Autoinhibition of the Platelet-derived Growth Factor β-Receptor Tyrosine Kinase by Its C-terminal Tail*

Federica Chiara‡, Subal Bishayee§, Carl-Henrik Heldin‡, and Jean-Baptiste Demoulin‡¶‖

From the ‡Ludwig Institute for Cancer Research, S-75124 Uppsala, Sweden, the ¶Christian de Duve Institute of Cellular Pathology, Université Catholique de Louvain, B-1200 Brussels, Belgium and the §Department of Pathology and Laboratory Medicine, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey 07103

In this report, we investigated the role of the C-terminal tail of the platelet-derived growth factor (PDGF) β-receptor in the control of the receptor kinase activity. Using a panel of PDGF β-receptor mutants with progressive C-terminal truncations, we observed that deletion of the last 46 residues, which contain a proline- and glutamic acid-rich motif, increased the autoactivation velocity *in vitro* and the V_{max} of the phosphotransfer reaction, in the absence of ligand, as compared with wild-type receptors. By contrast, the kinase activity of mutant and wild-type receptors that were pre-activated by treatment with PDGF was comparable. Using a conformation-sensitive antibody, we found that truncated receptors presented an active conformation even in the absence of PDGF. A soluble peptide containing the Pro/Glu-rich motif specifically inhibited the PDGF β-receptor kinase activity. Whereas deletion of this motif was not enough to confer ligand-independent transforming ability to the receptor, it dramatically enhanced the effect of the weakly activating D850N mutation in a focus formation assay. These findings indicate that allosteric inhibition of the PDGF β-receptor by its C-terminal tail is one of the mechanisms involved in keeping the receptor inactive in the absence of ligand.

The platelet-derived growth factor (PDGF)[1] receptors are receptor tyrosine kinases that trigger essential cellular responses such as proliferation, migration, and survival, particularly in the developing embryo (1). Four polypeptide chains, namely PDGF-A, -B, -C, and –D, form homodimeric and heterodimeric ligands that bind to two structurally related PDGF receptors, PDGFRα and PDGFRβ (2). Each receptor contains a large extracellular domain involved in growth factor binding and an intracellular region that includes a split tyrosine kinase domain flanked by a juxtamembrane domain and a C-terminal tail. PDGF stimulation induces receptor dimerization, which causes a dramatic increase in its kinase activity, resulting in the autophosphorylation of a number of tyrosine residues in the intracellular domain that act as docking sites for cytoplasmic signaling proteins (2).

PDGF participates in the development of certain tumors, as illustrated first by the observation that the simian sarcoma virus oncogene v-*sis* is functionally identical to PDGF-B (3, 4). Aberrant expression of PDGF ligands or receptors occurs in certain forms of neoplasia, such as gliomas. In addition, genetic alterations of the PDGF receptor genes have been found in different types of human cancer cells. These modifications produce ligand-independent activated receptors, which stimulate cell growth in an uncontrolled manner. For instance, in chronic monomyelocytic leukemia, chromosomal translocations lead to the production of oncogenic chimeric proteins in which the PDGFRβ kinase domain is fused to an oligomerization motif, such as the transcription factor Tel (5). Moreover, activating point mutations were described in the PDGFRα juxtamembrane domain and the activation loop in certain gastrointestinal stromal tumors (6).

The mechanism by which the PDGF receptor kinase is activated by ligand binding is not fully understood. In several other receptor tyrosine kinases, such as the insulin receptor, a structurally conserved mobile segment containing key regulatory tyrosines, called the activation loop, controls the access to the active site cleft. Dimerization of the kinase domain upon ligand binding provokes the *trans*-phosphorylation of the activation loop, which moves from a closed conformation to a position that allows substrate binding (7, 8). In the PDGF β-receptor, however, the role of the phosphorylation of the activation loop tyrosine, located at position 857, has been debated. The mutation of that residue has only a moderate effect on receptor phosphorylation (9), whereas the corresponding mutation in other tyrosine kinase receptors, such as the hepatocyte growth factor receptor (c-Met) and insulin receptors, severely impairs their kinase activities (10). In addition, introduction of the D850N mutation in the PDGFRβ activation loop is not enough to confer transforming potential (this report and footnote 2),[2] in contrast to the homologous mutations in the stem cell factor receptor (c-Kit) and c-Met, which have been found in tumors (11–14). Altogether, these observations suggest that additional mechanisms may control the activation of PDGFRβ. In this respect, the receptor juxtamembrane region, which resembles a WW domain, was suggested to play a self-inhibitory role in the receptor kinase activation (15). Finally, protein tyrosine phosphatases may also prevent receptor activation in the absence of ligand (16).

In this study, we reveal an additional intramolecular mech-

[1] The abbreviations used are: PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; MBP, myelin basic protein; NHERF, Na$^+$/H$^+$ exchanger regulatory factor; PAE cells, porcine aortic endothelial cells.

[2] F. Chiara and R. Heuchel, unpublished results.

anism that keeps PDGFRβ in an inactive state in the absence of PDGF. We show that deletion of the PDGFRβ C-terminal tail increases the autoactivation velocity and the V_{max} of the receptor kinase in the absence of ligand. The kinase activities of mutant and wild-type receptors were comparable when the receptors were subjected to ligand-mediated dimerization before the assay. The inhibitory function of the C-terminal tail could be mimicked by a soluble peptide comprising part of that domain. Deletion of the PDGFRβ C terminus did not confer ligand-independent activation in cells but cooperated with the D850N mutation in the activation loop to produce a transforming receptor.

EXPERIMENTAL PROCEDURES

Mutagenesis—Site-directed mutagenesis was performed on a cDNA encoding the full-length human PDGFRβ inserted into the pcDNA3 cloning vector (Invitrogen) using the QuikChange kit (Stratagene). The mutations were confirmed by sequencing.

Cell Culture, Reagents, and Transfection—COS-1 simian kidney epithelial cells (American Type Culture Collection, Manassas, VA) and porcine aortic epithelial (PAE) cells were cultured in Dulbecco's modified Eagle's medium and Ham's F12, respectively, supplemented with 10% fetal bovine serum (Sigma). NIH3T3 mouse fibroblasts and L6 myoblasts were grown in Dulbecco's modified Eagle's medium in the presence of 10% calf serum (Invitrogen). Anti-phosphotyrosine (pY99, catalog number sc-7020) was obtained from Santa Cruz Biotechnology. Polyclonal anti-PDGFRβ R3 and monoclonal antibodies B1 and B2, raised against the extracellular region of PDGFRβ, have been described elsewhere (16). Purified human PDGF-BB was generously provided by Amgen. Serum LW4, raised against fibroblast growth factor receptor-1 (FGFR-1) was kindly provided by Lena Claesson-Welsh.

