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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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Claudia Miranda‡, Michela Di Virgilio‡, Silvia Selleri‡, Giuseppe Zanotti§, Sonia Pagliardini‡, Marco A. Pierotti‡¶, and Angela Greco‡¶‖

From the ‡Department of Experimental Oncology, Istituto Nazionale Tumori, Via G. Venezian 1, Milan 20133, Italy and the §Department of Organic Chemistry, University of Padova, Via Marzolo 1, Padova 35131, Italy

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*[1] 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. 1*A*). Several studies have shown that the activity of the NTRK1 receptor requires the phosphorylation of five different tyrosine residues, located in the juxtamembrane domain (Tyr^{490}), in the tyrosine kinase domain (Tyr^{670}, Tyr^{674}, and Tyr^{675}), and in the C-terminal tail (Tyr^{785}). Tyr^{670}, Tyr^{674}, and Tyr^{675} are located in the activation loop (6) and play an important role in the receptor activation; they are also involved in the activation of APS, SH2B, and Grb2 (7, 8). Phosphorylated Tyr^{490} provides the docking site for Shc and FRS2 adaptor proteins and is also implicated in the activation of phosphatidylinositol 3-kinase (9–11). Phosphorylated Tyr^{785} recruits and activates PLC-γ (12). Multiple downstream pathways, such as the extracellular signal-regulated kinase and c-Jun N-terminal kinase cascades, are triggered by NTRK1 activation and mediate the differentiating and surviving effects of NGF (13).

Congenital insensitivity to pain with anhidrosis (CIPA; MIM 256800) (also known as hereditary sensory and autonomic neuropathy, or HSAN, type IV) is a rare autosomal recessive disorder associated with consanguinity (14, 15). Specific features of CIPA are 1) profound loss of pain sensitivity, leading to injuries, self-mutilation, and osteomyelitis; 2) defects in thermoregulation, causing anhidrosis and episodic fever with hyperpyrexia; and 3) mental retardation (16). CIPA is the consequence of a genetic defect in the differentiation and migration of neural crest elements. Recently, the genetic bases of CIPA have been identified. Mutations of the *NTRK1*/NGF receptor gene have been detected by several laboratories, including ours, in CIPA patients from different ethnic groups (17–23). Most mutations occur within the tyrosine kinase domain, and a few occur within the extracellular domain. Mutation types include frameshift, nonsense, splice site, and missense. Whereas the effect of the first three mutation types, producing aberrant proteins, is foreseeable, the consequences of single amino acid substitutions, caused by missense mutations, require functional studies to formally demonstrate their causative role in CIPA disease and to distinguish them from rare polymorphisms. We were aware of this and proposed an approach based on the analysis of the biological effect produced by CIPA mutations. In previous studies, we have demonstrated that two CIPA mutations, the R774P and the G571R, inactivate the NTRK1 receptor by interfering with the NGF-induced autophosphorylation (18, 24). In this paper, we extended our analysis to seven additional CIPA mutations. A preliminary characterization of the same mutations, investigating only the effect on receptor phosphorylation, has been recently reported (25). We performed a more exhaustive study, including bio-

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¶ Senior co-authors.
‖ To whom correspondence should be addressed: Istituto Nazionale Tumori, Dept. of Experimental Oncology, Via Venezian 1, 20133 Milan, Italy. Tel.: 39 02 23 90 3222; Fax: 39 02 23 90 2764; E-mail: greco@istitutotumori.mi.it.

[1] The abbreviations used are: NTRK1, neurotrophic tyrosine receptor kinase type 1; CIPA, congenital insensitivity to pain with anhidrosis; NGF, nerve growth factor; Shc, Src homology-containing protein; FRS2, fibroblast growth factor receptor substrate-2; TK, tyrosine kinase; PLC-γ, phospholipase C-γ; ER, endoplasmic reticulum; Endo H, endo-β-*N*-acetylglucosaminidase H; WT, wild type; PBS, phosphate-buffered saline.

