Tyrosine 981, a Novel Ret Autophosphorylation Site, Binds c-Src to Mediate Neuronal Survival*

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The glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) are neurotrophic factors that influence several aspects of the developing and injured nervous system. GFLs signal through a common receptor tyrosine kinase (Ret) and one of the four ligand-binding co-receptors (GFRα1 to 4). Ligand-induced translocation of Ret to lipid rafts, where it interacts with the nonreceptor tyrosine kinase Src, is a prerequisite for full biological activity of these neurotrophic factors. This interaction and subsequent activation of Src are required for GFL-mediated neuronal survival, neurite outgrowth, or cell proliferation. Here we show by multiple approaches that Ret tyrosine 981 constitutes the major binding site of the Src homology 2 domain of Src and therefore the primary residue responsible for Src activation upon Ret engagement. Other tyrosines such as 1015 and 1029 may contribute to the overall interaction between Ret and Src, as judged by overexpression experiments. By generating a phosphospecific antibody, we demonstrate that tyrosine 981 is a novel autophosphorylation site in Ret. Importantly, we also show that this tyrosine becomes phosphorylated in dissociated sympathetic neurons after ligand stimulation. Mutation of tyrosine 981 to phenylalanine reduces GDNF-mediated survival in a transfected cerebellar granule neuron paradigm.

The GDNF1 family ligands (GFLs) constitute a group of neurotrophic factors (GDNF, neurturin, persephin, and artemin) that regulate several aspects of the developing nervous system such as neuronal survival, differentiation, and precursor proliferation. First identified as survival-promoting factors in vitro, the GFLs support the survival of cultured sympathetic, sensory, motor, enteric, and midbrain dopaminergic neurons (1–5). GFLs signal via a multicomponent receptor system, consisting of a common transmembrane receptor tyrosine kinase (Ret) that does not bind GFLs directly and one of the four glycosylphosphatidylinositol-linked coreceptors (GFRα1 to α4), which provide ligand specificity. Ret is alternatively spliced to produce at least two variants of identical sequence up to residue 1063, followed by a unique C-terminal sequence composed of either 9 or 51 amino acids (Ret9 and Ret51) (reviewed in Ref. 6). Recent genetic experiments have confirmed the importance of GFLs for the development of the nervous system and revealed an unexpected role in kidney morphogenesis. Thus, targeted deletion of Ret (as well as GDNF or GFRα1) results in renal agenesis and lack of enteric neurons distal to the stomach, along with variable cell losses in the parasympathetic, sensory, and motor nervous systems (for reviews, see Refs. 7 and 8). Interestingly, a detailed analysis of knockout mice has expanded the roles of GFLs in vivo from neuronal survival to proliferation, migration, and axonal elongation of neuronal precursors (9, 10).

Upon ligand binding, receptor tyrosine kinases undergo autoprophosphorylation of selected tyrosine residues, which become docking sites for adapter proteins and enzymes that propagate the signal. Typically, these signaling proteins contain modular domains such as Src homology 2 (SH2) or phosphotyrosine-binding domains that only recognize phosphotyrosines surrounded by specific sequences. In the case of Ret, some of these tyrosines as well as their binding partners have been identified and characterized. Tyr1005 is conserved in many receptor tyrosine kinases and lies in the activation loop of the kinase domain. Mutation of this site causes a severe but not complete decrease in Ret activity. This tyrosine serves also as a binding site for Grb10 and Grb7 (11, 12). Phospholipase C-γ and Grb2 bind to Tyr1015 and Tyr1029, respectively (13, 14). Tyr1062 is a binding site for Shc, IRS-1, FRS-2, and Dok-δ if phosphorylated and binds Enigma in a phosphorylation-independent manner (15–22). Besides these residues, other tyrosines such as Tyr1028 have been identified as autoprophosphorylation sites in Ret, but their binding partners are currently unknown (23). The same work also suggested the presence of more potential autoprophosphorylated tyrosines in the receptor, which remain to be mapped.

We and others have shown that one important mediator of GFL bioactivity is the nonreceptor tyrosine kinase, Src. The activity of this enzyme is necessary for full GDNF-induced neuronal survival and differentiation (24) as well as Ret-mediated cell proliferation (25). The interaction between Src and Ret occurs only after the receptor translocates to specialized membrane microdomains known as lipid rafts, in response to ligand stimulation (26). At least part of the interaction is dependent on the direct binding of the SH2 domain of Src to one or more yet unidentified tyrosines on Ret (26).

