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The Full Oncogenic Activity of Ret/ptc2 Depends on Tyrosine 539, a Docking Site for Phospholipase Cγ

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***RET/PTC* oncogenes, generated by chromosomal rearrangements in papillary thyroid carcinomas, are constitutively activated versions of proto-*RET*, a gene coding for a receptor-type tyrosine kinase (TK) whose ligand is still unknown. *RET/PTC*s encode fusion proteins in which proto-*RET* TK and C-terminal domains are fused to different donor genes. The respective Ret/ptc oncoproteins display constitutive TK activity and tyrosine phosphorylation. We found that Ret/ptcs associate with and phosphorylate the SH2-containing transducer phospholipase Cγ (PLCγ). Two putative PLCγ docking sites, Tyr-505 and Tyr-539, have been identified on Ret/ptc2 by competition experiments using phosphorylated peptides modelled on Ret sequence. Transfection experiments and biochemical analysis using Tyr→Phe mutants of Ret/ptc2 allowed us to rule out Tyr-505 and to identify Tyr-539 as a functional PLCγ docking site in vivo. Moreover, kinetic measurements showed that Tyr-539 is able to mediate high-affinity interaction with PLCγ. Mutation of Tyr-539 resulted in a drastically reduced oncogenic activity of Ret/ptc2 on NIH 3T3 cells (75 to 90% reduction) both in vitro and in vivo, which correlates with impaired ability of Ret/ptc2 to activate PLCγ. In conclusion, this paper demonstrates that Tyr-539 of Ret/ptc2 (Tyr-761 on the proto-*RET* product) is an essential docking site for the full transforming potential of the oncogene. In addition, the present data identify PLCγ as a downstream effector of Ret/ptcs and suggest that this transducing molecule could play a crucial role in neoplastic signalling triggered by Ret/ptc oncoproteins.**

The proto-*RET* gene codes for a receptor-type tyrosine kinase (RTK) whose ligand is still unknown (39). Alterations of this gene have been found to be associated with five different diseases. In fact, different germ line point mutations of proto-*RET* have been recently identified in the inherited cancer syndromes multiple endocrine neoplasia types 2A and 2B and familial medullary thyroid carcinoma, as well as in a congenital malformation, Hirschsprung disease (reviewed in reference 42 and references therein). In contrast, somatic rearrangements of the gene generating *RET/PTC* oncogenes have been detected in about 35% of surgical specimens from papillary thyroid carcinomas (4, 5, 12). In transfection assays, *RET/PTC*s transform NIH 3T3 cells and differentiate the pheochromocytoma cell line PC12 (4, 5, 12, 27). *RET/PTC*s encode fusion proteins in which the tyrosine kinase (TK) and the C-terminal domains of Ret are fused to different donor genes: the previously unknown *H4* and *ELE1* sequences for *RET/PTC1* and *RET/PTC3*, respectively, and a portion of *RIα*, encoding the regulatory subunit of protein kinase A (PKA), for *RET/PTC2* (4, 5, 12). All the donor genes, being constitutively expressed in all tissues, are responsible for the ectopic expression of the oncogenes in thyroid cells, and, as suggested by sequence analysis and coimmunoprecipitation experiments (5), their products presumably promote a constitutive dimerization of the chimeric proteins. As a consequence, Ret/ptc oncoproteins have been demonstrated to be constitutively phosphorylated on tyrosine and to display constitutive activity (6, 16).

Extensive work has recently elucidated the principles of RTK signal transduction pathways. Following ligand binding and receptor dimerization and autophosphorylation, src homology 2 (SH2) domain-containing proteins are recruited to phosphorylated tyrosine residues on the receptor and are activated by a variety of mechanisms (reviewed in reference 28). One class of SH2 domain-containing proteins includes molecules with intrinsic enzymatic activity such as phospholipase Cγ1 (PLCγ1), Ras-GTPase-activating protein (GAP), src kinase, p91STAT, and Syp phosphotyrosine phosphatase. The other class is represented by adapter proteins such as Shc, Grb2, Nck, and p85, the regulatory subunit of phosphatidylinositol 3′ kinase (PI3 kinase). The SH2 domains mediate high-affinity interactions of cytoplasmic effectors with unique phosphotyrosine residues in TK receptors (reviewed in references 15 and 29). The molecular mechanisms by which each SH2 domain recognizes phosphotyrosines flanked by specific sequences have been investigated by using different approaches, including selection of the optimal sequence for each SH2 from a degenerate phosphopeptide library and X-ray crystallographic analyses. The three amino acids immediately following the phosphotyrosine were shown to be the primary determinants for SH2 specificity (8, 37, 45).

Many studies have been performed in the last few years in order to identify specific phosphotyrosine residues in TK receptors as major or unique binding sites for each one of the

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known SH2-containing proteins. In fact, specific docking sites for PLCγ, PI3 kinase, and Ras GAP have been identified in platelet-derived growth factor (PDGF), epidermal growth factor (EGF), colony-stimulating factor 1 (CSF1), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF) receptors (reviewed in reference 29) mainly by using the following complementary approaches: (i) competition assays for SH2 domains with synthetic phosphotyrosine-containing peptides modelled on receptor autophosphorylation sites and (ii) conversion by site-directed mutagenesis of the relevant tyrosine residues to phenylalanine, followed by biochemical and biological analysis of the mutants (e.g., see references 30 and 31).

In the case of Ret, the lack of ligand has hampered the analysis of its signal transduction pathway, which is relevant for the comprehension of the molecular basis of the different Ret-associated diseases. However, since Ret/ptc oncoproteins, maintaining the whole cytoplasmic portion of Ret protein, behave in a manner similar to that of a constitutively activated TK receptor, it is possible to study the biochemical basis of their transforming activity by using the approaches described above. In this context, we have previously shown that the Shc adapter proteins are phosphorylated and bound to Grb2 in cells expressing Ret/ptc oncoproteins (6).

In this report, we show that PLCγ is phosphorylated and coimmunoprecipitates with Ret/ptc oncoproteins in NIH 3T3 cells containing either the *RET/PTC1* or the *RET/PTC2* oncogene. PLCγ is an SH2-containing enzyme catalyzing the hydrolysis of phosphatidylinositol biphosphate (PIP2) to inositol triphosphate and diacyl glycerol, and its tyrosine phosphorylation status has been associated with its enzymatic activity (13, 21). On the basis of sequence analysis, a tyrosine residue in the C terminus of Ret, Tyr-539 in Ret/ptc2, has been predicted to be a docking site for PLCγ. We have shown that a phosphotyrosine-containing decapeptide encompassing Tyr-539 is able to block in vitro the binding between the N-terminal SH2 of PLCγ and the Ret/ptc2 oncoprotein. Kinetic measurements of the interaction between PLCγ SH2 domains and the Ret/ptc2 pY539 phosphopeptide indicate a high-affinity binding. Moreover, we have demonstrated that a *RET/PTC2*-Y539F (Y-to-F change at position 539) mutant oncogene, while maintaining its TK activity, displays not only a drastically reduced ability to activate PLCγ but also a strongly reduced oncogenic activity, as assessed both by an in vitro transfection assay with NIH 3T3 cells (75 to 90% reduction in number of foci) and by in vivo tumorigenicity in nude mice (about a 90% reduction of tumor mass formation).

MATERIALS AND METHODS

Site-directed mutagenesis and cloning of *RET/PTC2*-YF mutants. The cloning of the *RET/PTC2* cDNA has been previously reported (5) (EMBL data bank accession number L03357). The 2-kb *Xba*I cDNA insert was cloned into the pAlter vector (Promega). Site-directed mutagenesis was performed by using an in vitro oligonucleotide mutagenesis system (Altered Sites in vitro mutagenesis system; Promega). Oligonucleotides carrying the Tyr-539→Phe and the Tyr-505→Phe mutations were 5′-AGAGGAGAGACTTCTTGGACCTTGC-3′ and 5′-AGCGAGGAGATGTTCCGCCTGATGCT-3′, respectively, and were synthesized with an Applied Biosystems 391 apparatus. Since the introduced mutations did not destroy or create restriction sites, mutant clones were identified by selective PCR. Taking advantage of enzymatic discrimination against elongating mismatched termini (the AT terminus is extended 2,500 times faster than is the TT terminus), we have used the forward oligonucleotide GATGGTTAAGAG GAGAGACTA or, alternatively, GATGGTTAAGAGGAGAGACTT with a common reverse oligonucleotide, 50 μM deoxynucleoside triphosphates, and annealing and extension cycles of 65°C for 1 min to amplify a 200-bp fragment for the Y539F mutant. An analogous strategy was used to identify the Y505F mutants. Full-length *RET/PTC2* cDNA clones carrying the appropriate mutations were entirely sequenced. Wild-type (wt) and mutant cDNAs were then inserted into the *Xba*I sites of two different eukaryotic expression vectors, pRC-CMV (Invitrogen) and pMAMneo-BLUE (Clontech) under the control of the human cytomegalovirus (CMV) and mouse mammary tumor virus promoters, respectively.

Cell culture and transient transfection. Human HeLa and mouse NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 10% calf serum, respectively. Before protein extraction, NIH 3T3 cells and NIH 3T3-derived cell lines were serum starved for 48 h in DMEM plus 0.1% bovine serum albumin (BSA) plus 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer; in some cases, the cells were pretreated with 50 nM sodium orthovanadate (Na_3VO_4) for 3 h.

Transient transfection in HeLa cells (5×10^5 cells per 100-mm-diameter dish) was performed by the calcium phosphate method (46) with 10 μg of expression plasmid DNA. Ret/ptc2 proteins were extracted 48 h after transfection and processed for immunoblotting as described below.

Characterization of oncogenic properties. NIH 3T3 cells (2×10^5 cells per 100-mm-diameter dish) were transfected by calcium phosphate coprecipitation (46) by using 10 to 500 ng of the different recombinant plasmids and 25 μg of mouse carrier DNA. G418-resistant ($G418^R$) colonies were selected in DMEM plus 10% calf serum plus G418 antibiotic (650 μg ml^{-1}), and transformation foci were selected in DMEM plus 5% calf serum. NIH 3T3 $G418^R$ colonies and transformation foci were either fixed and counted or isolated for further studies 14 to 20 days after transfection. Alternatively, NIH 3T3 transfected pools were tested for their ability to growth either in soft agar or in nude mice. For an anchorage-independent assay, 10 days after the transfection 10^5 cells per 35-mm-diameter dish were plated, suspended in 0.33% agar solution containing DMEM plus 10% fetal calf serum, and overlaid onto 0.5% agar solution in the same medium. After 2 weeks in culture, clones were stained with *p*-iodonitrotetrazolium-violet (Sigma), counted, and photographed.