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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 autophosphorylation, 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, using as 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′-CTGGAGCTC**A**GTGATCTGAG-3′ for R85S; 5′-CGTGCTGC**C**GCGGTGCCAG-3′ for L213P; 5′-CCTCCGATCC**T**ATGGACCCG-3′ for H598Y; 5′-CTGCTGGCTG**T**TGGGGAGG-3′ for G607V; 5′-TTGTGCAC**T**GGGACCTGG-3′ for R643W; 5′-CATGAGCAGGTA**T**ATCTACAGCA-3′ for D668Y; and 5′-GGAGCTTC**A**GCGTGGTGC-3′ for G708S.

Mutant clones were identified by PCR followed by allele-specific oligonucleotide hybridization in the case of R85S, H598Y, G607V, and G708S. Clones carrying the R643W mutation were identified by *Msp*I digestion of a PCR fragment; the mutation abrogates a restriction site present in the WT. Clones carrying the D668Y mutation were identified by digestion with *Eco*RV, 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 G418 (400 μg/ml). NIH3T3 and derived cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. G418-resistant clones were cultured in the presence of the antibiotic. Monkey COS1 and human HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, PC12nnr5 cells in RPMI 1640 medium supplemented with 5% fetal calf serum, and 10% horse serum.

The NIH3T3 cells (2.5×10^5/10-cm plate) were transfected by the $CaPO_4$ method, as previously described (27), using 1 μg or 10 ng of plasmid DNA together with 30 μg of mouse 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. G418-resistant colonies and transformed foci were either fixed or isolated for further studies 2 weeks after transfection.

COS1 cells (8×10^5/10-cm plate) were transfected with the DEAE-dextran procedure, as previously described (24). One microgram of specific plasmid DNA was transfected with 19 μg of pRC/CMV DNA. Two days after transfection, cells were incubated overnight in 0.5% fetal calf serum and then treated or not with 50 ng/ml NGF for 10 min and then processed for immunoprecipitation.

PC12nnr5 cells were transfected using Cellfectin (Life Technologies, Inc.). Cells (2×10^5) were seeded on collagen-coated 12-multiwell plates and transfected with 100 ng of specific plasmid DNA together with 500 ng of plasmid carrier DNA. After incubation with the reagent, transfected cells were treated with NGF (50 ng/ml) and scored for neurite outgrowth 2 days later.

HeLa cells (5×10^5 cells/10-cm plate) were transfected with the $CaPO_4$ procedure. Two days after transfection cells were serum-starved overnight in 0.5% fetal calf serum medium. After treatment with 50 ng/ml NGF for 10 min, cells were processed for immunoprecipitation.

Immunoprecipitation, Pull-down, and Western Blot Analysis—Cells were lysed with PLCLB buffer (50 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM $Na_4P_2O_7$, 100 mM NaF) supplemented with aprotinin, pepstatin, leupeptin, phenylmethylsulfonyl fluoride, and Na_3VO_4. Cell extracts (0.4–1 mg) were precipitated with the appropriated antibodies or with p13suc1-agarose. The precipitates were washed three times with HNTG buffer (20 mM Hepes, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and suspended in Laemmli sample buffer. Protein samples were electrophoresed on SDS-PAGE (6.5%), transferred to nitrocellulose filters, and immunoblotted with the appropriated antibodies. The immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham Biosciences). The anti-TRK antibodies and the p13suc1-agarose were from Santa Cruz Biotechnology, Inc.; the anti-phosphotyrosine, anti-Shc, anti-FRS2, and anti-PLC-γ antibodies were from Upstate Biotechnology, Inc.; the MGR12 antibodies (28) were a kind gift of Dr. E. Tagliabue.

Immunokinase Assay—COS1 cells transiently expressing wild type and CIPA NTRK1 proteins were lysed in radioimmune precipitation buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40) supplemented with protease inhibitors. NTRK1 proteins were immunoprecipitated with MGR12 antibodies, adsorbed on protein A-Sepharose beads, and washed twice with radioimmune precipitation buffer. After one wash with incubation buffer (50 mM Hepes, 20 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol), the samples were incubated for 10 min at 30 °C in 50 μl of the same buffer containing 10 μM ATP and [γ-^{32}P]ATP (5000 Ci/mmol; Amersham Biosciences). After washing with radioimmune precipitation buffer, proteins were eluted and subjected to 6.5% SDS-PAGE. ^{32}P-Labeled proteins were revealed by autoradiography of the dried gel.