In the present work, we identify tyrosine 981 as the major Src-binding residue of the receptor. We also show that tyrosines 1015 and 1029 partially contribute to the overall binding of Src to Ret. By generating a phosphospecific antibody to...
this residue, we demonstrate that Tyr981 is a novel Ret auto-
phosphorylation site. Finally, mutation of tyrosine 981 tophe-
nylalanine reduces GDNF-mediated survival in transfected
cerebellar granule neurons in culture.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfection, and Plasmids—Human embryonic kidney
293 cells were grown in Dulbecco’s modified Eagle’s medium (Sigma)
supplemented with 10% fetal bovine serum (Sigma) and 1.4 mM t-
glutamine. NBL-S neuroblastoma cells were grown in Dulbecco’s mod-
ified Eagle’s medium/F-12 medium (Sigma) plus 10% fetal bovine
serum and 1.4 mM t-glutamine. 293 cells were seeded in 6-well dishes (Falcon;
Becton Dickinson, Franklin Lakes, NJ) and co-transfected with Ret
mutants, GFRα1, and Src by the calcium phosphate method. Twenty-
four hours after transfection, cells were serum-starved for 3 h, stimu-
lated with 50 ng/ml GDNF for 10 min, and processed for analysis. Ret
mutants were generated by PCR mutagenesis and confirmed by se-
quencing. The Δ1014 Ret mutant is truncated at amino acid 1014 of
human Ret and has a C-terminal Myc tag. The expression plasmids
coding for Src, GFRα1, Ret9, and Ret51 are described elsewhere (26,
24). The plasmid coding for the SH2 domain of Src fused to GST was a
generous gift of Dr. Christine A. Cartwright (Stanford University) (27).

Immunoprecipitation and GST Pull-down Assays—Cells were rinsed
with PBS and lysed on 1 ml of ice-cold co-immunoprecipitation buffer
(50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA,
24). The plasmid coding for the SH2 domain of Src fused to GST was
co-transfected with 250 ng of Ret and 500 ng of GFRα1 per well in
four-well dishes (Falcon, Naperville, IL). Two days after transfection,
the initial number of EGFP-positive cells in designated fields (usually 150–
200 cells) was counted. Cultures were rinsed three times in Dulbecco’s
modified Eagle’s medium and switched to high potassium plus serum
medium (K25 + S, basal medium Eagle (Invitrogen) plus 10% fetal bo-
vine serum and a final concentration of 25 mM KCl), low potassium
without serum medium (K25-S), or K25-S supplemented with 50 ng/ml
GDNF (R&D Systems, Minneapolis, MN) for 48 h. After this period,
the number of EGFP-positive cells remaining in the same fields was scored
in a blinded manner to obtain the percentage of neuronal survival.

RESULTS

Ret Kinase Activity Is Necessary but Not Sufficient for Src
Binding—We and others have demonstrated that Src can di-
rectly bind to activated Ret in a phosphotyrosine-dependent
manner (24–26). In an attempt to map the Src binding ty-
rosine(s) in Ret, we constructed different receptor mutants and
transiently co-transfected them together with Src in 293 cells.
Simple overexpression of wild type Ret induced ligand-inde-
pendent activation of its kinase activity, as judged by phospho-
tyrosine blotted of RET immunoprecipitates (Fig. 1A). How-
ever, optimal co-immunoprecipitation of Src was not achieved
unless cells were co-transfected with GFRα1 and stimulated
with GDNF, despite the fact that total Ret phosphorylation was
not significantly increased by the presence of ligand. In con-
trast, Far Western analysis using the SH2 domain of Src fused
to GST (GST-Src SH2) as a probe revealed the presence of the
Src docking tyrosine(s) in Ret in both control and GDNF-stim-
ulated cells, with no further effect of ligand (Fig. 1A). Finally,
tyrosines 905, 1015, 1062, and 1096 were also phosphorylated
to comparable extents under both conditions (data not shown),
as assessed by immunoblotting with phosphospecific antibodies
(28). These results are consistent with our previous observa-
tions, underscoring the necessity of ligand-mediated transloca-
tion of Ret to lipid rafts to achieve Src binding (and optimal
biological responses) but not for phosphorylation and gener-
ation of Src binding site(s) in the receptor (24, 26).