To test for the acquisition of in vivo tumorigenicity, 14 days after transfection 10^6 NIH 3T3 pool cells transfected with 500 ng of either *RET/PTC2*-wt or *RET/PTC2*-Y539F plasmid, suspended in 100 μl of phosphate-buffered saline (PBS), pH 7.5, were subcutaneously injected into 5- to 7-week-old athymic mice (CD1 *nu/nu*; Charles River). Mice were inspected for 6 weeks; tumor growth was evaluated at days 12 and 24 after injection.

Immunoprecipitation and Western blotting (immunoblotting). Protein samples were prepared as previously reported (6) and immunoprecipitated with the specified antisera. Antisera were anti-Ret common antiserum, raised in rabbits immunized with the synthetic peptide conjugated to ovoalbumin (Neosystem, Strasbourg, France); KRRDYLDLAASTPSDSL, corresponding to amino acids 535 to 551 of the C-terminal sequence common to the two Ret isoforms of Ret/ptc2 (6); antiphosphotyrosine (anti-PTyr), a commercially available monoclonal immunoglobulin G2b antiserum (Upstate Biotechnology Incorporated); anti-PLCγ1 antiserum (Upstate Biotechnology Incorporated); and anti-Shc (a gift from P. G. Pelicci and described previously [6]). Different amounts of protein extracts were used, as specified in the figures: for binding experiments, 1 mg; for coimmunoprecipitation experiments, about 3 to 4 mg; and for other immunoprecipitation experiments, about 0.5 mg. Immunoprecipitates were resolved by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6 or 7.5% polyacrylamide) gels. Proteins were transferred onto nitrocellulose filters, blocked with 5% BSA or 0.5% gelatin in Tris-buffered saline (TBS) (pH 7.6), and immunoblotted with the anti-Ret antiserum described above or with another affinity-purified anti-Ret common polyclonal antiserum (raised against the synthetic peptide DISKDLEKMMVKRRD, derived from amino acids 524 to 538 of the C-terminal sequence common to the two Ret isoforms), both designated anti-Ret (3.5 μg ml^{-1}), or with anti-PTyr (0.2 μg ml^{-1}) or anti-PLCγ1 (1 μg ml^{-1}) antisera (6). Immunoreactive bands were visualized by using ^{125}I-labeled protein A (Amersham) and then autoradiography or by using a horseradish peroxidase-conjugated anti-rabbit or anti-mouse antiserum and ECL detection reagents (Amersham). When ^{125}I-protein A was used, the filters were exposed to storage phosphor screen films and analyzed with a PhosphorImager apparatus (Molecular Dynamics) in order to quantify the counts per minute associated with the Ret-specific bands reacting with anti-Ret or anti-PTyr antisera.

In vitro immunocomplex kinase assay. The anti-Ret immunoprecipitates, absorbed on protein A-Sepharose beads, were washed twice with lysis buffer and once with incubation buffer (50 mM HEPES [pH 7.2], 20 mM $MnCl_2$, 5 mM phenylmethylsulfonyl fluoride) and incubated for 15 min at 4°C in 20 μl of the same buffer containing 0.5 mM dithiothreitol, 4 μCi of [γ-^{32}P]ATP (5,000 Ci $mmol^{-1}$; Amersham) diluted with unlabeled ATP to the final concentration of 26 pmol of ATP per sample, and 50 μM myelin basic protein (MBP). The reaction was stopped by addition of concentrated Laemmli buffer. Proteins were eluted and subjected to SDS–7.5 or 15% PAGE. ^{32}P-labeled bands were revealed by autoradiography of the dried gel and analyzed by using a PhosphorImager.

In vitro kinase assay using synthetic peptides. An in vitro kinase assay was performed as described in the previous paragraph, with the following modifications: 250 μM synthetic peptides instead of MBP was added, and the reaction was carried out at 37°C for 1 h and was stopped by adding 30% acetic acid. The peptides contained in the mixture of the immunokinase reaction were dried, resuspended in 25 μl of water, and separated by reverse-phase high-pressure liquid chromatography (HPLC) on a Waters DELTA PAK 18C-300Å column (particle size, 15 μm; 7.8 by 300 mm). Elution was performed at 1 ml min^{-1} with a linear gradient from 15 to 85% solvent B in 30 min (solvent A was 0.05%

trifluoroacetic acid in water-acetonitrile [95:5], and solvent B was 0.05% trifluoroacetic acid in water-acetonitrile [40:60]). The eluant was monitored by using a UV detector (Kontron Instruments) at 220 nm. The fractions were manually collected. The peptide-containing fractions (deduced by adding the synthetic cold phosphorylated reference peptide to the mixture, in a separate run) were dried, resuspended in 5 μl of 30% acetic acid, and spotted in 1-μl aliquots onto a cellulose thin-layer chromatography (TLC) plate (10 by 20 cm; Merck). TLC plates were eluted with water–pyridine–acetic acid–1-butanol (60:50:75:15), air dried, and exposed to X-ray film (Hyperfilm-MP; Amersham). In addition, the same plate was stained with ninhydrin to reveal the reference peptides.

In vitro binding experiments using GST-fused proteins and phosphopeptide competition. Bacterial cultures expressing pGEX vectors alone (Pharmacia) or recombinant pGEX containing the C-terminal or N-terminal SH2 of PLCγ (gifts from J. Schlessinger) or the SH2 domain of Shc or that of Grb2 (gifts from P. G. Pelicci) were grown in Luria broth (LB) containing 100 μg of ampicillin ml^{-1} and induced with 1 mM isopropyl β-D-thiogalactopyranoside for 3 to 6 h. The induced bacteria were lysed by sonication in PBS containing 10 μg each of aprotinin and leupeptin ml^{-1}. Glutathione *S*-transferase (GST) or GST-fused proteins were purified by using glutathione-Sepharose (Pharmacia) and retained in the immobilized form bound to glutathione-Sepharose. Cells were lysed in PLC-LB {50 mM HEPES [pH 7.5], 150 mM sodium chloride, 10% glycerol, 1% Triton X-100, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 1.5 mM magnesium chloride, 100 mM sodium fluoride, 10 mM sodium PP_i, and 10 μg each of aprotinin and leupeptin ml^{-1}}, and the clarified lysates were incubated with 2 to 5 μg of immobilized GST or GST-fused proteins for 90 min at 4°C. When required, 1% SDS was added and protein samples were treated for 5 min at 65 or 100°C and subsequently diluted to a 0.1% SDS concentration before incubation with the GST proteins. The protein complexes were washed three times with PLC-LB buffer and eluted by boiling for 5 min in Laemmli sample buffer. Protein complexes were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were blocked and probed as described above.

The peptide competition assay was performed by preincubating the GST-fused proteins bound to the glutathione-Sepharose with the indicated (see Fig. 2) concentrations of peptides and phosphopeptides dissolved in PLC-LB buffer before adding the cell lysates.

The phosphopeptides were obtained by solid-phase synthesis performed manually or with an automatic peptide synthesizer (Milligen 9050; Millipore). Chain elongation was carried out by using Fmoc (fluorenylmethyloxycarbonyl) chemistry and amino acid activation by 2-benzotriazolyl-tetramethyluronium-tetrafluoroborate in the presence of 1-hydroxybenzotriazole hydrate (10). *tert* butyl groups provided side chain protection, where necessary, and N-terminal amino acids were introduced as *tert*-butyloxycarbonyl derivatives. Tyrosine phosphorylation was performed on a solid phase (14) by using di-*tert*-butyl-*N,N*-diethylphosphoramidite and then *tert*-butylhydroperoxide for oxidation, with yields of >80%. Simultaneous deprotections and cleavage of peptides from the resin were achieved by using 90% trifluoroacetic acid and scavengers. All the phosphopeptides were purified by preparative reverse-phase HPLC and obtained as lyophilized powders; their purity was checked by analytical C_{18} reverse-phase HPLC and was >90% in every case. They were characterized by fast atom bombardment mass spectrometry and amino acid analysis.

BIAcore analysis. After purification, the SH2 domains were desalted through a Pharmacia column (Sephadex G-50 prepacked system) in order to achieve buffer exchange to the BIAcore running buffer, consisting of 20 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20. Phosphopeptides used were Ret/ptc2 pY539 (see Table 1) and HGF/scatter factor receptor (HGF/SF-R) pY1356 [HVNATY(p)VNVKOH].

Phosphopeptides as 0.5 mM solutions in 50 mM HEPES (pH 7.4)–2 M NaCl were immobilized on the surface of sensor chips under conditions previously described (9). Following phosphopeptide immobilization, purified desalted GST-SH2 fusion proteins at concentrations ranging from 20 to 300 nM were injected across the surface and the resonance signal was recorded. Both the association and dissociation rate constants (K_{on} and K_{off} values, respectively) were obtained from the resonance signal data and calculated by using BIA evaluation software (Biosensor AB; Pharmacia) according to equations previously described (9, 25). The K_{off} values were measured following injection of excess free phosphopeptide (100 μM) to prevent rebinding. The K_d values were obtained according the equation $K_d = K_{off}/K_{on}$.

Analysis of total IPs. Cells were grown in DMEM containing 10% calf serum. Forty hours before the extraction, subconfluent cells were washed and the medium was replaced with Basal Medium Eagle containing 0.5% calf serum and 1 μCi of [^{3}H]myoinositol (specific activity, 102 Ci $mmol^{-1}$; Amersham) ml^{-1}. Cells were collected, washed three times with Krebs-Ringer-HEPES (KRH) buffer (125 mM NaCl, 5 mM HCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM HEPES [pH 7.4], 2 mM $CaCl_2$, 6 mM glucose), and resuspended in KRH buffer containing 0.1% BSA.

Cells (0.5×10^6 per sample) were treated with 10 mM LiCl for 10 min and then with 50 ng of PDGF BB (GIBCO-BRL) ml^{-1} for 30 min at 37°C, and the reaction was stopped with 7.5% trichloroacetic acid. The trichloroacetic acid supernatants were extracted with diethyl ether, neutralized to pH 7.5, and fractionated on Dowex columns (3). The inositol phosphates (IPs) were eluted as described by Berridge et al. (3). Samples were scintillated and counted in a beta counter. Total phospholipid incorporation was checked for each sample, and IP formation was expressed as a percentage of the total phospholipids.