Peptides, corresponding to amino acids residues 1063–1088 (PE1/2), 1063–1077 (PE1), and 1078–1088 (PE2) of the human PDGFRβ C-terminal tail (amino acid sequences are PLEPQDEPEPEPQLELQVE-PEPELEQ, PLEPQDEPEPEPQLE, and LQVEPEPELEQ, respectively) or a peptide derived from the C-terminal tail of human FGFR1 (PSF-PDTRSSTCSSGEDSVFSHEPLPEEP) were synthesized on an Applied Biosystems 433A instrument using Fmoc (*N*-(9-fluorenyl)methoxy-carbonyl) chemistry and purified by preparative reversed phase chromatography.

Transient transfection of COS cells and stable transfection of PAE cells was performed using a DNA-calcium phosphate procedure (Cell-Phect transfection kit, Amersham Biosciences) or LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's instructions. PDGF-BB stimulation (10 ng/ml) was performed on serum-starved COS or PAE cells for 7 min at 37 °C. Focus formation assays were performed as described (17). Briefly, cells ($1.5 \cdot 10^5$ cells per 100-mm plate) were transfected with the appropriate pcDNA3 plasmid (10 μg/plate) by the calcium phosphate method in the presence of 10% calf serum (Colorado Serum Company, Denver, CO). After 24 h, serum concentration was reduced to 5%, and the cells were cultured for additional 21 days. The medium was changed every 3 days. Foci were scored after fixation and staining with Giemsa dye.

Western Blot Analysis—Protein extracts were obtained by solubilizing cells in lysis buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM sodium orthovanadate, 1% Trasylol, and 1 mM Pefabloc). Extracts were clarified by centrifugation, and protein concentration was determined by BCA protein assay system (Pierce).

Approximately 600–1000 μg of extracted protein were used per immunoprecipitation reaction. The PDGFRβ was immunoprecipitated using 3 μg of B1 and B2 monoclonal antibodies kindly provided by Dr. K. Rubin. Incubation of antibodies with extracts was performed for 2 h at 4 °C after pre-incubation of 40 μl of protein A-Sepharose beads (Pharmacia) with rabbit anti-mouse antibodies (Pierce) for 30 min at 4 °C. The beads were then washed four times with buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 150 mM NaCl), boiled for 5 min in 2× Laemmli SDS-sample buffer, and run on an 8% polyacrylamide gel. Proteins were then transferred to nitrocellulose membranes and probed with anti-phosphotyrosine antibodies or anti-PDGFRβ antibodies (Santa Cruz Biotechnology).

Kinase Assays—Transiently transfected COS cells were lysed in lysis buffer in the absence of sodium orthovanadate to allow dephosphorylation, and PDGFRβ was immunoprecipitated with anti-PDGFRβ antibodies. Immunopurified proteins were then washed three times with lysis buffer and two times with kinase buffer (25 mM HEPES, pH 7.1, 5
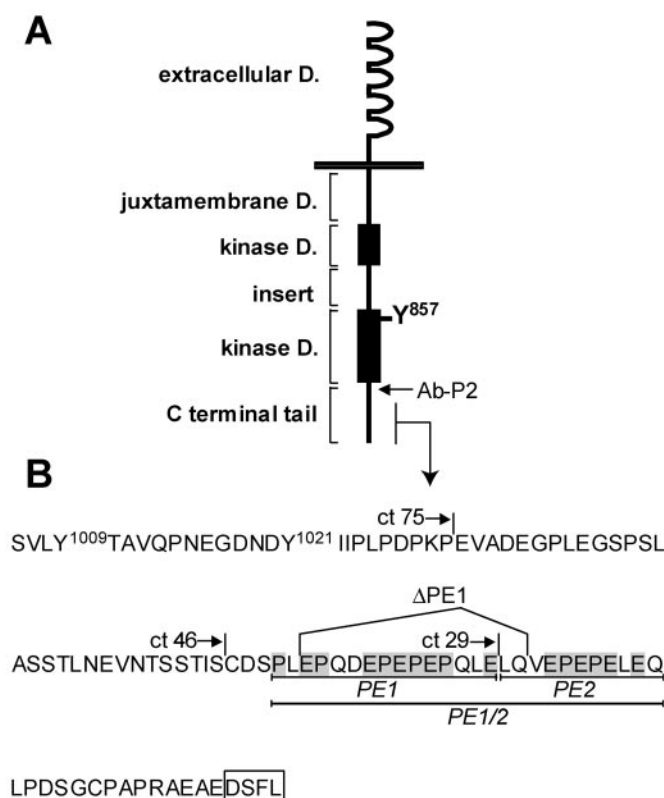

FIG. 1. **PDGFRβ domains and sequence of the C-terminal tail.** *A*, the various domains of PDGFRβ and the epitope recognized by the Ab-P2 antibody are shown. Tyr-857 (*Y857*) is a phosphorylated tyrosine residue of the activation loop of human PDGFRβ. *D.*, domain. The sequence of the C-terminal domain is shown in *panel B*. The P/E repeats are highlighted with a *gray background*. The four different deletion mutants used in this study are indicated (ct29, ct46, ct75, and ΔPE1). The sequences of the PE1/2, PE1, and PE2 peptides used for kinase experiments are underlined. The NHERF binding site is *boxed*.

mM MgCl$_2$, and 100 mM NaCl). The phosphorylation reaction was performed in kinase buffer with [γ-^{32}P]ATP (5 μCi) and 50 μM unlabeled ATP for increasing periods of time at 20 °C. The reaction was stopped by addition of boiling Laemmli SDS sample buffer. The samples were analyzed by 4–12% gradient SDS-PAGE (Invitrogen), Western immunoblotting with anti-PDGFRβ antibodies, and autoradiography. Signal quantification was performed using a PhosphorImager apparatus and Image Quant software (Amersham Biosciences).