The interaction of Src with Ret is dependent on the binding
of the Src SH2 domain to phosphorylated tyrosine(s) in Ret
(26). Moreover, at least one of these phosphotyrosines is
mapped within the juxtamembrane or the kinase domains
of one oncogenic form of Ret (25). To confirm the latter result
in the context of the Ret proto-oncogene, we used a mutant of
the receptor truncated at the end of the kinase domain (residue
1014) and, therefore, lacking several autophosphorylation ty-
rosines (residues 1015, 1062, and 1096). A Myc tag was added
at the C terminus of the mutant to facilitate its manipulation.
Pull-down assays using GST-Src SH2 revealed that the mutant receptor retained the ability to interact with this domain (Fig. 1B), confirming the existence of Src binding site(s) upstream of residue 1014. As expected, the introduction of a point mutation (K758M) in the ATP binding site of Ret kinase that renders a catalytically inactive receptor abolished the interaction (Fig. 1B), confirming that association of the Src SH2 domain with Ret depends on an intact kinase activity of the receptor.

Ret Tyrosine 981 Constitutes a Major Src Binding Site—The optimal consensus sequence for the SH2 domain of Src has been determined to be pYEEI by screening of a phosphopeptide library (34). In the same study, Met, Leu, and Val are also preferred residues at position +3 (three amino acids C-terminal to the phosphotyrosine). Moreover, structural studies suggest that the +3 residue is critical for SH2 binding to synthetic phosphopeptides (35). Since none of the tyrosines present in the juxtamembrane or the kinase domains of Ret fit the optimal sequence, we decided to analyze those that have one of the above residues at position +3 (Fig. 2). Thus, we generated tyrosine-to-phenylalanine point mutants of residues 752, 809, 952, and 981 in the context of Ret51. In addition, we used the catalytically compromised K758M and Y905F mutants to assess whether phosphorylation-independent binding of Src to Ret also occurred (by means of other Src domains such as the Src homology 3 or the unique domains). Fig. 2 shows a summary of the structure of Ret and the mutants used in this study.

Co-immunoprecipitation assays demonstrated that neither Tyr752 nor Tyr981 was relevant for Src binding, since mutation of these sites did not impair the interaction of Ret with Src. In contrast, the amount of Src co-immunoprecipitated by K758M and Y905F was greatly reduced as compared with wild type Ret, and Src relies on the binding of the Src SH2 domain to one or more phosphotyrosines in the receptor, with minor or no participation of other Src domains. Finally, Y982F and Y981F mutants showed a striking loss of interaction with Src, although some
residual binding was observed when compared with either K758M or Y905F (Fig. 3). However, the global phosphotyrosine content of Y952F mutant was severely reduced, making it difficult to interpret the role of this tyrosine in Src binding. This reduction in the phosphotyrosine content of Y952F was due to a decreased kinase activity of the mutated receptor, as revealed by a parallel reduction in the phosphorylation of Tyr981 (data not shown).

Binding of the SH2 domain of Src to phosphorylated receptor tyrosine kinases promotes Src activation, which can be monitored by assessing the level of Src autophosphorylation on tyrosine 416 (36). Therefore, we used the degree of phosphorylation of that tyrosine as an indicator of Src binding to Ret. Lysates from 293 cells expressing GFR1 and Src and stimulated with GDNF were resolved by SDS-PAGE and probed with a specific p-Y416-Src antibody (Fig. 4A, bottom two panels). Phosphorylation of this tyrosine correlated well with the degree of kinase activity of Ret mutants, except for Y981F mutation, which caused a marked but not complete reduction of Src phosphorylation, despite showing levels of kinase activity comparable with those of wild type Ret. In contrast, the Y952F receptor, despite its severely compromised kinase activity, induced levels of Src autophosphorylation similar to those promoted by Y981F, suggesting that tyrosine 952 does not contribute to Src phosphorylation (Fig. 4A, bottom two panels). Therefore, Tyr981 appears to be involved in Src binding by two independent approaches: co-immunoprecipitation and Src autophosphorylation.