RESULTS

In NIH 3T3 transformants expressing Ret/ptc1 or Ret/ptc2 oncoproteins PLCγ is phosphorylated and binds the oncoproteins. To elucidate the biochemical basis of the transforming activity associated with *RET/PTC* oncogenes, we have analyzed their ability to couple with known pathways implicated in mitogenic signal transduction. One such pathway, leading to increased PIP2 breakdown, is thought to be initiated by TK receptor-dependent recruitment of PLCγ. As shown in the left panel of Fig. 1A, because of differential splicing in the 3′ end of *RET*, *RET/PTC* oncogenes code for two isoforms: p59 and p64 for Ret/ptc1 and p76 and p81 for Ret/ptc2 (6). Ret/ptc oncoproteins have been previously demonstrated to be constitutively phosphorylated on tyrosine and able to activate signal transducers such as Shc (6). As shown in Fig. 1A, whereas in serum-starved NIH 3T3 cells PLCγ is not phosphorylated, NIH 3T3 cells expressing either Ret/ptc1 or Ret/ptc2 oncoproteins showed a high level of steady-state Tyr-phosphorylated PLCγ. This modification of PLCγ has been found to be essential for the activation of the enzyme and the hydrolysis of inositol phospholipid (13, 21). The coimmunoprecipitation of either the Ret/ptc1 or Ret/ptc2 phosphorylated oncoprotein with PLCγ is also shown (Fig. 1A, rightmost two panels), thus suggesting a direct interaction between the oncoproteins and the enzyme. Both the two Ret/ptc isoforms were coimmunoprecipitated by PLCγ, as shown in Fig. 1 and in additional experiments (data not shown).

To study the PLCγ-Ret interaction, SH2 domains of PLCγ fused to GST were used for in vitro binding experiments (Fig. 1B). Both PLCγ N-terminal and C-terminal SH2 domains, but not the GST control, were able to bind in vitro Ret/ptc2 oncoproteins. In addition, treatment of the lysates with boiling SDS did not abrogate the in vitro PLCγ-Ret/ptc2 binding (Fig. 1B), thus indicating direct interaction.

Identification of putative docking sites for PLCγ. To identify the binding sites of Ret with high-level affinity for the SH2 domains of PLCγ, a series of synthetic decapeptides corresponding to tyrosine residues of Ret TK and C-terminal domains surrounded by 4 to 5 amino acids on each side were synthesized. The tyrosine-phosphorylated peptides are listed in Table 1; in most cases, the corresponding unphosphorylated control peptides were produced also. These peptides were then examined for their ability to prevent the in vitro association between the constitutively autophosphorylated Ret/ptc2 oncoprotein isoforms and the SH2 domains of PLCγ contained in different GST fusion proteins. For the first screening, a final concentration of 100 μM for each peptide was used. The results of the screening are shown in Fig. 2A for the SH2 N terminus (N-SH2) and panel B for the SH2 C terminus (C-SH2) of PLCγ. Phosphopeptides pY505 and pY539 were able to block the binding of Ret/ptc2 to the N-SH2 of PLCγ (Fig. 2A). According to the prediction based on the analysis of a phosphopeptide library (37), the Ret peptide pY539 KRRD pYLDLAA would fit better than pY505 (SEEMpYRLMLQ) with the consensus of the docking site for N-SH2 of PLCγ, YL(3.8)D(1.8)L(3.2) (where the numbers in parentheses indicate the enrichment value of the amino acids selected), and is identical to the docking site for PLCγ identified on the FGF receptor (20, 30). pY539 was also able to inhibit the binding of Ret/ptc2 to C-SH2 of PLCγ (Fig. 2B). The slight inhibition with pY553 on C-SH2 of PLCγ seemed aspecific, not being dose dependent when decreasing amounts of phosphopeptide

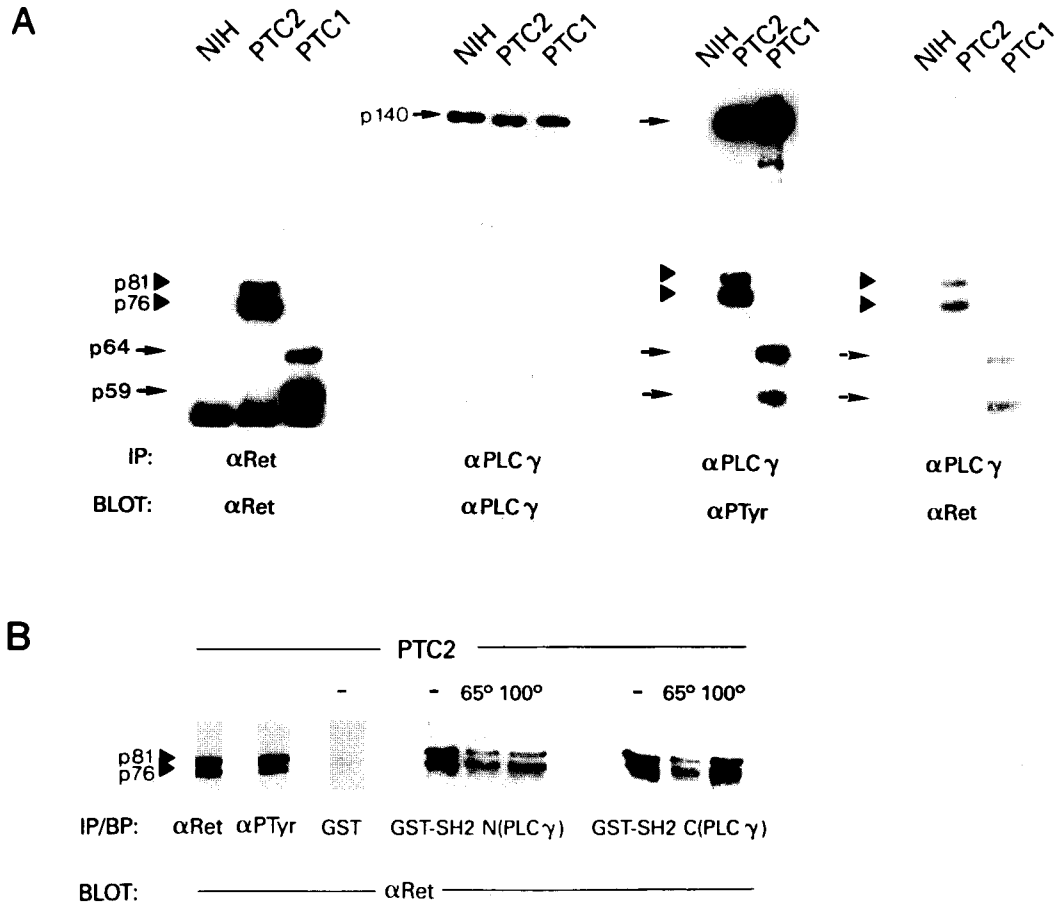

FIG. 1. PLCγ is phosphorylated in NIH 3T3 transformants containing *RET/PTC* oncogenes and coimmunoprecipitates with and directly binds Ret/ptc oncoproteins in vitro. (A) Western blot analysis with the indicated antisera of immunoprecipitates (IP) from NIH 3T3 cells (lane NIH) or NIH 3T3 cell lines expressing Ret/ptc1 (lane PTC1) or Ret/ptc2 (lane PTC2) oncoproteins. A 0.5-mg amount of protein extracts was used for immunoprecipitations shown in the first two panels, and 4 mg was used for the coimmunoprecipitations shown in the last two. Ret/ptc2 oncoprotein isoforms are indicated by arrowheads as p81 and p76, while Ret/ptc1 p64 and p59 isoforms and PLCγ p140 protein are indicated by arrows. αRet, anti-Ret. (B) Western blot analysis with anti-Ret (αRet) antiserum with a protein extract from the NIH 3T3 cell line expressing Ret/ptc2 oncoproteins (PTC2) in a binding experiment using 5 μg of Sepharose-bound GST fusion proteins (BP) bearing the SH2 C-terminal [C(PLCγ)] or the SH2 N-terminal [N(PLCγ)] domain of PLCγ or using 5 μg of the control GST protein only (lane GST). A 1-mg amount of protein extract in PLC-LB buffer was used. The samples were left untreated (lanes −) or heated in the presence of 1% SDS at 65°C (lane 65°) or 100°C (lane 100°) before binding. As a control, 0.3 mg of the same cell extract was immunoprecipitated with αRet or αPTyr antibodies (lanes αRet and αPTyr). IP, immunoprecipitates.

were used (data not shown). To roughly define the relative affinities of peptides pY505 and pY539 for PLCγ, in vitro binding experiments were performed with decreasing amounts of peptides. Both peptides displayed a significant inhibition of the binding between Ret/ptc2 and the N-SH2 of PLCγ. The best result was obtained with peptide Y539 (Fig. 2C), which under our experimental conditions (with 4 μg of GST-fused proteins and 1.5 mg of cell protein extract) abolished the binding at the concentration of 20 μM (Fig. 2C, central panel). None of the unphosphorylated peptides tested showed any ability to inhibit in vitro the association between the Ret/ptc2 oncoprotein and the SH2 domains of PLCγ (data not shown); Fig. 2 shows the unphosphorylated peptides Y539 (panel C), Y505 (panel A), and Y553 (panel B). Moreover, 100 μM pY539 but not pY505 also displayed a weak (30%) but consistent inhibition with the C-SH2 of PLCγ as the GST-fused protein (Fig. 2B and D). pY539 at 100 μM was then tested with fusion proteins containing the SH2 domain of Shc, Grb2, or src and the two SH2 domains of Syp. A weak inhibition (25%) was obtained only for the binding between Ret/ptc2 and Shc (Fig. 2C, rightmost panel, and data not shown).