For exogenous substrate phosphorylation assays, the phosphorylation reaction was performed in the presence of increasing concentrations of myelin basic protein (MBP) and 10 μM unlabeled ATP and [γ-^{32}P]ATP in kinase buffer for 30 min at 4 °C and blocked by the addition of EDTA. The samples were analyzed as above. Inhibitory peptides (diluted in kinase buffer, pH 8) were added to the reaction mixture at the indicated concentrations.

RESULTS

The C-terminal Tail of PDGFRβ Negatively Regulates Receptor Kinase Activity—To test if the PDGFRβ C-terminal tail is involved in a self-inhibitory mechanism, we constructed deletion mutants lacking the last 29, 46, and 75 amino acid residues of the receptor, referred to as ct29, ct46, and ct75, respectively (Fig. 1). We compared the kinetics of activation of these mutants with wild-type receptors, with or without activation by ligand stimulation.

We first performed *in vitro* autophosphorylation assays on immunopurified receptors from lysates of transiently transfected COS cells in the presence of [γ-^{32}P]ATP. To avoid ligand-independent receptor activation induced by over-expression, only limited amounts of expression vectors were used for transfection. Receptors were immunoprecipitated from cell lysates by using a monoclonal antibody against the extracellular part
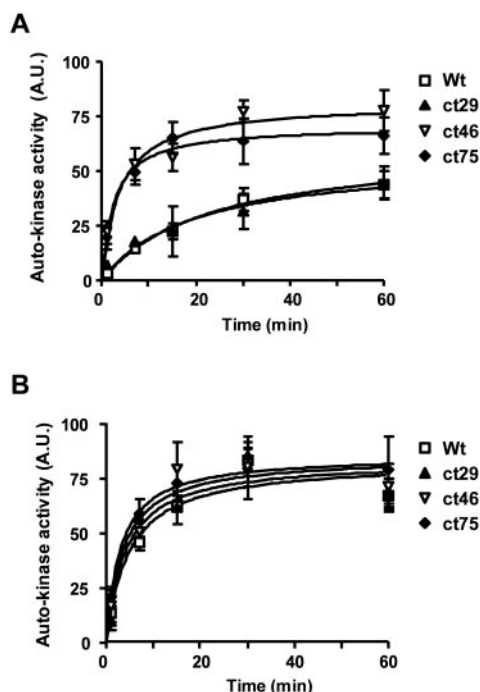
Autoinhibition by the PDGF β-Receptor C-terminal Tail

FIG. 2. **Partial deletion of the C-terminal tail increases PDGFRβ autophosphorylation in the absence of ligand.** Wild-type (*Wt*) or mutated PDGFRβ was transiently expressed in COS1. Cells were starved for 1 day in medium containing 0.1% bovine serum albumin and left untreated (*A*) or stimulated with PDGF-BB (*B*). Immunopurified receptors from COS lysates were incubated in the presence of [γ-^{32}P]ATP for increasing periods of time, and the extent of autophosphorylation was determined by SDS-PAGE and analyzed by a PhosphorImager. Total amounts of receptor were determined by Western blotting and used to normalize the values. The average of three independent experiments with S.D. is shown. *A.U.*, arbitrary units.

FIG. 3. **Partial deletion of the C-terminal tail increases the catalytic activity of the PDGFRβ toward an exogenous substrate.** Wild-type (*Wt*) or mutated PDGFRβ was immunoprecipitated from COS cells left untreated (*A*) or stimulated with PDGF-BB to pre-activate receptors (*B*), as described in the legend of Fig. 2. Immunoprecipitated proteins were incubated in the presence of [^{32}P]ATP and MBP for increasing periods of time. The extent of MBP phosphorylation was determined by SDS-PAGE followed by analysis by a PhosphorImager. Values were treated as described in the Fig. 2 legend. *A.U.*, arbitrary units.

of human PDGFRβ, as described under "Experimental Procedures." As illustrated in Fig. 2*A*, the receptor mutants ct46 and ct75, in the absence of ligand stimulation, were autophosphorylated at a substantially higher rates as compared with those of wild-type PDGFRβ and ct29; ct46 and ct75 reached full activation after 30 min of reaction at 20 °C, whereas wild-type PDGFRβ and ct29 were phosphorylated more slowly. The increased auto-activation velocity of the ct46 and ct75 receptor mutants was observed only in the absence of the ligand; ligand-dimerized receptors and receptor mutants had comparable kinetics of auto-activation (Fig. 2*B*).

We next compared the *in vitro* kinase activity of wild-type and deleted receptors toward an exogenous substrate, MBP. In the experiment presented in Fig. 3*A*, the unstimulated wild-type PDGFRβ and ct29 displayed an initial linear phase with a slow rate of MBP phosphorylation, gradually reaching the saturation at 60 min of reaction. By contrast, ct46 and ct75 rapidly phosphorylated MBP, reaching saturation after 30 min of reaction (Fig. 3*A*). Ligand stimulation enhanced the kinase activity of wild-type and ct29 mutant receptors, which reached maximal MBP phosphorylation at 30 min, whereas ct46 and ct75 reached full phosphorylation of the substrate after only 15 min (Fig. 3*B*). In this assay, ct75 was slightly more active than ct46. The relevance of that small difference was not clear, because it was not confirmed in other assays (see Fig. 2 and below).

To further characterize the mutant receptors, their kinase activities were measured in the presence of different concentrations of substrate. Fig. 4*A* shows that the extent of MBP phosphorylation was higher in unstimulated ct46 and ct75 mutants compared with that in the ct29 and wild-type recep-
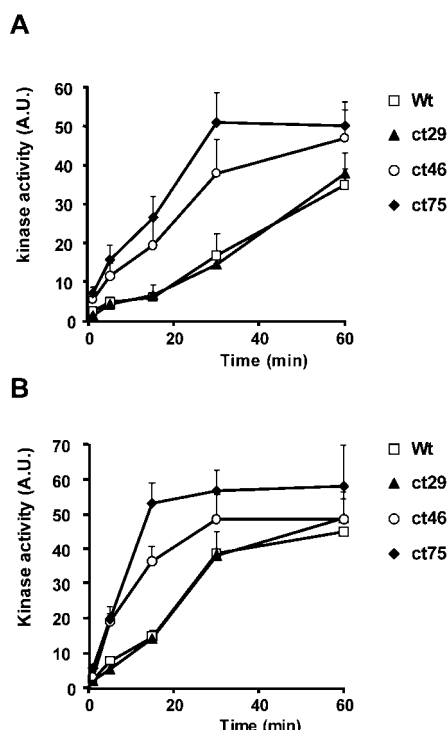
tors. Again, after PDGF stimulation there was no difference in phosphorylation rates between the wild-type and truncated receptors (Fig. 4*B*).

