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A single amino acid substitution in a WW-like domain of diverse members of the PDGF receptor subfamily of tyrosine kinases causes constitutive receptor activation

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Platelet-derived growth factor β receptor (PDGFβR) is a transmembrane receptor tyrosine kinase involved in a variety of cellular functions. We have generated a constitutively activated murine PDGFβR containing a valine to alanine substitution at residue 536, located in the cytoplasmic juxtamembrane domain. When this mutant receptor (PR-V536A) was expressed in Ba/F3 cells, it allowed the cells to survive and proliferate in the absence of IL-3 or PDGF, and tyrosine phosphorylation of PR-V536A was increased markedly compared with that of the wild-type PDGFβR in the absence of ligand and similar to that observed in ligand-activated PDGFβR. PR-V536A displayed increased tyrosine kinase activity *in vitro* toward an exogenous substrate, and the tyrosine kinase activity of the receptor was required for the constitutive activation of the mutant. This valine to alanine substitution also activated a PDGFβR mutant unable to bind PDGF. Alanine substitutions at positions homologous to V536 of the murine PDGFβR also activated other members of the PDGF receptor subfamily. The amino acid sequence of this region revealed a strong similarity to WW domains present in other signal transduction proteins. Furthermore, GST fusion proteins containing the juxtamembrane region of the PDGFR specifically associated with peptides containing the WW domain consensus recognition sequence PPXY. The results suggest that the cytoplasmic juxtamembrane domain plays a role in the regulation of receptor activity and function, perhaps by participating in protein–protein interactions.

Keywords: c-*kit*/oncogene/PDGF receptor/tyrosine kinase/WW domain

Introduction

The platelet-derived growth factor β receptor (PDGFβR) is a receptor tyrosine kinase (RTK) that belongs to the type III RTK subfamily. This group of RTKs also includes the PDGFαR, the colony-stimulating factor 1 receptor (CSF1-R) and the stem cell factor (SCF) receptor (also known as c-Kit). The current model of RTK activation involves ligand binding to the receptor extracellular domain, followed by dimerization of the receptor and autophosphorylation at specific tyrosine residues in its cytoplasmic domain (Ullrich and Schlessinger, 1990). These phosphorylated tyrosines serve as docking sites for intracellular signaling proteins, including phosphatidylinositol 3′ kinase (PI3K) and phospholipase C-γ (PLC-γ). After these proteins are recruited to the membrane-bound receptor, they initiate a cascade of signaling events that are responsible for the ligand-induced phenotype.

Several RTKs can be activated constitutively by point mutations. For example, a single amino acid substitution in the transmembrane domain of the RTK erbB-2 (Neu) results in oncogenicity (Bargmann *et al.*, 1986). This mutation appears to stimulate tyrosine kinase activity by facilitating receptor dimerization (Weiner *et al.*, 1989; Smith *et al.*, 1996). A point mutation in the extracellular domain of the human CSF1-R activates its transforming potential (Roussel *et al.*, 1988). A substitution of valine for aspartic acid at codon 816 in the cytoplasmic kinase domain of the human c-Kit protein results in constitutive activation; this mutant displays altered substrate specificity and induces the degradation of a negative regulator of the receptor, the tyrosine phosphatase SHP-1 (Piao *et al.*, 1996). These data indicate that point mutations in various domains of RTKs can activate these receptors by diverse mechanisms. However, no constitutively activated PDGFβR point mutants have been identified so far.

Although oncogenic versions of PDGFβR generated by point mutation have proven elusive, there are examples of genetic rearrangements involving the PDGFβR locus that result in receptor activation. Chimeric PDGFβR proteins have been found in chronic myelomonocytic leukemia cells, in which the cytoplasmic and transmembrane domains of the receptor were fused by a translocation event to either the dimerization domain of the TEL transcription factor (Golub *et al.*, 1994) or the leucine zipper domain of the Huntingtin-interacting protein 1 (Ross *et al.*, 1998). These chimeric proteins showed constitutive tyrosine phosphorylation when expressed in hematopoietic cell lines, and allowed growth factor-independent proliferation (Carroll *et al.*, 1996; Ross *et al.*, 1998). In addition, a truncated PDGFβR form that resulted from integration of an HTLV-I provirus into the PDGFβR gene was identified recently in C10/MJ cells and shown to have weak transforming activity in NIH-3T3 cells (Chi *et al.*, 1997). However, tyrosine phosphorylation of this truncated receptor was not examined. We showed that a chimeric PDGFβR containing the transmembrane domain of activated ErbB2 was constitutively tyrosine phosphorylated and transformed murine C127 and Ba/F3 cells (Petti *et al.*, 1998).

Here we demonstrate that a single amino acid substitution in the cytoplasmic juxtamembrane region of PDGFβR is sufficient to constitutively activate this receptor. Homologous substitutions in all tested members of the PDGFR subfamily of RTKs activated also these receptors, indicating that this protein region may play a general role in regulation of receptor activity. In addition, we have

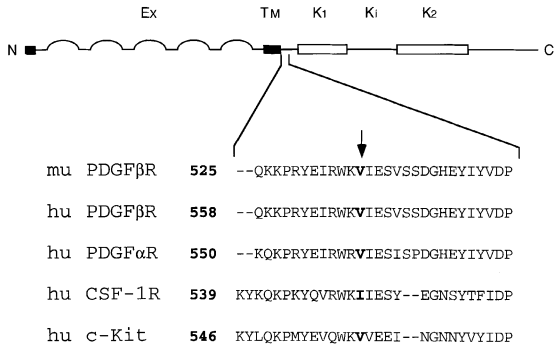

Fig. 1. Schematic diagram of the PDGFβ receptor and sequence alignment of the cytoplasmic, juxtamembrane domain of related receptors. The top shows the full-length receptor containing (from left to right) the N-terminus (N), signal sequence (filled-in box), five extracellular immunoglobulin-like domains (Ex), transmembrane domain (TM), and the tyrosine kinase domain (open boxes, K1 and K2) split by the kinase insert region (Ki). The bottom shows the sequence alignment of the cytoplasmic juxtamembrane domains of different receptors of the PDGFβR subfamily of tyrosine kinases, with the position of residue 536 shown by the arrow. The homologous residues in the other receptors are shown in bold. The numbers indicate the first amino acid shown in the sequence.

identified the juxtamembrane region of type III RTKs as a potential WW domain. WW domains are a class of protein–protein interaction domains that are present in a variety of signaling proteins (Einbond and Sudol, 1996; Sudol, 1996), but they have not been identified previously in RTKs.

Results

A PCR-based cloning procedure generated an activated form of the PDGF receptor

During a PCR-based cloning procedure using the *Taq* DNA polymerase, we generated a mutant PDGFβR that unexpectedly was constitutively activated (data not shown). Sequence analysis revealed that this mutant contained, in addition to the desired mutation, a T→C transition in codon 536 of the murine PDGFβR cDNA (Yarden *et al.*, 1986). This mutation, which was evidently introduced by misincorporation during PCR amplification, resulted in a valine to alanine substitution in the juxtamembrane cytoplasmic region of the PDGFβR. The amino acid sequence of this region of the receptor is highly conserved among several members of the type III subfamily of RTKs (Figure 1). This V536A mutation was reintroduced into an otherwise wild-type receptor and caused the activated phenotype described below. This reconstructed mutant receptor (PR-V536A) was used in the experiments described here.

The PR-V536A mutant transformed Ba/F3 cells to IL-3 independence

Ba/F3 cells are mouse hematopoietic cells that are strictly interleukin-3 (IL-3)-dependent for growth; removal of IL-3 from the culture medium leads to rapid apoptotic cell death (Palacios and Steinmetz, 1985). Ba/F3 cells do not express endogenous PDGFβR, and ectopic expression of wild-type PDGFβR alone does not rescue Ba/F3 cells from apoptosis after IL-3 deprivation (Drummond-Barbosa *et al.*, 1995; Petti *et al.*, 1997). However, PDGF treatment of Ba/F3 cells expressing the wild-type PDGFβR or co-expression of wild-type PDGFβR together with either v-*sis* (a form of PDGF) or BPV-E5 (a viral oncoprotein that activates the PDGFβR) prevents apoptosis and allows Ba/F3 cells to proliferate in the absence of IL-3 (Drummond-Barbosa *et al.*, 1995; P.M.Irusta and D.DiMaio, unpublished results).