TABLE 1. Sequences of Ret phosphopeptides

Phosphopeptide	Sequence
pY276	GRAGpYTTVAV
pY315	VIKLpYGACSQ
pY330+333	LIVEpYAKpYGSLRG
pY350	VGPGpYLGSGG
pY388	QGMQpYLAEMK
pY424	SRDVpYEEDSY
pY429	EEDSpYVKRSQ
pY452	FDHIpYTTQSD
pY476	GGNPpYPGIPP
pY505	SEEMpYRLMLQ
pY539	KRRDpYLDLAA
pY553	DSLIpYDDGLS
pY586	ENKLpYGMSDP

Kinetic measurements of the association of PLCγ N- and C-SH2 domains with Ret pY539 phosphopeptide. To provide a quantitative analysis of PLCγ-Ret/ptc2 interaction, we measured real-time binding of both PLCγ SH2 domains with the Ret-derived phosphopeptide pY539, corresponding to the putative docking site for PLCγ. This approach has been previ-

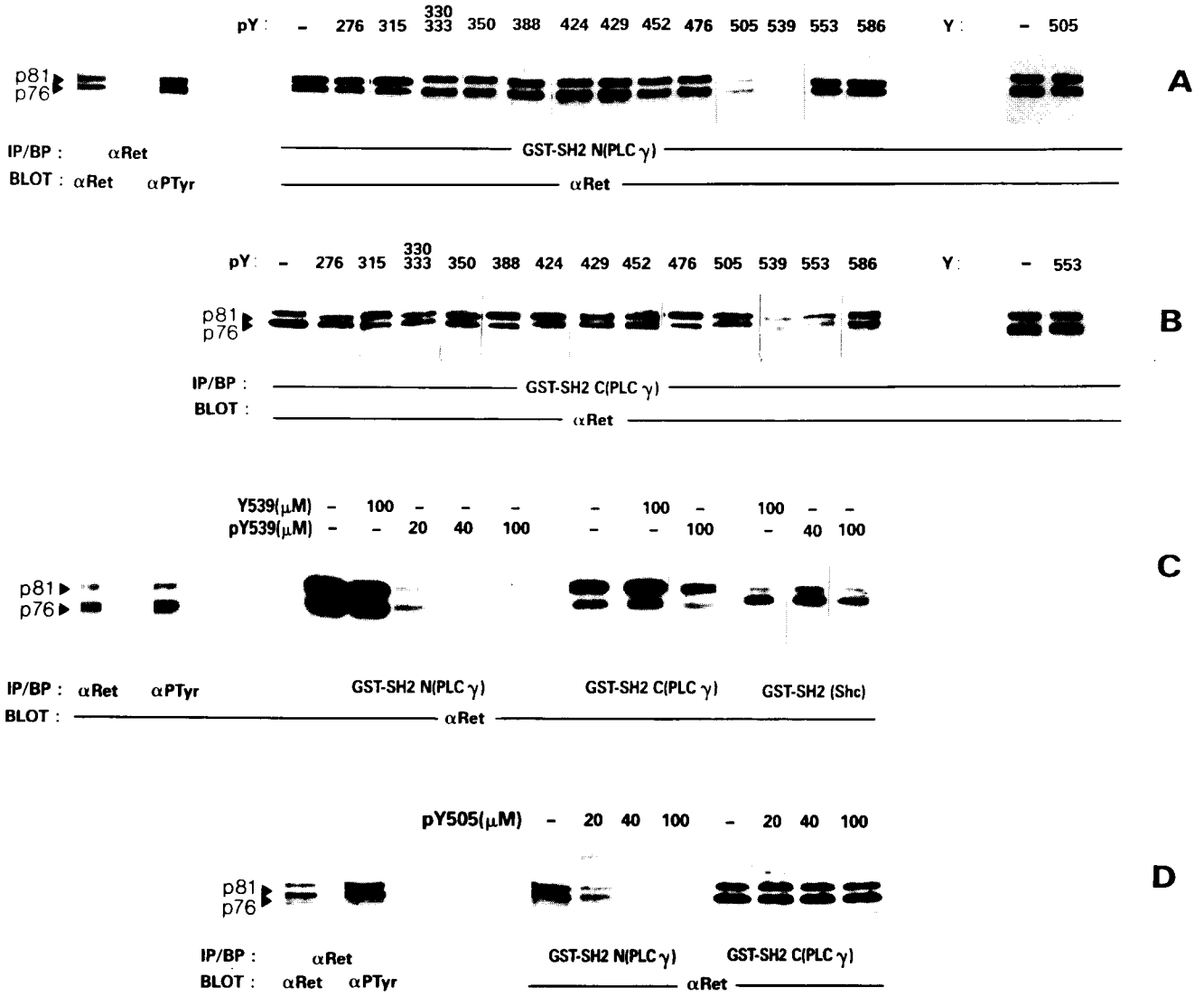

FIG. 2. Identification of putative PLCγ docking sites on Ret/ptc2 by phosphopeptide competition of in vitro binding. Shown is Western blot analysis with anti-Ret (αRet) antiserum on αRet or αPTyr immunoprecipitates (IP), as controls, or on GST-fused bound proteins (BP) with a cell extract from the NIH 3T3 cell line expressing Ret/ptc2 and the indicated GST fusion proteins. A 4-μg amount of GST-fused proteins and 1 mg of cell extract were used. (A and B) The indicated unphosphorylated (Y) or Tyr-phosphorylated (pY) decapeptides listed in Table 1 were used at 100 μM to inhibit the binding. –, no peptide. (C) Binding inhibition with Tyr-phosphorylated (pY539) and unphosphorylated (Y539) peptides at the indicated concentrations. –, no peptide. (D) Binding inhibition with the pY505 phosphopeptide at the indicated concentrations. –, no peptide.

ously used successfully with PDGF receptor-derived phosphopeptides and various SH2 domains (9, 17, 25).

The pY539 phosphopeptide of the Ret/ptc2 tail was immobilized on the BIAcore chip and allowed to interact with a range of concentrations of purified SH2 domains. The obtained sensorgrams were used to calculate association and dissociation constants (Table 2). Dissociation of bound protein was measured following injection of an excess of free phosphopeptide to prevent rebinding. The results of these experiments indicate that PLCγ binds Ret pY539 phosphopeptide with an affinity ($K_d = 10^{-8}$ M) comparable to that previously reported for the PDGF receptor system (17). Interestingly, when binding kinetics of the two PLCγ SH2 domains were compared, the N-SH2 domain displayed an affinity for the Ret phosphopeptide sixfold higher than that of the C-SH2 one (Table 2). These data suggest that the N-SH2 domain is primarily responsible for specific PLCγ binding to the Tyr-539 phosphorylation site in the tail of the Ret/ptc2 oncoprotein and are in agreement with a prediction based on screening of a degenerate phosphopeptide library (37) and with the phosphopeptide inhibition experiments (see above).

Although the sequence surrounding Tyr-539 in the Ret/ptc2

TABLE 2. Kinetic parameters of PLCγ SH2 domain association with Ret phosphopeptide spanning Tyr-539[a]

PLCγ domain	K_{on} (M^{-1} s^{-1}) (10^5)	K_{off} (s^{-1}) (10^{-1})	K_d (M) (10^{-8})
N-SH2	25.1	0.25	1.00
C-SH2	4.24	0.24	5.66

[a] K_{on} and K_{off} are the association and dissociation rate constants, respectively. The equilibrium dissociation constants (K_d) were obtained by dividing K_{on} by K_{off}.

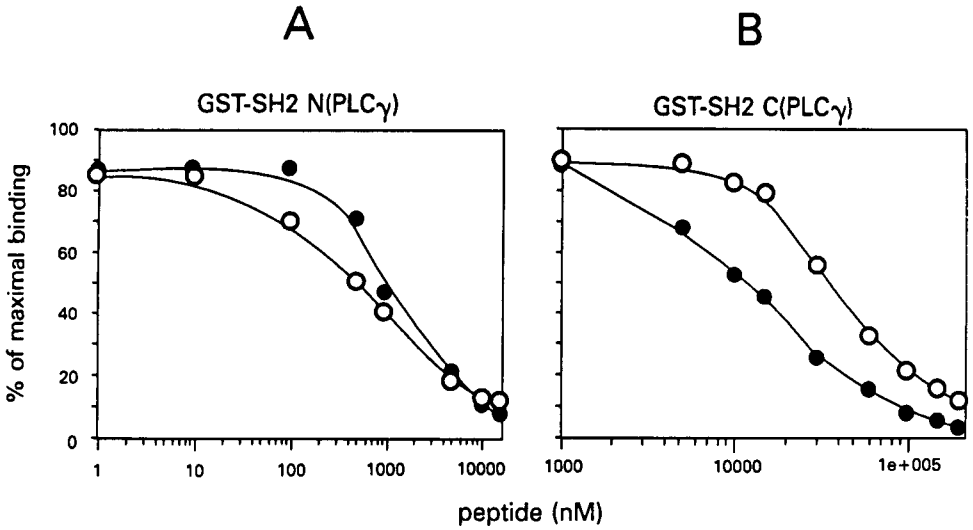

FIG. 3. Evaluation of relative affinities of Ret/ptc2 pY539 and HGF receptor pY1356 for PLCγ N-SH2 [N(PLCγ)] (A) and C-SH2 [C(PLCγ)] (B). Relative affinities were determined by measuring the ability of the corresponding phosphopeptide to inhibit the interaction of the PLCγ SH2 domains with an immobilized phosphopeptide [VNATY(p)VNVK, pY1356] which includes Tyr-1356 of the HGF receptor. The amount of PLCγ SH2 domain bound in the presence of increasing concentrations of pY539 or pY1356 phosphopeptides is expressed as a relative percentage of SH2 bound in the absence of phosphopeptides. ○, pY539 Ret peptide; ●, pY1356 Met peptide.

protein (YLDL) has been previously identified as an optimal consensus motif for PLCγ (N-SH2) binding on the FGF receptor (20, 30), another sequence [YV(H/N)V] recently has been shown to mediate the association of the HGF/SF-R with PLCγ (32). It was therefore interesting to directly compare the binding of PLCγ to the HGF-R phosphopeptide (pY1356) with the binding of PLCγ to the Ret/ptc2 phosphopeptide (pY539) (Fig. 3). In these experiments, a phosphopeptide spanning Tyr-1356 of the HGF-R tail was immobilized on the BIAcore chip and purified SH2 domains were injected in the presence of increasing concentrations of the Ret/ptc2 (pY539) or the HGF/SF-R (pY1356) phosphopeptide. Figure 3 shows the results of these measurements, expressed as percentages of maximal binding. Comparison of the values at which half-maximal binding was observed provided useful information about the relative affinities. For the PLCγ N-SH2 domain, the highest apparent affinity is displayed by the Ret/ptc2 phosphopeptide (Fig. 3A), while for the C-SH2 domain, the HGF/SF-R phosphopeptide has a higher apparent affinity than does the Ret/ptc2 phosphopeptide (Fig. 3B). These results are in agreement with the prediction based on screening of a degenerate phosphopeptide library which has indicated the sequence YL(D/E)L as a preferential consensus for the PLCγ N-SH2 domain and the motif YVI(P/V) as the consensus sequence for the PLCγ C-SH2 domain (37).

