

Definition of an Inhibitory Juxtamembrane WW-like Domain in the Platelet-derived Growth Factor β Receptor*

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A variety of tumors contain activating mutations in the cytoplasmic juxtamembrane domain of the type III family of receptor-tyrosine kinases, and some constructed mutations in this domain induce ligand-independent receptor activation. To explore the role of this domain in regulation of receptor activity, we subjected the juxtamembrane domain of the murine platelet-derived growth factor (PDGF) β receptor to alanine-scanning mutagenesis. The mutant receptors were expressed in Ba/F3 cells and tested for constitutive tyrosine phosphorylation, association with phosphatidylinositol 3'-kinase, and their ability to induce cell survival and proliferation in the absence of interleukin-3. The mutant receptors accumulated to similar levels and appeared to undergo a normal PDGF-induced increase in tyrosine phosphorylation. Alanine substitutions at numerous positions located throughout the juxtamembrane domain caused constitutive receptor activation, as did an alanine insertion in the membrane-proximal segment of the juxtamembrane domain and a six-amino acid deletion in the center of the domain. It is possible to model the PDGF receptor juxtamembrane domain as a short α -helix followed by a three-stranded β -sheet very similar to the known structures of WW domains. Strikingly, the activating mutations clustered in the central portions of the first and second β strands and along one face of the β -sheet, whereas the loops connecting the strands were largely devoid of mutationally sensitive positions. These findings provide strong support for the model that the activating mutations in the juxtamembrane region stimulate receptor activity by disrupting an inhibitory WW-like domain.

Receptor tyrosine kinases (RTKs)¹ are transmembrane proteins that regulate numerous aspects of cell physiology including proliferation and survival. Binding of a soluble ligand to the

extracellular domain of these receptors typically induces receptor dimerization and trans-phosphorylation of the cytoplasmic catalytic domain. This tyrosine phosphorylation stimulates the intrinsic tyrosine kinase activity of the receptor and generates binding sites for signaling proteins containing SH2 domains. Although ligand-induced dimerization is an important trigger of receptor activation, receptor activity is also subject to additional levels of regulation. For example, the cytoplasmic juxtamembrane region of receptor tyrosine kinases, which is located between the transmembrane domain and the kinase domain, has been implicated in regulation of receptor enzymatic activity (e.g. see Ref. 1–6).

The type III family of RTKs is defined by the presence of five extracellular immunoglobulin-like domains and a cytoplasmic kinase domain that is interrupted by a kinase insert segment (see Fig. 1). The cytoplasmic juxtamembrane domain is highly conserved between different members of this receptor family (Fig. 1), which include the platelet-derived growth factor (PDGF) α and β receptors, the colony-stimulating factor-1 (CSF-1) receptor, the stem cell factor receptor (c-kit), and FLT-3. Recently, a variety of tumors in humans and animals have been shown to harbor activating mutations in the juxtamembrane domain of c-kit and FLT-3. Various juxtamembrane c-kit mutations have been detected in sporadic and hereditary mast cell tumors, sinonasal lymphomas, and gastrointestinal stromal tumors (7–13). Most of these mutations are located in the membrane-proximal segment of the juxtamembrane domain, as is an activating mutation in a form of v-kit (14). Similarly, internal tandem duplications in the juxtamembrane domain of FLT-3 are present in some cases of acute myeloid leukemia (15, 16). The juxtamembrane c-kit and FLT-3 mutations recovered from tumors often result in increased receptor tyrosine phosphorylation, and the mutant receptors confer growth factor independence in various test cell systems.

There has been little systematic mutational analysis of the juxtamembrane domain of the type III RTKs. We reported previously that mutation of a juxtamembrane valine to alanine at position 536 in the murine PDGF β receptor caused constitutive receptor tyrosine phosphorylation (3). The biological activity of this mutant was tested in Ba/F3 cells, a murine hematopoietic cell line devoid of endogenous PDGF β receptor that normally requires interleukin-3 (IL-3) for survival and growth (17, 18). Expression of the constitutively active PDGF β receptor juxtamembrane mutant but not the wild-type PDGF β receptor allowed Ba/F3 cells to proliferate after IL-3 deprivation (3). Activation of the PDGF β receptor by this mutation did not depend on the ability of the receptor to bind PDGF and was associated with increased dimerization of the receptor and constitutive association with several SH2 domain-containing

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¹ The abbreviations used are: RTK, receptor-tyrosine kinase; PDGF, platelet-derived growth factor; CSF, colony-stimulating factor; IL, interleukin; MOPS, 4-morpholinepropanesulfonic acid.

signal transduction proteins. Alanine substitutions at the homologous position also activated c-kit, the CSF-1 receptor, and the human PDGF α and β receptors. Ma *et al.* (19) subsequently reported that alanine mutations at several positions in the membrane-proximal portion of the juxtamembrane domain of c-kit increased receptor tyrosine phosphorylation in COS cells overexpressing these mutants, but the biological activities of these mutants were not determined.

The molecular basis for receptor activation by the juxtamembrane mutations in type III RTKs is not known. The two tyrosines located in the center of the juxtamembrane domain are phosphorylated in response to treatment with ligand, thereby generating binding sites for SH2 domain signaling proteins (20, 21). Although mutation of these tyrosines to phenylalanine severely impairs ligand-induced PDGF β receptor kinase activity in porcine aortic endothelial cells and human hepatoma cells (1, 21), PDGF β receptors lacking these tyrosines can support PDGF- or *v-sis*-induced proliferation in other cell types, including Ba/F3 cells (18, 22). Ma *et al.* (19) proposed that a 10-amino acid membrane-proximal segment of the wild-type c-kit juxtamembrane domain adopts an α -helical conformation that inhibits receptor activity and that the activating mutations disrupted this inhibitory conformation. We noted previously that the sequence of the juxtamembrane domain of type III RTKs resembled the consensus sequence of a WW domain (3), a modular protein-protein interaction domain present in many signaling proteins (23).

Here we carried out a systematic alanine-scanning mutational analysis of the juxtamembrane domain of the murine PDGF β receptor and identified a number of positions that, when mutated, resulted in constitutive receptor activation. The results of this analysis, combined with molecular modeling, strongly suggest that the juxtamembrane sequence of the type III RTKs constitutes an inhibitory WW-like domain that can be disrupted by a large variety of mutations, resulting in receptor activation.