Since co-immunoprecipitation experiments cannot discriminate between direct or indirect association, we conducted both Far Western and GST pull-down experiments (using the GST-Src SH2 recombinant protein) to determine whether Tyr981 represents a direct binding residue for the SH2 domain of Src. Surprisingly, only a very modest reduction of this binding was observed by either method (Fig. 4A, top four panels), suggesting that additional sites outside the kinase domain of Ret may exist. Alternatively, Tyr981 might not constitute a direct binding site for Src. To distinguish between these two possibilities, we introduced a Y981F point mutation in the context of the Δ1014 truncated Ret and tested its ability to bind the SH2 domain of Src in GST pull-down assays. As shown in Fig. 4B, the binding of Δ1014/Y981F Ret to Src SH2 was virtually undetectable. For comparison, we also included a Δ1014/Y905F mutant, which bound with similar or even higher affinity to GST-Src SH2 than Δ1014/Y981F, although its total tyrosine phosphorylation was marginal. Taken together, these data strongly suggest that Tyr981 is a major Src binding site in Ret but additional sites exist beyond residue 1014.

Tyrosine 1096 Interacts with the SH2 Domain of Src in Vitro but Does Not Contribute to Src Binding in Vivo—In an attempt

![Figure 3](image1.png)

**Fig. 3.** Tyrosine 981 is a major Src binding site in Ret. 293 cells were co-transfected with the indicated Ret51 point mutants along with GFR1 and Src and stimulated with GDNF. Ret was immunoprecipitated and probed with the indicated antibodies to check loading (middle). The amount of Src that co-immunoprecipitated with each Ret mutant is shown at the top. Note that mutation of Tyr981 severely decreases Ret tyrosine phosphorylation. IP, immunoprecipitation; IB, immunoblot; WT, wild type.

![Figure 4](image2.png)

**Fig. 4.** A, mutation of tyrosine 981 in Ret51 causes only a modest loss of interaction with the SH2 domain of Src. 293 cells were co-transfected with the indicated Ret51 mutants, GFR1 and Src, and stimulated with GDNF. Ret was immunoprecipitated and probed with the indicated antibodies or the SH2 domain of Src fused to GST (top three panels). Another aliquot from the same lysate was subjected to GST pull-down with the SH2 domain of Src (middle panel). Finally, a phospho-Src immunoblot was performed with lysates from the same samples. The blot was stripped and reprobed for total Src. B, introduction of Y981F mutation in Δ1014 Ret abolishes its binding to Src SH2. GST pull-down with lysates from 293 cells transfected with the indicated point mutants in Δ1014 Ret (top panel). Immunoprecipitates from the same lysates with an extracellular domain (ECD) Ret antibody, probed with anti-phosphotyrosine (middle panel) or anti-Ret (bottom panel) are shown as a control. IP, immunoprecipitation; IB, immunoblot; WT, wild type.
We reasoned that perhaps overexpression of Ret was leading to its aberrant phosphorylation on additional residues that might be providing ectopic docking sites for Src. Therefore, we decided to use a more physiological model in which wild type, endogenous Ret was phosphorylated only in response to ligand. GDNF-stimulated NBL-S human neuroblastoma cells, which express both Ret and GFRA1, were used for this purpose. To test the role of specific tyrosines, a competitive GST pull-down assay was performed with GST-Src SH2 in the presence of phosphopeptides comprising the sequences around tyrosines 905, 952, 981, 1015, 1029, and 1096. Phosphopeptides containing tyrosines 952 and 981 competed with Ret for the binding of GST-Src SH2 with similar efficiency, whereas phosphopeptides for tyrosines 905, 1015, and 1062 peptides had no effect (Fig. 5B). Surprisingly, the phosphopeptide containing tyrosine 1096 was the best competitor, causing a complete blockade of the binding, although the Y1096F mutant did not show a decreased interaction with Src in co-immunoprecipitation experiments (Fig. 5, compare A and B). Thus, the binding of Src SH2 to Tyr1096 apparently takes place in vitro but not in intact cells (see “Discussion”).

Analysis of Point Mutations in the Ret9 Splice Isoform—The above result (strong binding of Src SH2 to tyrosine 1096 in vitro) may explain why the drastic reduction in Src co-immunoprecipitation seen with some Ret mutants did not translate to a similar decrease in binding to GST-Src SH2 in both pull-down and Far Western analysis (e.g. compare the effect of Y981F mutation in Figs. 3 and 4A). Since phospho-Tyr1096 tightly binds GST-Src SH2 in vitro, the contribution of other residues to the overall binding of Ret to that domain may be masked by that tyrosine (note, however, that co-immunoprecipitation indicated that Tyr1096 had no role in Src binding in intact cells). Therefore, we decided to conduct the previous experiments in the context of Ret9 splice isoform, which lacks tyrosine 1096. Accordingly, we generated Ret9 point mutants of tyrosines 981, 1015, 1029, and 1062 and performed GST pull-down experiments. In the context of Ret9, the Y981F mutation almost completely abolished the ability of GST-Src SH2 to interact with Ret. Mutation of tyrosines 1015, 1029, or 1062, on the other hand, did not decrease this interaction (Fig. 6A). Double mutants consisting of Y981F and either Y1015F or Y1029F further decreased the residual binding observed with Y981F alone, whereas Y981F/Y1062F had no effect (Fig. 6B).