Altogether, the kinetic data obtained with the Biosensor approach show that, at least in this in vitro system, Tyr-539 in the Ret/ptc2 tail mediates high-affinity interaction with PLCγ and suggest that the N-SH2 domain is primarily responsible for this association.

In vitro phosphorylation of Ret synthetic peptide Y539 by Ret/ptc2 oncoprotein. It has been recently demonstrated that by using synthetic peptides as substrates in an in vitro kinase reaction, the phosphorylation sites in tyrosine kinases can be predicted (32, 40). Therefore, to determine whether Tyr-539 of Ret/ptc2 could be phosphorylated by the Ret kinase, a decapeptide encompassing Tyr-539 was tested (Fig. 4). The products of immunokinase reaction on the Y539 peptide (KRRD YLDLAA) and on the negative-control unrelated peptide NC

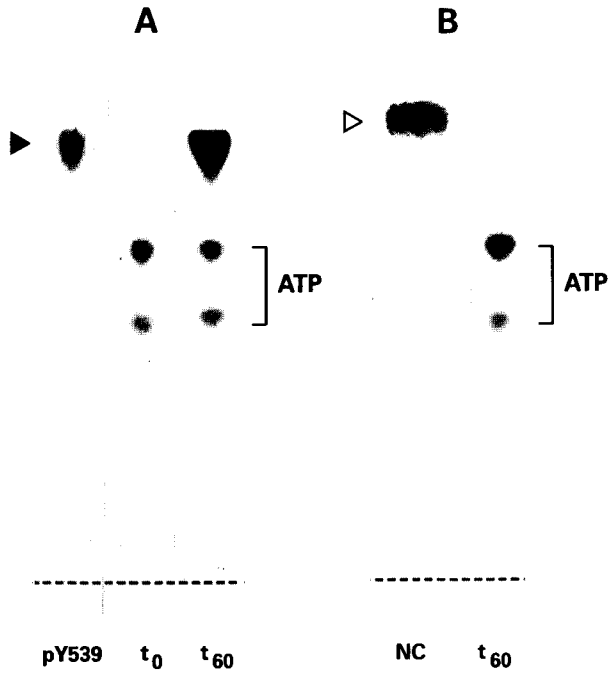

FIG. 4. Ret/ptc2 in vitro phosphorylates Y-539-containing peptide. An in vitro kinase reaction was carried out with immobilized Ret/ptc2 oncoprotein to phosphorylate the synthetic decapeptide Y539 (KRRDYLDLAA) (A) and the negative control peptide NC (EADNDFIIPLPD) (B). The kinase reaction mixtures containing either Y539 or NC peptide were partially purified by reverse-phase HPLC (see Materials and Methods). The peptide-containing fractions derived from the kinase reaction stopped immediately after the addition of the peptide (lane t_0) or from the sample subjected to 60 min of kinase reaction (lanes t_{60}) were analyzed by TLC, as described in Materials and Methods. The same plate was stained with ninhydrin to reveal the reference peptides (lanes pY539 and NC). Positions of the cold reference phosphorylated peptide pY539 and negative control peptide NC are indicated by arrowheads. The origin (dashed line) and unreacted ATP are also indicated.

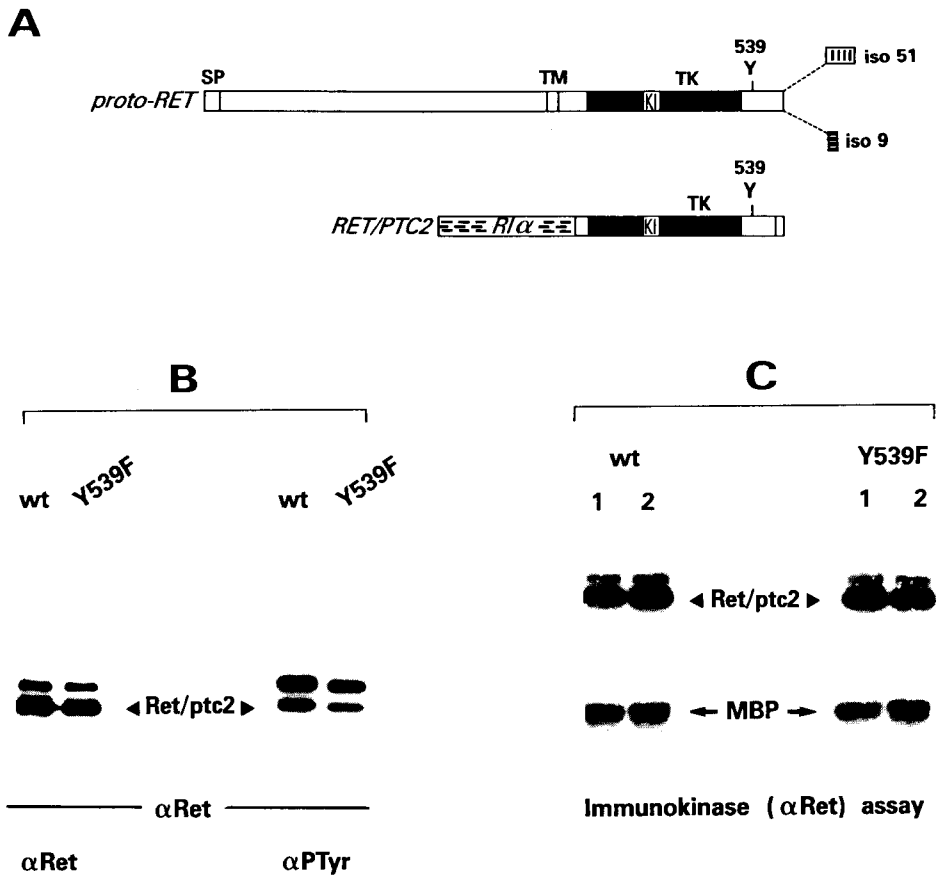

FIG. 5. Schematic representation and biochemical features of *RET/PTC2*-Y539F oncogene mutant. (A) Schematic representation of the introduced Y539F mutation on *RET/PTC2* and proto-*RET* genes. The two isoforms of Ret (iso 51 and iso 9) are also indicated. The portion of the Ret/ptc2 fusion protein contributed by *RI*α is barred. SP, signal peptide; TM, transmembrane domain; TK, tyrosine kinase domain; KI, kinase insert. (B) Expression and tyrosine phosphorylation of Ret/ptc2-wt (wt) and Ret/ptc2-Y539F (Y539F) proteins (indicated by arrowheads) transiently expressed in HeLa cells. αRet, anti-Ret; IP, immunoprecipitate. (C) In vitro immunocomplex kinase assay of Ret/ptc2-wt and Ret/ptc2-Y539F transiently expressed in HeLa cells. Shown are autophosphorylation and phosphorylation on the exogenous substrate MBP. Two replicates (indicated by wt-1 and -2 and Y539F-1 and -2) are shown.

(EADNDFIIPLPD), partially purified by reverse-phase HPLC to eliminate excess ATP, were subjected to TLC followed by autoradiography. A spot, comigrating with the cold phosphorylated peptide pY539 used as a reference, was present only on the peptide fraction of Y539 subjected to 60 min of kinase reaction (Fig. 4A, lane t_{60}). This result demonstrates that, at least in vitro, Tyr-539 is a target of phosphorylation for Ret kinase.

Construction and biological activity of *RET/PTC2*-Y539F mutant. Because of its high-affinity binding, Tyr-539 was chosen as the target for mutagenesis to eliminate the putative docking site pTyr-539 in the Ret/ptc2 oncoprotein. The Tyr-539→Phe mutation (Y539F) was introduced in the *RET/PTC2* cDNA encoding the short isoform (5) by site-directed mutagenesis (Fig. 5A; see Materials and Methods). A full-length *RET/PTC2*-Y539F cDNA clone was sequenced in its entirety and was demonstrated to differ from the *RET/PTC2*-wt only with respect to the introduced missense mutation (data not shown). The *RET/PTC2*-Y539F and the *RET/PTC2*-wt cDNAs were then cloned in the pRC-CMV and pMAMneo-BLUE eukaryotic expression vectors, both carrying the neomycin resistance gene.

The pRC-CMV constructs were used for transient expression in HeLa cells in order to verify the synthesis of the correctly sized (76-kDa) protein and to compare the phosphorylation levels of wt and mutant Ret/ptc2 proteins. As shown in Fig. 5B, both constructs express the expected Ret/ptc2 protein, reacting with anti-Ret antiserum, while untransfected HeLa cells or HeLa cells transfected with an antisense construct fail to reveal anti-Ret-reactive bands (data not shown). Wt and mutant Ret proteins display comparable steady-state Tyr phosphorylation levels. Moreover, the wt and mutant proteins, transiently expressed in HeLa cells and analyzed by an in vitro immunokinase assay, showed comparable autophosphorylation and substrate phosphorylation activities on MBP (Fig. 5C). An additional band of slightly higher molecular weight was also variably present in anti-Ret immunoprecipitates, most likely because of translation starting from the ATG present in the pRC-CMV vector 6 bp upstream of the cloning site and in frame with the *RET/PTC2* sequence (data not shown). Since this band was absent when the pMAMneo-BLUE vector was used (data not shown) and since we have shown previously (27) and shown in this paper (see below) that the different constructs have similar biological activities in transfected NIH 3T3 cells, we assume that the additional band could not affect the comparison between the wt and mutants.