EXPERIMENTAL PROCEDURES

Mutant Construction—pRV-mPR-V536A was described previously (3). pmPRV-3, a pLXSN-based retroviral vector carrying the wild-type murine PDGF β receptor (24), was used as a template in standard site-directed mutagenesis reactions (Quick-Change site-directed mutagenesis kit; Stratagene) to generate mutants PR-I532A, PR-R533A, PR-W561A, and PR-P564A. The other juxtamembrane mutations were subcloned as *SacII* fragments into the *SacII* site of pLXSN-mPR- Δ JM, a mutant in which DNA encoding the wild-type juxtamembrane domain from the proline at position 528 to the arginine at position 565 was deleted and replaced with a unique *SacII* site. These *SacII* fragments were generated either by polymerase chain reaction using a mutagenic primer or by annealing oligonucleotides containing the desired mutation. These procedures did not alter the protein sequence at the ends of the insertion. The DNA sequence of the entire juxtamembrane domain was determined for each mutant. Details of mutation construction are available from the authors on request.

Retrovirus Stocks and Cells—Retroviral vectors were packaged by calcium phosphate-mediated transfection of BOSC or Phoenix-Ampho packaging cells (obtained from the ATCC) (24). Two days after transfection, virus-containing supernatants were harvested and used to infect Ba/F3 cells (obtained from Alan D'Andrea, Dana-Farber Cancer Institute, Boston, MA), which were maintained in RPMI/IL-3 medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 5% WEHI conditioned medium to provide IL-3, 0.05 mM β -mercaptoethanol, and antibiotics). Cell lines expressing the various wild-type and mutant receptors were established as described before (18). Briefly, $\sim 10^5$ colony-forming units of retrovirus were incubated with 1×10^6 cells in 10 ml of RPMI/IL-3 containing 4 mg/ml of Polybrene for 2 days. 1 ml of the cells was then transferred into 10 ml of RPMI/IL-3 containing 1 mg/ml of G418 (Invitrogen). After three-four passages into medium containing G418, mock-infected cells had died, and pooled stable drug-resistant cell lines were obtained from infected cells.

IL-3 Independence Test—IL-3 independence tests were performed as described previously (24). Briefly, Ba/F3 derivative cell lines were

grown in RPMI/IL3 medium to density of $\sim 10^5$ cells/ml, harvested by low speed centrifugation, and washed once with phosphate-buffered saline. Cells were transferred to a T-25 flask containing 10 ml of RPMI/No IL-3 medium (RPMI 1640 formulated as above but without WEHI conditioned medium) at a density of 1.5×10^4 cells/ml. The cells were then incubated at 37 °C for 5 to 7 days, and viable cells were counted by using a hemocytometer to assess cell proliferation. For each IL-3 test, cells expressing wild-type PDGF β receptor and PR-V536A were included as controls. The number of viable cells is expressed in Fig. 3 and Fig. 6 as the percentage of cells arising in cultures expressing PR-V536A. The results determined for multiple cell lines of each genotype were averaged, and the standard deviation is shown.

Immunoprecipitation and Immunoblotting—Cells grown in medium containing IL-3 were harvested by low speed centrifugation and washed twice with cold phosphate-buffered saline. Protein extracts were obtained by lysing cells in radioimmune precipitation assay-MOPS buffer (20 mM MOPS, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, and 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 10 mg/ml leupeptin, and 10 mg/ml aprotinin. Saturating amounts of PDGF receptor antiserum were added to ~ 500 or 1000 μ g of extracted protein, respectively, for detection with anti-PDGF receptor or anti-phosphotyrosine antibody. For immunoprecipitation, antibodies were incubated with extracts at 4 °C for 4 h, followed by the addition of 50 μ l of protein A-Sepharose beads (Amersham Biosciences) (50% slurry in 10 mM Tris-HCl, pH 7.4, 165 mM NaCl, 10% bovine serum albumin) and incubation at 4 °C overnight. The beads were then washed five times with NET-N buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 0.1% (v/v) Tween 20), boiled for 5 min in 2 \times Laemmli sample buffer, and electrophoresed on an SDS-7.5% polyacrylamide gel as described previously (18). After transfer to nitrocellulose, total PDGF β receptor was visualized by probing with rabbit anti-PDGF receptor antibody followed by horseradish peroxidase protein A, and tyrosine-phosphorylated PDGF β receptor was visualized with PY100 monoclonal anti-phosphotyrosine antibody (Cell Signaling Technology, Inc.) and donkey anti-mouse immunoglobulin horseradish peroxidase. Following detection by ECL+ (Amersham Biosciences), bands were visualized, and fluorescent intensity was measured on a Storm 840 (Molecular Dynamics, Inc.).

For co-immunoprecipitation experiments, actively growing cells were washed once with phosphate-buffered saline, resuspended, and incubated in RPMI/IL-3 medium containing 0.5% fetal bovine serum for 24 h. Extracts were prepared in radioimmune precipitation assay-MOPS buffer. 50 μ g of extracted protein were immunoprecipitated with anti-PDGF receptor antiserum, electrophoresed, and transferred to filters as described above. To detect phosphatidylinositol 3-kinase associated with the PDGF β receptor, Western blotting was performed as described previously using anti-rat phosphatidylinositol 3-kinase rabbit polyclonal serum (catalog number 06-195; Upstate Biotechnology, Inc.) at a 1:1000 dilution (18).

To test the ability of PDGF to induce tyrosine phosphorylation of the PDGF β receptor, 4×10^7 cells were serum-starved and harvested as described above. Recombinant human PDGF-BB (catalog number 13244-033; Invitrogen) was added at 50 ng/ml for 15 min at room temperature and then protein extracts were obtained. PDGF β receptor immunoprecipitation and anti-phosphotyrosine immunoblotting were performed as above.

Molecular Modeling—The homology model of the WW domain was made using the program Modeler (Molecular Simulations Inc., San Diego, CA). The juxtamembrane domain sequence was aligned manually against the sequence of the designed WW domain whose structure has been determined by NMR spectroscopy (28). The program derives spatial restraints from the reference structure. The modeled structure is optimized using conjugate gradient energy minimization followed by restrained simulated annealing molecular dynamics. The figures were generated with the program InsightII (Molecular Simulations Inc., San Diego, CA). The secondary structure was defined by the Kabsch-Sander algorithm, which is based on hydrogen bonding patterns.