These data suggested a major regulatory role for the amino acid sequence located between positions 46 and 29 in the C terminus of PDGFRβ. This region contains a glutamic acid/proline repeat flanked by glutamine residues, referred to as the PE1 motif (see Fig. 1). Interestingly, a similar motif, named PE2, is also present in the last 29 amino acids of the receptor. To test the importance of the PE1 motif directly, we designed a new mutant receptor, called ΔPE1, in which 15 residues, including the PE1 motif, were deleted, and we compared its autokinase activation kinetics with wild-type and mutated receptors. As shown in Fig. 5*A*, in the absence of ligand stimulation the ΔPE1 mutant was autophosphorylated at the same rate as ct46 and ct75 and reached full activation after 30 min, whereas wild-type PDGFRβ and ct29 were phosphorylated more slowly. As observed previously, the advantage of deleted mutants was lost after ligand stimulation. Altogether, these data suggest that the region containing the PE1 motif in the C-terminal tail of the PDGFRβ is involved in an autoinhibition mechanism, which is cancelled by ligand binding to the receptor.

A Soluble Peptide Mimics the Inhibitory Function of the C-terminal Domain—To further test the role of the P/E-rich sequences in the C-terminal tail, we designed soluble peptides containing the PE1 motif, the PE2 motif, or both (PE1/2, see Fig. 1). The effect of these peptides on receptor kinase activity was tested in autophosphorylation assays performed *in vitro*. We observed that the PE1 and PE1/2 peptides efficiently inhibited PDGFRβ autophosphorylation (Fig. 6). The PE2 pep-
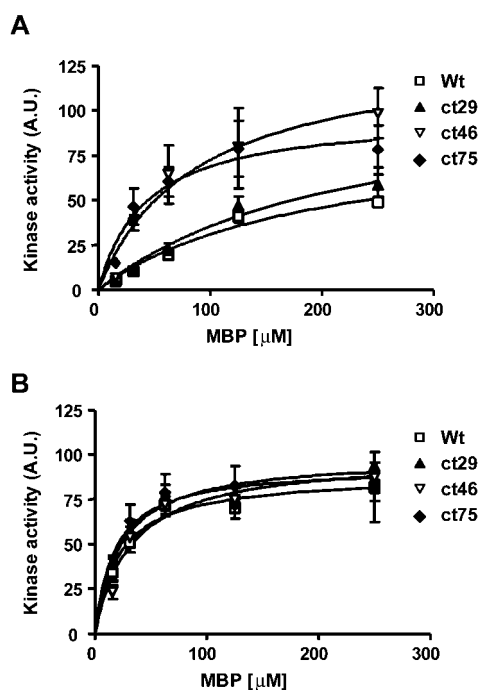
A

B

FIG. 4. **Removal of the C-terminal tail increases the affinity of the receptor for its substrate.** Wild-type (*Wt*) or mutated PDGFRβ receptors were transiently expressed in COS cells. Cells were left untreated (*A*) or stimulated with PDGF-BB (*B*), and the receptors were isolated by immunoprecipitation and used to phosphorylate MBP for 15 min at 20 °C. The amount of [γ-^{32}P]ATP incorporated into MBP at different substrate concentrations was determined by SDS-PAGE followed by analysis by a PhosphorImager. *A.U.*, arbitrary units.

A

B

FIG. 5. **Deletion of the PE1 motif enhanced the PDGFRβ kinase activity.** Wild-type (*Wt*) or mutated PDGFRβ was transiently expressed in COS1. Cells were starved for 1 day in a medium containing 0.1% bovine serum albumin and left untreated (*A*) or stimulated with PDGF-BB (*B*). Immunopurified ct46, ΔPE1, and ct75 deleted receptors from COS lysates were incubated in the presence of [γ-^{32}P]ATP for increasing periods of time, and the extent of autophosphorylation was determined by SDS-PAGE and PhosphorImager analysis. Total amounts of receptor were determined by Western blotting and used to normalize the values. One representative experiment of two is shown. *A.U.*, arbitrary units.

tide did not affect the kinase activity even at 1.25 mM, the highest concentration used. These results are in agreement with the data obtained on mutant receptors, because deletion of the PE2 motif (ct29 mutant) had no effect, whereas deletion of the PE1 motif enhanced the kinase activity (ct46 and ΔPE1 mutants). As an additional specificity control, a synthetic peptide containing a proline-rich sequence derived from the C-terminal tail of human FGFR1 was included in the experiments. This peptide had essentially no effect on the kinase activities of PDGFRβ (Fig. 6) and FGFR1 (data not shown). We also found that the PDGFRβ-derived peptides had no inhibitory activity on two other receptor tyrosine kinases, FGFR1 and EGFR (data not shown).

These results prompted us to test whether the addition of the PE1/2 peptide to deleted receptors could compensate for the loss of kinase self-inhibition during its activation in the absence of ligand stimulation. To explore this possibility, we performed an *in vitro* kinase assay on unstimulated receptors, which were immunoprecipitated from PAE cells stably expressing ct75, to measure their auto-phosphorylation kinetics and their ability to phosphorylate an exogenous substrate in the presence or in the absence of the PE1/2 peptide. We found that the auto-phosphorylation rate of the ct75 mutant was markedly decreased in the presence of PE1/2, reaching a rate similar to that of wild-type PDGFRβ in the absence of peptide. The inhibitory peptide also delayed the activation of the wild-type receptor, thus enhancing the inhibitory effect of the C-terminal tail, but did not affect the maximal auto-kinase activity. Thus, the ct75 mutant was more sensitive to inhibition by the PE1/2 peptide compared with the wild-type receptor, which was to be expected if this peptide mimics the region that is lacking in ct75 (Fig. 7*A*). Similar results were obtained in MBP phosphorylation experiments (Fig. 7*B*). In conclusion, these results further support the notion that a proline- and glutamic acid-
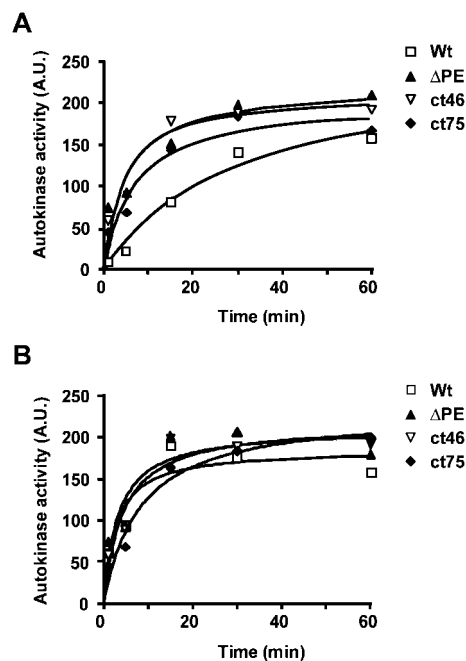
rich motif in the C-terminal part of PDGFRβ negatively regulates its kinase activity.