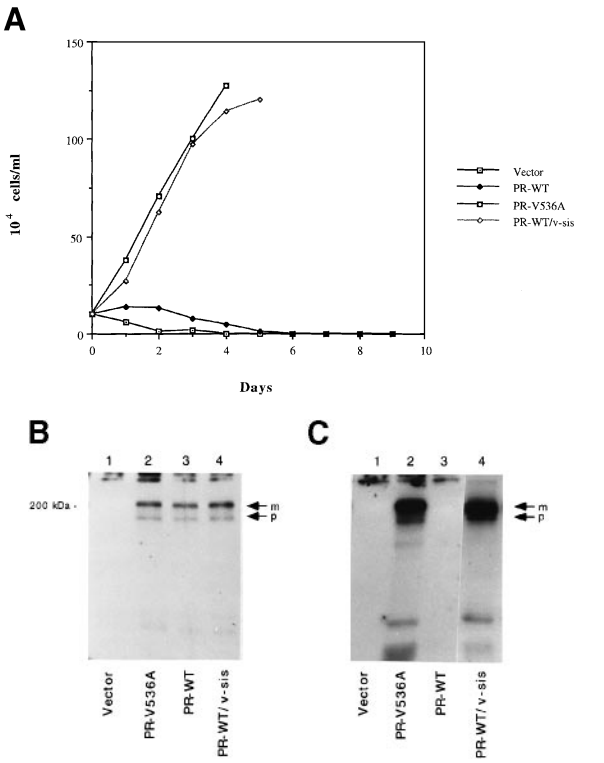

Fig. 2. Initial characterization of PR-V536A. (**A**) IL-3 independence test of Ba/F3-derivative cell lines. Approximately 10^5 cells/ml were seeded in RPMI media lacking IL-3, and viable cells were counted daily. The results are shown for cells infected with empty retroviruses (Vector) or expressing wild-type PDGFβR (PR-WT) as negative controls, cells expressing PR-V536A (PR-V536A), and cells co-expressing PDGFβR wild-type and v-*sis* (PR-WT/v-sis) as a positive control. (**B** and **C**) Levels of expression and tyrosine phosphorylation of PDGF receptors in Ba/F3 cells. Cell extracts of Ba/F3-derivative cell lines were immunoprecipitated with anti-PDGFR antibody and blotted with either the same antibody (B) or anti-phosphotyrosine monoclonal antibody (C). The precursor (p) and mature (m) forms of the PDGFβR are indicated by arrows. Migration of molecular weight marker (in kDa) is indicated.

Wild-type murine PDGFβR (PR-WT) and PR-V536A were cloned into the retroviral vector pLXSN, and ecotropic retrovirus stocks were used to generate stable Ba/F3-derivative cell lines. The cell lines obtained were then tested for IL-3-independent growth and characterized biochemically. As shown in Figure 2A, Ba/F3-derivative cell lines carrying an empty retroviral vector and cells expressing wild-type PDGFβR alone did not proliferate and died shortly after IL-3 removal. In contrast, cell lines expressing the mutant PR-V536A survived and proliferated in the absence of IL-3. The extent of IL-3-independent proliferation of these cells was comparable with that observed in Ba/F3 cells co-expressing the wild-type PDGFβR and the v-*sis* oncogene (Figure 2A). Thus, PR-V536A transformed Ba/F3 cells to an IL-3-independent phenotype, suggesting that the receptor signaling pathway was constitutively activated by this mutant.

The PR-V536A mutant was constitutively phosphorylated on tyrosine residues

The levels of tyrosine phosphorylation of the wild-type and mutant PDGFβR were analyzed by immunoblotting. Cell extracts of Ba/F3-derivative cell lines grown in the presence of IL-3 were immunoprecipitated with anti-PDGFR antibody and blotted with either the same antibody (to assess PDGFβR protein levels) or anti-phosphotyrosine monoclonal antibody (to assess the level of tyrosine phosphorylation of the receptors).

As shown in Figure 2B, Ba/F3 cells infected with the empty vector did not express PDGFβR, and all the other Ba/F3-derivative cell lines analyzed expressed the mature and the more rapidly migrating precursor forms of the receptor. The exogenous PDGFβR was expressed at similar levels in the various cell lines. Tyrosine phosphorylation of these receptors is shown in Figure 2C. There was little basal tyrosine phosphorylation of the wild-type PDGFβR (Figure 2C, lane 3). In contrast, the level of phosphotyrosine of the mutant PR-V536A was high (lane 2) and similar to that observed in ligand-activated receptors isolated from cells that co-expressed PR-WT and v-*sis* (lane 4). Both the mature form of PR-V536A and the precursor form with immature carbohydrates contained high levels of phosphotyrosine. Similar results were observed when the mutant receptor was stably expressed in human 293 epithelial cells (data not shown). Thus, in both Ba/F3 and 293 cells, PR-V536A was constitutively phosphorylated on tyrosine residues.

Dimerization of the tyrosine-phosphorylated PR-V536A

Activation of PDGFβR by PDGF is thought to be mediated by receptor dimerization. To determine whether PR-V536A constitutively formed dimeric complexes, *in vivo* cross-linking experiments were carried out in 293 cells expressing either wild-type or V536A PDGFβR. Intact cells expressing PR-WT and PR-V536A were treated with the membrane-impermeable chemical cross-linker bis (sulfosuccinimidyl)suberate (BS^3) and, after cell lysis, activated receptor was visualized by immunoprecipitation, gel electrophoresis and immunoblotting for phosphotyrosine. As shown in Figure 3, cross-linker addition to cells treated with PDGF resulted in the appearance of a higher molecular weight form of tyrosine-phosphorylated PDGFβR of approximately the size of PDGFβR dimers (Figure 3, lanes 3 and 6). The presence of tyrosine-phosphorylated monomeric receptor is presumably a reflection of the non-quantitative nature of this cross-linking reaction. Dimeric activated receptor complexes were also observed after cross-linker addition to cells expressing PR-V536A even in the absence of PDGF (Figure 3, lane 4). Dimeric complexes were not observed in the absence of cross-linker treatment (data not shown). This result indicated that *in vivo*, a fraction of the tyrosine-phosphorylated PR-V536A constitutively formed dimers at the cell surface.

In vitro kinase activity of PR-V536A

To determine the effect of the V536A mutation on the tyrosine kinase activity of the PDGFβR, we performed *in vitro* kinase assays. Cell lysates were prepared from Ba/F3 cells expressing the wild-type PDGFβR, the

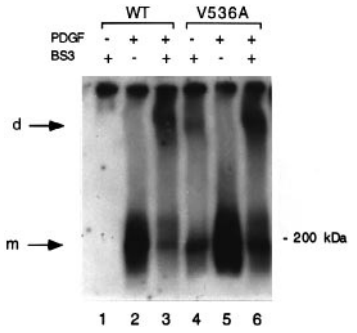

Fig. 3. *In vivo* chemical cross-linking analysis of PR-WT and PR-V536A. 293-derivative cells expressing the murine wild-type PDGFβR (lanes 1–3) and V536A mutant (lanes 4–6) receptors were incubated in the presence or absence of PDGF on ice for 90 min and were then treated with either vehicle alone or BS^3 for 90 min on ice. After quenching the reaction the cells were lysed. The PDGFβR was immunoprecipitated with C3a antibody and immunoblotted by using anti-phosphotyrosine antibody 4G10. m indicates the migration position of monomeric PDGFβR, and d indicates the position of the presumed dimeric receptor.

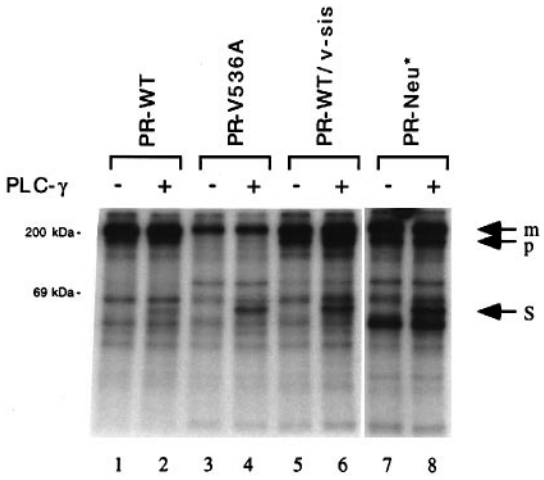

Fig. 4. *In vitro* kinase assays. PDGFβ receptors were immunoprecipitated from lysates of Ba/F3-derivative cell lines and their *in vitro* kinase activity was assessed as described in the text. Kinase reactions were carried out in the absence (–) or presence (+) of a GST–PLC-γ fusion protein as an exogenous substrate, as indicated. The precursor (p) and mature (m) forms of the PDGFβR as well as the GST–PLC-γ substrate (s) are indicated by arrows. The prominent 60 kDa tyrosine-phosphorylated protein generated in extracts from cells expressing PR-Neu* was noted previously (Petti *et al.*, 1998).

PDGFβR together with v-*sis*, the PR-V536A mutant, or PR-Neu*, a constitutively activated chimeric PDGFβR that contains the transmembrane domain of the oncogenic Neu protein (Petti *et al.*, 1998). These lysates were immunoprecipitated with anti-PDGFR antibody and incubated in kinase reaction buffer containing [γ-^{32}P]ATP in the absence or presence of an exogenous substrate containing the phosphorylation sequence of PLC-γ, a natural substrate of PDGFβR. PR-V536A phosphorylated this substrate to an ~2- to 3-fold greater extent than did unstimulated wild-type receptor, to an extent similar to that of wild-type receptor activated by v-*sis* (Figure 4, lanes 4 and 6). Interestingly, PR-V536A was ~2- to 3-fold less active for receptor autophosphorylation than were the other activated receptors. However, the other activated receptors did not display increased autokinase activity

compared with unstimulated wild-type receptor, suggesting that the ability of a receptor to catalyze autophosphorylation in this assay did not necessarily reflect the activity of the receptor in intact cells. Thus, the activating V536A mutation resulted in increased receptor tyrosine phosphorylation in intact cells and increased the *in vitro* kinase activity of the receptor when measured on an exogenous substrate, even though the mutant receptor did not appear to autophosphorylate efficiently *in vitro*.