RESULTS

Alanine-scanning Mutations of the Murine PDGF β Receptor Juxtamembrane Domain—To analyze the role of the juxtamembrane domain in regulation of PDGF β receptor activity, we performed an alanine-scanning mutational analysis of this region. We generated and analyzed a panel of mutants carrying single alanine substitutions at residues Arg-529 through Trp-561, a sequence that comprises almost the entire juxtamem-

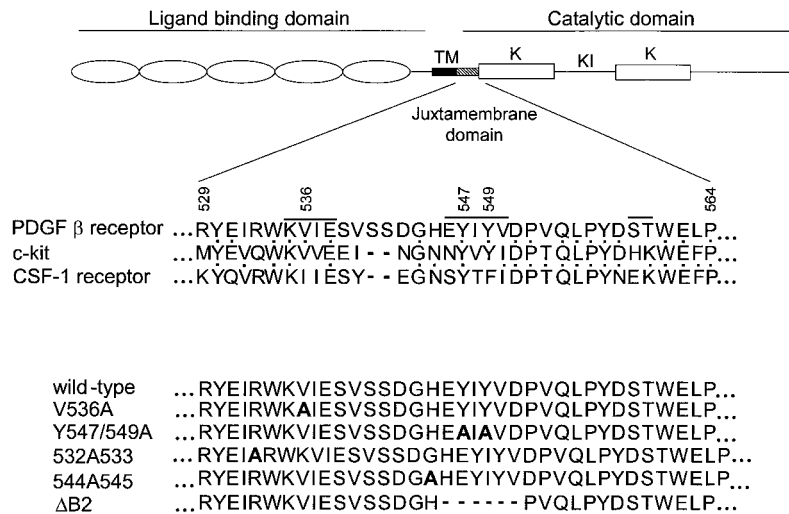


FIG. 1. PDGF β receptor juxtamembrane mutations. The upper portion shows a schematic diagram of the type III family of RTKs. The five immunoglobulin-like motifs in the extracellular ligand binding domain are shown as ellipses, the transmembrane (TM) domain as the black box, and the juxtamembrane domain as the hatched box. The intracellular catalytic domain contains the kinase domain (K) split by a kinase insert region (KI). The juxtamembrane domain is expanded to show the sequence of three members of the type III family of RTKs, the murine PDGF β receptor, human c-kit, and human CSF-1 receptor. The numbers indicate the positions of key amino acids in the murine PDGF β receptor numbering system. The lines over the PDGF β receptor sequence indicate the anti-parallel β -strands predicted by the molecular modeling. The lower portion of the figure shows the juxtamembrane domain sequence of the wild-type PDGF β receptor and selected mutants. The mutated residues are shown in boldface or, in the case of the deletion, by dashes.

brane domain of the murine PDGF β receptor. Tyr-547 and Tyr-549, which when phosphorylated are recognized by SH2 domain proteins such as src family kinases (21), were both changed to alanines in the mutant PR-Y547A/Y549A to eliminate any contribution of the binding of SH2 proteins to the juxtamembrane domain (Fig. 1). We did not mutate Ser-541 or Ser-542, because these residues appear to be inserted relative to c-kit and the CSF-1 receptor, even though the juxtamembrane sequences of these receptors are otherwise quite homologous to the PDGF β receptor (Fig. 1). Each of the mutants was cloned into a retrovirus vector containing a G418 resistance gene and introduced into Ba/F3 cells. Stable cell lines expressing the various receptors were generated by infection followed by selection for G418. The wild-type PDGF β receptor and PR-V536A, the original activated mutant, were used as controls.

Expression and Tyrosine Phosphorylation of the Alanine-scanning Mutants—The expression and phosphotyrosine content of the mutant receptors were analyzed by immunoblotting. Detergent extracts of cells were immunoprecipitated with anti-PDGF β receptor antibody and blotted with either the same antibody (to assess PDGF β receptor levels) or an anti-phosphotyrosine monoclonal antibody (to assess the level of tyrosine phosphorylation of the receptors). The mature and the more rapidly migrating precursor forms of the exogenous PDGF β receptor were expressed at similar levels in all the cell lines (representative examples are shown in the top panel of Fig. 2). Thus, none of the mutations had a significant effect on receptor stability or processing.

To determine whether the mutations induced constitutive activation of the PDGF β receptor, we examined the level of tyrosine phosphorylation of the mutant receptors in the absence of ligand stimulation (Fig. 2). The wild-type PDGF β receptor and many of the mutant receptors did not display significant tyrosine phosphorylation. In contrast, the previously identified activated mutant PR-V536A contained high levels of phosphotyrosine, as did PR-Y530A, PR-W534A, PR-I537A, PR-L555A, PR-Y557A, and the double mutant PR-Y547A/Y549A. PR-E531A and PR-D551A displayed a modest increase in tyrosine phosphorylation compared with the wild-

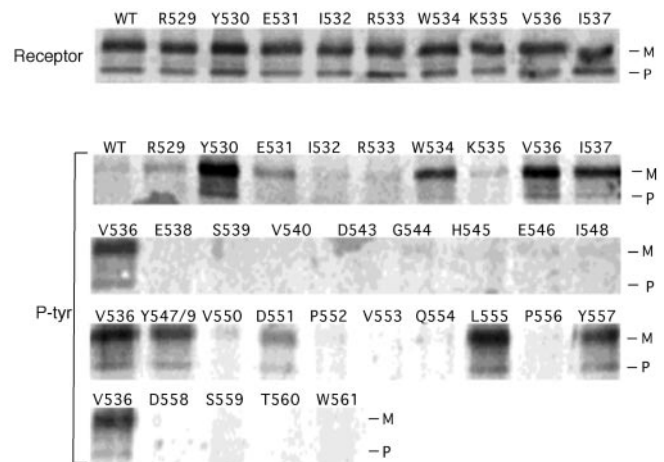


FIG. 2. Expression and tyrosine phosphorylation of alanine-scanning mutants. The top panel shows a PDGF β receptor immunoblot of representative PDGF β receptor immunoprecipitates. The other panels show anti-phosphotyrosine immunoblots for all the alanine-scanning mutants. The designations above the lane identify the wild-type PDGF β receptor (WT) or each mutant (designated by the single letter amino acid code for the wild-type amino acid and the position). In each group of mutants, cells expressing PR-V536A were processed in parallel. M, mature form of the PDGF β receptor; P, precursor form.

type receptor. Similar results were obtained when independent cell lines established with the same mutant were tested or when a single cell line was tested in multiple independent blots (data not shown). The results shown in Fig. 2 were quantitated and are summarized in Fig. 3. For this analysis, tyrosine phosphorylation of PR-V536A was measured simultaneously with each group of mutants and was arbitrarily set at 100%.

