

# Distinct structural elements in GDNF mediate binding to GFR $\alpha$ 1 and activation of the GFR $\alpha$ 1–c-Ret receptor complex

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**Ligand-induced receptor oligomerization is a widely accepted mechanism for activation of cell-surface receptors. We investigated ligand–receptor interactions in the glial cell-line derived neurotrophic factor (GDNF) receptor complex, formed by the c-Ret receptor tyrosine kinase and the glycosylphosphatidylinositol (GPI)-anchored subunit GDNF family receptor alpha-1 (GFR $\alpha$ 1). As only GFR $\alpha$ 1 can bind GDNF directly, receptor complex formation is thought to be initiated by GDNF binding to this receptor. Here we identify an interface in GDNF formed by exposed acidic and hydrophobic residues that is critical for binding to GFR $\alpha$ 1. Unexpectedly, several GDNF mutants deficient in GFR $\alpha$ 1 binding retained the ability to bind and activate c-Ret at normal levels. Although impaired in binding GFR $\alpha$ 1 efficiently, these mutants still required GFR $\alpha$ 1 for c-Ret activation. These findings support a role for c-Ret in ligand binding and indicate that GDNF does not initiate receptor complex formation, but rather interacts with a pre-assembled GFR $\alpha$ 1–c-Ret complex.**

**Keywords:** c-Ret/GDNF/GFR $\alpha$ 1/ligand–receptor interaction/site-directed mutagenesis

## Introduction

Most biological processes are governed by specific protein–protein interactions. When growth factors bind to their receptors, an extensive surface becomes buried in the binding interface, typically involving 10–30 amino acid residues from each protein (De Vos *et al.*, 1992; Banner *et al.*, 1993; Wiesmann *et al.*, 1997). However, in the few cases investigated directly, only a small and complementary set of contact residues maintains binding affinity between ligand and receptor. In the complex formed by human growth hormone and its receptor, for example, the functional epitope is formed by a central hydrophobic region surrounded by hydrophilic residues of lower importance (De Vos *et al.*, 1992; Clackson and Wells, 1995). Mutagenesis studies have shown that fewer than half of the residues buried in the binding interface of this complex contribute ~90% of the total binding

energy (Clackson and Wells, 1995). Similarly, in the neurotrophins, three basic residues provide the critical binding determinants for interaction with their p75 receptor (Ibáñez *et al.*, 1992; Rydén *et al.*, 1995). These and other examples indicate that functional studies of binding surfaces are required for a complete understanding of protein–protein interactions, in order to determine to what extent different exposed residues contribute to the overall binding energy of a complex.

Glial cell-line derived neurotrophic factor (GDNF) is a distant member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily that promotes survival and differentiation of subpopulations of central and peripheral neurons, including several groups of neurons that are compromised in many neurodegenerative diseases (Lapchak, 1996; Unsicker, 1996). GDNF is also an inducer and branching factor of ureteric buds during kidney development (Sariola and Sainio, 1997). The neurotrophic and morphogenic activities of GDNF are mediated by its interaction with a multicomponent receptor complex formed by the c-Ret receptor tyrosine kinase (Durbec *et al.*, 1996; Trupp *et al.*, 1996; Vega *et al.*, 1996; Worby *et al.*, 1996) and a glycosylphosphatidylinositol (GPI)-anchored ‘accessory’ receptor, GDNF family receptor alpha-1 (GFR $\alpha$ 1), which is required for ligand binding (Jing *et al.*, 1996; Treanor *et al.*, 1996). Complex formation is believed to result in c-Ret dimerization and activation of the c-Ret tyrosine kinase. The current model of GDNF signalling proposes a stringent division of labour between GFR $\alpha$ 1 and c-Ret receptors, in which the latter delivers the intracellular signal but cannot bind ligand on its own, whereas the former binds ligand but is thought not to signal in the absence of c-Ret. Three close mammalian homologues of GDNF have been identified, all of which utilize c-Ret as the signalling receptor component with the aid of different members (GFR $\alpha$ 1–4) of the GFR $\alpha$  family of GPI-linked accessory receptors (Buj-Bello *et al.*, 1997; Klein *et al.*, 1997; Baloh *et al.*, 1998; Enokido *et al.*, 1998; Trupp *et al.*, 1998). GFR $\alpha$  receptors have been shown to provide some degree of ligand specificity, although cross-talk between the different receptors is also possible (Ibáñez, 1998).

c-Ret is not alone among receptor tyrosine kinases in its dependence on an accessory receptor component, although it is the first known to utilize a GPI-anchored partner (Lindsay and Yancopoulos, 1996). It remains unclear as to how accessory components facilitate ligand binding to and dimerization of signalling components. One possibility is that the ligand and the accessory receptor present a combined surface for binding to the signalling components; alternatively, binding of ligand to the accessory component may change the conformation of either molecule allowing it to bind and activate the signalling receptor. Finally, it is also possible that accessory and

signalling components form a pre-associated complex to which ligands bind. Although it has been shown that c-Ret, GFR $\alpha$ 1 and GDNF can form a complex (Treanor *et al.*, 1996), the interactions required for its assembly and stabilization remain to be defined. Initial studies led to the suggestion that c-Ret may not contribute to the interaction of GDNF with the receptor complex (Jing *et al.*, 1996). However, GDNF can be chemically cross-linked to c-Ret with high efficiency (Trupp *et al.*, 1996), indicating that the two molecules contact each other in the complex. Moreover, c-Ret has been shown to enhance the binding of GDNF to other non-preferred members of the GFR $\alpha$  family, such as GFR $\alpha$ 2 and GFR $\alpha$ 3 (Sanicola *et al.*, 1997; Trupp *et al.*, 1998). Together, these observations suggest that the interaction between GDNF and c-Ret may play an important role in the assembly and stability of functional receptor complexes. Finally, it has also been shown that GFR $\alpha$  receptors can, to some extent, interact with c-Ret in the absence of ligands (Sanicola *et al.*, 1997; Trupp *et al.*, 1998), suggesting that GDNF could also function by stabilizing pre-formed complexes of GFR $\alpha$ 1 and c-Ret.

The GDNF–GFR $\alpha$ 1–c-Ret complex provides an attractive system in which to investigate protein–protein interactions involved in the assembly of multi-subunit receptor complexes. In this work, we investigated structure–function relationships in GDNF using alanine scanning mutagenesis of surface-exposed amino acid residues. Using cell lines expressing a defined complement of receptor components, we probed the ability of different GDNF mutants to bind GFR $\alpha$ 1 and to activate c-Ret. Our results define a hot spot in GDNF for binding to the GFR $\alpha$ 1 receptor, and suggest a new model for the assembly of the GDNF receptor complex.

