGGAGCG-3' for H598Y; 5'-CTGTTGGCTCTGTGGGAGG-3' for G607V; 5'-TTCTGAGCTGGAGGCTG-3' for R643W; 5'-CTAGGAGCATGTATCTACAGA-3' for D668Y; and 5'-GGAGGCTCAGCGGTGGT-3' for G708S.

Mutant clones were identified by PCR followed by allele-specific oligonucleotide hybridization in the case of R553S, H598Y, G607V, and G708S. Clones carrying the R643W mutation were identified by MspI digestion of a PCR fragment; the mutation abolishes a restriction site present in the WT. Clones carrying the D668Y mutation were identified by digestion with EcoRV, since a restriction site is abrogated by the mutation. Clones carrying the L213P mutation were identified by nucleotide sequence of a PCR fragment containing the mutation. All of the mutant clones were subjected to nucleotide sequence to exclude possible additional mutations accidentally occurring during the mutagenesis reaction.

Cell Culture and Transfection—E25 cells, expressing the WT NTRK1 receptor, have been previously described (26). N5.3 cell line has been constructed by transfecting the NTRK1/L213P mutant into NIH3T3 cells and selecting in the presence of G418 antibiotic (400 μg/ml) to determine the transfection efficiency and in medium containing 5% serum, to determine the transforming activity. N5.3 cell line has been constructed by transfecting the NTRK1/L213P mutant into NIH3T3 cells and selecting in the presence of G418 antibiotic (400 μg/ml) to determine the transforming activity. NIH3T3 and derived cell lines were selected in the presence of G418 (400 μg/ml). NIH3T3 and derived cell lines were selected in the presence of G418 (400 μg/ml). The NIH3T3 cells (2.5 × 10^5/10-cm plate) were transfected by the CaPO4 method, as previously described (27), using 1 μg or 10 ng of plasmid DNA together with 30 μg of linearized DNA. Transfected cells were selected in the presence of G418 antibiotic (400 μg/ml) to determine the transfection efficiency and in medium containing 5% serum, supplemented or not with NGF, to determine the transforming activity.

RESULTS

Selection of Mutations and Construction of CIPA Mutants—We have selected seven recently reported NTRK1 mutations associated with CIPA and involving different regions of the NTRK1 receptor (Fig. 1) (19, 22).

R855S occurs in the extracellular domain, within a leucinerich motif, it has been detected in association, on the same allele, with a point mutation of the 3' splicing site of intron 4. L213P occurs within the first Ig-like domain, and it has been found associated, on the second allele, with a 7-bp deletion causing a frameshift and a premature termination. H598Y and G607V, both in the TK domain, have been detected as triple mutations in association on the same chromosome with a mutation creating a stop codon at residue 9. R643W occurs within exon 15, in the TK domain; it has been found as homozygous mutation. D668Y, in the TK domain, has been detected in four different families and in association, on the same chromosome, with different mutations (splice site, frameshift, nonsense, missense). G708S, within the TK domain, has been detected as a homozygous mutation. As can be deduced from the above description, L213P, R643W, D668Y, and G708S display the features of pathogenic mutations, whereas the others may represent rare polymorphisms. Although the genetic analysis excludes any role of the latter mutations in CIPA disease, their study will formally prove their effect on NTRK1 receptor activity.

All of the mutations described above were introduced into the NTRK1 cDNA inserted into the pRC/CMV mammalian expression vector as described under “Experimental Procedures.”

Analysis of NTRK1/CIPA Proteins Transiently Expressed in COS1 Cells—The NTRK1/CIPA mutants, as well as the WT NTRK1 cDNA, were transiently transfected into COS1 cells. 