As another measure of receptor activation, we used co-immunoprecipitation to determine whether the tyrosine-phosphorylated alanine substitution mutants formed stable complexes with the p85 subunit of phosphatidylinositol 3'-kinase, which associates via its SH2 domain with specific phosphotyrosines in the kinase insert region (25). Detergent extracts were immunoprecipitated with anti-PDGF receptor antiserum and then

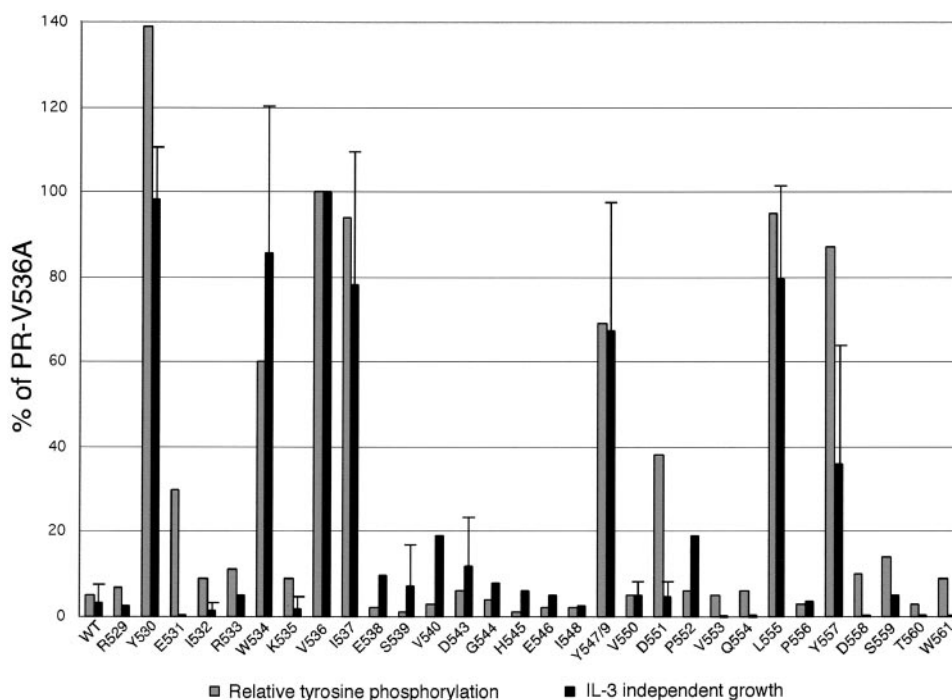


FIG. 3. **Tyrosine phosphorylation and induction of IL-3 independence by the alanine-scanning receptor mutants.** The results shown in Fig. 2 were quantitated by Storm 840 by using Imagequant software. Band intensity expressed as the percentage of the signal obtained for PR-V536A is shown in the *light bars*. As described under "Experimental Procedures," viable cells were counted approximately 1 week after IL-3 removal. The results in the *dark bars* are shown as a percentage of the number of cells in the PR-V536A cultures, with the standard errors indicated.

subjected to gel electrophoresis and immunoblot analysis with anti-p85 antiserum. As shown in Fig. 4, the wild-type PDGF β receptor did not associate with significant amounts of p85 unless the cells were treated with PDGF prior to extraction. Similarly, representative inactive mutants PR-K535A and PR-I548A did not constitutively associate with p85. In contrast, all of the activated mutants constitutively associated with p85.

Transforming Activity of Alanine-scanning Mutants—As a biological measure of constitutive receptor activation, we assessed the ability of the PDGF β receptor mutants to allow IL-3-independent proliferation of Ba/F3 cells. As expected, cells expressing wild-type PDGF β receptor did not proliferate following IL-3 removal, whereas cells expressing PR-V536A survived and proliferated to high density. The extent of IL-3-independent proliferation of cells expressing each mutant receptor was compared with the proliferation of PR-V536A cells tested in the same experiment. The averaged results obtained with multiple, independently derived cell lines for each mutant are shown in Fig. 3. The original activated mutant, PR-V536A, and several additional mutants, namely PR-Y530A, PR-W534A, PR-I537A, PR-L555A, and the double mutant PR-Y547A/Y549A, conferred IL-3 independence. Cells expressing PR-Y557A displayed a more variable IL-3-independent phenotype but in general grew to a lower saturation density or with a longer lag period than did cells expressing PR-V536A. The other mutants failed to confer significant IL-3 independence.

There was an excellent correlation between the level of constitutive tyrosine phosphorylation of the various mutant receptors and their ability to confer IL-3 independence (Fig. 3). In particular, PR-Y530A, PR-W534A, PR-V536A, PR-I537A, PR-L555A, PR-Y557A, and PR-Y547A/Y549A displayed substantially elevated phosphotyrosine and conferred IL-3 independence. Two mutants, PR-E531A and PR-D551A, which induced slightly elevated receptor tyrosine phosphorylation, failed to allow significant IL-3-independent growth. No mutants induced IL-3 independence in the absence of receptor tyrosine

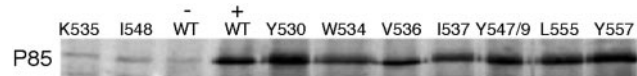


FIG. 4. **Complex formation between PDGF β receptor juxtamembrane mutants and phosphatidylinositol 3'-kinase.** Detergent extracts were prepared from cells expressing the wild-type PDGF β receptor (WT) in the presence (+) or absence (-) of PDGF treatment or from cells expressing the indicated mutants in the absence of PDGF treatment. After immunoprecipitation with anti-PDGF receptor antiserum, associated p85 subunit of phosphatidylinositol 3'-kinase was detected by immunoblotting.

phosphorylation. Thus, substitution of alanine at ~25% of the positions in the juxtamembrane region conferred IL-3 independence and increased receptor tyrosine phosphorylation and constitutive complex formation with phosphatidylinositol 3'-kinase. Furthermore, these activating positions were scattered throughout the juxtamembrane region.