## Results

### **Prominent features of the GDNF molecular surface**

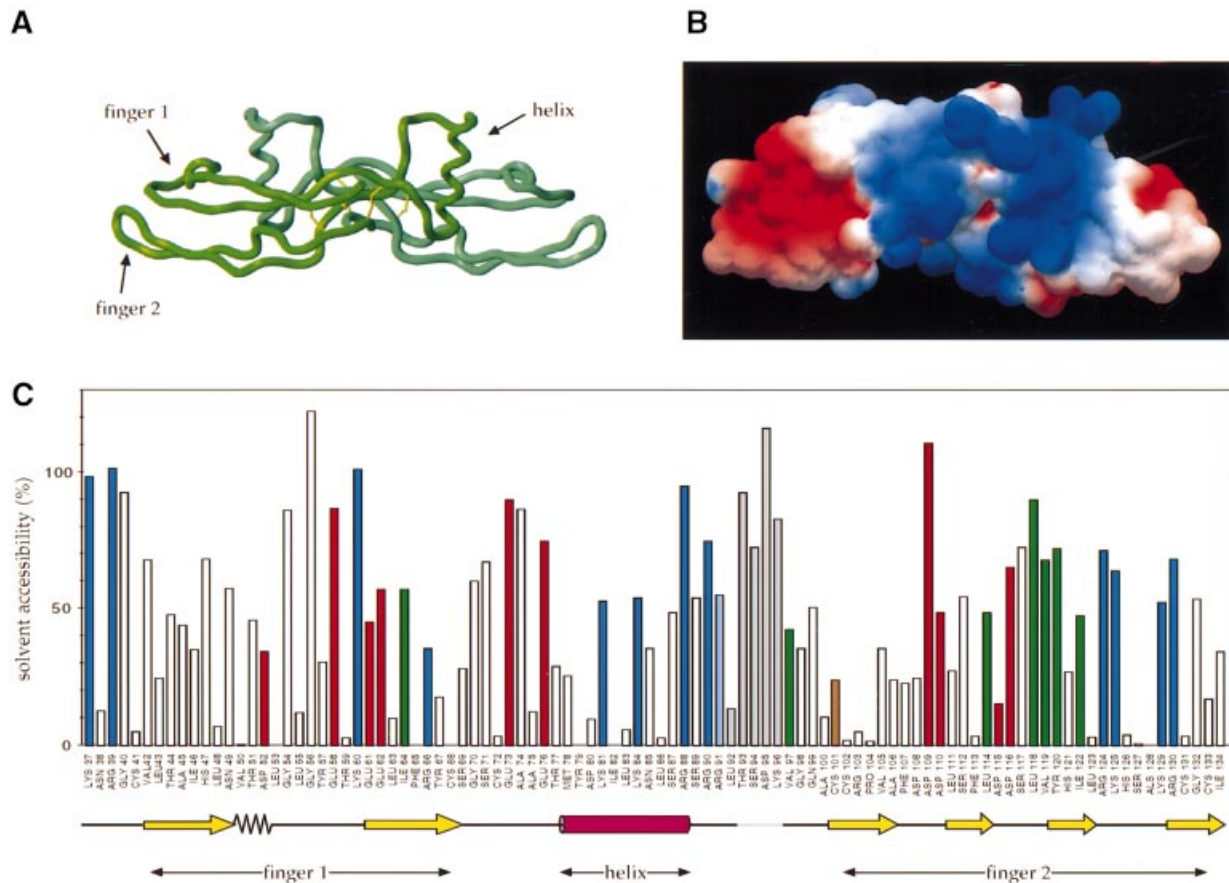
The three-dimensional structure of the GDNF monomer is characterized by two long fingers formed by pairs of anti-parallel  $\beta$ -strands connected by loops, and a helical region at the opposite end (Eigenbrot and Gerber, 1997). Both protomers associate in a tail-to-head orientation to form an elongated, cigar-shaped dimer with the two helices flanking a cysteine-knot motif at the centre of the structure (Figure 1A). The crystal structure of GDNF reflects its structural similarities to members of the TGF- $\beta$  superfamily, originally predicted from the conserved pattern of cysteine residues in the primary sequences of these two factors (Lin *et al.*, 1993). Negatively, positively and uncharged regions are well segregated in the GDNF dimer (Figure 1B). A continuous belt of net positive charge forms across the middle of the dimer, including Lys81, Lys84, Arg88, Arg90 and Arg91 from the exposed surface of the  $\alpha$ -helix, Lys37 and Arg39 from the N-terminal region of the first finger, and Lys129 and Arg130 from the second protomer (Figure 1B). Negatively charged residues, including Asp52, Glu58, Glu61 and Glu62 from the first finger and Asp109, Asp110, Asp115 and Asp116 from the second finger, cluster at the end of the elongated GDNF protomer forming a patch of negative electrostatic potential (Figure 1B). A symmetric patch is formed at the opposite end by the corresponding residues from the

second protomer. A plot of the solvent accessibility of the different amino acid residues in GDNF reveals several highly exposed hydrophobic residues including a prominent patch in the tip of the second finger formed by Leu114, Leu116, Leu118, Val119, Tyr120 and Ile122 (Figure 1C). The first 36 residues in the N-terminus, as well as four residues in a loop connecting the  $\alpha$ -helix with the second finger, could not be resolved in the crystal structure and probably represent highly flexible regions in the molecule.

### **Site-directed mutagenesis of solvent-accessible residues in GDNF**

We targeted different features of the GDNF molecular surface using site-directed mutagenesis. Highly exposed, positively charged (blue bars, Figure 1C), negatively charged (red bars, Figure 1C) and hydrophobic (green bars, Figure 1C) residues were mutated into alanine, either individually or in combinations of two to four residues. Alanine is best suited to the scanning approach because it can accommodate most elements of the secondary structure of proteins, so it conveniently combines small size with minimal structural distortion. Here, these mutants will be referred to by the wild-type residue(s) as single-letter code, followed by their position in the primary sequence of mature rat GDNF, followed by the replacing residue(s), in most cases A for alanine. The cysteine residue at position 101 involved in the disulfide bridge that connects the two protomers was also changed into Ala. The six residues in the flexible loop connecting the  $\alpha$ -helix with the second finger (RLTSDK, grey bars, Figure 1C) were replaced by topologically equivalent residues from TGF- $\beta$ 2 (TINPEA). Finally, we also generated a deletion of the N-terminal extension of GDNF, which, interestingly, is a unique feature of this molecule and is not present in the other members of the GDNF family.

Mutant GDNF proteins were produced in the conditioned medium of transiently transfected COS cells and quantified by Western blotting using different specific antipeptide antibodies and purified recombinant GDNF as standard. Two mutant proteins were purified from COS cell-conditioned medium for further analyses as indicated below; all other mutants were assayed directly from concentrated conditioned medium. Medium from mock-transfected cells had negligible effects on either binding or c-Ret phosphorylation. Most of the mutants were produced at levels comparable with wild-type, indicating that they undergo folding without major problems. The main exceptions were the triple mutant DDD108AAA, the double mutant DD115AA and the D115A mutation, which could not be detected in supernatants of transfected COS cells. Individual mutations of D109, D110 and D116 were, however, well tolerated. Binding to the GFR $\alpha$ 1 receptor was assessed by the ability of the mutants to displace radiolabelled GDNF from GFR $\alpha$ 1-binding sites in a MG87 fibroblast cell line stably transfected with a GFR $\alpha$ 1 cDNA (herein called M23 cells). M23 cells do not express detectable levels of c-Ret or any GFR $\alpha$  receptor other than GFR $\alpha$ 1. Receptor binding was quantified by direct measurement of radiolabelled GDNF bound to cells or by subsequent cross-linking, SDS–PAGE and phosphorimaging quantification of affinity-labelled receptor bands. The latter technique gave a relatively low non-