In order to investigate the biological effect of the mutant protein and to study its biochemical properties, NIH 3T3 cells were stably transfected with the above-described pRC-CMV constructs. The abilities of *RET/PTC2*-wt and *RET/PTC2*-

TABLE 3. Oncogenic activities of *RET/PTC2* and *RET/PTC2*-Y539F on NIH 3T3 cells

Transfected plasmid	Morphological transformation[a]		Tumorigenicity in nude mice[b]		
	Transformed foci/ $G418^R$ colonies	Relative transforming activity	Tumors/injection on day: 12	Tumors/injection on day: 24	Mean vol (mm^3) ± SD (*n*)
pRC/RET/PTC2	8.0×10^{-2}	1.00	12/12	12/12	332 ± 230 (12)
pRC/RET/PTC2 Y539F	1.4×10^{-2}	0.17	1/12	8/12	19 ± 19 (8)
Vector	$<1 \times 10^{-5}$		0/4	1/4	3.4 (1)

[a] NIH 3T3 $G418^R$ colonies and transformation foci were fixed and counted 14 days after transfection. Transfection experiments were done in triplicate with 200 ng of plasmid per plate. The vector plasmid was pRC-CMV.

[b] 10^6 NIH 3T3 pool cells transfected with 500 ng of the indicated plasmids were injected. Tumor appearance was scored every 3 days, and the tumor size was evaluated 24 days after injection. All the injected mice negative at day 24 developed only small tumors after 5 to 7 weeks.

Y539F to induce in NIH 3T3 cells morphological transformation (transformation foci), anchorage-independent growth in soft agar, and in vivo tumor formation were compared.

RET/PTC2-Y539F showed a strong reduction of transforming activity, evaluated as the number of transformation foci, in comparison with the activity of *RET/PTC2*-wt (Table 3 and Fig. 6). A dose-effect response is reported in Fig. 6: at doses ranging from 50 to 500 ng, *RET/PTC2*-Y539F showed a strong reduction of the transforming activity, producing only 11 to 24% of the transformation foci originated by *RET/PTC2*-wt. In Table 3, the transforming activities of the mutant and wt *RET/PTC2* are normalized for the number of $G418^R$ colonies selected from the same transfection experiment, to eliminate the variability due to different plasmid preparations. This analysis confirmed the strong reduction (83%) of transforming activity for the mutant oncogene. As an additional control, cell lysates from pools of $G418^R$ NIH 3T3 colonies transfected by *RET/PTC2*-wt or by *RET/PTC2*-Y539F were shown to express comparable amounts of Ret/ptc2 proteins (data not shown). Several individual $G418^R$ colonies containing either the *RET/PTC2*-wt or the *RET/PTC2*-Y539F gene were also isolated for the biochemical analyses described below. The strongly reduced transforming activity in a focus-forming assay shown by *RET/PTC2*-Y539F was confirmed by using the wt and mutant genes cloned in the pMAMneo-BLUE vector (data not shown).

Moreover, to test the in vivo tumorigenicity of the two constructs, NIH 3T3 cells transfected with either *RET/PTC2*-Y539F or *RET/PTC2*-wt were used to subcutaneously inject nude mice. As reported in Table 3, *RET/PTC2*-Y539F induced a lower number of tumors with a longer latency and, overall, with a significantly reduced volume, corresponding to about 6% of the average volume of the tumors induced by *RET/PTC2*-wt. In keeping with this result, when the anchorage-independent growth of NIH 3T3 cells transfected with either of the plasmids was examined, *RET/PTC2*-Y539F was found to show a significantly reduced (about 80%) colony-forming efficiency in comparison with that obtained with *RET/PTC2*-wt. Altogether, these results clearly demonstrate the dependence on Tyr-539 of the full oncogenic activity of the *RET/PTC2* oncogene.

Biochemical analysis of Ret/ptc2-Y539F mutant protein. Since the pY539 peptide was able to block the in vitro binding between Ret/ptc2 and N-SH2 of PLCγ, the in vitro ability of Ret/ptc2-Y539F to bind the PLCγ N-SH2 domain was first analyzed. Figure 7A shows that the binding between Ret/ptc2, transiently expressed in HeLa cells, and N-SH2 of PLCγ was significantly impaired but not abolished by the Y539F mutation. To verify whether Ret/ptc2-Y539F was still able to activate PLCγ, four independent NIH 3T3 $G418^R$ cell lines, expressing comparable amounts of Ret/ptc2-wt or of Ret/ptc2-Y539F proteins, were analyzed for their ability to induce Tyr phosphorylation of PLCγ. As shown in Fig. 7B, in two representative clones expressing Ret/ptc2-Y539F protein, the steady-state Tyr phosphorylation level of PLCγ was only about 10% of that present in cell lines expressing Ret/ptc2-wt, as measured by using a PhosphorImager (see Materials and Methods), thus suggesting that the residual in vitro binding of Ret/ptc2-Y539F was not fully effective in inducing PLCγ phosphorylation. Coimmunoprecipitation experiments, using anti-Ret or anti-PTyr as the developing antiserum on anti-PLCγ immunoprecipitates from the four cell lines, confirmed the interaction between PLCγ and Ret/ptc2-wt and showed low-level interaction, if any, for the Y539F mutant protein (data not shown). Moreover, to investigate whether the phosphorylation level of PLCγ correlates with its enzymatic activity, total IPs in serum-starved NIH 3T3 cells and in NIH 3T3 cell lines expressing either Ret/ptc2-wt or Ret/ptc2-Y539F were analyzed. We have evaluated IP formation in the absence of external stimuli and after 30 min of treatment with PDGF, in untransfected NIH 3T3 cells as well as in the four NIH 3T3 cell lines expressing either Ret/ptc2-wt or Ret/ptc2-Y539F proteins. It is known that PDGF stimulation of cells expressing the PDGF receptor activates PLCγ, which catalyzes the production of IPs (41). The histogram in Fig. 8 shows total IP production with and without PDGF, normalized for the total [^{3}H]myoinositol incorporation, for one representative experiment in untransfected NIH 3T3 cells and in Ret/ptc2-expressing NIH 3T3 cell lines. Similar results were obtained in a total of three independent experiments; the overall results are reported in Fig. 8. Stimulation of NIH 3T3 cells by PDGF under our experimental conditions led to the production of IPs at a level about 15 times higher than the basal level. In two NIH 3T3 cell lines expressing Ret/ptc2-wt protein, the ratio of PDGF-stimulated to basal-level IPs was only 2.5. On the other hand, two NIH 3T3 cell lines expressing Ret/ptc2-Y539F protein displayed basal and PDGF-activated IP levels similar to those detected in untransfected NIH 3T3 cells. These results suggest that less available PLCγ could be present in Ret/ptc2-wt-expressing cells than in those expressing the Y539F mutant protein.

Since we have previously demonstrated that Shc proteins are phosphorylated in cells expressing Ret/ptc2 (6) and because the peptide pY539 was able to slightly reduce Shc-Ret/ptc2 in vitro binding, NIH 3T3 cell lines expressing wt and mutant Ret/ptc2 proteins were analyzed for the Tyr phosphorylation level of Shc. All the cell lines showed similar levels of Tyr phosphorylation of Shc proteins and identical coimmunoprecipitations of Shc proteins with Ret/ptcs (data not shown), thus ruling out a significant role for Tyr-539 as a docking site for Shc. Finally, the same cell lines expressing wt or mutant Ret/ptc2 did not show gross differences in phosphotyrosine protein

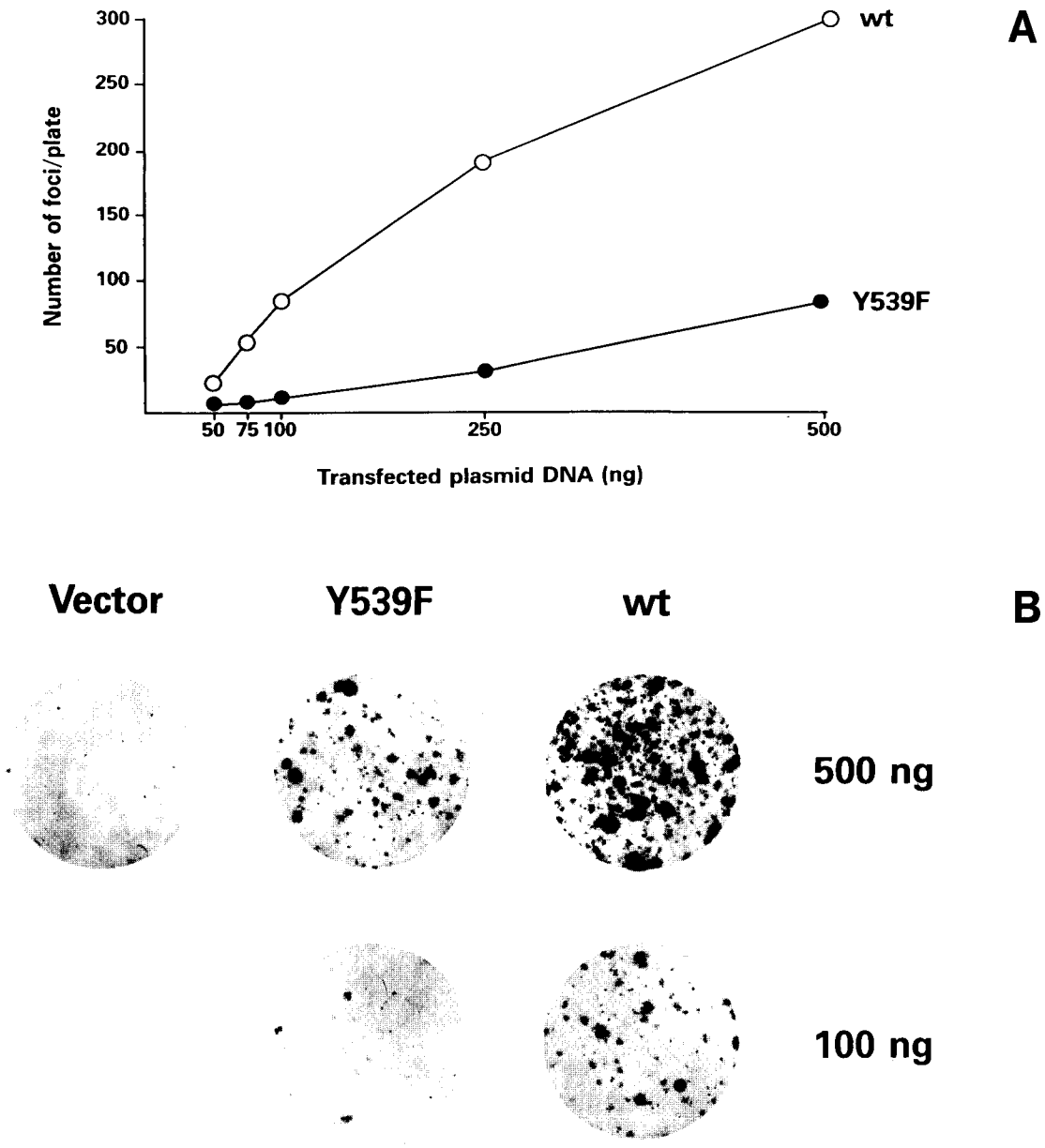

FIG. 6. Transforming activity of *RET/PTC2*-Y539F on NIH 3T3 cells analyzed by focus-forming assay. (A) The indicated amounts of plasmids were used to transfect NIH 3T3 cells as described in Materials and Methods. Transformation foci were fixed and counted 14 days after the transfection. Each point is the average for four plates derived from two independent DNA precipitations. (B) Representative plates of the focus formation assay shown in panel A with pRC-CMV (Vector) or the pRC-CMV constructs containing *RET/PTC2*-wt (wt) or *RET/PTC2*-Y539F (Y539F) at the indicated doses.

patterns, besides that of PLCγ, when analyzed by Western blotting with anti-PTyr on anti-PTyr immunoprecipitates (data not shown).