NTRK1/NGF Receptor Gene Mutations in CIPA

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Three days after transfection cells were treated or not with NGF for 10 min and then subjected to protein extraction. After immunoprecipitation with the MGR12 antibodies, directed against the NTRK1 extracellular portion (28), Western blot with anti-TRK and anti-phosphotyrosine antibodies was performed. The results are reported in Fig. 2A. The blot with anti-TRK antibodies showed that, similarly to the wild type, all of the CIPA mutants except for L213P produced the two NTRK1 proteins of 110 and 140 kDa, corresponding to the partially and completely glycosylated receptor, respectively. The NTRK1/L213P cDNA produced only the 110-kDa form. The Western blot with anti-phosphotyrosine showed a phosphorylation status similar to WT for mutants R85S, H598Y, G607V, and D668Y. A basal phosphorylation was detectable in the untreated cells, due to receptor self-activation caused by overexpression. Treatment with NGF further increased the phosphorylation level. On the contrary, no phosphorylation was detected in mutants R643W and G708S. The 110-kDa NTRK1/L213P protein showed a faint level of phosphorylation, which remained unmodified following the NGF treatment. Data similar to ours have been recently reported (25). The effect of the CIPA mutations on NTRK1 receptor activity was also investigated by the immunocomplex-autokinase assay reported in Fig. 2B. NTRK1 proteins expressed in COS1 cells were immunoprecipitated with the MGR12 antibodies and incubated with [γ-32P]ATP. Autokinase activity was detectable in WT, R85S, H598Y, G607V, D668Y receptors; it was barely visible in L213P and below the detection level in R643W and G708S mutants.

Biological Activity of NTRK1/CIPA Mutants—The ectopic expression of either constitutively activated TRK oncoproteins or wild type NTRK1 receptor in the presence of NGF leads to cellular transformation of NIH3T3 mouse fibroblasts, detectable as the formation of foci of transformed cells (29, 30). The NIH3T3 transfection/focus formation assay was used to investigate the effect of the different CIPA mutations on NTRK1 activity. High doses of plasmid DNA (1 μg/2 × 105 cells) and NGF (50 ng/ml) were used to detect also very low transforming activities. Transfected cells were selected in the presence of G418 antibiotic to determine the transfection efficiency and in medium containing 5% serum supplemented or not with NGF to determine the transforming activity. All of the constructs produced G418-resistant colonies with comparable efficiency, and none of them was able to induce foci formation in the absence of NGF (data not shown). In the presence of NGF, no transformation foci were detected in cells transfected with L213P, R643W, and G708S. On the contrary, R85S, H598Y, G607V, and D668Y mutants induced foci formation, similarly to the wild type (Fig. 3A and data not shown). For each transfection, several G418-resistant clones were isolated and analyzed by Western blot for the expression of the NTRK1 proteins (data not shown). Selected clones were treated with NGF and subjected to biochemical and morphological analysis (Fig. 3B and data not shown). NGF treatment induced the phosphorylation of NTRK1 receptor in clones expressing WT, R85S, H598Y, G607V, and D668Y mutants, similarly to the wild type (Fig. 3A and data not shown). For each transfection, several G418-resistant clones were isolated and analyzed by Western blot for the expression of the NTRK1 proteins (data not shown). Selected clones were treated with NGF and subjected to biochemical and morphological analysis (Fig. 3B and data not shown). NGF treatment induced the phosphorylation of NTRK1 receptor in clones expressing WT, R85S, H598Y, G607V, and D668Y mutants. Concomitantly, cells displayed the typical transformed phenotype, being spindle-shaped and less adherent. On the contrary, no effect of NGF was detected in clones expressing L213P, R643W, and G708S mutants.

To study the effect of NTRK1/CIPA mutants in a physiologically relevant cellular context, we used the PC12mnrr5 mutant, derived from the rat pheochromocytoma PC12 cell line, that...
does not express endogenous NTRK1 and does not differentiate in response to NGF (31). Transfection of NTRK1 receptor in PC12nnr5 cells restores NGF responsiveness (32). PC12nnr5 cells were transfected with the CIPA mutants and scored for neurite outgrowth. No differentiation was observed in un-treated cells. Treatment with NGF induced neurite formation in cells transfected with WT, R85S, H598Y, G607V, and D668Y receptors but not in those transfected with L213P, R643W, and G708S mutants (Fig. 3C and data not shown). Western blot analysis showed a comparable expression for all the constructs (data not shown).

Altogether the biochemical and biological data reported...
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above indicate a clear loss of function for L213P, R643W, and G708S but not for the remaining mutants. With respect to the latter, as indicated by genetic analysis, R585S, H598Y, and G607V are most likely polymorphisms, whereas the D668Y has the features of a pathogenic mutation (19, 22). This suggests that a novel pathogenetic mechanism can be exerted by D668Y.