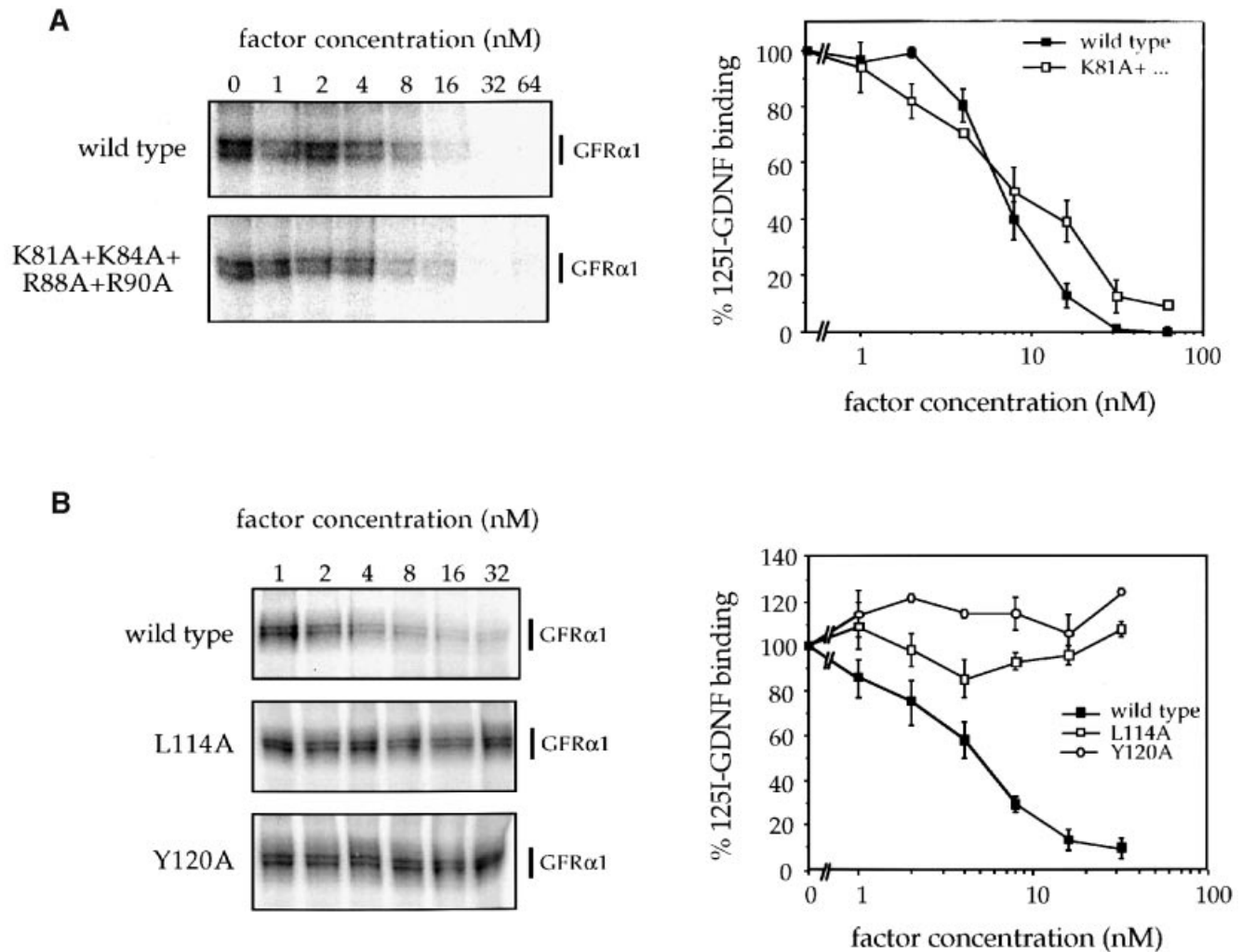
**Fig. 1.** Features of the GDNF molecular surface. (A) Alpha-carbon chain representation of the GDNF dimer viewed perpendicular to a vertical 2-fold symmetry axis. Several features of the secondary structure are indicated. The two protomers are coloured bright green and pale green, respectively. Disulfide bridges forming the ‘cysteine knot’ are in yellow. (B) Electrostatic potential on the GDNF surface. Same view as in (A). Negative potential is shown in red, positive in blue and neutral in white. This image was generated using the program GRASP (Nicholls *et al.*, 1991). (C) Solvent accessibility plot of GDNF residues visible in the X-ray crystal structure (Eigenbrot and Gerber, 1997), calculated with NACCESS (Hubbard and Thornton, 1993). This program calculates the atomic accessible surface defined by rolling a probe of 1.40 Å around a van der Waals surface. Coloured bars (blue, basic; red, acidic; green, hydrophobic; brown, cysteine) indicate residues targeted by site-directed mutagenesis. In one of the mutant molecules, hatched residues at positions 91–96 (RLTSDK) were replaced by the corresponding residues in TGF- $\beta$ 2 (TINPEA). Elements of the secondary structure are indicated below. Arrows,  $\beta$ -strands; spring, turn; cylinder,  $\alpha$ -helix.

specific background signal (<5%), at the same time allowing us to distinguish the contribution of either the GFR $\alpha$ 1 or c-Ret receptor subunits to GDNF binding (see below), and this was the method used in most assays. In all experiments, wild-type GDNF produced and quantified under the same conditions was used as an internal standard, and all binding data are expressed as percentage relative to wild-type. Since the concentration of radiolabelled GDNF used as a tracer was close to the  $K_d$  of binding (i.e.  $4 \times 10^{-10}$  M), the values reported are good estimates of the relative binding affinity of the mutant molecules to the GFR $\alpha$ 1 receptor. Figure 2 shows representative examples of displacement binding assays analysed by cross-linking, all the results are summarized in Table I.

Although positively charged residues constitute a prominent feature of the GDNF molecular surface (Figure 1B), they are dispensable for GDNF binding to the GFR $\alpha$ 1 receptor (Table I). In particular, although the quadruple mutation K81A + K84A + R88A + R90A removes almost all the positive charges from the centre of the dimer, this molecule has almost equal binding affinity to wild-type GDNF (Figure 2A and Table I). Several negatively charged residues, however, are critical for GFR $\alpha$ 1

binding, including D52, E61 and E62 in finger 1, and D116 in finger 2 (Table I). These residues contribute most of the negative electrostatic potential located at the two symmetric ends of the elongated GDNF dimer (Figure 1B), suggesting that the distal ends of the two fingers in GDNF are points of contact with GFR $\alpha$ 1 receptors. This notion is strengthened by our analyses of exposed hydrophobic residues in this region. Four hydrophobic residues appear to be crucial for GDNF binding to GFR $\alpha$ 1, all are located at the distal ends of fingers 1 and 2, including I64 in finger 1, and L114, Y120 and I122 in finger 2 (Figure 2B and Table I). Although less important, mutation of L118 also had an effect on GFR $\alpha$ 1 binding (Table I). Together, these data indicate that GFR $\alpha$ 1 binds to GDNF by contacting exposed negatively charged and hydrophobic residues in the distal ends of fingers 1 and 2.

Replacement of six residues in the central loop connecting the  $\alpha$ -helix with the second finger by equivalent residues from TGF- $\beta$ 2 did not have any major effect on GDNF binding to GFR $\alpha$ 1 (Table I). This is in agreement with the GFR $\alpha$ 1-binding sites being localized at the two ends of the GDNF dimer. In contrast, mutation of C101, involved in an interprotomer disulfide bridge, or deletion



**Fig. 2.** GFR $\alpha$ 1-binding activities of GDNF mutants analysed by cross-linking. Autoradiograms showing affinity-labelled GFR $\alpha$ 1 receptors after cross-linking of iodinated GDNF to M23 cells in the presence of increasing concentrations of unlabelled competitors (left). Displacement binding curves obtained from phosphorimaging quantification are shown on the right. All experiments were performed in duplicate and repeated two to four times with identical results. Values are expressed as mean  $\pm$  SEM. (A) Positive charges in the  $\alpha$ -helix of GDNF are not required for binding to GFR $\alpha$ 1. (B) Hydrophobic residues in finger 2 are critical for GDNF binding to GFR $\alpha$ 1.

of the N-terminal extension of GDNF, reduced binding to GFR $\alpha$ 1 by  $\sim$ 20-fold (Table I).