Immunofluorescence—Cells were grown on 13-mm coverslides at 70% confluence and fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. After three washes with PBS, one set of slides was incubated for 1 h at 37 °C with MGR12 antibodies at the final concentration of 10 μg/ml in PBS; after three washes with PBS, slides were incubated with fluorescein isothiocyanate-conjugated mouse IgG (Alexa) diluted 1:500 in PBS. The other set of slides was permeabilized in 0.1% sodium citrate, 0.1% Triton in PBS for 10 min at room temperature; washed three times with PBS and incubated for 1 h at 37 °C with 10 μg/ml anti-TRK antibodies; washed; and then incubated with fluorescein isothiocyanate-conjugated rabbit IgG (Meloy) diluted 1:50 in PBS. After washing, the slides were mounted with DABCO-Mowiol and analyzed with a fluorescent microscope using a ×63 objective lens.

Endoglycosidase H Treatment—Wild type and L213P NTRK1 proteins were immunoprecipitated from E25 and N5.3 cell lines, respectively, using the MGR12 antibodies as described above. Immunocomplexes were washed three times with HTNG buffer and once with deionized water and then digested overnight with endoglycosidase H (Endo H; ProZyme), according to the manufacturer's instructions. Reactions were stopped by adding Laemmli sample buffer, boiled for 5 min, and then separated on 7.5% SDS-PAGE.

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).

R85S occurs in the extracellular domain, within a leucine-rich 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 other 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.

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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. 2*A*. 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. 2*B*. NTRK1 proteins expressed in COS1 cells were immunoprecipitated with the MGR12 antibodies and incubated with [γ-^{32}P]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.

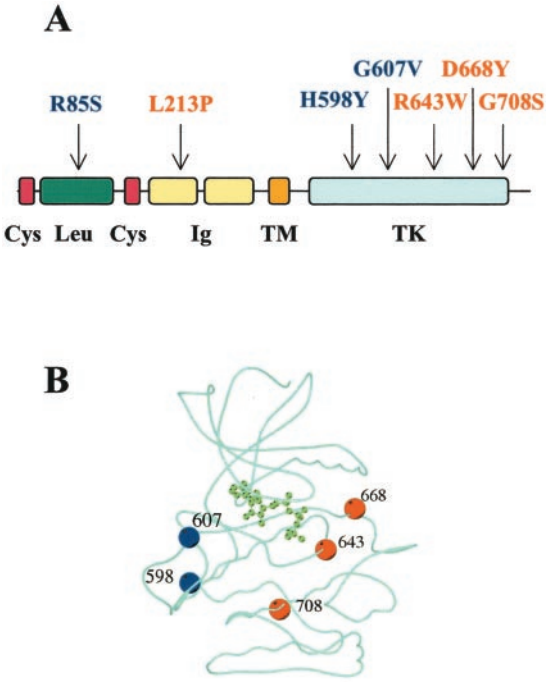

FIG. 1. *A*, structure of the NTRK1/NGF receptor and localization of the CIPA missense mutations analyzed in this study. *Red*, mutations with pathogenic features; *blue*, mutations that may represent polymorphisms. Cys- and Leu-rich regions, Ig-like, transmembrane (*TM*), and TK domains are shown. *B*, model of the catalytic domain of the receptor; the mutations analyzed in this study are indicated. Models of the active and inactive forms of the enzyme were produced with the Pro-Mod server (43) in analogy with the insulin receptor kinase (Protein Data Bank codes 1IR3 and 1IRK). Only the active conformation is shown. *Red*, the three mutations that display features of pathogenic mutations; *blue*, the mutations that may represent polymorphisms. The *ball-and-stick model* in *green* represents the ATP bound in the nucleotide-binding site.