Effect of Mutations Predicted to Perturb the Structure of the Juxtamembrane Domain—To explore the requirements for receptor activation in more detail, we tested the effects of several other mutations in the juxtamembrane domain of the PDGF β receptor. Mutations near Val-536 activated the receptor, but an alanine substitution at the adjacent lysine 535 did not activate (Fig. 3). To test whether the receptor was activated by mutations that reversed the charge of this lysine or reduced rotational freedom at this position, lysine 535 was replaced with aspartic acid and proline to generate PR-K535D and PR-K535P, respectively, and the mutant receptors were tested as described above. Even though the alanine substitution at position 535 did not activate the receptor, proline and aspartic acid at this position did activate, as assessed by induction of IL-3 independence and receptor tyrosine phosphorylation (see Fig. 5 and Fig. 6).

Because insertion of a single amino acid alters the relative orientation of the α -helical segments upstream and downstream of the site of insertion, we tested whether the insertion

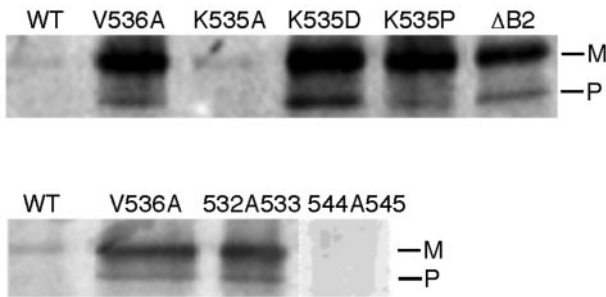


FIG. 5. Expression and tyrosine phosphorylation of additional juxtamembrane mutants. Extracts from cells expressing the wild-type (WT) PDGF β receptor and the indicated receptor mutants were immunoprecipitated with anti-PDGF receptor antiserum. After gel electrophoresis, levels of phosphotyrosine were measured by immunoblotting with anti-phosphotyrosine antibody. *M*, mature form of the PDGF β receptor; *P*, precursor form.

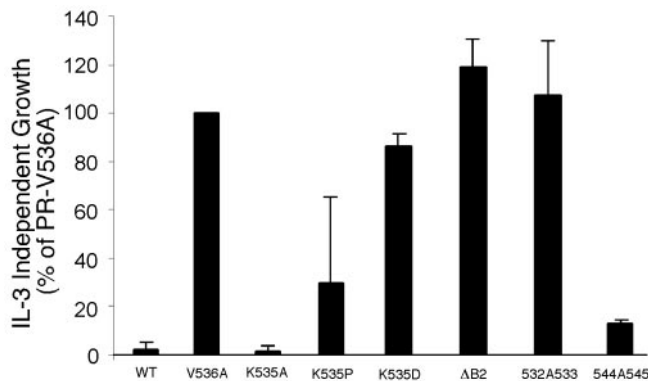


FIG. 6. IL-3-independent proliferation of additional juxtamembrane mutants. IL-3 independent proliferation was determined as in Fig. 3. WT, wild-type.

of an additional alanine residue in the juxtamembrane domain resulted in receptor activation. An alanine residue was inserted between positions 532 and 533 and between 544 and 545 to generate mutants PR-I532-A-R533 and PR-G544-A-H545, respectively (Fig. 1). As shown in Fig. 6, cells expressing PR-G544-A-H545 did not proliferate after IL-3 removal, but cells expressing PR-I532-A-R533 proliferated well in the absence of IL-3. Similarly, PR-G544-A-H545 did not contain significant levels of tyrosine phosphorylation, whereas PR-I532-A-R533 displayed high tyrosine phosphorylation (Fig. 5). Thus, the PDGF β receptor was activated by an alanine insertion in the membrane-proximal segment of the juxtamembrane region but not by an insertion near the middle of the region.

Finally, we constructed and tested a mutant receptor, PR- Δ B2, containing a six-amino acid deletion (residues 546 to 551) that removed the central tyrosines and flanking amino acids (Fig. 1). This mutant receptor was constitutively tyrosine-phosphorylated and induced IL-3 independence, indicating that deletion of the central portion of the juxtamembrane domain caused receptor activation (see Fig. 5 and Fig. 6). The exogenous PDGF β receptor was expressed at similar levels in cells expressing each of the mutants described in this section (data not shown).

Response of PDGF β Receptor Mutants to PDGF—The analysis described above identified several mutant receptors that did not confer IL-3 independence or display increased tyrosine phosphorylation. To establish that the juxtamembrane mutations did not merely inactivate the tyrosine kinase activity of these mutants or prevent their proper localization, we tested the ability of PDGF to induce tyrosine phosphorylation of the wild-type receptor and the mutant PDGF β receptors that

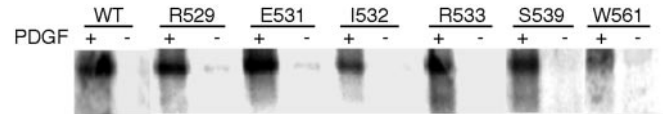


FIG. 7. PDGF-stimulated tyrosine phosphorylation of PDGF β receptor. Cells expressing the wild-type (WT) PDGF β receptor or the indicated receptor mutants were treated with PDGF as described under "Experimental Procedures" (+) or left untreated (-). Protein extracts were then prepared and tested by immunoblotting for PDGF receptor tyrosine phosphorylation.

harbored non-activating mutations. Cells expressing these receptors were deprived of serum for 24 h and then treated with PDGF-BB or left untreated. Cell extracts were immunoprecipitated with anti-PDGF receptor antiserum and immunoblotted with an anti-phosphotyrosine monoclonal antibody. The mature, cell-surface form of the non-activated mutant receptors became tyrosine-phosphorylated after PDGF treatment (Fig. 7, and data not shown), indicating that the mutant receptors transited to the cell surface and that the mutations did not abolish tyrosine kinase activity. PR-W561A reproducibly displayed less of an increase in tyrosine phosphorylation than the other mutants, presumably because this mutation impinged on the beginning of the kinase domain.

We also tested the ability of a limited set of the non-activated mutants to support proliferation of cells in the presence of PDGF following IL-3 deprivation. All of the tested mutants allowed proliferation in the presence of PDGF, but the proliferation of cells expressing PR-W561A was impaired compared with cells expressing the wild-type PDGF β receptor or the other tested mutants (data not shown).