Active Conformation of Truncated PDGFRβ in the Absence of Ligand—Bishayee and colleagues (18) have suggested that the PDGFRβ C-terminal tail may undergo a conformational change upon ligand binding, based on experiments performed with a conformation-sensitive antibody, called Ab-P2, raised against the unphosphorylated peptide corresponding to amino acid residues 932–947 located close to the kinase domain (Fig. 1 and Ref. 18). This antibody is able to recognize the native wild-type receptor in its activated form only, indicating that the epitope is buried in the inactive receptor and becomes accessible upon ligand binding. The Ab-P2 antiserum was used to immunoprecipitate wild-type PDGFRβ and ct75 from PAE cells. As shown in Fig. 8*A* (*upper panel*), Ab-P2 recognized ct75 even in the absence of PDGF. By contrast, the wild-type receptor was immunoprecipitated only after ligand stimulation, as expected. By a parallel immunoprecipitation with the monoclonal anti-PDGFRβ antibodies B1 and B2, which are directed against the extracellular domain, we confirmed equal receptor expression (Fig. 8*A*, *lower panel*). Thus, the change of conformation of the C-terminal region of PDGFRβ, which is induced by ligand binding, is mimicked by truncation of the last 75 amino acid residues; it is possible that this region interacts with the kinase domain or the juxtamembrane domain and thereby restricts the availability of the active site of the kinase domain.

We next asked whether receptor dimerization was enough to displace the tail from its inhibitory conformation or whether receptor phosphorylation is needed. To address this issue, we analyzed the conformation of a receptor devoid of kinase activity (K634A mutant), which dimerizes upon ligand binding but is not phosphorylated. Using Ab-P2, we could not immunopre-
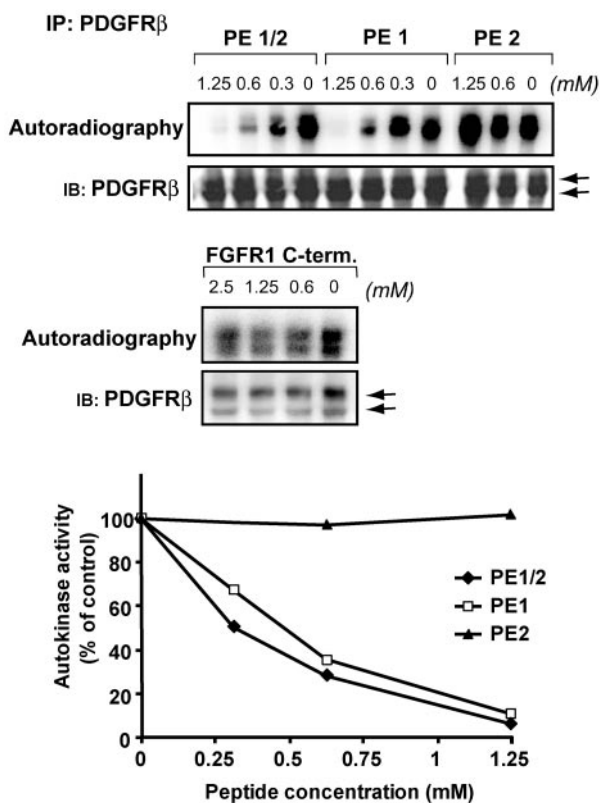
FIG. 6. **Peptides containing the PE1 motif inhibit PDGFRβ kinase activity.** PDGFRβ was immunoprecipitated (*IP*) from transiently transfected COS cells, starved for 24 h, and stimulated with PDGF-BB. Immunocomplexes were subjected to *in vitro* kinase assay in the presence of the indicated peptide concentrations and [γ-^{32}P]ATP under conditions described under "Experimental Procedures." Autophosphorylated receptors were separated by SDS-PAGE and analyzed by autoradiography and immunoblotting (*IB*). After PhosphorImager analysis, values were normalized by dividing by the total amount of receptor. The *arrows* indicate the 190-kDa mature and 170-kDa immature forms of PDGFRβ. *C-term.*, C terminus.

FIG. 7. **The PE1/2 peptide mimics the PDGFRβ C-terminal tail.** PDGFRβ was immunoprecipitated from transiently transfected COS-1 cells, starved for 24 h in starvation medium containing 0.1% bovine serum albumin, and immunocomplexes were subjected to *in vitro* kinase assay for various periods of time in the presence of the indicated peptide (0.6 mM) and [γ-^{32}P]ATP to evaluate the receptor autophosphorylation (*A*) or MBP to measure the activity toward an exogenous substrate (*B*). Proteins were separated by SDS-PAGE and analyzed by a PhosphorImager. Shown are results from one representative experiment of two performed. *Wt*, wild-type; *A.U.*, arbitrary units.

cipitate K634A receptors from PDGF-stimulated transfected COS cells, in contrast to the stimulated wild-type receptor (Fig. 8*B*, *upper panel*). These data suggest that the change in conformation of the C-terminal region is dependent on phosphorylation. We also tested the importance of the phosphorylation of tyrosine 857 in the activation loop. First, we observed that deletion of the C-terminal tail did not modify the phosphorylation of tyrosine 857, as tested by Western blotting with a phospho-specific antibody (data not shown). Mutation of this residue reduces but does not abolish receptor phosphorylation (9). The Ab-P2 antibody did not recognize the Y857F receptor mutant, not even after PDGF stimulation, although the stimulated receptor showed, as described previously (9), some residual auto-phosphorylation (Fig. 8*B*). Altogether, these results suggest that tyrosine 857 phosphorylation is a prerequisite to induce the change of conformation of the PDGFRβ C-terminal domain. This conformational change likely removes the autoinhibition of the PDGFRβ kinase, because it can be mimicked by deletion of the C-terminal inhibitory motif.