#### Discrepancy between GFR $\alpha$ 1 binding and c-Ret phosphorylation

The ability of wild-type and mutant GDNF molecules to recruit the c-Ret receptor tyrosine kinase to the receptor complex was determined by assessing c-Ret tyrosine phosphorylation in fibroblast cells co-expressing GFR $\alpha$ 1 and c-Ret (M23–Ret). Figure 3 shows representative examples of c-Ret phosphorylation assays; all the results are summarized in Table I. Because GDNF cannot bind or activate c-Ret in the absence of GFR $\alpha$  receptors, we expected a good correlation between the ability of GDNF mutants to bind GFR $\alpha$ 1 and their ability to induce c-Ret tyrosine phosphorylation. In agreement with this, mutation of the acidic residues in finger 1, which disrupted binding to GFR $\alpha$ 1, i.e. D52A and E61A, as well as mutation of the dimerizing Cys101, resulted in a pronounced reduction ( $>$ 10- and 4-fold, respectively) in ligand-stimulated c-Ret phosphorylation (Figure 3A and Table I). Surprisingly, however, none of the residues located in finger 2 that are

critical for GFR $\alpha$ 1 binding appears to be necessary for stimulation of c-Ret phosphorylation (Figure 3B and Table I). These include negatively charged residues, such as D116, as well as hydrophobic residues, such as L114 and Y120 (Figure 3B), whose mutation into Ala significantly reduced binding to GFR $\alpha$ 1. Dose–response analyses of c-Ret tyrosine phosphorylation induced by different GDNF molecules indicated a fully normal response of L114A, D116A and Y120A mutants at a broad range of concentrations (Figure 3B). The same was true for the GDNF mutant with a deletion in the N-terminus, which stimulated c-Ret phosphorylation at wild-type levels, despite having 20-fold lower affinity for GFR $\alpha$ 1 (Table I). However, we did not find any GDNF mutant that was capable of binding GFR $\alpha$ 1 but unable to induce c-Ret phosphorylation (Table I).

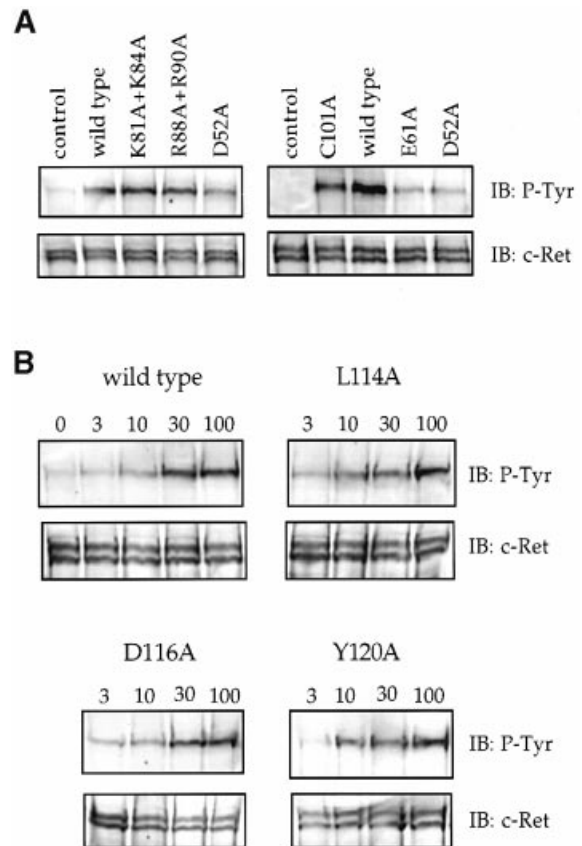
The ability of some of the GDNF mutants deficient in GFR $\alpha$ 1 binding to stimulate c-Ret phosphorylation at normal levels indicated that they are capable of interacting with c-Ret or with a protein complex containing this receptor. We, therefore, tested the ability of several of these mutants to displace radiolabelled GDNF from

**Table 1.** GFR $\alpha$ 1 binding and c-Ret tyrosine phosphorylation activities of GDNF mutants

GDNF variant	GFR $\alpha$ 1 binding (% wild-type)	c-Ret phosphorylation (% wild-type)
Wild-type	100	100
<b>Basic</b>		
K37A + R39A	77	$\geq 50$
R66A	25	$\geq 50$
K81A + K84A	83	$\geq 50$
R88A + R90A	50	$\geq 50$
K81A + K84A + R88A + R90A	80	$\geq 50$
R91A	77	$\geq 50$
R124A + K125A	56	$\geq 50$
R124A + K125A + H126A	45	$\geq 50$
K129A + R130A	45	$\geq 50$
<b>Acidic</b>		
<u>D52A</u>	<u>6</u>	$\leq 10$
E58A	60	$\geq 50$
E58A + K60A	36	$\geq 50$
<u>E61A</u>	<u>2</u>	$\leq 10$
<u>E62A</u>	<u>15</u>	$\geq 50$
<u>E61A + E62A</u>	<u>0</u>	$\leq 10$
E73A	39	$\geq 50$
E76A	29	$\geq 50$
D109A	100	$\geq 50$
D110A	50	$\geq 50$
<u>D116A</u>	<u>0</u>	$\geq 50$
<b>Hydrophobic</b>		
<u>I64A</u>	<u>10</u>	$\geq 50$
V97A	60	$\geq 50$
<u>L114A</u>	<u>0</u>	$\geq 50$
<u>L118A</u>	<u>21</u>	$\geq 50$
V119A	74	$\geq 50$
<u>Y120A</u>	<u>0</u>	$\geq 50$
<u>I122A</u>	<u>11</u>	$\geq 50$
<b>Other</b>		
<u>C101A</u>	<u>5</u>	<u>25</u>
<u><math>\Delta</math>N</u>	<u>6</u>	$\geq 50$
TGF- $\beta$ 2 loop	50	25

Underlining highlights major effects of the mutations. Binding is expressed as percentage of wild-type using the equation:  $100 \times (\text{mutant IC}_{50} / \text{wild-type IC}_{50})$ . The numbers shown are averages of at least three independent experiments each from duplicate or quadruplicate wells. All phosphorylation assays were performed in dose–response similar to those shown in Figure 3B and quantified using ImageQuant software (Materials and methods). The results shown are representative of two or three independent experiments.

M23–Ret cells co-expressing GFR $\alpha$ 1 and c-Ret receptors. These experiments indicated that L114A, D116A and Y120A mutants are still unable to displace iodinated GDNF from GFR $\alpha$ 1-binding sites on M23–Ret cells (Figure 4A and B; data not shown). However, the three mutants are capable of displacing all radiolabelled GDNF from c-Ret with a dose–response profile comparable with wild-type (Figure 4A and C; data not shown). These data indicate that although they show diminished binding to GFR $\alpha$ 1, these GDNF mutants still retain almost wild-type affinity for c-Ret, which is in agreement with their ability to stimulate c-Ret phosphorylation at normal levels. Together, our results show that acidic and hydrophobic residues in fingers 1 and 2 of GDNF are required for binding to the GFR $\alpha$ 1 receptor, whereas only residues in finger 1 appear to be necessary for binding to and activation of c-Ret.