Cellular Localization of NTRK1/L213P Protein—The biochemical analysis reported in the previous paragraph showed that the L213P mutation produces only the 110-kDa, partially glycosylated NTRK1 protein, indicating that the mutation interferes with the receptor processing. To determine the cellular localization of the L213P protein we performed the immunofluorescence experiments shown in Fig. 4A. As shown in Fig. 4A, the N5.3 cell line, derived from NIH3T3 transfected with the L213P mutant, was compared with the E25 cell line, expressing the wild type NTRK1 receptor (26). Staining of permeabilized cells with anti-TRK antibodies (reacting with the NTRK1 C terminus) showed cytoplasmic and perinuclear distributions of the WT NTRK1 protein. On the contrary, the L213P protein showed mostly a perinuclear reticular pattern. The staining of nonpermeabilized cells with the MGR12 antibodies (reacting with the NTRK1 extracellular portion) emphasized the different localization of L213P with respect to the WT receptor. The cells expressing the WT receptor showed a membrane pattern, whereas no reactivity above the background was detected in cells expressing the L213P protein. These data demonstrate that the NTRK1/L213P receptor is not located in the plasma membrane.

To address whether the NTRK1/L213P receptor might be retained in the endoplasmic reticulum (ER), we performed digestion of L213P and WT receptors with Endo H. This enzyme cleaves proteins with early high mannose forms characteristic of the ER species. Wild type and L213P receptors immunoprecipitated from E25 and N5.3 cell extracts, respectively, were subjected to Endo H treatment followed by Western blot analysis with anti-TRK antibodies. As shown in Fig. 4B, the 140-kDa fully glycosylated protein encoded by the wild type receptor was insensitive to the enzyme. On the contrary, after Endo H treatment, the partially glycosylated 110-kDa L213P protein, similarly to the equivalent form of the wild type receptor, was reduced to 80 kDa, corresponding to the NTRK1 core protein. Altogether, our data indicate that the L213P 110-kDa receptor does not reach the plasma membrane because it is retained in the ER.

Signaling and Biological Activity of NTRK1/D668Y Receptor—Although at the genetic level the D668Y has the features of a pathogenic mutation (22), our biochemical and biological analysis indicated that it does not abrogate the NTRK1 activity. To test whether the mutation could interfere with the receptor activation of downstream signal transducers, we analyzed the capability of the NTRK1/D668Y receptor to activate PLC-γ, FRS2, and Shc. HeLa cells were transiently transfected with WT or R643W, D668Y, and G708S mutant receptors and treated with NGF. Cell extracts were incubated with anti-PLC-γ and anti-Shc antibodies or with the FRS2-interacting protein p13Suc-1 conjugated to agarose beads. The results of Western blot with anti-phosphotyrosine antibodies (Fig. 5) showed that the D668Y mutant receptor is able to induce PLC-γ, Shc, and FRS2 tyrosine phosphorylation, similarly to WT. As expected, no activation of the three signal transducers was detected in the presence of R643W and G708S mutant receptors. As control, the expression levels of PLC-γ, FRS2, Shc, and NTRK1 are shown. Our data indicate that the D668Y mutant receptor does not differ from wild type in the recruitment of Shc, PLC-γ, and FRS2.

We also considered the possibility that the D668Y mutation might cause a partial inactivation of the NTRK1 receptor not detectable in the experiments above reported, mostly based on overexpression. We thus compared the transforming activity of WT and D668Y receptors at low doses of plasmid DNA and NGF. We transfected 10 ng of DNA/2 × 10⁵ cells and selected foci in different NGF concentrations (range 0–10 ng/ml). In these conditions, we have previously been able to detect transforming activity of the WT NTRK1. As shown in Fig. 5B, the transforming activity of the D668Y receptor was reduced with respect to the WT at all of the NGF concentrations analyzed. A similar reduction was also observed when 20 and 50 ng of DNA/2 × 10⁵ cells were transfected (data not shown), thus supporting the role of D668Y in CIPA.