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 $\times$ 10^5 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. 3*A* 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. 3*B* and data not shown). NGF treatment induced the phosphorylation of NTRK1 receptor in clones expressing WT, R85S, H598Y, G607V, and D668Y. 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 PC12nnr5 mutant, derived from the rat pheochromocytoma PC12 cell line, that

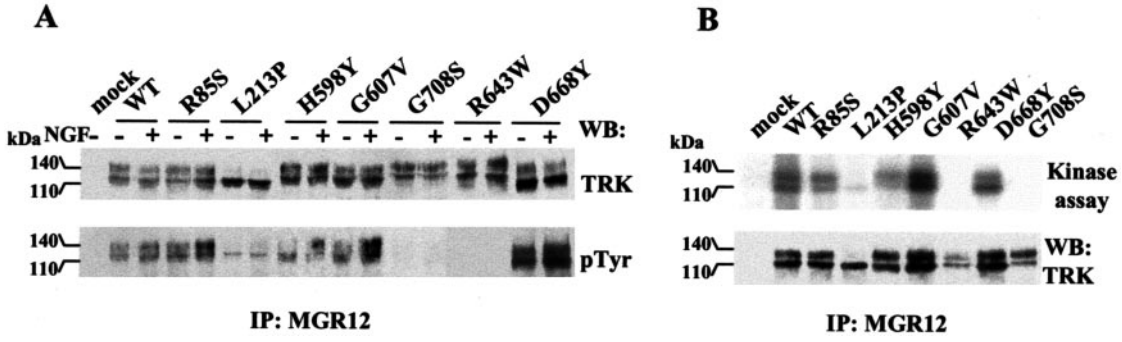

FIG. 2. **Tyrosine phosphorylation (*A*) and kinase activity (*B*) of NTRK1/CIPA mutants transiently expressed in COS1 cells.** *A*, transfected COS1 cells were treated or not with 50 ng/ml NGF for 10 min. Cell extracts were immunoprecipitated with MGR12 antibodies and then subjected to Western blot with anti-TRK antibodies (*upper panel*) or anti-phosphotyrosine antibodies (*lower panel*). *B*, cell extracts from transfected COS1 cells treated with NGF were immunoprecipitated with MGR12 antibodies and subjected to a kinase assay as described under "Experimental Procedures" (*upper panel*). NTRK1 expression levels are shown in the *lower panel*. The 140- and 110-kDa NTRK1 isoforms are indicated.

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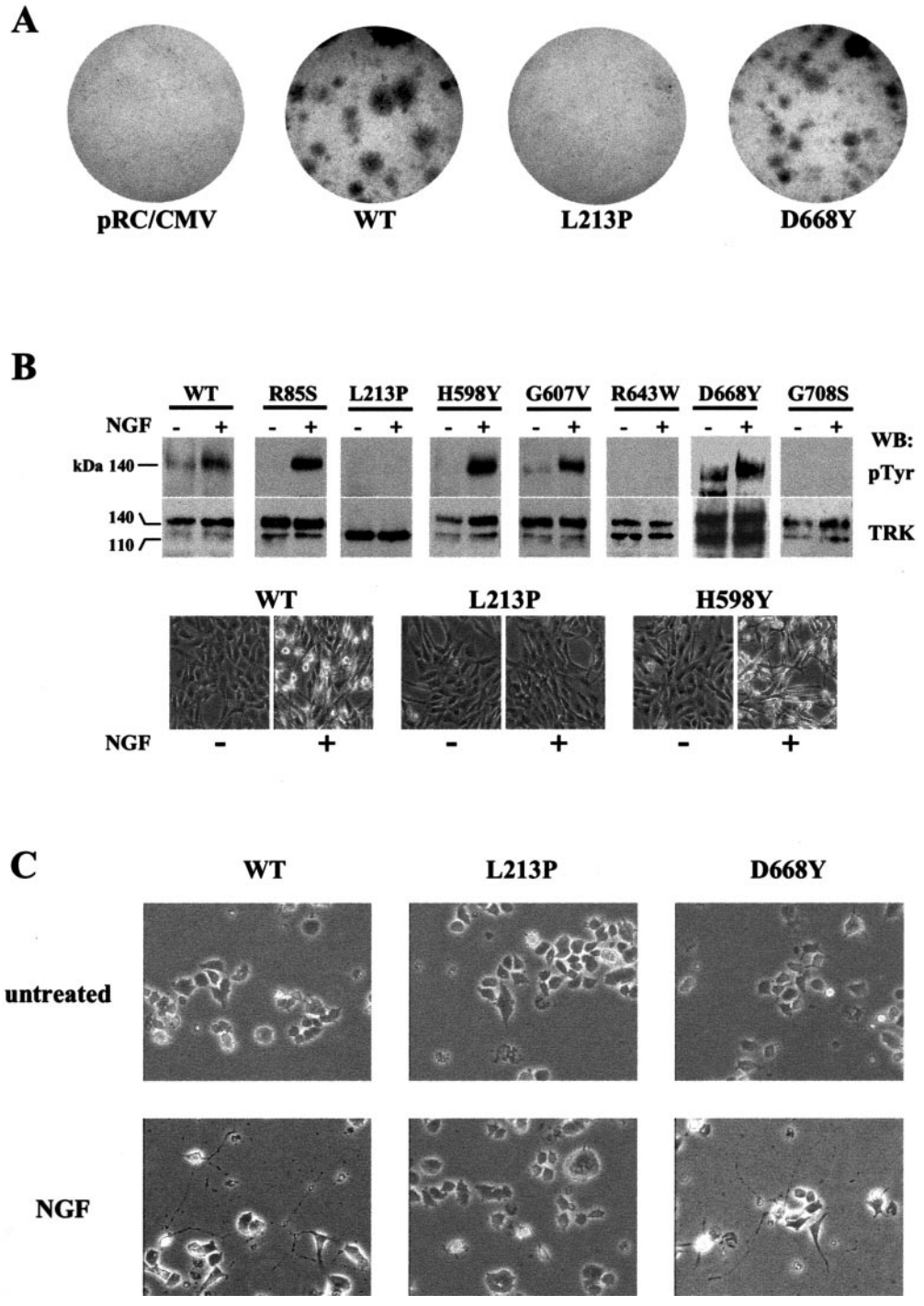