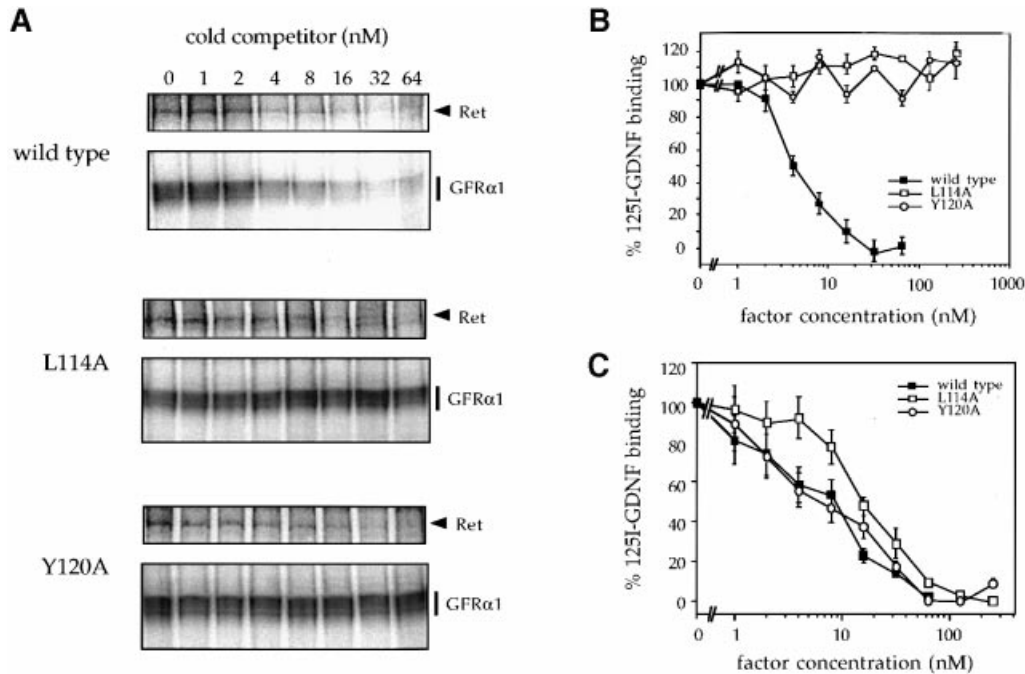


**Fig. 3.** Stimulation of c-Ret tyrosine phosphorylation by GDNF mutants. (A) After stimulation of M23–Ret cells with the indicated ligands at 30 ng/ml ( $\sim 1.2$  nM), c-Ret was immunoprecipitated and filter blots probed with anti-phosphotyrosine antibodies (upper) and re-probed with anti-c-Ret antibodies (lower). The lower band in c-Ret blots corresponds to a cytoplasmic glycosylation intermediate of this receptor. (B) Same procedure as (A) using different concentrations of the indicated ligands in ng/ml. Note that D116A, L114A and Y120A are all able to stimulate c-Ret phosphorylation at normal levels despite their inability to bind to GFR $\alpha$ 1 efficiently. All phosphorylation assays were repeated at least three times with identical results.

#### GDNF mutants deficient in GFR $\alpha$ 1 binding still require GFR $\alpha$ 1 for c-Ret activation

Are these GDNF mutants able to interact with c-Ret directly, without the aid of GFR $\alpha$ 1 receptors? To address this point, we purified the Y120A mutant from the conditioned medium of transiently transfected COS cells and tested its ability to stimulate c-Ret phosphorylation in M23–Ret cells that had previously been treated with phosphatidylinositol phospholipase C (PIPLC), which removes all GPI-anchored proteins from the cell membrane, including GFR $\alpha$  receptors. Figure 5A shows that pretreatment of M23–Ret cells with PIPLC abolishes the ability of both wild-type and Y120A mutant GDNF to stimulate c-Ret phosphorylation. Thus, although the Y120A mutant cannot bind to GFR $\alpha$ 1 directly, it still requires GFR $\alpha$ 1 receptors on the cell membrane to stimulate c-Ret phosphorylation in M23–Ret cells.

Finally, we investigated the activities of wild-type and Y120A mutant in cells expressing only c-Ret (MG87–Ret). The absence of GFR $\alpha$  receptors in these cells results in a slight increase of basal ligand-independent c-Ret phosphorylation compared with M23–Ret cells, as reported previously (Trupp *et al.*, 1998). In agreement with the



**Fig. 4.** Displacement binding assays in M23-Ret cells co-expressing GFR $\alpha$ 1 and c-Ret receptors. (A) Autoradiograms of cross-linking binding assays using increasing amounts of unlabelled wild-type and mutant GDNF competitors. The affinity labelled bands corresponding to GFR $\alpha$ 1 and c-Ret come from the same gel, but different exposures are shown for better detection of the c-Ret band. Note that the mutants displace iodinated GDNF from c-Ret, but not from GFR $\alpha$ 1, to the same extent as wild-type GDNF. (B) Displacement binding curves obtained from phosphorimaging quantification of affinity-labelled GFR $\alpha$ 1. Note that the mutants do not significantly displace iodinated GDNF from this receptor. (C) Displacement binding curves obtained from phosphorimaging quantification of affinity-labelled c-Ret. Note that the curve for the mutants, Y120A in particular, superimposes quite well onto that of wild-type GDNF. All experiments were performed in duplicate. Values are expressed as mean  $\pm$  SEM.

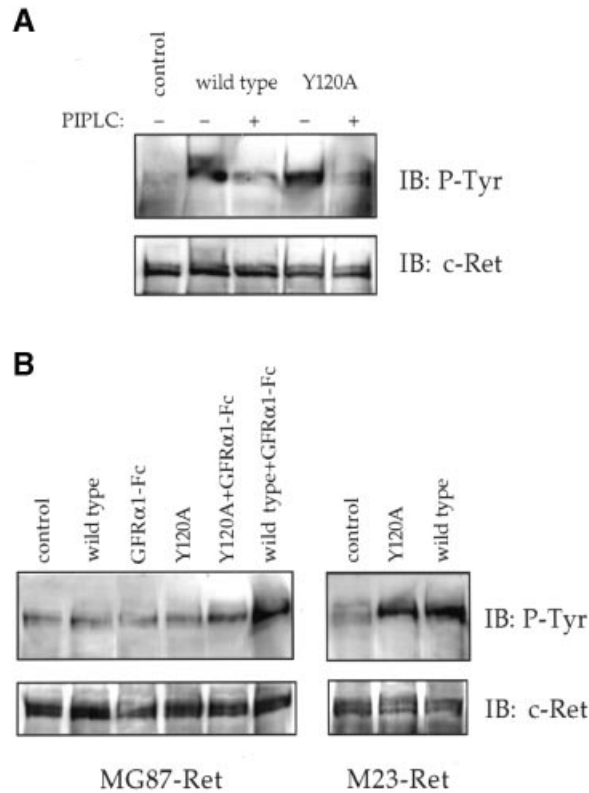
PIPLC experiment, neither wild-type GDNF nor the Y120A mutant are able to stimulate c-Ret phosphorylation above background levels in these cells (Figure 5B), corroborating the requirement of GFR $\alpha$ 1 for c-Ret activation by the Y120A mutant. This result also demonstrates that the stimulation of c-Ret phosphorylation elicited by the Y120A mutant in M23-Ret cells could not have been mediated through interaction with low amounts of GFR $\alpha$  receptors endogenously expressed by the parental MG87 line, if such receptors exist.

As reported previously by others (Jing *et al.*, 1996; Treanor *et al.*, 1996; Yu *et al.*, 1998), soluble GFR $\alpha$ 1 provided *in trans* reinstates the ability of wild-type GDNF to stimulate c-Ret phosphorylation in cells lacking endogenous GFR $\alpha$ 1 receptors (Figure 5B, left). The Y120A mutant is, however, much less efficient at stimulating c-Ret phosphorylation using soluble GFR $\alpha$ 1, only a small increase over background levels could be seen (Figure 5B, left). In a parallel experiment performed in M23-Ret cells, however, the Y120A mutant was as efficacious as wild-type GDNF at stimulating c-Ret phosphorylation (Figure 5B, right). Thus, the impaired ability of the Y120A mutant to bind to GFR $\alpha$ 1 prevents it from utilizing soluble GFR $\alpha$ 1 receptors to stimulate c-Ret activation in c-Ret-only cells, suggesting that, in contrast to membrane-bound receptors, soluble GFR $\alpha$ 1 molecules first form a complex with GDNF, and this complex subsequently binds to and activates membrane-bound c-Ret.