2 C. Miranda and A. Greco, unpublished results.
**DISCUSSION**

Point mutations affecting the NTRK1/NGF receptor gene have been associated with the genetic disorder CIPA (17–23). By functional analysis, we have previously demonstrated that two of such mutations, the R774P and the G571R, lead to the inactivation of the NTRK1 receptor (18, 24) and thus exert a pathogenic role in CIPA disease. In our opinion, functional analysis represents a unique tool to distinguish pathogenic CIPA mutations from rare polymorphisms. Moreover, this approach allows the unveiling of the mechanism responsible for NTRK1 receptor inactivation. We have applied this type of study to seven missense mutations detected in CIPA patients; some of them were detected as double or triple mutations, being associated on the same allele with other clearly inactivating mutations. Transient expression into COS1 cells of NTRK1/CIPA cDNAs produced evidence of the processing and the phosphorylation status of the mutated proteins. The L213P mutation causes a processing defect, giving rise to a protein of 110 kDa unable to exit the ER and to reach the cell membrane. All of the other mutated NTRK1 receptors are processed similarly to the wild type. NGF-induced phosphorylation and autokinase activity were detected in mutants R85S, H598Y, G607V, and D668Y but not in mutants L213P, R643W, and G708S. Expression of CIPA mutants in NIH3T3 and PC12nnr5 cells allowed the investigation of transforming and differentiating activity, respectively. In the presence of NGF, mutants R85S, H598Y, G607V, and D668Y produced NIH3T3-transformed foci and induced neurite outgrowth similarly to the WT NTRK1 receptor. On the contrary, transforming and differentiating activities were completely abrogated by mutations L213P, R643W, and G708S. Analysis of stable NIH3T3 clones expressing the CIPA mutants showed results similar to those obtained with transient expression with respect to protein processing and NGF-dependent phosphorylation. Altogether, our results showed a clear inactivating effect for mutations L213P, R643W, and G708S but not for mutations R85S, H598Y, G607V, and D668Y. With respect to the latter group, mutations R85S, H598Y, and G607V have been classified as polymorphisms, whereas D668Y has the features of pathogenic mutation.

Leu213 is located within the first Ig-like domain of the NTRK1 extracellular portion. Mutant L213P produced only the partially glycosylated protein, thus suggesting that the mutation interferes with the receptor processing. Indeed, we showed that the NTRK1/L213P protein does not reach the plasma membrane but displays a perinuclear distribution. This result, together with the sensitivity to Endo H digestion, indicates that mutant L213P is retained in the ER. There are several genetic diseases in which mutations result in protein misfolding and ER retention (33–35). Indeed, reticulum retention is a mechanism of quality control by which misfolded proteins fail to exit the ER, remain associated with chaperon proteins, and are degraded in proteasome complexes (36, 37). Interestingly, another CIPA mutation, the L93P, has been recently shown to produce only the 110-kDa NTRK1 form (25), thus suggesting that processing alterations might be common in CIPA. The retention of L213P protein in the ER, its possible involvement in degradation pathways, and the possibility of rescuing the NTRK1/L213P receptor from the ER remain to be fully investigated.

Arg643 is located in the first Ig-like domain of the receptor. In our model, whereas in the inactive form of the enzyme the Arg643 side chain points toward the solvent, in its active conformation...
it is a charge partner of Tyr(P)\(^{675}\), as also shown by functional-structure analysis (38). Substitution with the neutral Trp would destabilize the active conformation of the enzyme, thus causing the observed loss of activity.

Gly\(^{708}\) is located within an a-helix of the C-terminal domain of the kinase and the effect of a substitution with Ser is not obvious. Since the inactivating effect of the mutation detected in our study suggests a critical role for Gly\(^{708}\) in the NTRK1 receptor activity, a possible explanation is that the Ser side chain in a hydrophobic environment perturbs the conformation, destabilizing the structure and, indirectly, its active site.

The most intriguing mutation is D668Y. Although having all of the features of a pathogenic mutation, it did not cause inactivation of the NTRK1 receptor. Asp\(^{668}\) is positioned in the activation loop of the kinase, close to phosphorylated residues Tyr\(^{670}\), Tyr\(^{674}\), and Tyr\(^{675}\). The Asp\(^{668}\) residue is highly conserved among receptor tyrosine kinases. Mutation of homologous residues has been shown to cause constitutive activation of c-Kit and c-Met. Substitutions to Val and to Tyr in c-Kit have been detected in human and mouse mastocytosis (39); substitution to Asn and to His in c-Met have been found in human papillary renal carcinoma (40). At variance, we went further on the biological characterization and on the analysis of biological effects. A novel inactivating mechanism, based on the ER retention of unprocessed receptor, has been unveiled. More importantly, a putative novel pathogenic mechanism, based on the reduction of activity, can be ascribed to mutation D668Y. The definition of molecular bases of these novel pathogenic mechanisms, however, will require further investigation. Our results strongly support the need for functional analysis, following mutation detection, in order to assess their role in CIPA pathogenesis.

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