Molecular Modeling—The juxtamembrane sequence likely adopts a well-defined structure that connects the transmembrane and the kinase domains, because these domains are known to be rotationally coupled in RTKs (26, 27). Our mutational analysis, which showed that sensitive amino acids tended to cluster in discrete regions of the juxtamembrane sequence, supports this idea. To explore whether the juxtamembrane domain of the type III RTKs has the potential to adopt a WW domain fold, we modeled the segment from Tyr-530 to Trp-561 based on the NMR structure of a designed WW domain derived from a consensus WW domain sequence (28) (Fig. 8a). We first aligned the juxtamembrane sequence of the PDGF β receptor with that of the consensus WW domain. Using the high resolution NMR structure as a template, the program Modeler then built an initial model, which was refined by energy minimization followed by restrained simulated annealing molecular dynamics.

This analysis showed that the juxtamembrane sequence between the two Trp residues can form three hydrogen-bonded anti-parallel β -strands characteristic of a WW domain (see Fig. 1 and Fig. 8c). The distribution of amino acids in the model is reasonable from a structural standpoint, with the central portions of the three β -strands being largely hydrophobic, whereas the charged and polar residues are solvent accessible. The core of the domain is formed by Trp-534, which is packed between Tyr-530 and Val-550. As is the case for known WW domains, two central aromatic amino acids, in this case tyrosines 547 and 549, project from the concave face of the domain (Fig. 9), which is the face that contacts the protein ligand in solved WW domain structures. The juxtamembrane sequences of the c-kit and CSF-1 receptors also yielded similar WW domain folds when modeled by this program (data not shown). Importantly, the unrelated juxtamembrane sequence of the EphB2 receptor did not converge into a three-stranded β -sheet structure when homology modeled as a WW domain (Fig. 8b). Although the modeling program threads the EphB2 juxtamembrane se-

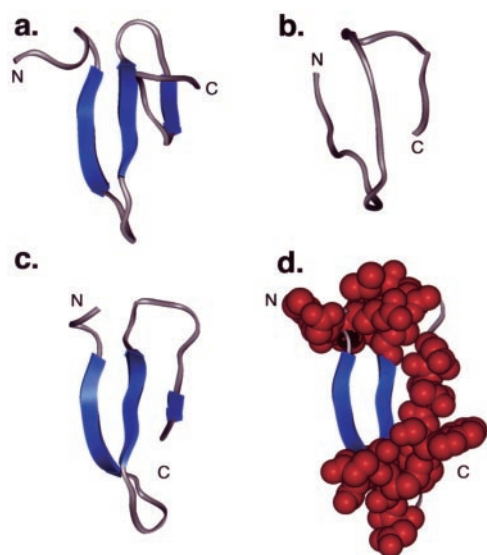


FIG. 8. Molecular modeling of the juxtamembrane sequence of the PDGF β receptor. Panel *a* shows a representation of the high resolution NMR structure of a designed WW domain (Protein Data Bank access code 1EOM) in which the trace of the peptide backbone is shown in gray, and the three characteristic anti-parallel β -strands are shown as flat blue arrows. A homology model of the PDGF β receptor juxtamembrane domain (amino acids Tyr-530 to Trp-561) was built on the structure shown in panel *a* and exhibits the characteristic WW domain fold and β -strands (panel *c*). PDGF β receptor amino acids where two or more consecutive positions are insensitive to alanine substitution are shown as red van der Waals surfaces (panel *d*). The insensitive amino acids are clustered in the loops connecting the β -strands and in the third β -strand. The juxtamembrane sequence of the EphB2 receptor cannot be modeled as a three-stranded anti-parallel β -sheet (panel *b*). *N* and *C* refer to the amino and carboxyl terminus of the juxtamembrane domain, respectively. The models shown in panels *b-d* are viewed from the membrane surface.

quence along the backbone carbon trace of the WW domain structure, proline residues in the sequence disrupt the β -sheet by preventing the formation of interstrand hydrogen bonds during the dynamics simulations. This is consistent with the sequence and crystal structure of the EphB2 receptor, which do not predict the existence of a WW domain in the juxtamembrane region (6). Moreover, the juxtamembrane sequences of type III family receptors cannot be modeled into the known structure of the EphB2 receptor because of the occurrence of a proline residue (Pro-556 in the murine PDGF β receptor) at the position of the β -helix in the EphB2 structure (6).

The juxtamembrane sequence of the PDGF β receptor between the two tryptophans is five amino acids longer than the consensus WW domain sequence. However, the inserts of two and three amino acids occur at the two turns in the model, where they are predicted not to perturb the overall domain fold (Fig. 1). One of the unusual features of the PDGF β receptor juxtamembrane sequence, which is conserved in other members of the type III RTK family, is the presence of prolines at positions 552 and 556 that fall in the turn between the second and third β -strands and are likely to facilitate formation of the turn.

The orientation and structure of the N-terminal and C-terminal residues in the model are consistent with the juxtamembrane region connecting the transmembrane and kinase domains of the receptor. The four residues (Tyr-530–Arg-533) that are immediately N-terminal to Trp-534 are modeled as α -helix and link the juxtamembrane region to the α -helical transmembrane domain (Fig. 9*a*). This helical secondary structure results in Tyr-530 packing on Trp-534 in an orientation having stabilizing π - π interactions between the aromatic rings. The C-terminal residues in our model, which connect the