FIG. 3. **Biological activity of NTRK1/CIPA mutants.** *A*, transforming activity. NIH3T3 cells were transfected with WT and mutated NTRK1 receptors. Transfected cells were selected in the presence of the G418 antibiotic to determine the transfection efficiency. Foci selection was performed in the presence of 50 ng/ml NGF for 2 weeks. Plates deriving from transfections of representative mutants are shown. *B*, effect of NGF on NTRK1 phosphorylation and cell morphology in NIH3T3 cell lines expressing the NTRK1/CIPA mutants. NIH3T3 G418-resistant colonies were isolated and analyzed for the expression of the NTRK1 proteins. For each mutant, a selected positive clone was used for the Western blot analysis of NTRK1 phosphorylation upon NGF treatment. As control, the Western blot hybridization with the anti-TRK antibodies is shown (*top*). Cells were also scored for morphology changes induced by NGF. Pictures of representative clones, taken after 48 h of NGF treatment, are shown. *C*, differentiating activity of NTRK1/CIPA mutants. PC12nnr5 were transfected with WT and mutated receptors and treated or not with 50 ng/ml NGF. Cells were scored for neurites, and pictures were taken 3 days after transfection. Pictures deriving from transfections of representative mutants are shown.

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 untreated 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. 3*C* 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, R85S, 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. 4*A*. 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. 4*B*, 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 recruitment/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