Tyrosine 981 Is a Novel Ret Autophosphorylation Site—The above data indicate that Tyr981 is the major Src binding site in Ret, with some potential, minor contribution of Tyr1015 and Tyr1029. The decreased interaction of the Y981F mutant with the SH2 domain of Src also suggests that this tyrosine is a novel autophosphorylation residue in Ret, since SH2 domains interact only with phosphotyrosines. To test this hypothesis directly, we generated a polyclonal antibody raised against phospho-Tyr981 Ret and evaluated its specificity using 293 cells transfected with wild type, K758M, or Y981F receptors. Both crude serum and affinity-purified antibodies were specific for this phosphotyrosine, as revealed by immunoblot of total cell lysates. The antibody did not recognize the K758M mutant, proving its affinity for phosphorylated Ret. The introduction of Y981F mutation also abolished the signal (Fig. 7A). To test whether this tyrosine was phosphorylated in response to ligand in neurons, extracts from dissociated superior cervical ganglion neurons stimulated with GDNF for different times were immunoblotted with the phospho-Tyr981 antibody. Fig. 7B shows that tyrosine 981 was phosphorylated in response to GDNF, with a time course that peaked after 15 min of exposure to ligand and decreased to baseline levels after 8 h.

Mutation of Tyrosine 981 Reduces GDNF-mediated Survival—We then analyzed the impact of Y981F mutation on neuronal...
survival, using dissociated cerebellar granule neurons as a model system (Fig. 8). These neurons do not express either Ret or GFRα1 and can be transfected with low efficiency. Therefore, the Ret/GFRα1 system can be reconstituted by ectopically overexpressing wild type or mutated versions of their components (24, 26). Neurons were co-transfected with either wild type or Y981F Ret9, together with GFRα1 and EGFP as a marker, and the survival of transfected cells was monitored before and 48 h after treatment with GDNF, as described under “Experimental Procedures.” The survival in high potassium, serum-containing medium (K25+S) was set to 100%, whereas medium containing low potassium and no serum (K5-S) served as a negative control. GDNF promoted the survival of 70.5 ± 2.7% (average ± S.E. of three independent experiments performed in triplicate) of neurons transfected with wild type Ret, whereas the survival of cells transfected with Y981F was decreased to 54.9 ± 3.2% (p < 0.005). Since about 25% of the neurons kept in K5-S survived after 48 h, this reduction represents an ∼30% decrease in survival as compared with wild type Ret. Thus, mutation of tyrosine 981 significantly, but not completely, reversed the survival-promoting effects of GDNF in these cells.

Fig. 6. A, analysis of point mutations in the Ret9 isoform. Lysates from 293 cells transfected with the indicated Ret9 point mutants were subjected to GST pull-down using the SH2 domain of Src and probed with a Ret9 antibody (top panel). An aliquot from the same lysates was saved before GST pull-down and probed with anti-Ret9 (middle panel) or anti-pY905 Ret antibodies to check for the kinase activity of the mutants (bottom panel). B, the same as in A but using the indicated double mutants. IP, immunoprecipitation; IB, immunoblot; WT, wild type.

Fig. 7. A, Ret tyrosine 981 is a novel autophosphorylation site. Cytoplasmic cell lysates from 293 cells transfected with the indicated Ret mutants were probed with crude serum or affinity-purified anti-Tyr(P)981 Ret antibody. Ret immunoprecipitates from the same lysates were probed with anti-phosphotyrosine (left) or anti-Ret antibodies (right). B, tyrosine 981 is phosphorylated in response to GDNF in sympathetic neurons. Nine days in vitro (DIV) rat sympathetic neurons were stimulated with 50 ng/ml GDNF for the indicated times, and lysates were probed with affinity-purified anti-pY981 Ret (top) or anti-Ret antibodies (bottom). IP, immunoprecipitation; IB, immunoblot; WT, wild type.