## Discussion

In this study we identified the structural elements in the molecular surface of GDNF responsible for its interaction

with the GFR $\alpha$ 1 receptor. Several features of this surface were investigated using site-directed mutagenesis, including exposed positively and negatively charged residues, exposed hydrophobic residues, a 36-residue N-terminal extension and a loop region in the middle of the molecule. This analysis revealed a set of eight residues, four negatively charged (Asp52, Glu61, Glu62, Asp116) and four hydrophobic (Ile64, Leu114, Tyr120, Ile122), that form a hot spot for GDNF binding to the GFR $\alpha$ 1 receptor (Figure 6A and B). Individual mutation of any of these residues into Ala had a major effect on the binding affinity of GDNF to GFR $\alpha$ 1, indicating that each of them makes an important contribution to the binding energy of the GDNF-GFR $\alpha$ 1 complex. Similar to other ligand-receptor complexes, many more residues are probably buried in the GDNF-GFR $\alpha$ 1 binding interface. These may include neighbouring residues whose individual mutation into Ala showed a smaller effect on binding, such as Glu58, Lys60 and Leu118, and residues contributing low binding energy, which is revealed only in the context of other mutations. Together, these residues define a surface for binding to GFR $\alpha$ 1 localized at the distal end of the elongated GDNF molecule (Figure 6C). Owing to the 2-fold symmetry of the GDNF dimer, identical binding surfaces are formed on both sides of the molecule, each composed of structural elements from a single protomer (Figure 6A and B). Indirect evidence supports a 1:2 stoichiometry for the GDNF-GFR $\alpha$ 1 complex (Jing *et al.*, 1996), so it is easy to envision how two molecules of GFR $\alpha$ 1 may each associate with the GDNF dimer through these two symmetrically related sites. We, therefore, propose that GDNF binds with its 2-fold symmetry axis perpendicular to the cell membrane (orientation shown in Figure 6A) to a



**Fig. 5.** GDNF mutants deficient in GFR $\alpha$ 1 binding still require GFR $\alpha$ 1 for c-Ret activation. (A) M23-Ret cells were treated with PIPLC prior to stimulation with the indicated ligands at 30 ng/ml. Cell lysates were processed for detection of c-Ret tyrosine phosphorylation as indicated above. (B) MG87-Ret cells (expressing only c-Ret) or M23-Ret cells (expressing GFR $\alpha$ 1 and c-Ret) were stimulated with the indicated purified ligands at 100 ng/ml. Where indicated, purified soluble GFR $\alpha$ 1-Fc fusion protein (R&D Systems) was also added at 300 ng/ml, corresponding to ~1:1 GDNF:GFR $\alpha$ 1-Fc molar ratio. These experiments were repeated two or three times with identical results.

GFR $\alpha$ 1 dimer that clamps the two GDNF protomers through symmetrically related surfaces located in the distal part of the molecule (Figure 6A and B).

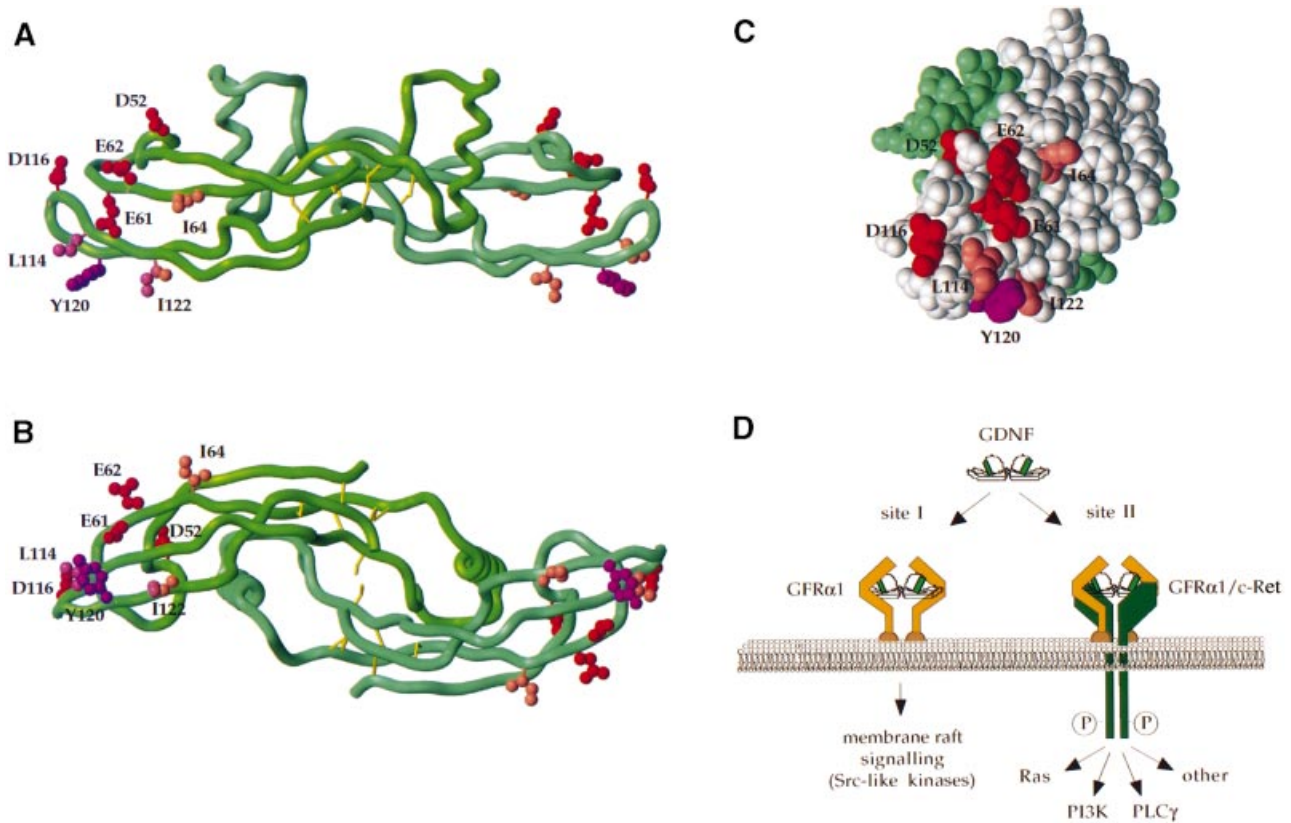
Deletion of the N-terminal extension of GDNF also affected binding to GFR $\alpha$ 1, indicating that some of the residues in this domain contribute to the GDNF–GFR $\alpha$ 1 interaction. In contrast, the effect of the C101A mutation on both GFR $\alpha$ 1 binding and c-Ret activation possibly involves conformational changes and/or partial destabilization of the GDNF dimer. Interestingly, a TGF- $\beta$ 1 mutant with the corresponding dimerizing Cys replaced by Ser also showed reduced, but still detectable, biological activity (Amatayakul-Chantler *et al.*, 1994). Several of the positions identified at the site of GDNF binding to the GFR $\alpha$ 1 receptor correspond to variable residues in other members of the GDNF family, including Glu62, Asp116, Leu118 and Ile122 (Table II), suggesting that these, as well as other neighbouring variable residues, could represent determinants of receptor binding specificity in this group of molecules.