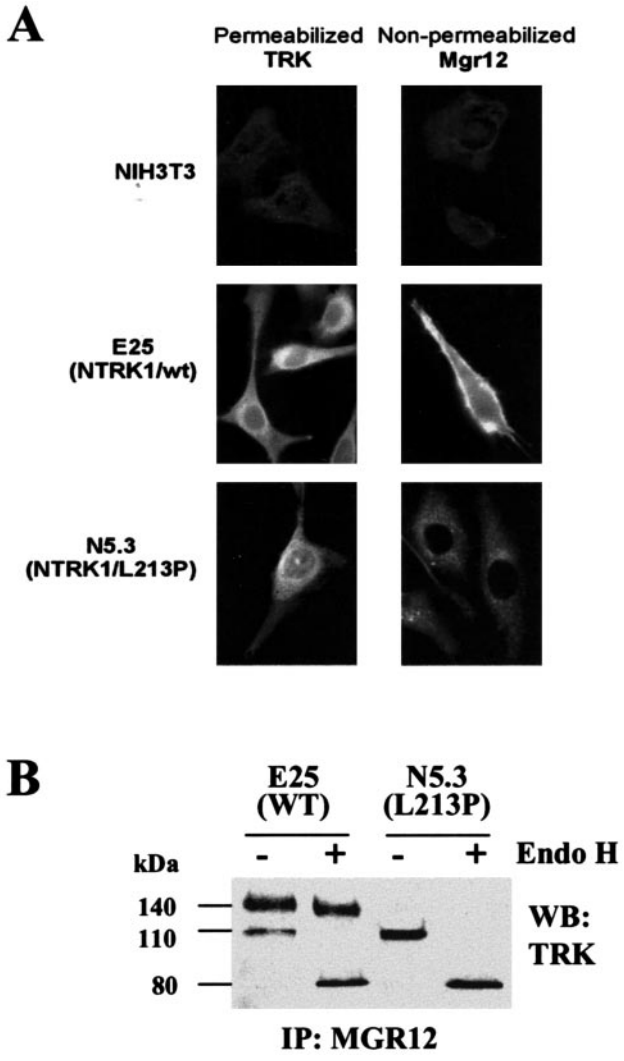

FIG. 4. **Cellular localization of WT and L213P proteins.** *A*, detection of NTRK1 WT and L213P proteins by immunofluorescence. NIH3T3, E25, and N5.3 cell lines were fixed and processed as described under "Experimental Procedures." Immunostaining with anti-TRK antibodies (directed to the NTRK1 C terminus) were performed after membrane permeabilization. Immunostaining with MGR12 antibodies (directed against the extracellular portion of NTRK1) was performed on nonpermeabilized cells. *B*, Endo H digestion. Wild type and L213P NTRK1 receptors were immunoprecipitated with MGR12 antibodies from E25 and N5.3 cell lines, respectively, and subjected or not to Endo H digestion, as reported under "Experimental Procedures." The samples were separated on 7.5% SDS-PAGE and visualized by Western blot hybridization with anti-TRK antibodies. The glycosylated 140- and 110-kDa NTRK1 isoforms and the 80-kDa core protein are indicated.

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 × 10^5 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.[2] As shown in Fig. 5*B*, 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 × 10^5 cells were transfected (data not shown), thus supporting the role of D668Y in CIPA.

[2] C. Miranda and A. Greco, unpublished results.

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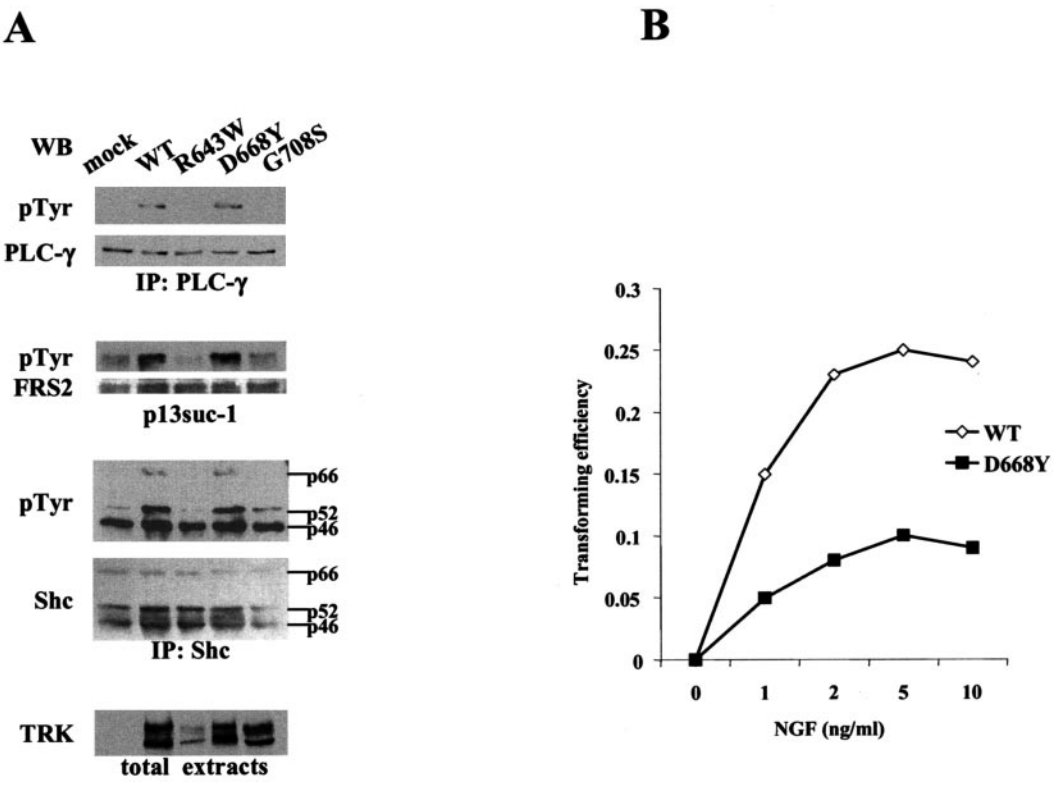

