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Functional characterization of mutations in the *GDNF* gene of patients with Hirschsprung disease

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Hirschsprung disease (HSCR) is a congenital disorder characterized by the absence of enteric nervous plexuses in hind gut. Ten to forty percent of HSCR patients carry a dominant loss-of-function mutation in the gene encoding the receptor tyrosine kinase RET, a receptor for glial cell line-derived neurotrophic factor (GDNF). Although several mutations have also been found in the *GDNF* gene of HSCR patients, their impact on GDNF function is unknown. In this study, we have characterized the effect of these mutations on the ability of GDNF to bind and activate its receptors. Although none of the four mutations analyzed appeared to affect the ability of GDNF to activate RET, two of them resulted in a significant reduction in the binding affinity of GDNF for the binding subunit of the receptor complex, GFRα1. Our results indicate that, although none of the GDNF mutations identified so far in HSCR patients are *per se* likely to result in HSCR, two of these mutations (i.e. D150N and I211M) may, in conjunction with other genetic lesions, contribute to the pathogenesis of this disease.

INTRODUCTION

Hirschsprung disease (HSCR), also known as congenital aganglionic megacolon, is a common congenital abnormality leading to bowel obstruction. HSCR is characterized by the absence of enteric ganglion cells in all or parts of the hind gut and occurs in 1/5000 live births. It is a disease with a complex genetic background and several genes have been implicated in its pathogenesis. Endothelin-3 (EDN3), the endothelin B receptor (EDNRB), endothelin-converting enzyme-1, and the transcription factor SOX10 have each been implicated in less than 5% of HSCR cases (1). On the other hand, mutations in the gene encoding the receptor tyrosine kinase RET are associated with HSCR in up to 40% of familial cases and in ~5% of sporadic cases. Screening of a population-based series including 62 sporadic and seven familial HSCR cases with single-strand conformation polymorphism found five mutations in *RET* (2). RET is a receptor for members of the glial cell line-derived neurotrophic factor (GDNF) family of ligands. In order to bind ligand, RET necessitates the presence of a member of the GFRα family of glycosyl-phosphatidylinositol (GPI)-anchored accessory receptors (3).

The GDNF family consists of four members, GDNF, neurturin (NTN), artemin (ART) and persephin (PSP), which interact with four distinct GFRα receptors, i.e. GFRα1–4 (4). Since all members of the GDNF family interact with RET, all these genes are possible targets of mutations contributing to HSCR. GDNF is produced as a 211 residue long precursor that is subsequently cleaved to give a 134 residue long mature polypeptide which assembles into a dimeric protein. The crystal structure of GDNF revealed that the protomer has two long fingers formed by pairs of anti-parallel β-strands connected by loops and a helical region at the opposite end (5). In the dimer, the two protomers are positioned in a head-to-tail orientation giving the molecule an elongated form with the two helices flanking a cysteine-knot motif at the center of the structure. Extensive site-directed mutagenesis revealed that negatively charged and hydrophobic residues located on the tips of the two fingers are important for GDNF binding to the GFRα1 receptor (6).

Several mutations have been found in the *GDNF* gene of patients with HSCR, although only five of them are missense mutations, resulting in a change in the amino acid sequence. These mutations are summarized in Table 1. The replacement of Arg93 with Trp (R93W) has been reported in HSCR patients that also carried RET mutations (7,8). The R93W mutation has also been found in sporadic pheochromocytoma, a cancer form that is associated with gain-of-function mutations in the *RET* gene normally found in multiple endocrine neoplasia type 2A (MEN 2A) (9). The R93W mutation has also been detected in one case of Ondine's curse or congenital central hypoventilation syndrome (CCHS), which often co-segregates with HSCR (10). In a case of sporadic HSCR in a patient with Down's syndrome, replacement of Asp150 with Asn (D150N) was reported in the absence of any *RET* mutations (8). Replacement of Thr154 with Ser (T154S) was found as a *de novo* mutation in a case of sporadic HSCR without any accompanying mutations in *RET* (11). Salomon *et al.* (8) reported a mutation in Pro21, P21S, located near the proteolytic cleavage of the GDNF pre-pro-hormone which does not form part of the mature GDNF polypeptide, but that could play a role in the post-translation processing of the protein. Finally, mutation of Ile211 to Met has also been reported in the literature, although no data concerning this mutation have been shown as yet

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Table 1. Mutations in GDNF reported in HSCR patients