Transforming Ability of PDGFRβ by Concomitant Deletion of the C Terminus and Mutation of the Activation Loop—The observation that the truncated PDGFRβ mutants show increased kinase activities in the absence of PDGF led us to investigate whether they could be constitutively activated *in vivo*. For a comparison, we introduced in the PDGFRβ activation loop a D850N mutation, which corresponds to mutations found in oncogenetic versions of c-Kit and c-Met (11, 12, 19). We
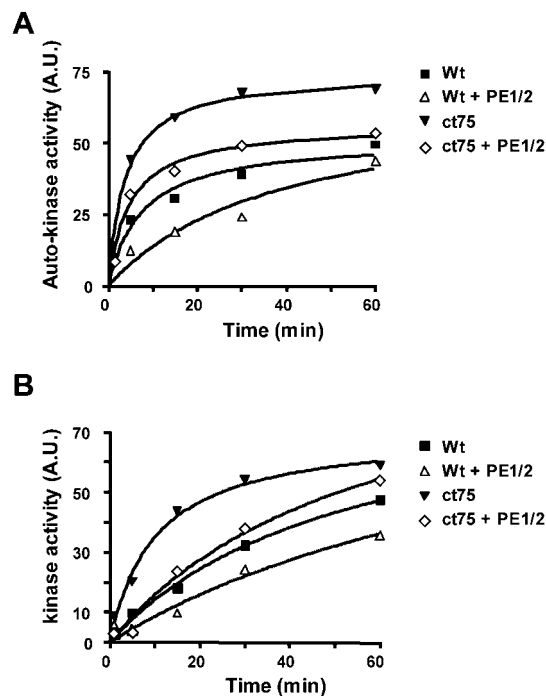
first investigated tyrosine phosphorylation of immunoprecipitated receptors isolated from transfected COS cells that were left untreated or stimulated with PDGF-BB. All mutants displayed a strong ligand-dependent phosphorylation with little background in unstimulated cells (Fig. 9*A*). Because neither ct46 nor D850N were constitutively phosphorylated to any appreciable extent, we produced a double mutant, ct46-D850N, harboring both modifications; its phosphorylation was still dependent on PDGF stimulation (Fig. 9*A*).

The four most C-terminal amino acid residues in the PDGFRβ, DFSL, have been shown to interact with the PDZ domains of NHERF, which was claimed to play a role in the activation of the receptor (20, 21). To investigate whether NHERF binding is important for receptor activation in our system, we added back a DFSL motif at the end of the ct46 receptor, which restored NHERF binding as expected (Fig. 9*B*). However, addition of the DFSL motif did not change the kinase activation parameters *in vitro* (data not shown) and had no major effect on the phosphorylation of the receptor in COS cells.

The transforming potential of deleted PDGF receptors was assessed by a focus formation assay in NIH3T3. Each receptor was transfected alone or in combination with a plasmid encoding the PDGF-B ligand, creating an autocrine stimulation loop. Co-transfection of wild-type PDGFRβ with PDGF-B gave rise to a basal number of transformed colonies (Fig. 9*C*). The ct46 or D850N mutations increased the number of transformed colonies to some extent, but still showed strong ligand dependences. By contrast, the double mutant ct46-D850N increased focus formation in NIH3T3 cell cultures even the absence of PDGF to an extent that was almost comparable to transfection with an activated Ras isoform (M-Ras Q71K; Ref. 22). The
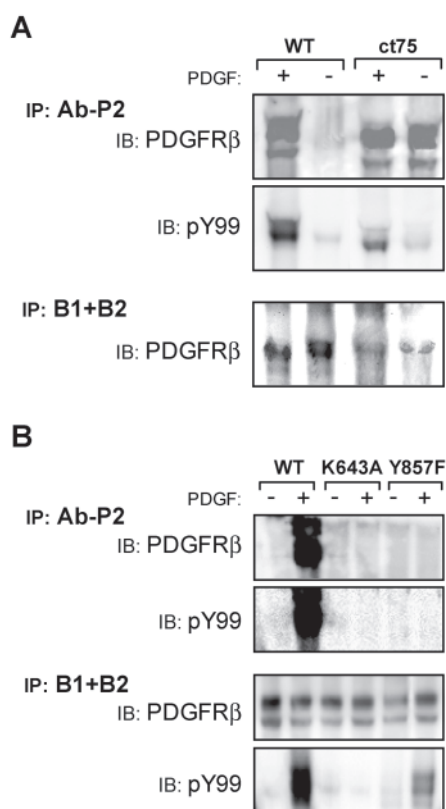
FIG. 8. **Ct75 presents an active conformation in the absence of ligand.** *A*, PAE cells stably expressing the wild-type (*WT*) PDGFRβ or ct75 mutant were starved for 24 h and stimulated with PDGF-BB. Cell lysates were subjected to immunoprecipitation (*IP*) with immunopurified Ab-P2 antiserum or B1 and B2 monoclonal antibodies, followed by SDS-PAGE and immunoblotting (*IB*) with PDGFRβ antiserum R3 or antibody anti-phosphotyrosine (*pY99*). *B*, COS cells were transiently transfected with wild-type PDGFRβ or with K634A (kinase dead) or Y857F mutated receptors, treated, and analyzed as described above.

addition of the DFSL motif to these receptors did not change their abilities to promote the formation of foci (Fig. 9*D*), suggesting that NHERF is not important in the process. In conclusion, these results further illustrate the inhibitory role of the PDGFRβ C-terminal tail and show that several mechanisms collaborate to keep the receptor inactive in the absence of PDGF.