Requirement for tyrosine kinase activity

Experiments were also carried out to assess the role of the kinase activity of PR-V536A in the constitutively active phenotype of this mutant. We introduced a lysine to arginine substitution at position 602 in the kinase domain of the PR-V536A, a mutation previously shown to eliminate tyrosine kinase activity (Escobedo *et al.*, 1988). This kinase-defective receptor (PR-V536A-NK) was not capable of conferring IL-3 independence (Figure 5A), and it did not display constitutive tyrosine phosphorylation (Figure 5B). These results demonstrated that a functional kinase domain was essential for the transforming activity of PR-V536A. We also tested the effect of AG1295, a specific inhibitor of the PDGFR tyrosine kinase (Kovalenko *et al.*, 1994). Treatment of Ba/F3 cells with this inhibitor caused a rapid and profound decrease in the level of tyrosine phosphorylation of ligand-stimulated wild-type PDGFβR and of PR-V536A (Figure 5C), and prevented IL-3-independent growth of cells expressing either the wild-type or the mutant PDGFβR, but had no effect on the growth of cells in the presence of IL-3 (data not shown). Therefore, both the mutant and the inhibitor studies demonstrated that the intrinsic tyrosine kinase activity of PR-V536A was required for constitutive receptor activation.

Role of the ligand-binding domain in PDGF receptor activation by V536A mutation

Since the medium used to grow the Ba/F3 cells contains 10% fetal calf serum (FCS), it was possible that the V536A mutation was acting by increasing the sensitivity of the receptor to its natural ligand so that levels of PDGF not sufficient to stimulate the wild-type receptor were sufficient to activate the mutant. Therefore, we tested the effect of the V536A mutation in the context of a receptor unable to respond to ligand. We first deleted most of the ligand-binding domain to construct a truncated PDGFβR with a wild-type cytoplasmic domain (PR-ΔEx-WT) (L.Petti and D.DiMaio, unpublished results). This truncation mutant was neither tyrosine phosphorylated nor able to transform Ba/F3 cells to IL-3 independence when it was expressed alone or when it was co-expressed with v-*sis*, demonstrating that this receptor was not constitutively activated and could no longer respond to PDGF (Figure 6B; data not shown). However, PR-ΔEx-WT was tyrosine phosphorylated and able to induce IL-3 independence when co-expressed with BPV-E5, which binds to the transmembrane region of the receptor (Petti *et al.*, 1997; L.Petti and D.DiMaio, unpublished results). Thus, the tyrosine kinase activity and signaling capacity of this truncated receptor were intact.

We introduced the V536A mutation into this truncated receptor, generating PR-ΔEx-V536A, and tested its ability to transform Ba/F3 cells. As shown in Figure 6A, receptors carrying the extracellular deletion in combination with the V536A mutation were still able to deliver a proliferative

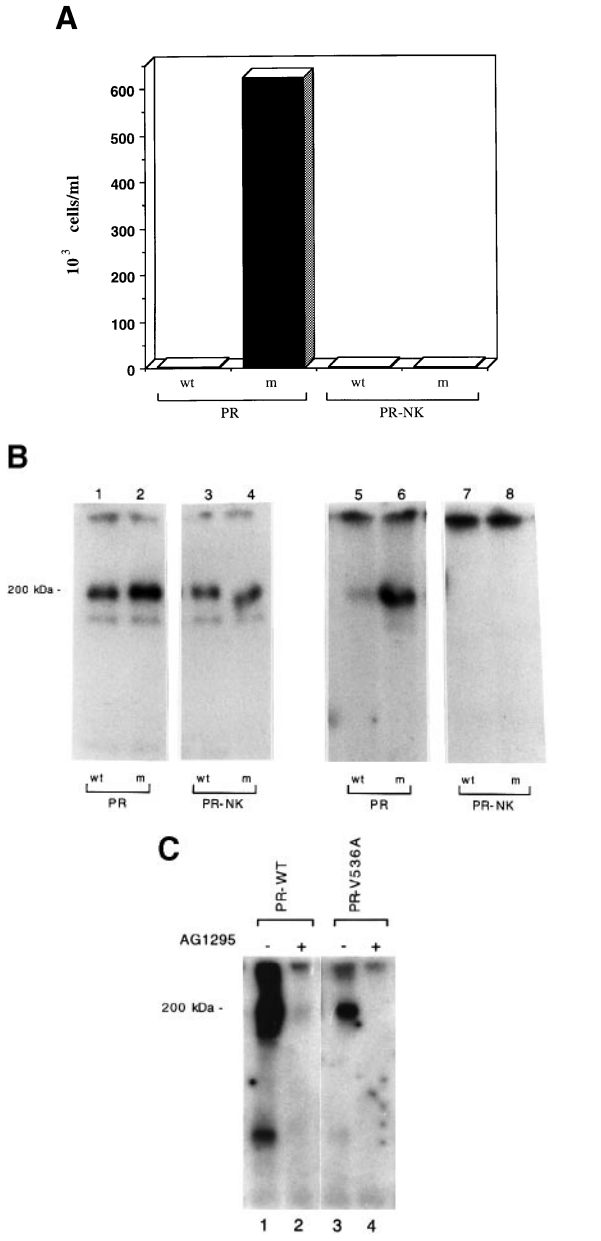

Fig. 5. A functional kinase domain is essential for PR-V536A activation. (**A**) A K602R substitution was introduced into the wild-type and V536A mutant receptors, eliminating kinase activity. The ability of the indicated kinase-competent (PR) or kinase-defective (PR-NK) receptors to transform Ba/F3 cells in the absence of IL-3 was analyzed. Viable cell densities at day 10 after IL-3 deprivation are shown. In this and subsequent figures, wt indicates a receptor containing a wild-type juxtamembrane domain, and m indicates a receptor containing the V536A mutation (or the alanine substitution at the homologous position in related receptors). (**B**) Protein (lanes 1–4) and phosphotyrosine (lanes 5–8) levels of the indicated receptors were assessed as described before. (**C**) Effect of PDGFR-specific kinase inhibitor AG1295 on receptor phosphorylation *in vivo*. Ba/F3-derivative cells expressing kinase-competent PR-WT or PR-V536A were treated for 5 min at 37°C with either vehicle alone (–) or 100 μM AG1295 (+), as indicated. Cells expressing PR-WT were then stimulated with 50 ng/ml of PDGF BB for 5 min at 37°C, whereas cells expressing PR-V536A were not stimulated with PDGF. The cells were then lysed, and the phosphotyrosine levels of the indicated receptors were analyzed as described in previous figures.

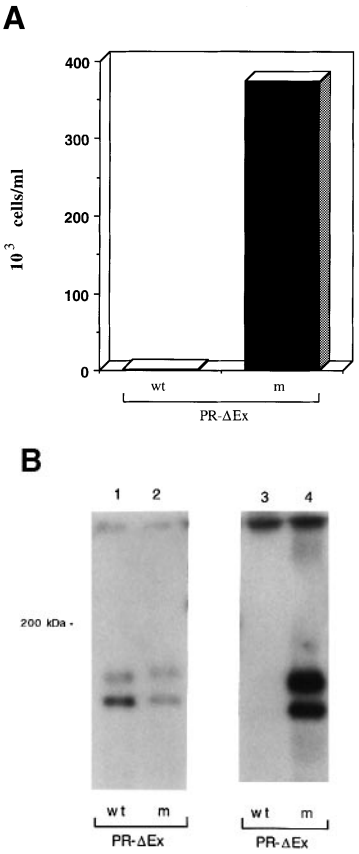

Fig. 6. Dispensability of the ligand-binding domain for PR-V536A activation. A truncated PDGFβR (PR-ΔEx-WT) was generated by removing part of the receptor's extracellular domain; subsequently a V536A mutation was introduced generating PR-ΔEx-V536A. (**A**) The ability of the indicated truncated receptors to transform Ba/F3 cells in a IL-3 independence test was measured. The figure shows the concentration of viable cells after 10 days incubation in the absence of IL-3. (**B**) Total PDGF receptor (lanes 1 and 2) and phosphotyrosine levels (lanes 3 and 4) of cells expressing the indicated receptors were analyzed as described in Figure 2.

signal to Ba/F3 cells. Furthermore, as shown in Figure 6B, whereas PR-ΔEx-WT contained no detectable phosphotyrosine (lane 3), PR-ΔEx-V536A was highly tyrosine phosphorylated (lane 4). Therefore, the ability of the V536A mutation to activate the PDGFβR was not dependent on a functional ligand-binding domain, demonstrating that the mutation did not cause activation by increasing the sensitivity to PDGF or by some other PDGF-mediated mechanism.