Fig. 8. Ret tyrosine 981 mediates neuronal survival. Cerebellar granule cells were transfected with the indicated Ret mutants, together with GFRα1 and EGFP as a marker. Neuronal survival was evaluated after 48 h in the presence of GDNF as described under “Experimental Procedures.” The survival in high potassium, serum-containing medium (K25+S) was set to 100%, whereas medium containing low potassium, serum-free medium (K5-S) served as a negative control. The asterisk denotes a significant difference between wild type (WT) and Y981F Ret-expressing neurons treated with GDNF (p < 0.005 by Student’s t test).
In this work, we have identified Ret tyrosine 981 as the major Src binding site in the receptor. Other residues such as Tyr^{1015} and Tyr^{1029} may contribute modestly to the overall binding. By generating a phosphospecific antibody, we demonstrate that Tyr^{981} represents a novel Ret phosphorylation site. Mutation of this residue to phenylalanine reduces GDNF-mediated neuronal survival in transfected cerebellar granule cells.

We used multiple approaches to determine the tyrosine(s) that constitute the Src binding site of Ret. Co-immunoprecipitation detects supramolecular complexes assembled in intact cells, involving multiple interactions between different proteins and domains. Therefore, the binding reactions occur in a cellular context, but the success of the procedure depends on the stability of such complexes after cell lysis and during the immunoprecipitation process. Moreover, co-immunoprecipitation does not discriminate between direct or indirect association between the two molecules tested. Approaches using recombinant SH2 domains, on the other hand, identify direct association between molecules but under binding conditions that do not resemble those inside intact cells. In particular, the use of microgram amounts of recombinant protein domains might displace endogenous proteins that bind to their cognate targets with higher affinity in cells. The problem of specificity is especially a concern for the Far Western assay, in which the target protein is denatured and separated by SDS-PAGE. Accordingly, it has been shown that the specificity of Far Western assays is greatly restricted if performed under competitive conditions (i.e., in the presence of unlabeled SH2 domains from other proteins) (37). Thus, techniques using SH2 domains are helpful to determine potential interactions that have to be confirmed in a cellular context. Finally, we have used Src phosphorylation as a correlate of Src binding. This technique is the least "invasive," since no further manipulation is required after cell lysis. However, other mechanisms besides SH2 displacement can influence Src autophosphorylation, such as phosphorylation/dephosphorylation of its C-terminal regulatory tyrosine (36). Therefore, the use of multiple approaches is clearly required to determine the critical residues involved in the interaction between two molecules. Of the many tyrosines analyzed, only tyrosine 981 was identified as a Src binding site by all the above criteria.

Is Tyrosine 952 an Autophosphorylation Site in Ret?—The role of Tyr^{952} in Src binding remains uncertain because mutation of this residue severely decreased the catalytic activity of the receptor. The use of phosphopeptides allowed us, to some degree, to circumvent this problem by analyzing the contribution of this tyrosine in the wild type receptor. Whereas that experiment showed that a phosphopeptide containing the sequence around Tyr^{952} is a good ligand for Src SH2 (Fig. 5A), whether this tyrosine is actually phosphorylated in Ret is not clear. In fact, we favor the hypothesis that this tyrosine is not an autophosphorylation site in the receptor for three reasons. First, analysis of the autophosphorylation sites of the FGF receptor, which shares the highest sequence identity with Ret (38), demonstrates that Tyr^{730} (equivalent to Ret Tyr^{952}) is an autophosphorylation site, whereas phosphorylation of Tyr^{701}, equivalent to Tyr^{962}, is never detected (39). Second, prediction software (NetPhos) (40) identifies Tyr^{981} as an autophosphorylation residue with high likelihood (score = 0.911 in a 0–1 range), only below that of Tyr^{905} (score = 0.989), whereas Tyr^{952} is not predicted to be phosphorylated (score = 0.109). Third, we and others have failed to develop a phosphospecific antibody against this tyrosine (41) (data not shown). More specifically, after two rounds of both positive and negative affinity purification (using the 952 phosphopeptide and a mixture of all other phosphopeptides, respectively), the resulting antibody no longer detected wild type, fully phosphorylated Ret in Western blot experiments. The same procedure yielded specific antibodies for Ret tyrosines 905, 1015, 1062, and 1096 (50). Although not conclusive, these considerations strongly suggest that tyrosine 952 is not phosphorylated in Ret and, therefore, does not have a role in Src binding.