GDNF mutation	Accompanying RET mutations	Family	Other comments	Reference
P21S	No	Affected family members shared the same haplotypes at the *RET* locus	Mutation lies near the consensus sequence for proteolytic cleavage	Salomon *et al.* (8)
R93W (R16W)[a]	Yes	Found in the family, but not in any unrelated controls	Also found in an Ondine's curse patient, without RET mutation	Angrist *et al.* (7), Salomon *et al.* (8), Amiel *et al.* (10)
D150N (E73N)[a]	No	Inherited from healthy father. Not found in controls	Patient also had Down's syndrome	Salomon *et al.* (8)
T154S (T77S)[a]	No	*De novo* HSCR	Not detected in normal controls	Ivanchuk *et al.* (11)
I211M (I134M)[a]	Not known			Martucciello *et al.* (13)

[a]Corresponding native and mutated amino acid residue in the primary sequence of mature rat GDNF.

(12,13). With the exception of T154S, all mutations co-segregate with mutations in the *RET* gene or are also found in unaffected members of HSCR families, indicating that these mutations in the *GDNF* gene are neither necessary nor sufficient to cause HSCR. However, several of these mutations could still contribute to the HSCR phenotype via interaction with other susceptibility loci, such as *RET* or genes in the endothelin pathway.

In this study, we have characterized the effects of the four HSCR mutations affecting the mature region of the GDNF polypeptide on the ability of this protein to bind and activate its receptors. Our results demonstrate that two of these mutations alter binding of GDNF to the GFRα1 receptor and may, in conjunction with other genetic lesions, contribute to the pathogenesis of HSCR.

RESULTS

R93W, D150N, T154S and I211M are the four GDNF mutations reported in patients with HSCR affecting the mature region of the protein. Herein, these mutants will be referred to by their position in the primary sequence of mature rat GDNF. Thus, R93W is R16W, D150N is E73N (note that Glu replaces Asp at this position in rat GDNF), T154S is T77S, and I211M is I134M. We have analyzed the effects of these mutations on the ability of GDNF to bind to the GFRα1 receptor and to induce RET tyrosine phosphorylation.

Mutations were introduced in the *GDNF* gene by site-directed mutagenesis. Mutant GDNF proteins were produced in the conditioned medium of transiently transfected COS cells and quantified by western blotting using different specific anti-peptide antibodies and purified recombinant GDNF as standard. Medium from mock transfected cells had negligible effects on either binding or RET phosphorylation.

Binding to the GFRα1 receptor was assessed by the ability of the mutants to displace radiolabeled GDNF from GFRα1-binding sites in a MG87 fibroblast cell line stably transfected with a GFRα1 cDNA (MG87-α1 cells). MG87-α1 cells do not express detectable levels of RET nor any GFRα receptor, other than GFRα1. Receptor binding was measured in displacement binding assays using chemical cross-linking, SDS–PAGE, phosphorimaging autoradiography, and quantification of affinity-labeled receptor bands. In all the experiments, wild-type GDNF produced and quantified under the same conditions was used as an internal standard. These experiments indicated that two of the four mutations, i.e. E73N and I134M, had a perceptible effect on GDNF binding to GFRα1, with an estimated 70–75% reduction in binding affinity (Fig. 1 and Table 2). A moderate effect, i.e. 35% reduction, was observed for T77S, whereas no significant effect could be detected for R16W (Table 2).

The ability of wild-type and mutant GDNF molecules to activate the RET receptor tyrosine kinase was determined by evaluating RET tyrosine phosphorylation in fibroblast cells co-expressing GFRα1 and RET (MG87-α1/Ret). In contrast to the binding assays on GFRα1-expressing cells, we could not detect any significant difference between mutant and wild-type GDNF molecules in their ability to induce RET tyrosine phosphorylation (Fig. 2 and Table 2).

DISCUSSION

Previous studies have shown that GDNF binding to GFRα1 is mediated by negatively charged and hydrophobic residues in fingers 1 and 2 of the GDNF protein (6,14). These studies also showed that deletion of the 36 residues in the extended N-terminus of the GDNF molecule reduced binding to GFRα1 but had no significant effect on RET autophosphorylation (6). This is presumably a region of high flexibility in the molecule which could not be resolved in the GDNF X-ray crystal structure (5). Arg16 is located within this segment and could therefore be involved in contacts contributing to the stabilization of the GNDF–GFRα1 interaction. However, we found no effect of the R16W mutation either in GFRα1 binding or RET phosphorylation, indicating that, if anything, this mutation may have a more subtle effect on GDNF activity below the detection threshold of our assays.