Effect of alanine substitutions at the homologous position of related receptor tyrosine kinases

Since the V536A mutation is located in a region of the murine PDGFβR that is highly conserved among other members of the type III family of RTKs (Figure 1), we tested the effect of similar amino acid substitutions at the homologous position in other receptors. First, we replaced Val569 in the human PDGFβR with an alanine. In contrast to the wild-type human PDGFβR, the resulting mutant receptor (huPRβ-V569A) transformed Ba/F3 cells to an IL-3-independent phenotype (Figure 7A) and was constitutively tyrosine phosphorylated (Figure 7B, lane 6). This increase in receptor phosphorylation was also observed in

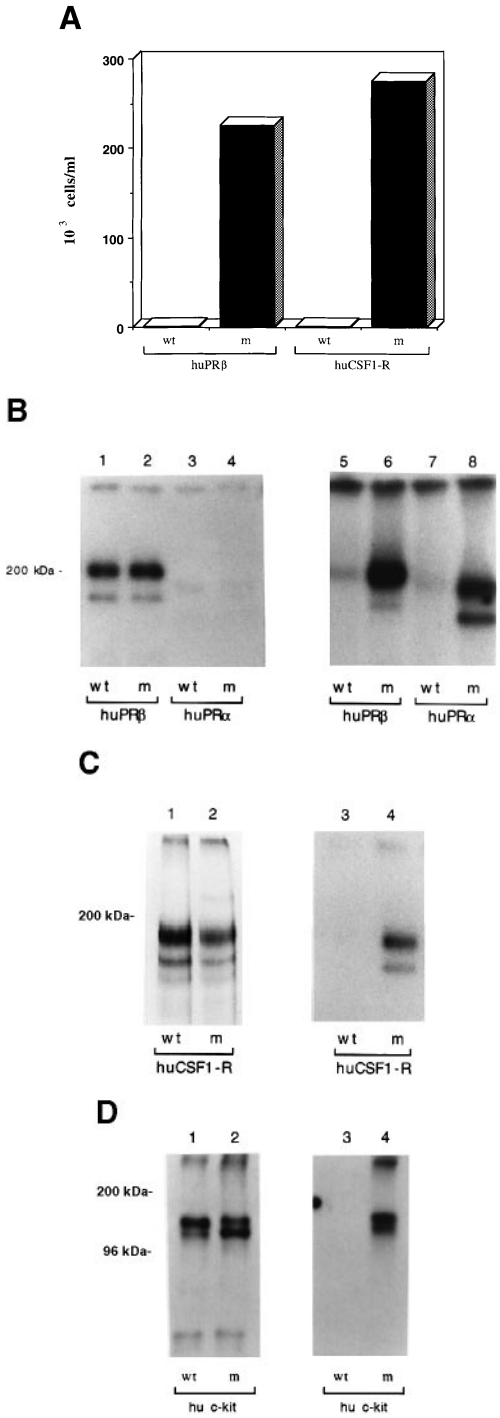

Fig. 7. Activation of type III RTKs by alanine substitutions at homologous positions. (**A**) IL-3-independent growth of cells expressing the wild-type (wt) and mutant (m) versions of the human PDGFβR (huPRβ), and the human CSF1-R (huCSF1-R) was assayed as described in Figure 5A. (**B**) Receptor levels (lanes 1–4) and phosphotyrosine levels (lanes 5–8) of the wild-type and mutant versions of huPRβ and huPRα are shown. (**C**) Receptor levels (lanes 1 and 2) and phosphotyrosine levels (lanes 3 and 4) of the wild-type and mutant versions of CSF1-R are shown. (**D**) Receptor levels (lanes 1 and 2) and phosphotyrosine levels (lanes 3 and 4) of wild-type and mutant versions of c-Kit receptors expressed in 293 cells are shown.

293 cells (data not shown). Thus, the mutation activated human as well as mouse PDGFβR.

Secondly, a valine to alanine substitution was introduced at residue 561 of the closely related human PDGFαR

to generate PRα-V561A. Although only low levels of exogenous PDGFαR were detectable in Ba/F3 cells (Figure 7B, lanes 3 and 4), tyrosine phosphorylation of PRα-V561A was greatly increased compared with that of the wild-type PDGFαR (Figure 7B, lanes 7 and 8). Thus, a valine to alanine substitution in the juxtamembrane domain of the human PDGFαR was sufficient to induce autophosphorylation. IL-3-independent growth was observed in cells expressing either the wild-type or mutant PRα receptor, making the analysis of the biological effects of this mutant receptor difficult (data not shown).

Thirdly, we engineered an isoleucine to alanine change at position 552 of the human CSF1-R, generating CSF1-R-I552A. As shown in Figure 7A, CSF1-R-I552A but not the wild-type CSF1-R rendered Ba/F3 cells IL-3 independent. In addition, this mutant receptor exhibited much higher phosphotyrosine levels than the wild-type CSF1-R (Figure 7C). This increase in phosphorylation was also observed when the receptors were expressed in a 293-derivative cell line (data not shown).

Finally, a valine to alanine substitution was constructed at position 559 of the human c-Kit receptor, generating c-Kit-V559A. Figure 7D shows that the wild-type c-Kit receptor was not detectably tyrosine phosphorylated when expressed in a 293-derivative cell line (lane 3). In contrast, expression of c-Kit-V559A resulted in high levels of constitutive tyrosine phosphorylation of the mutant receptor (Figure 7D, lane 4), indicating that this substitution was also sufficient to activate this RTK.

Constitutive association of downstream effector molecules with the activated receptors

After RTK activation by ligand, a series of SH2-containing proteins are recruited to the receptor complex by binding to specific phosphorylated tyrosine residues on the receptor. To determine whether similar interactions occurred constitutively with the mutant PR-V536A, we performed co-immunoprecipitation experiments. In these experiments, wild-type and mutant PDGFβRs were immunoprecipitated from extracts of Ba/F3 cells that had been stimulated previously with PDGF or left untreated, and receptor-associated proteins were detected by immunoblotting. As shown in Figure 8A, PDGF stimulation of PR-WT resulted in a marked increase in the amount of PLC-γ, ras GTPase-activating protein (GAP) and PI3K associated with the receptor (compare lanes 1 and 2), reflecting the ligand-induced recruitment of these proteins to the signaling complex. This increase was ~4-fold for PLC-γ, 2- to 3-fold for GAP and 10-fold for PI3K, as quantitated by PhosphorImager analysis. When PR-V536A was examined in the absence of PDGF treatment, the amount of these three proteins co-immunoprecipitated with the mutant receptor was increased compared with unstimulated PR-WT. The constitutive level of GAP and PI3K binding to the mutant was similar to the amount observed for ligand-stimulated wild-type receptor, and PLC-γ binding was increased 2- to 3-fold (Figure 8A, lane 3). These results indicated that PLC-γ, GAP and PI3K were constitutively associated with PR-V536A in transformed cells, suggesting that these effector molecules were involved in mediating the proliferative signal that results in Ba/F3 cell growth.

To test whether the activating mutations were triggering

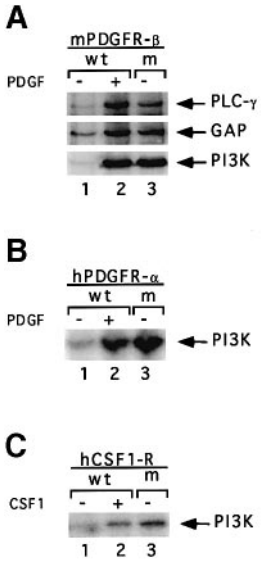

Fig. 8. Association of downstream effector molecules with constitutively activated receptors. (**A**) Association of effector proteins with PDGFβR. Wild-type and mutant mPDGFβR were immunoprecipitated from extracts of Ba/F3 cells that had been treated previously with 50 ng/ml of PDGF-BB for 5 min at 37°C (lane 2) or left untreated (lanes 1 and 3), and receptor-associated proteins were detected by immunoblotting. Results obtained with antibodies against PLC-γ (top panel), ras-GAP (middle panel) and PI3K (bottom panel) are shown. (**B**) Association of PI3K with hPDGFαR. Co-immunoprecipitation experiments using extracts of Ba/F3 cells expressing the wild-type and mutant versions of hPDGFαR were performed as described in (A). (**C**) Association of PI3K with hCSF1-R. Receptors were immunoprecipitated from extracts of Ba/F3 cells that had been treated previously with 50 ng/ml of CSF1 for 5 min at 37°C (lane 2) or left untreated (lanes 1 and 3), and the amount of receptor-associated PI3K was detected by immunoblotting as described above.

similar pathways in Ba/F3 cells expressing the other type III RTK mutants, we performed similar experiments using the wild-type and mutated versions of the hPDGFαR and the hCSF1-R. Since the results with PR-V536A were most clear-cut with PI3K and since activation of this substrate has been shown to be sufficient for PDGF-mediated cell transformation in diverse cell lines (Valius and Kazlauskas, 1993), we focused our analysis on this downstream effector molecule. As shown in Figure 8B and C, receptor stimulation by their respective ligands resulted in PI3K recruitment to both hPDGFαR (an increase of ~3- to 4-fold compared with unstimulated receptor) and hCSF1-R (~5-fold) (Figure 8B and C, lanes 1 and 2). The mutant versions of these receptors displayed constitutive binding to PI3K (Figure 8B and C, lane 3) to levels comparable with ligand-stimulated receptors (after correction for the amount of extract analyzed), indicating that the activating mutations in these RTKs also triggered this signaling pathway.