Unexpectedly, binding to GFR $\alpha$ 1 and activation of the c-Ret receptor tyrosine kinase could be dissociated in several of the GDNF mutants generated. The Y120A mutant, for example, is not able to utilize GFR $\alpha$ 1 or c-Ret if these are expressed independently, but displaces the

binding of iodinated GDNF to c-Ret and stimulates normal c-Ret activation in cells that co-express the two receptors. This finding has at least two implications for the mode of action of GDNF and the way in which active GDNF receptor complexes are assembled.

In the first place, c-Ret possibly plays a much more important role in ligand binding than thought previously. This notion is also supported by cross-linking experiments indicating a direct association between c-Ret and GDNF, and by the functional promiscuity displayed by GDNF family ligands in the presence of c-Ret. Thus, for example, although GDNF does not normally bind to GFR $\alpha$ 3, it can be cross-linked to this receptor in cells co-expressing c-Ret (Trupp *et al.*, 1998). Moreover, the GDNF homologue artemin (ART) was recently found to be capable of stimulating c-Ret-dependent signalling in cells co-expressing GFR $\alpha$ 1 and c-Ret, despite being unable to bind to isolated GFR $\alpha$ 1 (Baloh *et al.*, 1998). Although no dose–response analysis was made in that study, the behaviour of ART is not unlike that of the Y120A mutant, which can activate the c-Ret receptor normally despite its inability to displace GDNF binding from GFR $\alpha$ 1 in GFR $\alpha$ 1-only cells. The binding energy of the c-Ret–GDNF interaction is clearly not sufficient for c-Ret to bind ligand on its own, so it is likely that each component in the complex is interacting with all other subunits through multiple contacts.

The second implication of our findings relates to the nature of the GDNF receptors present in cells co-expressing GFR $\alpha$ 1 and c-Ret. The fact that the Y120A mutant can bind and activate c-Ret as efficiently as wild-type GDNF, but still necessitates the presence of the GFR $\alpha$ 1 receptor in the cell membrane, suggests that this mutant interacts with a binding site formed by a pre-associated GFR $\alpha$ 1–c-Ret complex. There is evidence in the literature supporting the capacity of GFR $\alpha$  and c-Ret receptors to associate with each other in the absence of ligand. Using co-immunoprecipitation experiments, Treanor *et al.* (1996) and Klein *et al.* (1997) have shown that GFR $\alpha$ 1 and GFR $\alpha$ 2 can associate with c-Ret in the absence of ligand. The amount of this complex that is recovered could be increased significantly by the addition of GDNF or neurturin (NTN), respectively, suggesting that the ligand stabilizes the association of GFR $\alpha$  receptors with c-Ret. In another study, Sanicola *et al.* (1997) independently isolated GFR $\alpha$ 1 by utilizing an expression cloning strategy in which the probe was a soluble c-Ret–Ig fusion protein. A fixation step was found to be necessary for detection of GFR $\alpha$ 1 by the c-Ret fusion, indicating that the interaction between the two molecules is of relatively low affinity (Sanicola *et al.*, 1997). Finally, we have shown previously that co-expression of GFR $\alpha$ 1 and c-Ret in COS cells diminishes constitutive c-Ret phosphorylation in a dose-dependent manner (Trupp *et al.*, 1998), suggesting a ligand-independent interaction between GFR $\alpha$ 1 and c-Ret in the cell membrane. Based on this evidence and our present results, we propose the existence of at least two distinct binding sites for GDNF in cells co-expressing GFR $\alpha$ 1 and c-Ret receptors (Figure 6D). The first site is formed exclusively by GFR $\alpha$ 1 subunits. GDNF binding to this site requires acidic and hydrophobic residues in fingers 1 and 2, as well as residues in the N-terminus of GDNF. Several of the mutants



**Fig. 6.** The GFR $\alpha$ 1-binding site in GDNF. **(A)** Alpha carbon representation of the GDNF dimer in the same orientation as in Figure 1A, i.e. perpendicular to a vertical 2-fold symmetry axis, with critical residues for GFR $\alpha$ 1 binding labelled and highlighted in colour. Red, Glu and Asp; magenta, Tyr; light brown, Ile and Leu. **(B)** View rotated 90° from (A) to look along the 2-fold axis of the dimer. Note that most residues in the GFR $\alpha$ 1-binding site are exposed in this view. **(C)** Van der Waals surface model of GDNF viewed from one of its ends, with residues in the GFR $\alpha$ 1-binding site highlighted in colour. The two GDNF protomers are in white and green, respectively. **(D)** Two distinct binding sites for GDNF. Site I consists exclusively of GFR $\alpha$ 1 receptors. GDNF binding to this site requires negatively charged and hydrophobic residues in fingers 1 and 2 and in the N-terminus. Signalling downstream of this complex appears to include activation of Src-like kinases in membrane rafts (Trupp *et al.*, 1999). Site II consists of a pre-associated GFR $\alpha$ 1–c-Ret complex. The actual stoichiometry of this complex, i.e. heterodimer versus heterotetramer, is unknown. GDNF binding to this site requires negatively charged residues in finger 1, but not in finger 2 or in the N-terminus. Signalling downstream of this complex includes activation of the Ras, PI3K, PLC $\gamma$  and other pathways (Trupp *et al.*, 1999).

**Table II.** Variability and conservation of amino acid residues in the GFR $\alpha$ 1 binding site among members of the GDNF ligand family: rat GDNF, mouse NTN, rat PSP and mouse ART

GDNF	NTN	PSP	ART
<u>Asp52</u>	<u>Glu</u>	<u>Glu</u>	Ala
<u>Glu61</u>	<u>Glu</u>	<u>Glu</u>	<u>Glu</u>
Glu62	Thr	Lys	Leu
<u>Ile64</u>	<u>Leu</u>	<u>Ile</u>	Arg
<u>Leu114</u>	<u>Leu</u>	<u>Leu</u>	Met
Asp116	Val	Asp	Val
Leu118	Ser	His	Ser
<u>Tyr120</u>	<u>Tyr</u>	<u>Trp</u>	<u>Trp</u>
<u>Ile122</u>	<u>Thr</u>	<u>Gln</u>	<u>Thr</u>

Conserved residues are indicated by underlining.

generated in this study, such as Y120A, cannot bind to this site efficiently. The second site consists of a pre-associated GFR $\alpha$ 1–c-Ret complex. GDNF binding to this site requires acidic residues in finger 1, but not finger 2, of GDNF. Mutations in finger 2 residues do not affect the interaction of GDNF with this second site and allow c-Ret tyrosine phosphorylation at normal levels. The apparent inability of the Y120A mutant to displace iodinated GDNF from GFR $\alpha$ 1 in M23–Ret cells indicates that only a

relatively small fraction of GFR $\alpha$ 1 receptors are pre-associated with c-Ret in these cells.

This model could also explain the reported cases of promiscuity in the interaction of GDNF family members with GFR $\alpha$  receptors. Thus, for example, NTN promotes the survival of dopaminergic neurons, which express GFR $\alpha$ 1 but not the cognate NTN receptor GFR $\alpha$ 2 (Horger *et al.*, 1998), and is also able to induce c-Ret phosphorylation in fibroblasts expressing GFR $\alpha$ 1 (Baloh *et al.*, 1997). In contrast, survival of submandibular ganglion neurons by NTN is unaffected by elimination of GFR $\alpha$ 1 (Horger *et al.*, 1998), as this activity of NTN is known to be mediated by GFR $\alpha$ 2 (Rossi *et al.*, 1999). In addition, ART has been shown to elicit transcriptional responses in transfected cells expressing c-Ret and GFR $\alpha$ 1 in the absence of GFR $\alpha$ 3, its preferred receptor (Baloh *et al.*, 1998). Whereas neither NTN nor ART are able to bind to GFR $\alpha$ 1 with high affinity, it is possible that, like some of the GDNF mutants described here, these factors are capable of interacting with a pre-formed GFR $\alpha$ 1–c-Ret complex.