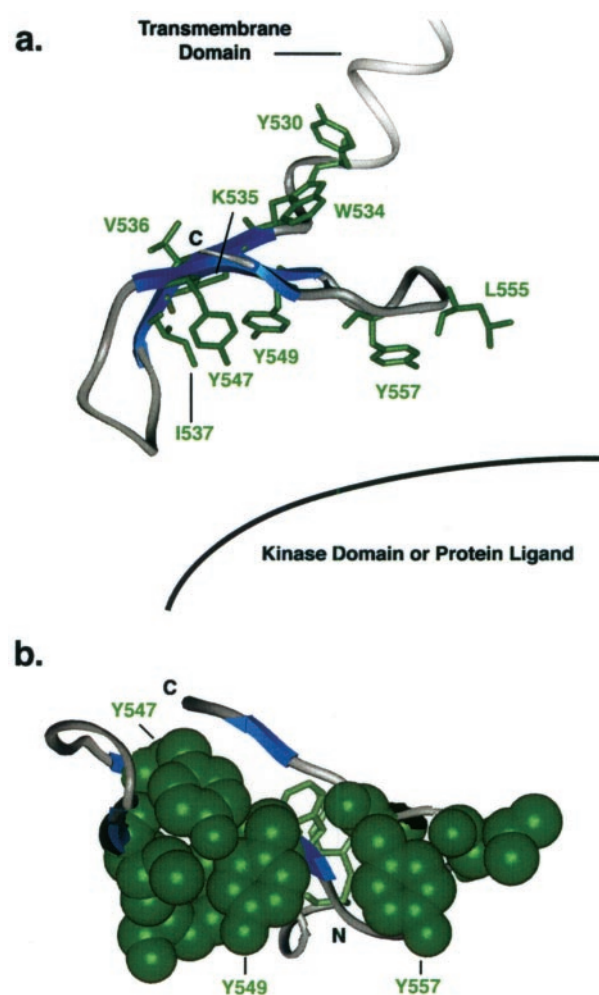


FIG. 9. Molecular model of the juxtamembrane sequence of the PDGF β receptor. Panel *a* presents an edge-on view of the model of the PDGF β receptor juxtamembrane domain shown in Fig. 8*c*, in which the side chains of the amino acids that confer activation upon substitution are shown in green. The predicted position of the transmembrane helix is shown as a light gray ribbon. Activating mutations between the two tryptophans fall largely on the first and second β -strands and line the concave face of the WW-like domain (in the region of Fig. 8*d* that is not occluded by the red van der Waals spheres). The concave face is hydrophobic and may interact with the kinase domain of the receptor or with a protein ligand recognizing the WW-like domain fold. Panel *b* shows the concave surface of the juxtamembrane domain model viewed from the cytoplasmic interior, produced by rotating the structure in panel *a* by 90°. Ile-537, Tyr-547, Tyr-549, Tyr-557, and Leu-555 are shown as green van der Waals spheres. These residues form a nearly continuous hydrophobic surface, which we propose interacts with the kinase domain or a protein ligand. *N* and *C* refer to the amino and carboxyl terminus of the juxtamembrane domain, respectively.

juxtamembrane sequence to the kinase domain, are in extended β -structure. The sequence from Leu-555 to Asp-566 is well conserved between the PDGF β receptor and the fibroblast growth factor receptor 1. Importantly, these residues are resolved in the crystal structure of the kinase domain of the FGF receptor and adopt extended structure consistent with our model. In the crystal structure, the Trp corresponding to Trp-561 in the PDGF β receptor is oriented toward and packed against the kinase domain (29).

Mutational Evaluation of the Proposed Juxtamembrane Model—The effect of mutations in the juxtamembrane domain is consistent with the model that the juxtamembrane domain forms a short α -helical segment followed by a WW domain fold. The receptor was activated by mutations at Tyr-530 and Trp-534, which are absolutely conserved in the other type III RTKs

with juxtamembrane WW-like domains (Fig. 1). Although alanine substitutions at the intervening residues did not activate the receptor, an alanine insertion between Ile-532 and Arg-533 did activate. This is the pattern expected if helical secondary structure occurs between Tyr-530 and Trp-534 and if these two residues need to maintain their stacked orientation. Furthermore, in studies of WW domain stability, the first Trp in the sequence (corresponding to Trp-534) is buried in the hydrophobic core of the domain and is critical for protein folding (30).

In Fig. 9, the side chains where substitutions caused constitutive receptor activation are shown in *green*. Most of the activating mutations were clustered in close proximity in the β -strands in the central core of the WW domain structure and along the surface that is predicted to bind to a protein ligand. The side chains of these amino acids extend from the same face of the β -sheet except for Trp-534, which forms the core of the domain and stacks with Tyr-530 in the short α -helix that precedes the WW domain fold, and Val-536. We note that the activating V536A mutation replaced the β -branched amino acid valine, which is favored in β -strands, with alanine, which is not. Therefore, this mutation is likely to activate by disrupting the overall WW fold rather than by removing a side chain that contributes to ligand binding. In addition, the activating six-amino acid deletion removed the second β -strand.

In contrast, the positions that did not result in activation are largely located in the loops away from the proposed central core. This is illustrated in Fig. 8*d* where the clusters of insensitive positions are highlighted in *red*. As noted above, the receptor was not activated by alanine substitutions in the N-terminal α -helix between the stacked aromatic residues (amino acids 531 to 533). Similarly, activation was not caused by alanine substitutions or an alanine insertion in the long loop between the first and second β -sheets (amino acids 538 to 546) or by alanine substitutions in the N-terminal portion of the loop between the second and third β -sheets (amino acids 550 to 554). Finally, substitutions at or downstream of Asp-558 did not activate. This latter sequence corresponds to the third strand in known WW domains but is proposed to be the boundary between the juxtamembrane region and the kinase domain in the PDGF β receptor.

DISCUSSION

We undertook a systematic mutational analysis of the murine PDGF β receptor cytoplasmic juxtamembrane domain extending from near the membrane interface to the border of the kinase domain. Activating mutations were scattered throughout the juxtamembrane domain and included numerous amino acid substitutions, an insertion of a single alanine, and a six-amino acid deletion. These findings imply that the juxtamembrane domain normally inhibits receptor activity in the absence of ligand and that the activating mutations remove this constraint. This conclusion is consistent with the previous analysis of a small segment of the juxtamembrane domain of c-kit and with the diversity of activating mutations in tumors. The distribution of activating mutations throughout the entire length of the juxtamembrane domain suggests that the overall conformation of this region, rather than a short, linear amino acid sequence, is required for inhibition.

The results of our mutational analysis, combined with molecular modeling, provide strong support for our earlier suggestion that the juxtamembrane domains of the type III RTKs adopt a WW domain fold. First, as noted previously, the primary amino acid sequence of the juxtamembrane domain closely resembles known WW domain sequences (3). Second, our molecular modeling indicated that the juxtamembrane domain of the type III RTKs can adopt a plausible three- β -stranded conformation characteristic of WW domains. Third,

the mutational analysis provided strong genetic evidence that the wild-type domain adopts an inhibitory WW-like domain fold. Most of the activating mutations are located at positions on the ligand binding face of the proposed structure, and the mutations that did not activate are located primarily in the loops of the predicted structure. These results suggest that the WW domain normally restrains receptor activity in the absence of ligand, presumably by engaging in protein-protein interactions that inhibit receptor activity, and that the activating mutations disrupt the structure of the domain or directly prevent these inhibitory interactions.