The juxtamembrane region of PDGFR: a WW domain?

Inspection of the amino acid sequence of the juxtamembrane region of the murine PDGFβR around residue 536 revealed the presence of a potential WW domain (Figure 9). WW domains are independently folded protein modules that mediate protein–protein interactions by binding to proline-rich motifs of the form PPXY (where X represents

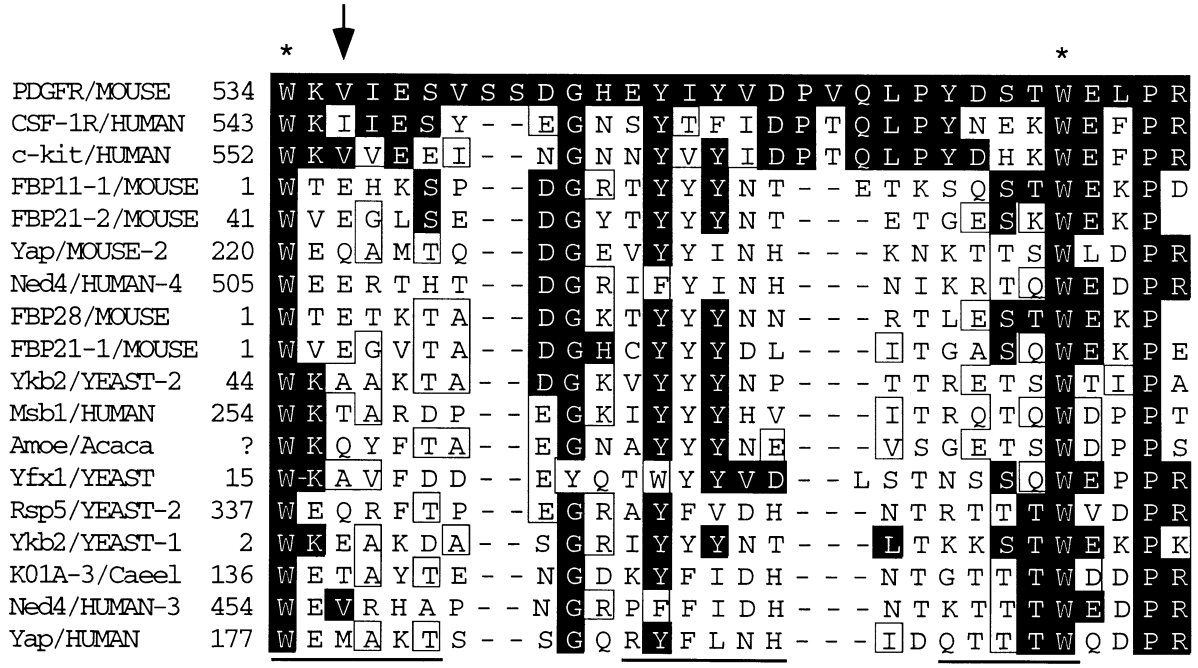

Fig. 9. Sequence alignment of the juxtamembrane region of three members of the type III subfamily of RTKs and selected WW domains. WW domain sequences were obtained from the WW domain web page (http://www.embl-heidelberg.de/~bork/ww1.html/). Several WW domains are compared with the murine PDGFβR, human CSF1-R and the human c-Kit receptor juxtamembrane sequences. The three known β-sheet regions of the WW domain of the human Yap protein are underlined. Black, identical amino acids to PDGFβR; boxed, similar amino acids. The numbers indicate the first amino acid shown in the sequence. V536 in PDGFβR is shown by the arrow, and the defining tryptophans of the WW domain are indicated by asterisks.

any amino acid). WW domains are defined by two highly conserved tryptophan residues, a central core of aromatic and hydrophobic residues, and a proline located three residues downstream of the second tryptophan (Sudol, 1996). All three of these features are present in the juxtamembrane region of the PDGFβR and other receptors in the type III RTK family, with tyrosines 547 and 549 of the mPDGFβR constituting the central aromatic core. The spacing of these elements in the receptors differs slightly from the canonical WW domain. Specifically, 12 residues separate the first tryptophan from the central core in the PDGFβR, whereas there are usually 10 residues in other WW domains. However, CSF1-R and c-Kit contain the canonical distance of 10 residues, and some recognized WW domains contain 11 residues. The distance between the aromatic core and the second tryptophan is 11 residues in type III receptors compared with the canonical 8–9 residues of most other WW domains (although this region is 17 residues in the transmembrane/juxtamembrane WW domain of the CD45-AP protein) (Cahir McFarland and Thomas, 1995). In addition to the absolutely conserved residues, the putative WW domains of the class III RTK family also display several features in common with most other WW domains. These include a charged amino acid one position downstream of the first tryptophan, an acidic residue or an asparagine followed by a glycine three positions upstream of the central aromatic core, an acidic residue one position downstream of the second tryptophan, and an arginine one position downstream of the conserved proline.

Association of the juxtamembrane domain of the PDGFβR with peptides containing the sequence PPXY

We tested the ability of the PDGFR juxtamembrane region to associate with amino acid sequences containing the consensus motif PPXY. The juxtamembrane domains of the wild-type murine PDGFβR and PR-V536A were fused to GST and expressed in *Escherichia coli*. These fusion proteins were labeled with [γ-^{32}P]ATP and used to probe diverse proline-rich peptides synthesized on nitrocellulose membranes by the SPOTs synthesis technique (Genosys Biotechnologies). These peptides included known and putative WW domain-binding motifs (i.e. peptides containing PPXY cores) as well as sequences predicted not to associate with WW domains (i.e. peptides containing PPXX or XPPY cores) (Figure 10A). As a positive control, we used a GST fusion protein containing the first WW domain of rat NEDD-4 and tested its association with proline-rich peptides derived from the epithelial sodium channel (ENaC), its natural partner.

Results obtained with a representative group of peptides are shown in Figure 10B. Filters probed with ^{32}P-labeled GST did not show strong association with any of the tested peptides (upper left panel). The WW domain of NEDD-4 associated strongly with a peptide containing the proline-rich sequence of the ENaC-γ (upper right panel, spot 3) as well as with other PPXY-containing peptides (spots 2 and 6 of same panel). In contrast, GST–NEDD4-WW failed to associate with three different peptides that lacked a PPXY motif (upper right panel, spots 1, 4 and