Construction of *RET/PTC2*-Y505F mutant and analysis of PLCγ activation by the mutant protein. Since the pY505 phosphopeptide was able to block the in vitro binding between Ret/ptc2 and the N-SH2 of PLCγ and since a residual in vitro binding between Ret/ptc2-Y539F and N-SH2 of PLCγ was detected, we mutagenized Tyr-505 of Ret/ptc2 to phenylalanine to investigate the in vivo involvement of Tyr-505 in PLCγ activation. The three residues downstream of Tyr-505 do not show any specificity for the N-terminal SH2 of PLCγ (Table 1) (37), but recently the +4 and +5 residues were suggested to be important for PLCγ binding. In particular, PLCγ SH2 domains can accommodate a +3 Met residue, provided that the appropriate Leu residue is present at the +4 position (17), as it is indeed for Tyr-505.

The mutation was introduced by site-directed mutagenesis, and a *RET/PTC2*-Y505F cDNA clone was entirely sequenced and cloned in the eukaryotic expression vector pRC-CMV, carrying the neomycin resistance gene. The pRC-CMV construct, analyzed by transient expression in HeLa cells, was shown to express a Ret/ptc2 protein of the expected size displaying tyrosine phosphorylation and a capability to bind the N-SH2 of PLCγ comparable to that of the Ret/ptc2-wt protein (Fig. 9A).

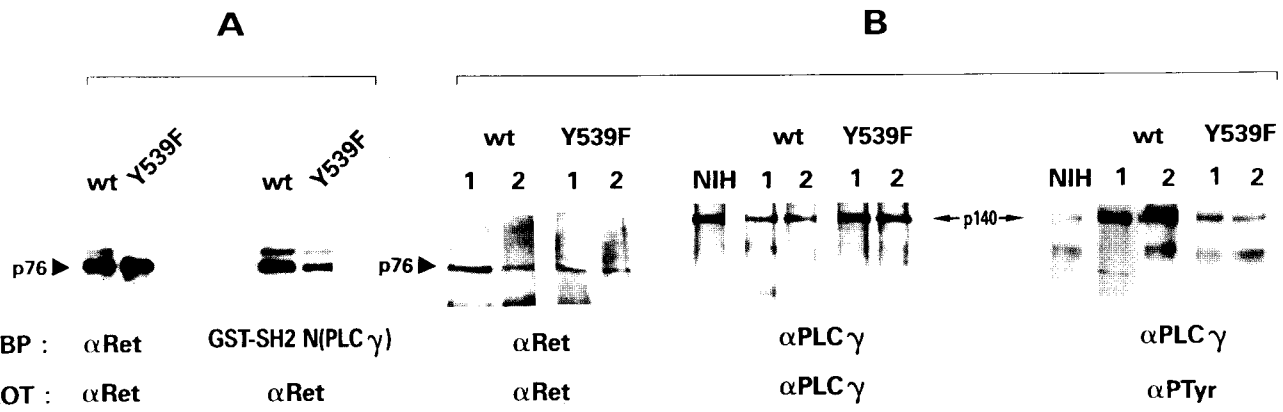

FIG. 7. PLCγ tyrosine phosphorylation in NIH 3T3 cell lines expressing Ret/ptc2-wt or Ret/ptc2-Y539F and in vitro binding between the SH2 N-terminal domains of PLCγ [N(PLCγ)] and Ret/ptc2 proteins. (A) Proteins were transiently expressed in HeLa cells; (B) four independent cell lines (indicated as wt-1 and -2 and Y539F-1 and -2) expressing either Ret/ptc2-wt (wt) or Ret/ptc2-Y539F (Y539F) proteins were analyzed. The protein extracts were immunoprecipitated (IP) with the indicated antisera (B) or used for binding experiments (A) with 5 μg of the indicated GST-fused protein (BP). αRet, anti-Ret.

In order to investigate PLCγ activation by Ret/ptc2, NIH 3T3 cells were stably transfected with the *RET/PTC2*-Y505F mutant construct to isolate NIH 3T3 cell lines containing the mutant gene. In Fig. 9B, the tyrosine phosphorylation of PLCγ in representative NIH 3T3 cell lines expressing either Ret/ptc2-wt or Ret/ptc2-Y505F is shown. No reduction of PLCγ phosphorylation was observed. In addition, the enzymatic activity of PLCγ in the same NIH 3T3 cell lines expressing Ret/ptc2-Y505F was analyzed. In Fig. 8 it is shown that the basal and PDGF-induced levels of IPs in cell lines expressing either Ret/ptc2-Y505F or Ret/ptc2-wt protein are indistinguishable, thus suggesting that Tyr-505 is not a functional docking site for PLCγ. However, *RET/PTC2*-Y505F displayed a 40% reduction of transforming activity in comparison with that of the wt, as evaluated by a focus-forming assay with NIH 3T3 cells, thus suggesting a yet-uncharacterized biological role for this tyrosine residue, not involving the recruitment of PLCγ.

DISCUSSION

RET/PTC oncogenes, generated by somatic rearrangements in a significant fraction of human papillary thyroid carcinomas, are genetic elements capable of transforming NIH 3T3 cells and are thought to be associated with the pathogenetic mechanisms affecting the original thyroid cells (4, 5, 12, 34). The Ret/ptc oncoproteins, in which the TK and C-terminal domains of *RET* are fused to 5′ regions from different donor genes, are constitutively phosphorylated on tyrosine and display a constitutive TK activity (5, 6, 16). To investigate the biochemical basis of transforming activity of *RET/PTC* oncogenes, we have analyzed their signal transduction. We have identified a ty-

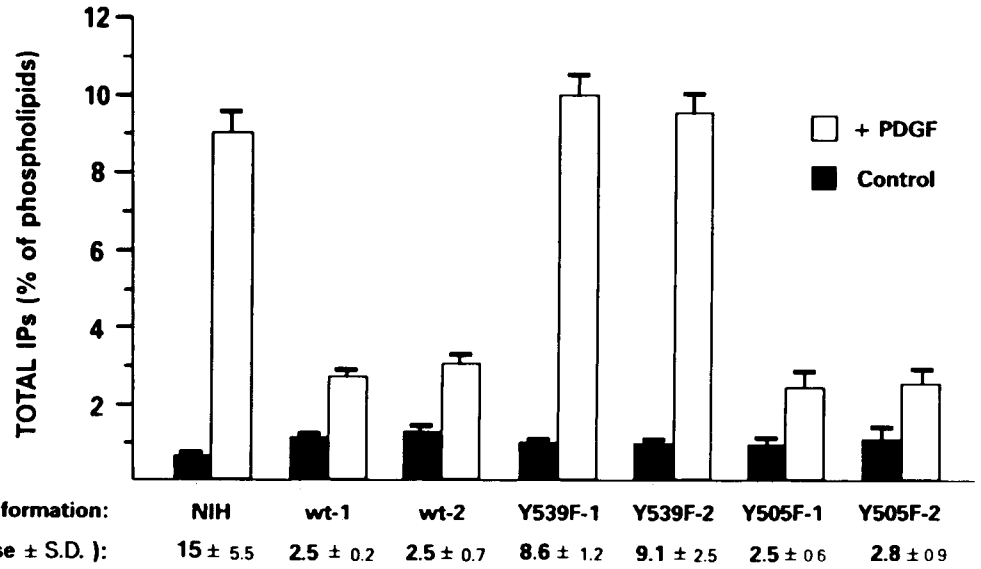

FIG. 8. Basal-level and PDGF-induced IP production. Resting NIH 3T3 cells (NIH) or NIH 3T3 cell lines expressing Ret/ptc2-wt (wt-1 and wt-2) or Ret/ptc2 mutant proteins carrying the indicated mutations (Y539F-1, Y539F-2, Y505F-1, and Y505F-2) were labeled with [^{3}H]myoinositol and then treated (□) with PDGF or not treated (■) in the presence of LiCl. Total IPs were purified on an AG1-X8 column. The data are expressed as percentages of the total cellular phospholipid. The experiment was done in triplicate, and the bars indicate standard deviations (S.D.). The ratios with standard deviations for three independent experiments are reported at the bottom.