FIG. 5. **Signal transduction (*A*) and transforming activity (*B*) of D668Y mutant.** *A*, HeLa cells transfected with WT, R643W, D668Y, and G708S NTRK1 receptors were treated with NGF. Immunoprecipitates with anti-PLC-γ or anti-Shc antibodies, p13suc-1 protein complexes, and total extracts were run on gel and blotted with anti-phosphotyrosine, anti-PLC-γ, anti-FRS2, anti-Shc, and anti-TRK antibodies. The three Shc isoforms (p46, p52, and p66) are indicated. *B*, transforming activity of WT and D668Y NTRK1 receptors in the presence of NGF. NIH3T3 transfection/focus formation assay was performed using low amount of plasmid DNA (10 ng/2 × 10^5 cells) and low NGF concentrations (0–10 ng/ml). Transforming activity was calculated by normalizing the number of transformed foci for that of G418-resistant colonies.

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.

Leu^{213} 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.

Arg^{643} is located in a loop close to the active site. In our model, whereas in the inactive form of the enzyme the Arg^{643} side chain points toward the solvent, in its active conformation

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it is a charge partner of Tyr(P)^{675}, as also shown by function-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 α-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, the NTRK1 D668Y mutation is not activating, since no biological activity can be detected in the absence of NGF. Interestingly, even the mutations to Val and Asn did not cause the expected ligand-independent NTRK1 activation.[3] This would suggest that, although occurring at a conserved residue, mutations of the Asp^{668} cause different structural alterations in NTRK1 *versus* other receptor tyrosine kinases. On the other hand, the D668Y mutation did not cause inactivation of the NTRK1 receptor, as one would foresee based on its association with CIPA disease. Our preliminary results showed a reduced biological activity of D668Y with respect to the wild type NTRK1 receptor. This is consistent with the observation that the D668Y mutation has never been detected as homozygous but always in a heterozygous compound with other mutations. Most likely, the NTRK1/D668Y receptor activity in the homozygous status could be still sufficient for a proper neuronal development, and a complete inactivation of the other allele is required in order to produce the disease.

Alternatively, the D668Y mutation might cause a shift of substrata, with effects detectable in developing neurons but not in NIH3T3 and PC12 cells. Our preliminary studies indicated that Shc, PLC-γ, and FRS2 are activated similarly to the wild type. However, the possibility of recruitment of novel substrata and activation of alternative pathways, based also on the consideration that Tyr^{668} itself could be phosphorylated and act as docking site, must be taken into account. In this respect, it is worth mentioning that the analogous mutation (D814Y) in the murine Kit causes alteration in substrate recognition (41). Similarly, the human D816Y mutant has been recently shown to activate signal transducer and activator of transcription STAT3, at variance with the SCF-stimulated WT receptor (42).

Moreover, the D668Y mutation could be a rare polymorphism associated to a transcriptional defect. In this respect, the expression of *NTRK1* mRNA in patients carrying the D668Y mutation should be investigated.

In conclusion, we have analyzed the effect of several putative CIPA missense mutations on the biological and biochemical properties of the NTRK1 receptor. As anticipated earlier, a partial functional study of the same mutations, based on the analysis of phosphorylation level of NTRK1 mutants, was recently reported (25). At variance, we went further on the biochemical 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 pathogenetic 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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[3] C. Miranda and A. Greco, manuscript in preparation.

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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

Claudia Miranda, Michela Di Virgilio, Silvia Selleri, Giuseppe Zanotti, Sonia Pagliardini, Marco A. Pierotti and Angela Greco

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