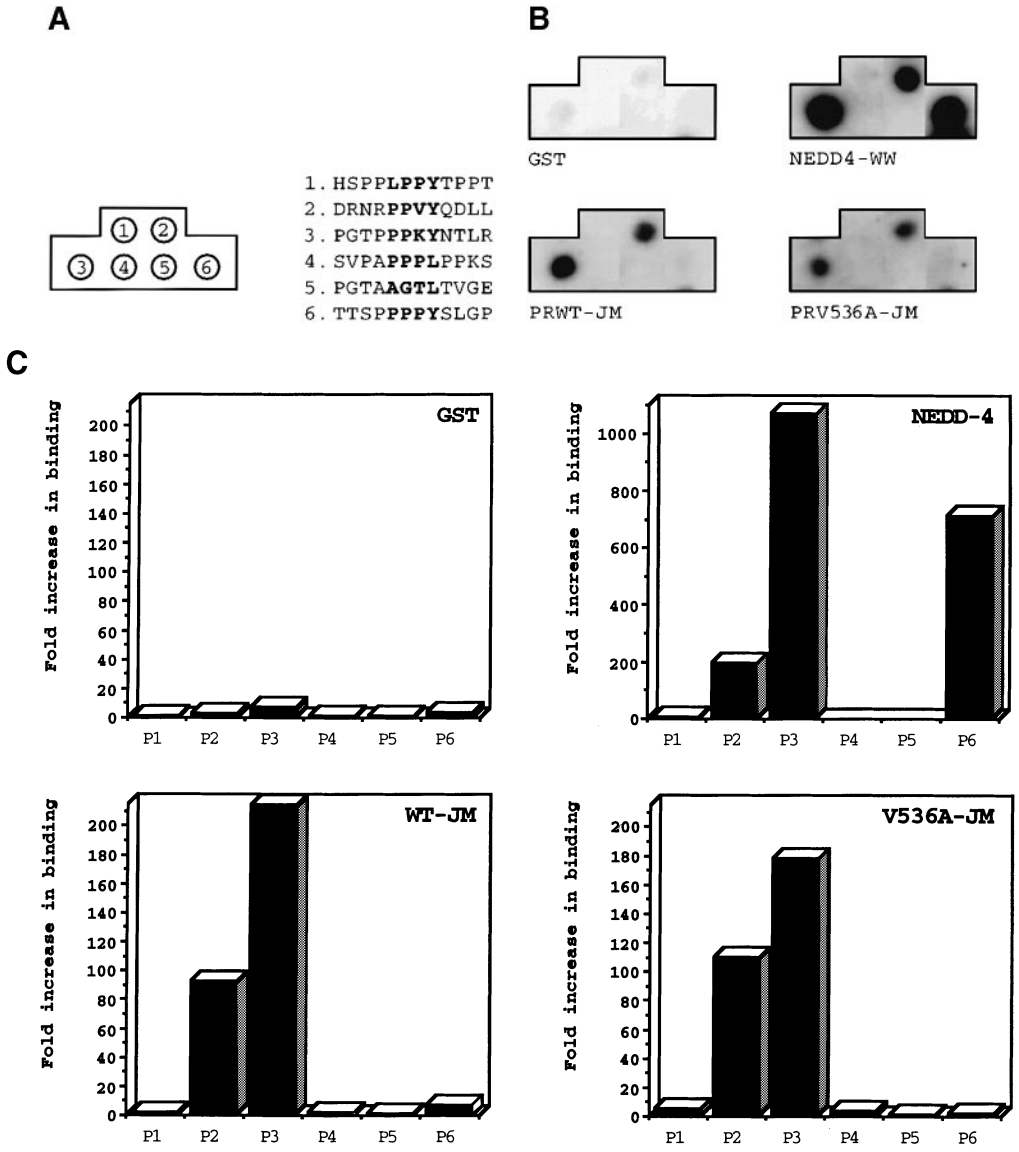

Fig. 10. Peptide-binding assay. Several 12mer peptides were synthesized on replicate nitrocellulose filters which subsequently were probed with the indicated ^{32}P-labeled GST fusion proteins. (**A**) Orientation and sequences of the peptides which were derived from the following proteins: renal chloride channel CLCN5 (peptide 1), IL7-R (peptide 2), ENaC-γ (peptide 3), CrK (peptide 4) and IL6-Rα (peptide 6). The proline-rich sequences are shown in bold. Peptide 5 is a negative control. (**B**) Association of GST fusion proteins with peptides. Filters containing the indicated peptides were incubated overnight at 4°C with ^{32}P-labeled GST (upper left panel), GST fused to the first WW domain of rat NEDD-4 (upper right panel), GST fused to the juxtamembrane domain of the wild-type PDGFβR (lower left panel), or GST fused to the juxtamembrane domain of mutant PR-V536A (lower right panel). After washes, the filters were exposed in a PhosphorImager cassette and analyzed using Molecular Dynamics PhosphorImager IQ software. All filters were exposed for the same length of time. The panels shown are a composite of spots derived from a single experiment; the same results were obtained in one or more additional experiments. (**C**) Quantification of binding to proline-containing peptides. The intensity of the spots in (B) was measured as described in Materials and methods, and the results are shown for the indicated ^{32}P-labeled fusion protein probes. Note that the scale for GST–NEDD-4 is different from that of the other probes.

5). Quantification of spots 2, 3 and 6 showed an increase in binding intensity of ~200- to 1000-fold over background levels (Figure 10C, upper right panel). Strikingly, GST fusion proteins containing the PDGFβR wild-type or V536A juxtamembrane region also recognized PPXY-containing peptides in this assay (Figure 10B, bottom panels, spots 2 and 3), and failed to recognize peptides lacking the PPXY motif (spots 1, 4 and 5). Quantification of positive spots 2 and 3 revealed a 100- to 200-fold increase in signal intensity (Figure 10C, lower panels). Unlike the NEDD-4 WW domain, the receptor juxtamembrane region did not recognize peptide 6. The wild-type and mutant versions of the juxtamembrane domain did not display differential association with the particular peptides tested.

Discussion

The results reported here demonstrated that single amino acid substitutions 12–14 residues downstream of the transmembrane (TM) domain of the PDGFβR and related RTKs caused constitutive receptor activation. These activated receptors transformed Ba/F3 cells to IL-3 independence and were constitutively tyrosine phosphorylated in

Ba/F3 and 293 cells. The valine to alanine substitution activated the PDGFβR through a ligand-independent mechanism, since a receptor mutant unable to respond to PDGF was also activated by the mutation. In addition, the identity of the TM domain was not important for the activity of the point mutant receptor, since the V536A substitution also caused constitutive activation of a chimeric PDGFβR carrying the TM domain of glycophorin A (P.Irusta, L.Petti and D.DiMaio, unpublished results). Analysis of the kinase-defective mutant receptor and studies with a specific inhibitor of the PDGF receptor kinase demonstrated that the intrinsic kinase activity of the receptor was required for signaling and tyrosine phosphorylation of PR-V536A.

Dimerization of the receptor and stimulation of its intrinsic tyrosine kinase activity appear to be central events in RTK activation by ligand binding or by many activating mutations (Heldin, 1992). In the case of the PDGFβR, activation by PDGF, BPV-E5 or fusion to the dimerization domain of TEL or the transmembrane domain of Neu* is believed to be mediated by receptor dimerization (Kelly *et al.*, 1991; Golub *et al.*, 1994; Lai *et al.*, 1998; Petti *et al.*, 1998). In these cases, dimerization is associated with increased tyrosine kinase activity of the receptor *in vitro*. The V536A mutation also directly or indirectly resulted in constitutive receptor dimerization, and PR-V536A displayed increased tyrosine kinase activity *in vitro* on an exogenous substrate. This result suggests that the mutation may be acting by stimulating dimerization, thereby increasing the intrinsic kinase activity of the receptor. According to this view, the V536A mutation may create a new site that nucleates or stabilizes dimer formation, cause a conformational change that facilitates dimerization and stimulates kinase activity, or inactivate a sequence that normally inhibits dimerization or catalytic activity in the absence of ligand. However, although the V536A mutation resulted in elevated receptor tyrosine phosphorylation in cells and increased *in vitro* kinase activity on an exogenous substrate, the mutant did not display increased ability to autophosphorylate *in vitro*. There are situations where the level of receptor tyrosine phosphorylation *in vivo* does not correlate with *in vitro* kinase activity. For example, a mutant PDGF receptor has been described that resulted in elevated receptor tyrosine phosphorylation in intact cells upon ligand binding but did not detectably increase the kinase activity of the receptor *in vitro* (Baxter *et al.*, 1998). In addition, others have described activating mutations that affect the specificity of RTKs toward exogenous substrates or alter autophosphorylation site selection (Santoro *et al.*, 1995; Sonyang *et al.*, 1995; Piao *et al.*, 1996).

On the other hand, it remains possible that the increased receptor tyrosine phosphorylation observed in cells is not a consequence of elevated receptor autokinase activity. For example, a basal level of receptor-catalyzed autophosphorylation may be sufficient to recruit another kinase, which catalyzes further tyrosine phosphorylation of the receptor. The interaction of such a putative kinase with the PDGFβR may be altered by the V536A mutation, resulting in abundant receptor phosphorylation and propagation of the mitogenic signal. Although Src family tyrosine kinases associate with the juxtamembrane region of the PDGFβR and catalyze receptor phosphorylation (Hansen *et al.*, 1996), the tyrosines largely responsible for Src binding are not required for receptor activation by this mutation (unpublished results). Alternatively, the juxtamembrane region of PDGFβR may normally be involved in binding a negative regulator of PDGFβR signaling, and receptor mutations that prevent or inhibit this association may result in activation. For example, if the V536A mutation inhibits the binding of PDGFβR to tyrosine phosphatases or proteins involved in receptor down-regulation, the receptor may display increased tyrosine phosphorylation with basal or even reduced levels of autokinase activity. Negative regulators of PDGFβR signaling have not been described, but precedents for such mechanisms exist for other type III RTKs. For example, genetic and biochemical studies indicate that the tyrosine phosphatase SHP1 negatively regulates signaling by both c-Kit and CSF1-R (Chen *et al.*, 1996; Lorenz *et al.*, 1996; Paulson *et al.*, 1996). In the case of c-Kit, tyrosine phosphatases SHP1 and SHP2 have been shown to associate with phosphotyrosines 567 and 569 in the juxtamembrane region of the murine receptor and to down-regulate c-Kit signaling after ligand stimulation (Kozlowski *et al.*, 1998). Although c-Kit mutants carrying phenylalanine substitutions in these residues exhibited enhanced proliferative response to SCF, these mutants were not constitutively activated. Similarly, analogous substitutions in PDGFβR failed to cause significant activation (Drummond-Barbosa *et al.*, 1995; unpublished results), and we have not been able to detect differential association of SHP1 with V536A compared with wild-type PDGFβR (data not shown). Therefore, it does not appear that the V536A mutation induces activation by impairing binding of SHP1.