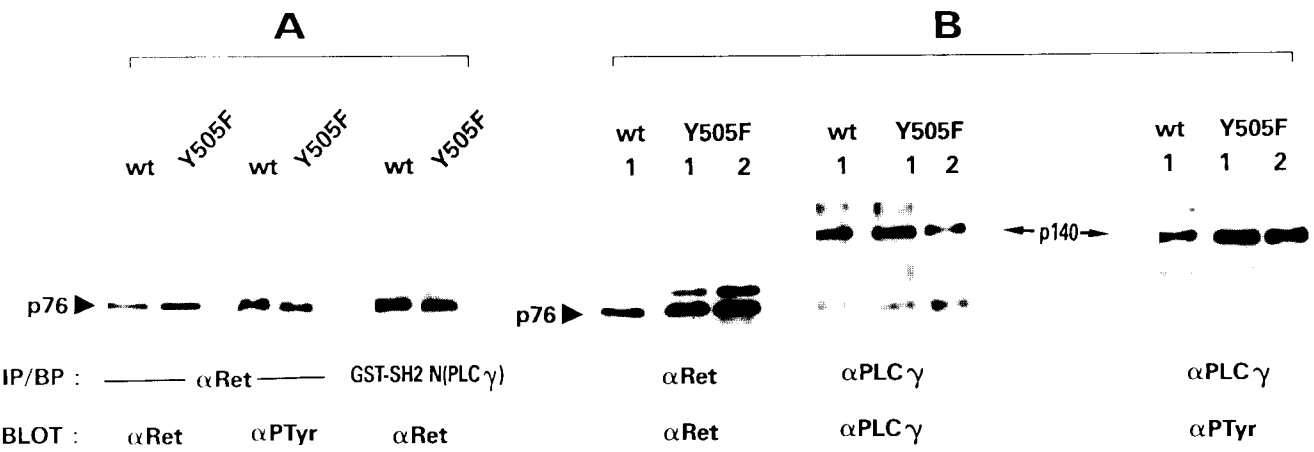

FIG. 9. PLCγ phosphorylation in NIH 3T3 cell lines expressing Ret/ptc2-wt or Ret/ptc2-Y505F proteins and in vitro binding between the SH2 N-terminal domain of PLCγ [N(PLCγ)] and Ret/ptc2 proteins. (A) Proteins were transiently expressed in HeLa cells; (B) three independent NIH 3T3 cell lines (indicated as wt-1 and Y505F-1 and -2) expressing either Ret/ptc2-wt (wt) or Ret/ptc2-Y505F (Y505F) proteins were analyzed. The protein extracts were immunoprecipitated (IP) with the indicated antisera (B) or used for binding experiments (A) with 5 μg of the specified GST-fused protein (BP).

rosine residue (Tyr-539 in Ret/ptc2, corresponding to Tyr-761 in the proto-*RET* product) essential for full transforming activity by Ret/ptc2 oncoproteins. In fact, substitution of Tyr-539 with phenylalanine left the kinase activity unchanged but strongly reduced (75 to 90%) both the in vitro and the in vivo oncogenicities of *RET/PTC2*, thus suggesting that this tyrosine is a docking site for SH2-bearing molecules essential for signalling pathways driving neoplastic cell transformation.

Whereas a number of substrates interacting with RTKs have been identified (reviewed in reference 28), little is known about substrate interaction mediating neoplastic transformation by RTK-derived oncoproteins. Beside *RET/PTC* oncogenes, other structurally related oncogenes, originating by fusion of a TK domain with an unrelated donor gene, have been isolated: *TRK* oncogenes (references 6 and 18 and references therein) derived from the nerve growth factor (NGF) receptor, *NTRK1* (proto-*TRK*), and the *TPR-MET* oncogene derived from the HGF/SF-R (26). The donor genes are able to promote a constitutive dimerization by homodimer formation, thus mimicking the effect of ligand binding to the relative RTK, as demonstrated for Tpr-met (33) and for Ret/ptc2 (7) oncoproteins.

The Ret/ptc oncoproteins, in contrast to the proto-*RET* gene product, are localized in the cytoplasm (16), as are the abovementioned Trk oncoproteins (19). In the latter case, the same cellular targets were suggested to be involved both for transmembrane receptor and for receptor-derived cytoplasmic oncoproteins. We have in fact demonstrated Shc activation in NIH 3T3 cells expressing the proto-Trk receptor and treated with NGF and in untreated NIH 3T3 cell lines containing Trk oncoproteins (6). Analogously, Omichi et al. (24) have detected constitutive phosphorylation of PLCγ in cells containing a Trk oncoprotein, similar to what has been observed for NGF-activated proto-Trk by other investigators (23, 44). In addition, Tpr-met oncoprotein coimmunoprecipitates with p85, the PI3 kinase regulative subunit, and with the adapter protein Grb2 (11), as the corresponding HGF receptor activated by its ligand does (32).

In contrast to the situation for NGF and HGF receptors, the signal transduction triggered by proto-Ret is largely unknown, except for some evidence deriving from studies of a chimeric *EGFR-RET* receptor (35, 43) and of *RET/PTC* oncogenes (6). Information about Ret/ptc biochemical pathways could also help in understanding the biochemical mechanisms involved in the different *RET*-associated diseases. In fact, besides somatic tumor-specific rearrangements of *RET* detected in 35% of papillary thyroid carcinomas (4), different germ line missense mutations of this gene have been associated with inherited predispositions to cancer (MEN2A, FMTC, and MEN2B), as well as with the congenital malformation Hirschsprung disease (reviewed in reference 42).

Tyr-539 of Ret/ptc2 was selected for site-directed mutagenesis because the corresponding phosphorylated peptide, pY539, was capable of inhibiting in vitro the binding between Ret/ptc2 and both SH2 domains of PLCγ, displaying a high affinity for the N-SH2 domain, comparable to that reported for the PDGF receptor (17). In addition, the peptide containing Tyr-539 was demonstrated in vitro to constitute a target of phosphorylation by Ret kinase itself, thus indicating the potential phosphorylation of this residue in the intact Ret/ptc2 protein. Moreover, PLCγ is likely to be directly recruited by Ret/ptc2 oncoproteins; in fact, it is phosphorylated on tyrosine, coimmunoprecipitates with the oncoproteins in intact cells, and binds Ret/ptc2 directly in vitro. The PLCγ recruitment and phosphorylation by Ret/ptc oncoproteins were comparable to those elicited by Trk oncoproteins (data not shown). The results of phosphoinositol analysis with cell lines expressing wt or Y539F Ret/ptc2 oncoproteins were in agreement with a constitutive activation of PLCγ, as demonstrated by high-level Tyr phosphorylation, only in the cell lines expressing Ret/ptc2-wt. Since Ret/ptc oncoproteins are constitutively activated, it was not feasible to study IP production by a conventional ligand stimulation approach. However, it has been reported that spontaneously transformed cells or NIH 3T3 cells transformed by the *TRK* oncogene showed basal IP levels similar to those of NIH 3T3 cells, but they were not able to increase IP production in response to serum, PDGF, or bombesin as untransformed parental cells were (1). We have therefore analyzed the ratio of IPs in PDGF-stimulated and unstimulated cells. Ret/ptc2-expressing cell lines and untransfected NIH 3T3 were therefore assayed. By this approach, we showed that in control untransfected NIH 3T3 cells PDGF stimulation induced IP production corresponding to about 15-fold the basal level. On the contrary, in NIH 3T3 cells expressing Ret/ptc2-wt the same stimulation caused only 2.5-fold IP production. This result could be explained by presuming that PLCγ, constitutively recruited by

Ret/ptc2, was not available to be activated by stimulated PDGF receptor. On the contrary, the cells expressing Ret/ptc2-Y539F behave as the parental NIH 3T3 cells, thus suggesting a weak basal PLCγ activation by this mutant protein.

By phosphopeptide competition experiments, we have identified Tyr-505 as a second putative docking site for PLCγ within the Ret sequence. However, when Tyr-505 of Ret/ptc2 was mutagenized to phenylalanine, NIH 3T3 cell lines expressing this mutant protein showed tyrosine phosphorylation and enzymatic activity of PLCγ indistinguishable from those of Ret/ptc2-wt-expressing cell lines. Similar findings have already been reported. The elimination of a tyrosine on the HGF receptor, identified by phosphopeptide competition as a docking site for p85-PI3 kinase, did not affect the association with this signal transducer (31). We have also demonstrated that, by eliminating Tyr-539, the resulting Ret/ptc2 mutant protein induces a low level of PLCγ phosphorylation in the expressing cells. Tyr phosphorylation of PLCγ was estimated to be reduced to about 10% of that displayed by cell lines expressing Ret/ptc2-wt, thus suggesting that Tyr-539 is the major docking site of Ret/ptc2 for PLCγ. Moreover, the mutagenized oncogene showed a significantly reduced oncogenic activity, revealed not only by a strong reduction in the number of transformation foci but also by the number, latency, and size of tumors generated in nude mice. The result that a single autophosphorylation site is necessary for the expression of full oncogenicity has been reported also for *NEU/ERBB2* and *TPR-MET* oncogenes (2, 32). On the other hand, when the ligand-induced mitogenicity was analyzed, a single autophosphorylation site appeared sufficient to restore the activity of a mutated RTK, as found, for instance, with the PLCγ or PI3 kinase docking site on the PDGF receptor (41). These studies and the present results suggest that mitogenic pathways could be more redundant than oncogenic ones.

The strong reduction of transforming activity obtained by eliminating the putative docking site Tyr-539 and the good correlation observed between transforming activity and PLCγ activation in NIH 3T3 cell lines expressing Ret/ptc2-wt or Ret/ptc2-Y539F protein clearly indicate that activation of PLCγ could be an essential step for the oncogenicity of Ret/ptc2 protein. The possibility that PLCγ plays a direct role in mitogenic signal transduction is supported by the observation that microinjection of purified PLCγ enzyme initiates a DNA synthesis response (36). In addition, PLCγ is involved in signal transduction by a variety of cell surface receptors and for some (i.e., PDGFR [41]) but not for all (i.e., FGFR [30]) is able to couple to mitogenic signalling pathways. Recently, PLCγ was also suggested to play a role in neoplastic transformation triggered by middle T antigen of polyomavirus (38).

We cannot exclude the possibility that Tyr-539 could be, alternatively, a docking site for multiple oncogenically relevant SH2-containing transducing proteins. In fact, it was previously demonstrated with other systems that a single phosphotyrosine could bind a number of SH2-containing proteins (22, 32). For this reason, we have tested the phosphorylation status of the adapter protein Shc, already demonstrated to be activated in cells expressing Ret/ptc oncoproteins (6) and whose binding to Ret/ptc2 was slightly inhibited by pY539 peptide. No variation in the phosphorylation of Shc was demonstrated when Ret/ptc2-Y539F instead of the wt was present in the cell. This fact was not surprising, since in other systems also Shc does not have a strict phosphorylation site requirement (47). In addition, although we cannot rule out the possibility that Tyr-539 could be a binding site for other transducing molecules, we have shown that the pY539 phosphopeptide does not inhibit the binding between Ret/ptc2 and the SH2 domains of Syp, src, and Grb2 (data not shown).

In conclusion, this paper demonstrates that Tyr-539 of Ret/ptc2 (Tyr-761 on the proto-*RET* product) is an essential docking site for the oncogenic potential of this transforming gene. Our results also suggest that PLCγ is a crucial transducer of the neoplastic signal triggered by the Ret/ptc oncoproteins.

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