Role of Tyrosines in the C Terminus of Ret—We observed that mutation of tyrosines 1015, 1029, and, to a lesser extent, 1062, decreased co-immunoprecipitation of Src with Ret. However, an effect of these mutations was not observed in GST pull-down experiments or phospho-Src immunoblots, even in the context of Ret9. Phosphopeptides containing tyrosines 1015 and 1062 also failed to compete with Src SH2 in GST pull-down assays. A slight but reproducible effect of residues 1015 and 1029 in the binding to Src SH2 was only unmasked if double mutants were used. This apparent paradox is not unprecedented; e.g., mutation of the Src binding site of TrkB in vivo reduces the amount of the receptor that is co-immunoprecipitated with phospholipase C-γ (42) without affecting the ability of the SH2 domain of phospholipase C-γ to precipitate the receptor in a pull-down assay (43). We believe that these observations reflect the highly interrelated nature of the signaling complexes assembled around activated receptor tyrosine kinases, in which all of their components may establish contact with each other directly or indirectly.

As for the role of tyrosine 1096, the SH2 domain of Src bound it with high affinity in vitro, whereas co-immunoprecipitation showed no effect of the Y1096F mutation in the association with Src. It is important to note that the sequence surrounding this tyrosine (pYANW) does not fit the consensus for the SH2 domain of Src (34) but actually functions as a Grb2 SH2 site (14). However, a recent report demonstrates that, at least in solution, the Src SH2 domain displays dual specificity for both Src and Grb2 motifs (44). A similar situation is described for the receptor protein tyrosine phosphatase α. Tyrosine 789 of this molecule, which displays a close sequence similarity (pY-ANF) to that of Ret Tyr^{1096}, binds both Grb2 and Src SH2 domains in vitro but is saturated by Grb2 in intact cells (45, 46). Therefore, we suggest that although Src SH2 can potentially bind to Tyr^{1096}, Grb2 outcompetes Src for the association with that tyrosine in cells.

Src-binding Tyrosines in Other Receptor Tyrosine Kinases—Our data demonstrate that Tyr^{981} of Ret is the major Src binding site. The sequence around this residue (pYRLM) resembles only distantly the pYEE1 consensus and is actually closer to the motif recognized by the p85 subunit of phosphatidylinositol 3-kinase. However, p85 does not bind Ret directly but through Gab1/2 and/or IRS-1 (18, 47). Examination of the binding sites for Src in other receptors reveals that their sequences are also divergent from the consensus. Relatives of Ret include the platelet-derived growth factor receptor, the colony-stimulating factor-1 receptor (CSFIR/mfs), and the stem cell factor receptor, c-kit (38). For all of them, the site responsible for Src binding is a conserved tyrosine located in the juxtamembrane domain of the receptors (e.g., Tyr^{579} for the platelet-derived growth factor receptor). The sequences C-terminal to this tyrosine are pYTVV for platelet-derived growth factor receptor, pYTFFI for CSFIR/mfs, and pYVYI for c-kit (48–50). Interestingly, this conserved tyrosine is not present in either Ret or its closest relative, FGF receptor. With respect to the FGF receptor, reports are contradictory on its ability to interact with Src. Zhan et al. (51) showed that FGF receptor and Src co-immunoprecipitate, whereas another report failed to detect such interaction (52). A recent work using immortal-
ized fibroblasts derived from embryos null for Src, Fyn, and Yes (SYF cells) has established that FGF-mediated proliferation actually requires Src family kinase activity (53), suggesting that the FGF receptor binds to and/or activates it. In any case, the tyrosine responsible for this putative binding has not been mapped.

**Biological Effects of Mutation of Tyrosine 981**—When ectopically overexpressed in cerebellar granule cells, the Y981F mutant caused a modest but highly statistically significant decrease in GDNF-mediated neuronal survival. The moderate magnitude of this effect was somewhat surprising, since we have previously demonstrated, using pharmacological and dominant negative approaches, that blockade of Src activity completely prevents neuronal survival in the same culture system (24). However, the residual Src binding observed in the Y981F mutant might be sufficient to promote survival in our system, in which Ret is overexpressed and therefore present in completely prevented by mutation of Tyr981, whereas mutation of Y981F mutant might be sufficient to promote survival in our system (24). However, the residual Src binding observed in the Y981F mutant might be sufficient to promote survival in our system, in which Ret is overexpressed and therefore present in completely prevented by mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, whereas mutation of Tyr981, 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