The notion that the V536A mutation disrupts a function of the juxtamembrane region is consistent with the finding that alanine substitutions at several residues close to V536 also activated the PDGFβR (unpublished results). In fact, this region of type III RTKs appears to be particularly sensitive to mutations. A valine to glycine substitution at residue 559 of the murine c-Kit receptor (located one residue downstream of the human c-Kit V559A mutation analyzed here) causes constitutive activation (Kanakura *et al.*, 1994; Kitayama *et al.*, 1995). During the preparation of this manuscript, Hirota *et al.* (1998) reported the isolation of activated forms of c-Kit from human gastrointestinal stromal tumors. The activating mutations were localized within the juxtamembrane region of c-Kit, and included several in-frame deletions and a valine to aspartic acid substitution at the residue homologous to V536 in the PDGFβR.

Regardless of the mechanism by which the juxtamembrane mutations activate the type III RTKs, our results suggest that this region of the receptor may comprise a discrete protein domain. The amino acid sequence of the juxtamembrane region of PDGFβR and related receptors closely resembles known WW domains, and the ability of GST fusion proteins carrying the juxtamembrane region of PDGFβR to recognize specifically peptides containing the sequence PPXY provides strong support for the identification of this region as a WW domain. The association of the PDGFβR juxtamembrane domain with the PPXY peptides appeared weaker than that observed for a known WW domain–PPXY peptide pair (NEDD-4/ENaC),

probably because the peptides tested here are almost certainly not the natural partners of the WW-like domain of PDGFβR. The RTK WW-like domains present several differences compared with canonical WW domains, which might influence substrate recognition. In fact, even canonical WW domains display considerable selectivity in their recognition of particular proline-rich peptides. For example, Pirozzi *et al.* (1997) have reported WW domains that bind some PPXY-containing peptides but not others. Thus, critical surrounding residues and the core motif of authentic substrates could differ from those contained in the peptides tested here. Similarly, the lack of differential binding between the wild-type and mutant PDGFβR juxtamembrane domains may reflect the fact that the authentic binding partners were not tested. We are currently screening cDNA libraries for physiologically relevant proteins able to interact with the juxtamembrane region of PDGFR. Finally, it is also possible that the receptor juxtamembrane domain serves several functions, and that the valine to alanine mutation induces activation without affecting the ability of the WW-like domain to recognize its proline-rich targets.

The central aromatic/hydrophobic core of canonical WW domains generally contains two contiguous aromatic residues followed by either another aromatic residue or a hydrophobic one, whereas the juxtamembrane region of the type III RTKs contains two aromatic residues separated by a single amino acid. In the case of PDGFβR, it is known that tyrosines Y547 and Y549 in the juxtamembrane domain, when phosphorylated, form the binding site for Src family tyrosine kinases (Mori *et al.*, 1993). Since the sequence context of these tyrosines is likely to be critical for their recognition and phosphorylation by the PDGFβR kinase domain and for the specificity of the association with the Src SH2 domain, the absence of two consecutive aromatic residues in the WW-like domain of RTKs may be dictated by the need to preserve these interactions. Since most WW domains were identified through data bank searching using an algorithm that demands two consecutive aromatic residues in the central core, it is likely that other WW domain-like modules were missed in these searches and may be present in a variety of other cellular proteins.

The identification of a WW-like domain in receptor tyrosine kinases may provide new insight into receptor function. For example, the proline-rich proteins to which WW domains bind are predicted to include a number of serine/threonine kinases and cytokine receptors which might regulate RTK signaling (Einbond and Sudol, 1996). In addition, the presence of tyrosines involved in Src binding in the putative WW domain itself raises new possibilities for regulation of receptor function. Phosphorylation of these tyrosines may directly influence binding to proline-rich ligands, may induce conformational changes that affect ligand binding or may indirectly influence function by generating a binding site for SH2-containing proteins such as Src that may compete with the proline-rich partner of the WW motif. Conversely, it has been suggested that phosphorylation of the tyrosine residue present in the proline-rich consensus sequence of WW domain ligands may negatively regulate the binding of these proteins to WW domains (Sudol, 1996). Thus, receptor-catalyzed phosphorylation of tyrosines present in the juxtamembrane domain or in the proline-rich ligand may provide a novel feedback mechanism to regulate WW domain function during receptor activation. WW-like domains are not present in the juxtamembrane region of other RTKs, suggesting that this sequence motif plays a role specific to the type III RTK family.

PR-V536A is, to our knowledge, the first constitutively activated PDGFβR generated by point mutation. This mutant receptor may prove to be a useful reagent for the study of PDGFβR signaling, as well as for the identification of natural and synthetic inhibitors of this signaling pathway. The location of activating mutations in the WW-like domain that comprises the juxtamembrane region of PDGFβR and related RTKs indicates that this region may play a general role in the regulation of signaling, perhaps by mediating important protein–protein interactions.

Materials and methods

DNAs and retroviral stocks

pmPRRV-3, a retroviral vector pLXSN carrying the wild-type murine PDGFβR (Petti *et al.*, 1997), was used as a template in standard site-directed mutagenesis reactions (Quick-Change site-directed mutagenesis kit, Stratagene, CA) to generate the various murine PDGFβR mutants described here. pLXSN constructs containing the wild-type human PDGFβR or PDGFαR (a generous gift of A.Kazlauskas) were used to generate phuPRβ-V569A and phuPRα-V561A, respectively, by the same methodology. pRV-PR-ΔEx-WT was generated by digestion of pmPRRV-3 with *Eco*RV and *Pml*I, followed by self-ligation of the large fragment to generate an in-frame deletion which removed nucleotides 402–1401 (L.Petti and D.DiMaio, unpublished results). This construct was used to generate pRV-PR-ΔEx-V536A by site-directed mutagenesis. Expression of these muPDGFβR genes resulted in the synthesis of truncated receptors lacking amino acids 135–467. The wild-type human CSF1-R gene was subcloned by standard cloning techniques from the vector psv7d-CSF1-R (Petti and DiMaio, 1994) into pLXSN to generate pRV-CSF1-R-WT. This construct was used as a template for the generation of pRV-CSF1-R-I552A by site-directed mutagenesis as described above.

Retroviral vectors were used to transfect BOSC cells (a 293 cell-derivative retrovirus packaging cell line kindly provided by Dr Warren Pear) (Pear *et al.*, 1993) by a standard calcium phosphate method as described elsewhere (Petti and DiMaio, 1994). Two days after transfection, virus-containing supernatants were harvested and used to infect Ba/F3 cells as described in the next section.

A vector containing the wild-type human c-Kit gene (pcDNA3-huKit-WT), a generous gift from J.Longley (Yale University), was used as a template for site-directed mutagenesis to generate pcDNA3-huKit-V559A. These plasmids were used to transiently express wild-type and mutant c-Kit receptor in BOSC cells by calcium phosphate transfection.

Cell lines

293 cells and BOSC cells were grown in DMEM-10 medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics). Stable 293 cell lines expressing exogenous wild-type and mutant receptors were obtained by calcium phosphate transfection followed by selection of resistant colonies in the presence of G418 as described (Petti *et al.*, 1997).

Ba/F3 cells were obtained from Alan D'Andrea (Dana-Farber Cancer Institute) and maintained in RPMI/IL-3 media [RPMI 1640 supplemented with 10% heat-inactivated FCS (hiFCS), 10% WEHI conditioned medium, 0.05% mM β-mercaptoethanol and antibiotics]. Ba/F3-derivative cell lines expressing the various wild-type and mutant receptors were established as described before (Drummond-Barbosa *et al.*, 1995). Briefly, ~10^5 colony-forming units (c.f.u.) of retrovirus were incubated with 5×10^6 cells in 10 ml of RPMI/IL-3 containing 4 μg/ml of Polybrene for a period of 2 days. Then, 1 ml of the cells was transferred into 10 ml of RPMI/IL-3 containing 1 mg/ml of G418 (Gibco-BRL) and passaged 1:10 every few days in the same media. After 3–4 passages, mock-infected cells had died and drug-resistant stable cell lines were obtained.

IL-3-independence tests were performed as previously described (Petti *et al.*, 1997). Briefly, Ba/F3 derivative cell lines were grown in RPMI/

IL-3 media to a density of ~10^6 cells/ml, washed once with phosphate-buffered saline (PBS), and transferred to a T-25 flask containing 10 ml of RPMI/No IL-3 medium (RPMI 1640 formulated as described above but without WEHI conditioned media) at a final density of 10^5 cells/ml. The cells were then incubated at 37°C, and viable cells were counted periodically in a hemocytometer to assess cell proliferation.