E73N and T77S are at the base of finger 1 just before the α-helix, in a region previously proposed to contribute to ligand specificity in the GDNF family (14). In addition, our own previous work has also indicated that Glu73 contributes to GFRα1 binding by the analysis of a E73A mutant with a 60% reduction in binding affinity to this receptor (6). Interestingly, mutation of the neighboring residue Glu76 to Ala also reduced GDNF binding to GFRα1 by ~70% (6), indicating that several residues in the region around Glu73 and Thr77 are functionally implicated in receptor binding. Ile134 is the last amino acid in the mature GDNF polypeptide, and we have found that its

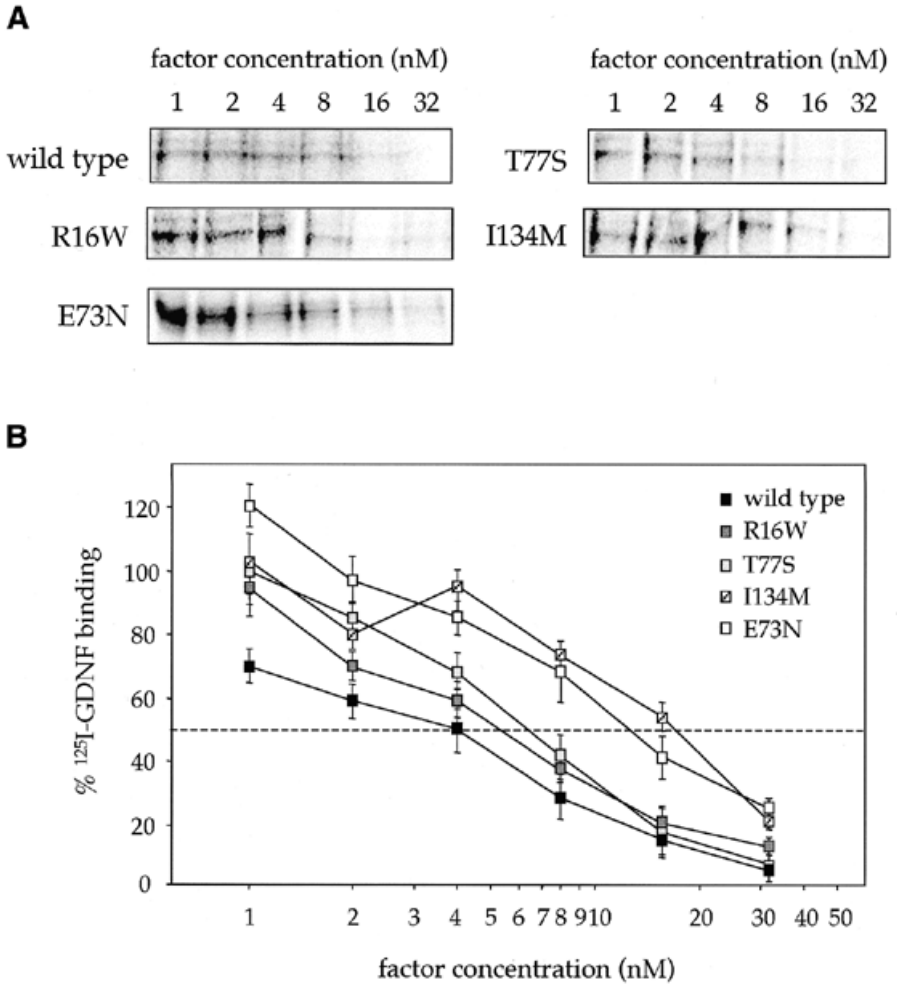

Figure 1. GFRα1-binding activities of GDNF mutants analyzed by cross-linking. (**A**) Autoradiograms showing affinity-labeled GFRα1 receptors after cross-linking of iodinated GDNF to MG87-α1 cells in the presence of increasing concentrations of unlabeled competitors. (**B**) Displacement binding curves obtained from phosphorimaging quantification. On the *y* axis, percent binding is plotted relative to that obtained in the absence of any unlabeled factor (set to 100%). The level of 50% displacement used to calculate IC_{50} values is indicated by a dashed horizontal line. The R16W mutation does not affect the binding to GFRα1, the T77S mutant retains 70% of the wild-type binding, and the E73N and I134M mutations reduce the binding to 30% of wild-type. The data shown are means ± SEM of three independent experiments each performed in duplicate.

replacement with Met reduced the protein ability to bind to GFRα1 by 70%. However, there is at present no evidence linking this residue to a functional surface in the GDNF molecule. Finally, a mutation in the *NTN* gene has recently been linked to HSCR (15). This mutation replaces the first residue in the mature sequence of NTN and may therefore influence the rate of cleavage of the NTN pro-hormone.