Ma *et al.* (19) proposed that the membrane-proximal segment of the c-kit juxtamembrane domain, corresponding to PDGF β receptor residues 530 to 540, forms an amphipathic α -helix that inhibits receptor activity. Alanine substitutions at all tested positions in this segment of the juxtamembrane domain had identical effects on tyrosine phosphorylation of the PDGF β receptor and c-kit, and both receptors were activated by substituting a proline for the lysine in this segment (19). These results indicate that the juxtamembrane domains of PDGF β receptor and c-kit restrain kinase activity by the same mechanism. Our modeling and mutational analysis suggests that the juxtamembrane sequence upstream of Trp-534 forms an α -helix and that the juxtamembrane sequence downstream of this position adopts the WW domain fold.

There are several possible mechanisms by which the wild-type juxtamembrane domain of the type III RTK family can inhibit receptor activity. The juxtamembrane domain may impair the ability of the receptor to dimerize in the absence of ligand. Given the diversity of the activating mutations, it seems unlikely that they generate a new dimerization interface, although it is possible that these mutations unmask an interface elsewhere in the cytoplasmic domain. Alternatively, the juxtamembrane domain may bind proteins that inhibit dimerization or kinase activity, and the activating mutations may disrupt binding. To account for their finding that the juxtamembrane sequence of the Flt-1 RTK contains elements that repress Flt-1 signaling, Gille *et al.* (2) proposed that a WW-like juxtamembrane domain in this receptor binds an unidentified protein ligand. They modeled the juxtamembrane sequence as a WW domain and proposed that its concave surface interacts with an inhibitory WW ligand and that its convex surface interacts with the kinase domain. In contrast to the Flt-1 juxtamembrane sequence, the juxtamembrane domain of the PDGF β receptor contains two tyrosines at positions 547 and 549, which are phosphorylated upon ligand stimulation, forming binding sites for cytoplasmic SH2 domain-containing signaling proteins, including *src* family kinases, STAT5, SLAP, and the tyrosine phosphatase, SHP-1 (20–22, 31–34). However, it seems unlikely that the juxtamembrane mutations activated the receptor by eliminating binding of an inhibitory SH2 domain protein. First, most activating mutations did not affect the central two tyrosines or neighboring residues. Second, although receptor activation was caused by mutation of the other two juxtamembrane tyrosines, Tyr-530 and Tyr-557, these do not appear to be sites of tyrosine phosphorylation (25). Finally, mutations at positions adjacent to the tyrosines, which are predicted to disrupt specific SH2 domain recognition (35), did not activate the receptor.

In the WW domain model proposed here, the juxtamembrane tyrosines project from the concave surface that is predicted to interact with a protein ligand. Deletion or substitution of these tyrosines leads to receptor activation. These observations raise the possibility that the wild-type juxtamembrane domain of the type III RTKs engages in an inhibitory intramolecular interaction with the kinase domain, as is the case for the EphB2

receptor, a non-type III RTK. Although the EphB2 juxtamembrane domain does not resemble a WW domain, two regulatory tyrosine residues occur at roughly the same position in the sequence as in the PDGF receptor, and they interact with the kinase domain. Wybenga-Groot *et al.* (6) propose that the phosphorylation of these tyrosines destabilizes the juxtamembrane structure and causes it to dissociate from the kinase domain, thereby removing inhibitory interactions and stimulating kinase activity. A similar mechanism may be operating in the PDGF β receptor and other type III RTKs that contain tyrosine residues in the putative central β -strand of the WW-like domain. It is also possible that the juxtamembrane region forms a conformational switch. When unphosphorylated, this domain forms inhibitory interactions with the surface of the kinase domain or with another protein ligand. When phosphorylated, the juxtamembrane domain may dissociate from the protein ligand or the kinase domain and interact with an SH2 domain.

The mutational results presented here support the conclusion that the juxtamembrane domain forms a WW-like structure, but they do not definitively distinguish between intermolecular and intramolecular inhibitory mechanisms. However, we note that the charged residues Glu-538, Asp-543, Glu-546, and Asp-558, which are insensitive to mutations, lie on the convex face of the domain and consequently should be solvent-exposed. Thus, it is unlikely that this surface interacts with the kinase domain as proposed by Gille *et al.* (2) for the Flt-1 receptor. In addition, in our model, the transmembrane and kinase segments of the PDGF receptor are located on opposite faces of the WW domain. This arrangement seems plausible, because it places the juxtamembrane domain between the membrane surface and the larger cytoplasmic kinase domain. In contrast, the model of Gille *et al.* (2) places the transmembrane and kinase segments on the same face of the WW domain. Overall, we favor the model that the juxtamembrane region imposes inhibition by binding to another segment of the receptor cytoplasmic domain.

We reported previously that glutathione *S*-transferase fusion proteins containing the PDGF β receptor juxtamembrane domain bound in a filter assay to certain arbitrarily chosen peptides that contained recognition sites for known WW domains (3). More recent experiments revealed that some (but not all) glutathione *S*-transferase fusion proteins lacking the juxtamembrane domain showed a similar level and specificity of binding to these peptides as did the original glutathione *S*-transferase fusion protein containing the juxtamembrane domain.² Therefore, this *in vitro* assay does not give a valid indication of the ability of the PDGF β receptor juxtamembrane domain to bind peptide ligands. Because we have not yet identified authentic binding partners of the juxtamembrane domain, we are not able to determine whether the mutations described here affected the protein binding activity of the domain.

In summary, these experiments indicated that the juxtamembrane domain of the PDGF β receptor normally restrains receptor activity in the absence of ligand, raising the possibility that ligand addition stimulates the activity of the receptor by relieving the inhibition imposed by the juxtamembrane domain. We also provided evidence that this sequence forms an inhibitory WW-like domain and identified specific amino acids required for inhibition. The mutants described here should be valuable reagents for elucidating the structural

and biochemical basis for the inhibition imposed by the juxtamembrane domain. Because similar mutations activate type III RTKs in human tumors, these experiments will provide insight not only into regulation of growth factor receptor function but also into carcinogenesis.

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