DISCUSSION

Uncontrolled activation of receptors has dramatic consequences *in vivo*. In the case of receptor tyrosine kinases, several mechanisms concur to silence receptors in the absence of ligand. In the present report, we demonstrate that, in PDGFRβ, one such inhibitory control is exerted by the C-terminal part of the receptor. This conclusion is based on three lines of evidence. First, deletion of the PDGFRβ C terminus enhanced its *in vitro* kinase activity toward itself or an exogenous substrate. Second, peptides corresponding to a Glu/Pro-rich motif present in the C-terminal tail specifically inhibited PDGFRβ activity; and finally, introduction of a C-terminal truncation increased the transforming potential of the D850N PDGFRβ mutant.

We speculate that the PDGFRβ C terminus moves from an inhibitory conformation to a more permissive position upon PDGF binding. Using the Ab-P2 conformation-sensitive antibody, Bishayee and colleagues had already suggested that a region located just after the PDGFRβ kinase domain undergoes a conformational change that, upon ligand binding, unmasks the epitope of the antibody (18). We show that Ab-P2 was able
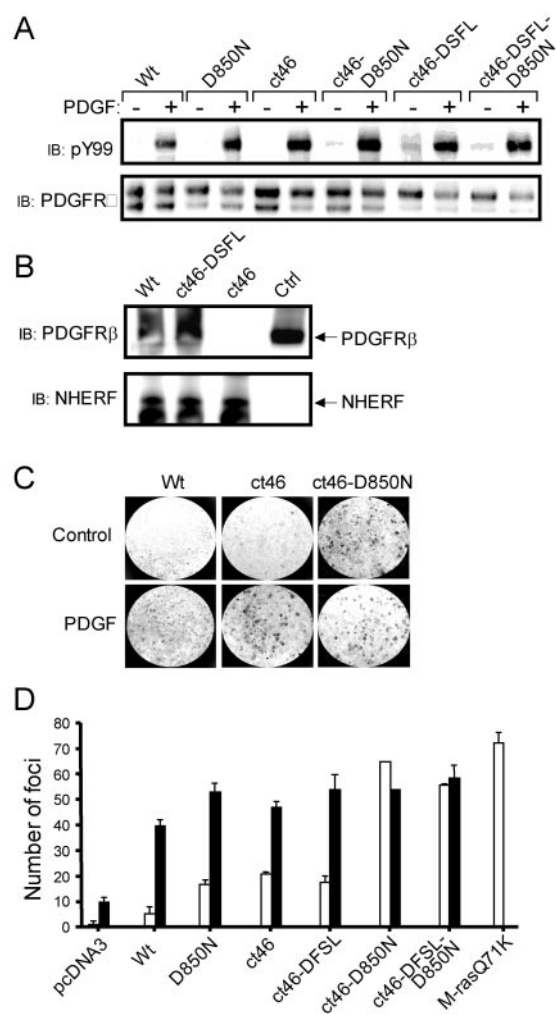

FIG. 9. **Concomitant deletion of the PDGFRβ C terminus and mutation of the activation loop produce a potent ligand-independent transforming receptor.** *A*, wild-type (*Wt*) and PDGFRβ mutants were immunopurified from lysates of COS cells transiently transfected with the indicated construct, starved, and then stimulated with PDGF-BB or left untreated. Immunoprecipitated proteins were separated by SDS-PAGE using a 4–12% gradient polyacrylamide gel and analyzed by Western blotting (*IB*) using anti-phosphotyrosine (*pY99*) and anti-PDGFRβ antibodies. *B*, NHERF protein was immunoprecipitated from lysates of HEK293T cells transiently transfected with wild-type PDGFRβ, ct46, and ct46DSFL. As a control, we immunoprecipitated PDGFRβ from transiently transfected cells with B1 and B2 monoclonal antibodies (*Ctrl*). Samples were separated on a 4–15% SDS-PAGE gel and analyzed by Western blot using anti-NHERF and anti-PDGFRβ antibodies. *C* and *D*, NIH3T3 cells were transfected with the indicated receptor alone (*open bars*) or co-transfected with a PDGF-B plasmid (*closed bars*). Cells were then grown in medium containing 5% serum for 3 weeks and then were fixed and stained. The number of foci was scored for each 10-cm dish.

to recognize ct75 in the absence of PDGF, suggesting that the C-terminal tail of PDGFRβ is required to stabilize the inactive conformation. Ab-P2 binding also depended on PDGFRβ tyrosine phosphorylation, particularly at Tyr-857 in the activation loop. It is possible that the PE1 motif directly interacts with the activation loop, which is reminiscent of what was proposed for the insulin receptor (23). In this respect, the crystal structure of another receptor tyrosine kinase, TIE2, revealed that its C terminus is located close to the active cleft (24). However, we can not exclude the possibility that mutation of Tyr-857 has an indirect effect on the conformation of the C-terminal domain by controlling the phosphorylation of other residues. The PDGFR tail could also interact with the juxtamembrane domain, which resembles a WW domain, and may therefore bind to proline

rich sequences such as the PE1 motif. Further work is required to elucidate the exact structure of the PDGFRβ C-terminal domain and to determine whether it folds back over the kinase domain or the juxtamembrane domain.

Recently, we and others showed that the PDGFRβ C terminus acts as a binding site for the PDZ domain of NHERF (20, 21). NHERF recruitment to PDGFRβ was suggested to potentiate the receptor activity in cells overexpressing the receptor (20), which was not observed in cells expressing both proteins at physiological levels (21). Our results further suggest that NHERF does not play a major role in PDGFRβ activation, because the addition of a functional NHERF-binding site to the ct46 mutant did not change its activity and transforming potential. Interestingly, a change in conformation of the PDGFRβ C terminus upon PDGF stimulation could provide an explanation for the observation that NHERF recruitment is ligand-dependent (21).

The deregulated activation of receptor tyrosine kinases can mediate cell transformation. In this report, we show that the deletion of 46 amino acid residues of the C-terminal tail of the PDGFRβ confers a transforming potential in a focus formation assay in the presence of the ligand, whereas the concomitant deletion of the C terminus and mutation of the activation loop results in full ligand-independent oncogenic activity. Previous studies have shown that one modification of a receptor is sometimes not enough. For example, the v-*kit* oncogene found in the acute transforming feline retrovirus HZ4-FeSV and amino acid residues in the juxtamembrane domain and the C-terminal sequence are deleted, compared with c-*kit* (25). The activated form of c-Met, D1228N, which was found in tumors, mediates both transformation and tumorigenicity but still requires a ligand to generate foci in a focus-forming assay (26). This mutation overcomes the otherwise required phosphorylation of both tyrosines in the activation loop, thus reducing the threshold for activation of the Met receptor (17); introduction of a second mutation makes the receptor fully transforming and ligand-independent (27). Nevertheless, single mutations that fully activate tyrosine kinase receptors have also been described, *e.g.* in PDGFRα and c-Kit (6, 11, 12).