GDNF is the main RET ligand in the enteric nervous system. Both the RET and GDNF knock-outs lack enteric neurons. Therefore, it is somewhat surprising that no more mutations in the *GDNF* gene have been linked to HSCR. Why is *RET* the main target of HSCR mutations in this signaling system? Clearly homozygous loss-of-function mutations in the *GDNF* gene would be lethal and will therefore not be found among HSCR patients. It may be possible that half a dose of GDNF, unlike RET, has no major effect on the development of enteric neurons. Alternatively, GDNF may have additional, RET-independent functions that keep a high selection pressure against loss-of function mutations, even in heterozygous form. Recent evidence obtained in RET-deficient cell lines and sensory neurons isolated from RET knock-out mice indicates the existence of alternative signaling mechanisms independent of RET (16,17), suggesting that GDNF could, in fact, have important functions that are not mediated by this receptor.

A recurrent theme in mutagenesis studies of GDNF and the GFRα1 receptor is the apparent dissociation between GDNF–GFRα1 interactions and RET activation. Several mutations in GDNF and GFRα1 that have a profound detrimental effect on the interaction between these two molecules do not affect the ability of GDNF to induce RET phosphorylation (6,18). Moreover, several members of the GDNF family are able to activate RET in the presence of GFRα1, despite being themselves very poor binders of this receptor (19). Here, we also observed that mutations that affected binding of GDNF to GFRα1 had no apparent effect on the ability of GDNF to induce RET phosphorylation (Table 2). It is possible that the binding affinity of GDNF for the GFRα1 receptor greatly surpasses the requirements for cellular

Table 2. GFRα1 binding and RET tyrosine phosphorylation activities of HSCR GDNF mutants

GDNF mutation	GFRα1 binding (% wild-type)[a]	RET phosphorylation (% wild-type)
Wild-type	100	100
R16W	85 ± 13	≥80
E73N	30 ± 0.4	≥80
T77S	65 ± 6.3	≥80
I134M	25 ± 4.5	≥80

[a]Relative binding is expressed as percentage of wild-type using the equation: 100 × (mutant IC_{50}/wild-type IC_{50}). The data shown are means ± SEM of three independent experiments each performed in duplicate.

activity, so that a very low number of occupied binding sites are enough for eliciting a full biological response, as recently reported for growth hormone (20). Another possibility is that, in the presence of RET, a novel binding site is generated by the two receptors which can now accommodate mutations in either the ligand or the GFRα subunit which, in the absence of RET, compromise ligand binding (6). Indeed, cooperation between GFRα1 and RET is a very appealing model which has experimental support from a number of recent studies (21). In addition, a reduced affinity for GFRα1 may affect the ability of GDNF to utilize the soluble form of this receptor for activation of RET in *trans* (22). Recently, this has been proposed as an important mechanism contributing to the effects of GDNF on survival and development of enteric neurons (23).

Simple Mendelian inheritance is rarely seen in HSCR and for almost every HSCR gene an incomplete penetrance has been observed, suggesting the existence of genetic modifier loci. Thus, it appears that the genetic background has a great influence on the phenotypic outcome of a given mutation in the genes most frequently affected in HSCR. Mutations in modifier genes may either compensate or enhance the effects of known HSCR mutations, leading several researchers in the field to regard HSCR as a model for complex polygenic disorders. In this context, although mutations in the *GDNF* and *NTN* genes have been found in only a minority of HSCR cases, they may have an important role in the development of the disease when combined with mutations in *RET* or in another gene contributing to HSCR, as has often been the case (1,24). The recent finding that a locus on chromosome 9q31 contributes to HSCR in conjunction with weak *RET* mutations, supports the view that HSCR is not a simple Mendelian trait but rather has a polygenic pattern of inheritance (25).

Without independent biochemical and biological evidence, it may be difficult to predict the phenotypic effect of a particular mutation. Here we have analyzed the effects of mutations in the *GDNF* gene found in patients with HSCR on the ability of this ligand to bind to GFRα1 and activate the GFRα1/RET complex. We found that some of the mutations, in particular E73N and I134M, resulted in a significant reduction in the ability of GDNF to bind to GFRα1. Our results predict that these and other similar mutations in the *GDNF* gene may be genetic alterations with a modulatory role in the pathogenesis of HSCR.