Immunoprecipitation and immunoblotting

Protein extracts were obtained by lysing cells in EBC buffer (50 mM Tris–HCl pH 8.0, 120 mM NaCl, 0.5% NP-40) as described previously (Drummond-Barbosa *et al.*, 1995; Petti *et al.*, 1997). Approximately 600–1000 μg of extracted protein were used per immunoprecipitation reaction. The PDGFβR and PDGFαR were immunoprecipitated using 10 μl of α-PR-C3a antibody (Drummond-Barbosa *et al.*, 1995; Petti *et al.*, 1997). The CSF1-R and c-Kit receptors were immunoprecipitated using 7 μl of α-fms (UBI, cat. #06-457) and 10 μl of α-c-Kit antibody (Santa Cruz, cat. #sc-168), respectively. Incubation of antibodies with extracts was performed for 2 h in ice, followed by the addition of 60 μl of protein A–Sepharose beads (Pharmacia) [50% slurry in 10 mM Tris–HCl pH 7.4, 165 mM NaCl, 10% bovine serum albumin (BSA)] and incubation for 30 min at 4°C. The beads were then washed four 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× Laemmli sample buffer, and electrophoresed on an SDS–7.5% polyacrylamide gel as described previously (Drummond-Barbosa *et al.*, 1995). Proteins were then transferred to nitrocellulose membranes at 100 V for 2 h and probed with anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc.) and anti-PR-C3a antibody as described (Drummond-Barbosa *et al.*, 1995). For anti-c-Kit immunoblots, α-c-Kit antibody (Santa Cruz, cat. #sc-168) was used at a 1/100 dilution in blocking buffer (5% milk–TNE-T) (10 mM Tris–HCl pH 7.5, 2.5 mM EDTA, 50 mM NaCl, 0.1% Tween-20) (Drummond-Barbosa *et al.*, 1995).

CSF1-R protein levels were assessed by immunoprecipitation of [^{35}S]methionine-labeled Ba/F3 cells. Briefly, cells were grown to a density of ~10^6 cells/ml in RPMI/IL3 media, washed with PBS and starved for 2 h in RPMI/IL-3 medium lacking methionine. Cells were then labeled by addition of 0.25 mCi/ml of [^{35}S]methionine (Amersham) for 6 h. Protein extracts were made and CSF1-R was immunoprecipitated as described above.

In co-immunoprecipitation experiments, immunoprecipitated RTKs were electrophoresed as described above. Detection of receptor-associated proteins was performed by Western blotting as previously described (Drummond-Barbosa *et al.*, 1995). For PLC-γ, a pool of anti-bovine PLC-γ monoclonal antibodies (UBI, cat. #05-163) was used at a 1:1000 dilution. For GAP, anti-Ras GAP monoclonal antibody (Santa Cruz, cat. #sc-63) was used at a 1:500 dilution. For both PLC-γ and Ras GAP detection, a step using rabbit polyclonal anti-mouse IgG at a 1:1000 dilution was included. For PI3K detection, anti-rat PI3K rabbit polyclonal serum (UBI, cat. #06-195) was used at a 1:1000 dilution. For Figure 8B and C, twice as much extract was immunoprecipitated for lane 3 compared with the other lanes. Band intensities were quantitated on a PhosphorImager and corrected for amount of extract analyzed.

Chemical cross-linking experiments

Stable 293 cell lines expressing either the murine wild-type PDGFβR or PR-V536A were grown to confluence, washed twice with ice-cold PBS and incubated in PBS (containing 1 mg/ml BSA) with or without PDGF (50 ng/ml, Calbiochem) for 90 min in ice. The cells were then washed three times with ice-cold PBS and incubated in PBS or PBS containing 2 mM BS^3 (Pierce) for 90 min in ice. The reaction was quenched by washing once with ice-cold PBS, followed by the incubation in 50 mM Tris–HCl (pH 7.4) for 15 min in ice. All buffers used after stimulation with or without PDGF contained sodium orthovanadate (Na_3VO_4, at a final concentration of 100 μM) to prevent receptor dephosphorylation. The cells were then lysed in EBC buffer and PDGFβR was immunoprecipitated as described above. Immunoprecipitates were subjected to SDS–PAGE (5% polyacrylamide gel) and phosphorylated monomeric and dimeric receptor complexes were visualized by immunoblotting using anti-phosphotyrosine monoclonal antibody 4G10 as described above.

In vitro kinase assay

Ba/F3 derivative cell lines expressing PR-WT, PR-WT together with v-*sis*, PR-V536A or the chimeric receptor PR-Neu* (Petti *et al.*, 1998) were serum starved for ~16 h in RPMI/IL-3 medium containing 1% hiFCS. The cells were then lysed in EBC buffer, and ~1 mg of extracted protein was immunoprecipitated with 10 μl of anti-PR-C3a antibody. The immunoprecipitates were then subjected to an *in vitro* kinase assay as described (Petti *et al.*, 1998). Briefly, ~1/10 of the immunoprecipitates was incubated for 10 min at 30°C in UKB buffer [20 mM PIPES (pH 7), 10 mM $MgCl_2$, 20 μg/ml of aprotinin] containing 0.2 μCi/μl of [γ-^{32}P]ATP in the presence or absence of 1 μg of the substrate GST–PLC-γ fusion protein, which includes amino acids 580–850 from rat PLC-γ (Valius *et al.*, 1995). The reaction was stopped by addition of 2× Laemmli sample buffer and boiling. The samples were then subjected to SDS–PAGE (10% polyacrylamide gel), and the gel was dried and exposed to X-ray film. Quantification of the bands corresponding to phosphorylated receptor was performed by PhosphorImager analysis. Immunoblotting anti-PDGFR was performed in parallel to normalize the kinase activity observed for the amount of receptor present in each sample.

Inhibition of PDGFR kinase activity by AG1295

Ba/F3-derivative cell lines expressing either PR-WT or PR-V536A were incubated for 5 min at 37°C in the absence or presence of the PDGFR blocker AG1295 (100 μM final concentration) (gift of S.Courtneidge) (Kovalenko *et al.*, 1994). Cells expressing PR-WT were then stimulated for 5 min at 37°C with PDGF BB (50 ng/ml final concentration), whereas cells expressing PR-V536A were left unstimulated. The cells were then collected and lysed, and the phosphotyrosine content of the receptor was analyzed as described above in the immunoprecipitation and immunoblotting section.

Production and labeling of GST fusion proteins

The juxtamembrane region of the murine PR-WT and PR-V536A (amino acids 520–580) as well as the first WW domain of rat NEDD-4 (amino acids 301–355, derived from a clone kindly provided by Dr Cecilia Canessa, Yale University, CT) were amplified by standard PCR techniques using *Pfu*I DNA polymerase (Stratagene), and cloned into the vector pGEX-2TK (Pharmacia). GST fusion proteins were purified from *E.coli* DH5-α expressing the different plasmids as described previously (Frangioni and Neel, 1993). Briefly, cells were lysed by sonication in ice-cold STE buffer [10 mM Tris (pH 8), 150 mM NaCl, 1 mM EDTA] containing 100 μg/ml of lysozyme, 5 mM dithiothreitol (DTT) and 0.75% Sarkosyl. After centrifugation, Triton X-100 was added to the supernatant to a final concentration of 2%, and the solubilized GST fusion proteins were then isolated using glutathione-conjugated Sepharose beads (Pharmacia) as described (Frangioni and Neel, 1993).

The purified GST fusion proteins were ^{32}P-labeled as previously described (Kaelin *et al.*, 1992). Briefly, Sepharose beads containing the desired GST fusion protein were washed once with HMK buffer [20 mM Tris (pH 7.5), 100 mM NaCl, 12 mM $MgCl_2$], and resuspended in 2–3 bead volumes of HMK buffer containing 1 mM DTT, 1 μCi/μl of [γ-^{32}P]ATP (4500 Ci/mmol, 10 mCi/ml, ICN) and 1 U/μl of the catalytic subunit of cAMP-dependent protein kinase (Sigma). The kinase reaction was performed at 4°C for 30 min and terminated by the addition of 1 ml of HMK Stop buffer [10 mM sodium phosphate (pH 8), 10 mM sodium pyrophosphate, 10 mM EDTA]. The beads were then washed five times with PBS, and the labeled GST fusion proteins were eluted in 4–8 bead volumes of 25 mM reduced glutathione, 50 mM Tris (pH 8), 150 mM NaCl, 0.1% Triton X-100 and 0.75% Sarkosyl.

Hybridization of SPOTs membranes

SPOTs filters containing diverse 12 amino acid long peptides were obtained from Genosys Biotechnologies (The Woodlands, TX). Filters were blocked overnight at room temperature with blocking buffer (TBST–5% milk), and binding reactions were carried out overnight at 4°C using as probes ~300 000 c.p.m./ml of ^{32}P-labeled GST fusion protein diluted in blocking buffer. Filters were then washed three times (15 min each) with blocking buffer, covered with Saran Wrap, and exposed overnight in a PhosphorImager cassette. Quantification of spots was performed using Molecular Dynamics PhosphorImager IQ software. For each filter, the intensity of the signal at peptide 5 was considered background binding and assigned the value of 1. The signal intensities obtained with the remaining peptides were expressed as the fold increase over the intensity obtained with peptide 5.

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