The PDGFRβ C-terminal sequence is poorly conserved in the other members of the family, *i.e.* PDGFRα, c-Kit, Flt-3, and the CSF-1 receptor, in contrast to the case in other domains. We could not identify any similar Glu/Pro-rich sequence in other tyrosine kinase receptors. In addition, the PE1 peptide had no effect on EGFR and FGFR1. Therefore, if similar mechanisms for autoinhibition occur in other tyrosine kinase receptors, completely different epitopes are involved. In this respect, the PE1 peptide used in this report could serve as a basis for designing specific PDGFR inhibitors. Observations suggesting that the C-terminal tail may prevent activation of tyrosine kinase receptors were also provided in studies of the insulin receptor and TIE2 (24).

In conclusion, we provide evidence that a 15-residue-long Glu/Pro-rich motif in the C terminus of PDGFRβ negatively regulates the activation of the receptor kinase. Future studies will have to determine how this mechanism cooperates with the activation loop, the juxtamembrane domain, or other parts of the intracellular domain of the receptor in keeping the receptor inactive in the absence of ligand.

REFERENCES

1. Heldin, C. H., and Westermark, B. (1999) *Physiol. Rev.* **79,** 1283–1316
2. Heldin, C. H., Östman, A., and Rönnstrand, L. (1998) *Biochim. Biophys. Acta* **1378,** F79–F113
3. Robbins, K. C., Antoniades, H. N., Devare, S. G., Hunkapiller, M. W., and Aaronson, S. A. (1983) *Nature* **305,** 605–608
4. Östman, A., and Heldin, C. H. (2001) *Adv. Cancer Res.* **80,** 1–38
5. Jousset, C., Carron, C., Boureux, A., Quang, C. T., Oury, C., Dusanter-Fourt, I., Charon, M., Levin, J., Bernard, O., and Ghysdael, J. (1997) *EMBO J.* **16,** 69–82
6. Heinrich, M. C., Corless, C. L., Duensing, A., McGreevey, L., Chen, C. J., Joseph, N., Singer, S., Griffith, D. J., Haley, A., Town, A., Demetri, G. D., Fletcher, C. D., and Fletcher, J. A. (2003) *Science* **299,** 708–710
7. Schlessinger, J. (2000) *Cell* **103,** 211–225
8. Weiss, A., and Schlessinger, J. (1998) *Cell* **94,** 277–280
9. Baxter, R. M., Secrist, J. P., Vaillancourt, R. R., and Kazlauskas, A. (1998) *J. Biol. Chem.* **273,** 17050–17055
10. Longati, P., Bardelli, A., Ponzetto, C., Naldini, L., and Comoglio, P. M. (1994) *Oncogene* **9,** 49–57
11. Nagata, H., Okada, T., Worobec, A. S., Semere, T., and Metcalfe, D. D. (1997) *Int. Arch. Allergy Immunol.* **113,** 184–186
12. Longley, B. J., Tyrrell, L., Lu, S. Z., Ma, Y. S., Langley, K., Ding, T. G., Duffy, T., Jacobs, P., Tang, L. H., and Modlin, I. (1996) *Nat. Genet.* **12,** 312–314
13. Schmidt, L., Junker, K., Nakaigawa, N., Kinjerski, T., Weirich, G., Miller, M., Lubensky, I., Neumann, H. P., Brauch, H., Decker, J., Vocke, C., Brown, J. A., Jenkins, R., Richard, S., Bergerheim, U., Gerrard, B., Dean, M., Linehan, W. M., and Zbar, B. (1999) *Oncogene* **18,** 2343–2350
14. Bardelli, A., Longati, P., Gramaglia, D., Basilico, C., Tamagnone, L., Giordano, S., Ballinari, D., Michieli, P., and Comoglio, P. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 14379–14383
15. Irusta, P. M., Luo, Y., Bakht, O., Lai, C. C., Smith, S. O., and DiMaio, D. (2002) *J. Biol. Chem.* **277,** 38627–38634
16. Shimizu, A., Persson, C., Heldin, C.-H., and Östman, A. (2001) *J. Biol. Chem.* **276,** 27749–27752
17. Chiara, F., Michieli, P., Pugliese, L., and Comoglio, P. M. (2003) *J. Biol. Chem.* **278,** 29352–29358
18. Bishayee, S., Majumdar, S., Scher, C. D., and Khan, S. (1988) *Mol. Cell. Biol.* **8,** 3696–3702
19. Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E., and Birchmeier, C. (1995) *Nature* **373,** 699–702
20. Maudsley, S., Zamah, A. M., Rahman, N., Blitzer, J. T., Luttrell, L. M., Lefkowitz, R. J., and Hall, R. A. (2000) *Mol. Cell. Biol.* **20,** 8352–8363
21. Demoulin, J. B., Seo, J. K., Ekman, S., Grapengiesser, E., Hellman, U., Ronnstrand, L., and Heldin, C. H. (2003) *Biochem. J.* **376,** 505–510
22. Louahed, J., Grasso, L., De Smet, C., Van Roost, E., Wildmann, C., Nicolaides, N. C., Levitt, R. C., and Renauld, J. C. (1999) *Blood* **94,** 1701–1710
23. Noelle, V., Tennagels, N., and Klein, H. W. (2000) *Biochemistry* **39,** 7170–7177
24. Shewchuk, L. M., Hassell, A. M., Ellis, B., Holmes, W. D., Davis, R., Horne, E. L., Kadwell, S. H., McKee, D. D., and Moore, J. T. (2000) *Structure Fold. Des.* **8,** 1105–1113
25. Qiu, F. H., Ray, P., Brown, K., Barker, P. E., Jhanwar, S., Ruddle, F. H., and Besmer, P. (1988) *EMBO J.* **7,** 1003–1011
26. Michieli, P., Basilico, C., Pennacchietti, S., Maffe, A., Tamagnone, L., Giordano, S., Bardelli, A., and Comoglio, P. M. (1999) *Oncogene* **18,** 5221–5231
27. Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C. P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude, G. F. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 11445–11450