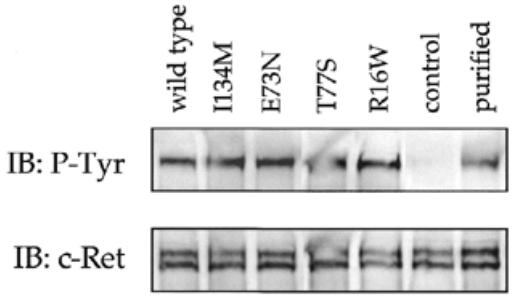

Figure 2. Stimulation of RET tyrosine phosphorylation by GDNF mutants. Wild-type and the indicated GDNF mutants were produced in supernatants of transfected COS cells. Conditioned medium of control transfected cells was also used (control), as well as purified GDNF protein (purified). After stimulation of MG87-α1/Ret cells with the indicated ligands at 30 ng/ml, RET was immunoprecipitated and filters probed with anti-phosphotyrosine antibodies (top) and re-probed with anti-RET antibodies (bottom). The GDNF mutations found in HSCR patients did not significantly affect the proteins ability to phosphorylate RET in MG87-α1/Ret cells.

MATERIALS AND METHODS

Site-directed mutagenesis and production of GDNF mutants

Site-directed mutagenesis was performed according to Kunkel (26) on full-length rat GDNF cDNA subcloned in pCDNA3 vector (Invitrogen). The mutations were confirmed by automated DNA sequence analysis. Mutant protein was obtained in the conditioned media of COS cells transfected by the DEAE–dextran–chloroquine method. One day after transfection the complete medium was changed to serum-free DMEM supplemented with insulin and transferrin. Four days after transfection the medium was harvested and subsequently concentrated 50–80 times by ultrafiltration through Centriprep 10 cartridges (Amicon). The concentration of mutant GDNF in the conditioned medium was estimated by western blotting against standards of purified recombinant GDNF, obtained from Sf21 insect cells (27) or from a commercial source (R&D). Anti-GDNF antibodies against GDNF were either from our own laboratory (27) or the D-20 antibody from Santa Cruz Biotechnology. Western blots were developed by enhanced chemifluorescence (ECF; Amersham), analyzed in a STORM 840 fluorimager and quantified using the ImageQuant software (Molecular Dynamics).

GDNF iodination and binding assays

For iodination, we used rat GDNF produced in insect cells as described by Trupp *et al.* (27). Iodination was performed by the lactoperoxidase method to a mean specific activity of 5×10^7 c.p.m./μg and the product purified by size-exclusion chromatography through a Sephadex G25 column. For binding assays, the cell-line MG87-α1 (28) expressing rat GFRα1, but no RET, was used. Cells were plated in 12-well plates and exposed to 10 ng/ml ^{125}I-GDNF in phosphate-buffered saline (PBS) supplemented with 1 mg/ml BSA, 1 mM $MgCl_2$, and 0.5 mM $CaCl_2$, in the presence or absence of serial dilutions of unlabeled competitors. Binding was allowed to occur at 4°C, with gentle rocking. After 4 h, 0.5 mM bis-(sulfosuccinimidyl) suberate (BS3) was added to chemically cross-link interacting

proteins. Cross-linking was allowed to proceed for 30 min at room temperature and was stopped by the addition of 50 mM glycine in PBS. Cells were washed three times with PBS and lysed with NP-40 lysis buffer. Cell lysates were analyzed with SDS–PAGE, gels fixed, dried, exposed to phosphorscreens (Molecular Dynamics) and analyzed in a STORM 840 phosphorimager. Quantifications of ^{125}I-GDNF to GFRα1 were done with ImageQuant software. The signal intensity obtained without unlabeled displacing factors was set to 100% binding; excess (i.e. 200-fold) unlabeled GDNF was used to determine maximal displacement which was set to 0% binding. All other measurements were referred to this scale and are plotted in Figure 1B. The results correspond to three independent experiments each performed in duplicate. Relative affinity (Table 2) was calculated as percentage of wild-type using the equation: 100 × (mutant IC_{50}/wild-type IC_{50}), where IC_{50} indicates the concentration of unlabeled factor required to obtain 50% displacement of ^{125}I-GDNF.

RET phosphorylation assays

RET phosphorylation was analyzed by stimulating cells expressing both RET and GFRα1, MG87-α1/Ret cells. Cells were stimulated for 15 min at 37°C, lysed with NP-40 lysis buffer (with protease inhibitors and tyrosine phosphatase inhibitor), debris was spun down and the lysate was immunoprecipitated with anti-RET-antibodies (C-20 and T-20, Santa Cruz Biotechnology). Immunoprecipitates were fractionated by SDS–PAGE and analyzed by western blotting with anti-phosphotyrosine antibodies (Upstate Biotechnology or Santa Cruz Biotechnology). Western blots were developed as described above. The membrane was subsequently striped and re-probed for RET. Bands were quantified with the ImageQuant software.

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