Current models of c-Ret activation propose that GDNF first binds to GFR $\alpha$ 1 and that c-Ret is subsequently recruited to the GDNF–GFR $\alpha$ 1 complex (Jing *et al.*, 1996; Treanor *et al.*, 1996). The fact that several GDNF mutants



deficient in GFR $\alpha$ 1 binding are still able to activate c-Ret normally suggests that the current model cannot be the predominant mechanism for c-Ret activation, and that the majority of c-Ret signalling sites may consist of a pre-associated GFR $\alpha$ 1–c-Ret complex. Interestingly, our data indicate that the Y120A mutant cannot utilize soluble GFR $\alpha$ 1 for activation of c-Ret as efficiently as wild-type GDNF. Whereas soluble GFR $\alpha$ 1 has access to a three-dimensional space, membrane-anchored GFR $\alpha$ 1 is restricted to the plane of the cell membrane. A higher concentration of soluble GFR $\alpha$ 1 receptors might therefore be required to allow c-Ret activation by the Y120A mutant.

Finally, we would like to draw attention to the possibility that the two GDNF-binding sites mentioned above may, in addition, have different functional capabilities. Whereas signalling by the GFR $\alpha$ 1–c-Ret complex utilizes the well-known Ras, phosphatidylinositol-3 kinase (PI3K), phospholipase C $\gamma$  (PLC $\gamma$ ) and probably other pathways characteristic of receptor tyrosine kinases, we have recently demonstrated the existence of an alternative mechanism of GDNF signalling mediated by GFR $\alpha$ 1 receptors acting in a cell-autonomous manner independently of c-Ret (Trupp *et al.*, 1999). This pathway involves the association of GFR $\alpha$ 1 with members of the Src family of cytoplasmic tyrosine kinases, Src-like kinase activation and phosphorylation of downstream substrates (Trupp *et al.*, 1999). A similar signalling mechanism has also been described for other GPI-anchored receptors and, like many of those receptors, GFR $\alpha$ 1 is present in detergent-insoluble membrane rafts that are also rich in Src-like kinases (C.F. Ibáñez, unpublished observations). Whether GFR $\alpha$ 1 is able to signal on its own or in association with other, as yet unknown, transmembrane proteins is still unclear.

In conclusion, we have identified structural elements mediating the interaction of GDNF with the GFR $\alpha$ 1 receptor and have generated GDNF mutants that have a reduced capacity to bind to this receptor, but retain the ability to induce normal c-Ret phosphorylation. Because these mutants still require GFR $\alpha$ 1 to activate c-Ret, we propose the existence of two distinct binding sites for GDNF, one formed by GFR $\alpha$ 1 alone and another formed by a pre-associated GFR $\alpha$ 1–c-Ret complex. These two sites may have different signalling capabilities which can now be dissected with the help of the GDNF mutants described in this study.

## Materials and methods

### Cells, antibodies and site-directed mutagenesis

MG87 fibroblasts are derived from mouse NIH 3T3 cells. Introduction of rat GFR $\alpha$ 1 receptors by stable transfection resulted in the M23 cell line. Introduction of human c-Ret (long isoform) receptors into M23 cells by stable transfection resulted in the M23–Ret cell line. M23 and M23–Ret cells express comparable levels of GFR $\alpha$ 1 receptors. Introduction of human c-Ret (long isoform) receptors into MG87 cells by retroviral infection resulted in the MG87–Ret cell line. MG87–Ret and M23–Ret cells express comparable levels of c-Ret receptors. Anti-phosphotyrosine monoclonal antibodies and anti-human c-Ret antibodies were obtained from Santa Cruz. Anti-GDNF antipeptide antibodies were either generated in our laboratory as described previously (Trupp *et al.*, 1995) or obtained from Santa Cruz. A cDNA fragment containing the full-length sequence of rat GDNF was subcloned into pCDNA3 (Invitrogen). Single-stranded DNA from this plasmid was used as a template for oligonucleotide-based site-directed mutagenesis as described previously (Kunkel, 1985). All mutations were confirmed by DNA sequence analysis.

### Protein production, purification, quantification and iodination

Rat GDNF used for iodination was produced and purified from baculovirus-infected Sf21 insect cells as described previously (Trupp *et al.*, 1995). Iodination was performed by the chloramine-T method to an average specific activity of  $5 \times 10^7$  c.p.m./ $\mu$ g. Iodinated GDNF was purified by size-exclusion chromatography through a Sephadex G25 column. Mutant GDNFs were produced in the conditioned medium of COS cells transiently transfected using the DEAE–dextran–chloroquine method. One day after transfection, complete medium was changed to serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 5  $\mu$ g/ml each of insulin and transferrin. Three days after this change, conditioned medium was harvested and concentrated 50- to 80-fold by ultrafiltration through Centriprep 10 cartridges (Amicon). The amount of GDNF present in the conditioned medium was quantified by Western blotting against standards of purified recombinant GDNF produced in insect cells (see above) or from commercial sources (PeProtech). Special care was taken to utilize anti-GDNF antisera that were raised against peptides from a region outside the mutations under study. Western blots were developed by enhanced chemifluorescence (ECF, Amersham), analysed in a STORM 840 fluorimager and quantified with ImageQuant software (Molecular Dynamics). Two mutants were purified from COS-cell-conditioned medium, K81A + K84A and Y120A. Five hundred millilitres of COS-cell-conditioned medium, processed as above, were filtered and purified by subsequent steps of ion-exchange, size-exclusion and reverse-phase chromatography using Poros columns in a Biocad Sprint workstation (PerSeptive Biosystems).

### Binding and c-Ret phosphorylation assays

For steady-state competitive binding assays, cells were plated in 96-well plates and exposed to 10 ng/ml  $^{125}$ I-GDNF ( $\sim 4 \times 10^{-10}$  M) in phosphate-buffered saline (PBS) supplemented with 1 mg/ml bovine serum albumin (BSA), 1 mM MgCl $_2$  and 0.5 mM CaCl $_2$ , in the presence or absence of serial dilutions of unlabelled competitors. Binding was allowed to occur with gentle rocking at 4°C for at least 4 h, followed by three washes with ice-cold PBS and the addition of scintillation cocktail. Plates were then read in a MicroBeta gamma counter (Wallac). Background binding was determined using a 200-fold excess of unlabelled GDNF. For chemical cross-linking, the same procedure was followed except that cells were plated in 12-well plates. After 4 h at 4°C, cross-linking was started by the addition of 0.5 mM Bis-(sulfosuccinimidyl) suberate (BS $^3$ ). Cross-linking was allowed to proceed for 30 min at room temperature and stopped by the addition of 50 mM glycine in PBS. Wells were washed three times with PBS and then lysed with NP-40 lysis buffer as described previously (Trupp *et al.*, 1998). After SDS–PAGE, gels were fixed, dried, exposed to phosphorscreens (KODAK/Molecular Dynamics) and analysed in a STORM 840 phosphorimager. Bands were quantified using ImageQuant software (Molecular Dynamics). c-Ret phosphorylation assays were performed as described previously (Trupp *et al.*, 1998, 1999) using ECF and fluorimaging detection as above. c-Ret phosphorylation was quantified using ImageQuant software. Levels of c-Ret phosphorylation were normalized to the total amount of c-Ret in each lane, quantified as above after reprobing of the polyvinylidene fluoride membranes with anti-c-Ret antibodies. For PIPLC treatments, cell monolayers were washed in serum-free medium and then incubated with 1 U/ml PIPLC (Sigma) in DMEM for 60 min at 37°C, followed by phosphorylation assay as described.

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