A Gain of Function Mutation in the Activation Loop of Platelet-derived Growth Factor β-Receptor Deregulates Its Kinase Activity*\[S\]

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The platelet-derived growth factor receptors (PDGFRs) are receptor tyrosine kinases implicated in multiple aspects of cell growth, differentiation, and survival. Recently, a gain of function mutation in the activation loop of the human PDGFRβ, namely an exchange of aspartic acid for asparagine at amino acid position 849 (D849N), confers transforming characteristics to embryonic fibroblasts from mutant mice, generated by a knock-in strategy. By comparing the enzymatic properties of the wild-type versus the mutant receptor protein, we demonstrate that the D849N mutation lowers the threshold for kinase activation, causes a dramatic alteration in the pattern of tyrosine phosphorylation kinetics following ligand stimulation, and induces a ligand-independent phosphorylation of several tyrosine residues. These changes result in deregulated recruitment of specific signal transducers. The GTPase-activating protein for Ras (RasGAP), a negative regulator of the Ras mitogenic pathway, displayed a delayed binding to the mutant receptor. Moreover, we have observed enhanced ligand-independent ERK1/2 activation and an increased proliferation of mutant cells. The p85 regulatory subunit of the phosphatidylinositol 3'-kinase was constitutively associated with the mutant receptor, and this ligand-independent activation of the phosphatidylinositol 3'-kinase pathway may explain the observed strong protection against apoptosis and increased motility in cellular woundng assays. Our findings support a model whereby an activating point mutation results in a deregulated PDGFRβ with oncogenic predisposition.

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§ The abbreviations used are: PDGF, platelet-derived growth factor receptor; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3'-kinase; ES, embryonic stem; MEF, mouse embryonic fibroblast; FCS, fetal calf serum; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; ERK, extracellular signal-regulated kinase; STS, staurosporine.

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creases cellular transforming capacity in vitro, and leads to alterations in cell motility and proliferation.

EXPERIMENTAL PROCEDURES

Generation of Mutant Mice—The genomic DNA used as homology arm for the targeting vector was cut out of genomic clones previously described (14). The mutation of aspartic acid 849 to asparagine was introduced into subcloned genomic DNA by oligonucleotide-directed mutagenesis using the QuikChange kit (Stratagene) according to the manufacturer’s recommendations. The mutation primers were as follows: 5′-GACCTGGCGTCAATCTAAATGAGCAGCTA-3′ and 5′-TCTAAGTTTGTGATCCCTTATAATATTGCGACGAGGGCGAATTGCT-3′ (base exchange resulting in amino acid mutation indicated in lowercase letters). The mutation was finally confirmed by sequencing of a PCR-amplified DNA fragment from targeted embryonic stem (ES) cells.

The targeting vector consisted of a 1.7-kb EcoRV-SpeI genomic 5′-fragment, followed by a PGKneoamA expression cassette flanked by loxP sites, a 5-kb Spel-XhoI genomic 3′-fragment containing the point mutated exon 18, and a herpes simplex virus thymidine kinase expression cassette in pBluescript SK(+) by a single Cre recombinase mediated recombination event. Positive ES clones were isolated after selection (15) and genotyped with G418 and gancyclovir. Homologous recombination events were confirmed by PCR, as described (14), using primers for the targeting construct (5′-CCGAAATGTGTACCAGTCTGAAA-3′) and the neo gene (5′-TGGCTACCCGGTGATTGCTG-3′) and sequence analysis of PCR products. Negative ES clones were removed, since its presence at that specific position in intron 15 had been shown previously to be without effect on the expression of the phosphoinositide 3-kinase (PI3K) regulatory subunit of PI3K antiserum was from Upstate Biotechnology, Inc. (Lake Placid, NY).

Antibodies against phosphorylated tyrosines Tyr(P)727, Tyr(P)725, Tyr(P)731, and Tyr(P)736 of the human PDGFRβ have been described recently (19). In addition, we have validated specific antibodies against Tyr(P)727 and Tyr(P)1002 using wheat germ agglutinin pull-downs from lysates prepared from PAE cells transfected with either wild-type PDGFRβ or a PDGF β-receptor mutated at the respective amino acid. The site-specific antibodies yielded a specific signal only for wild-type receptors after stimulation by ligand (Supplemental Fig. 1).

Kinase Assays—For kinase assays, MEFs were lysed in the absence of protein orthovanadate to allow dephosphorylation, and the various PDGFRβ proteins were immunoprecipitated with anti-PDGFRβ antibodies. Immunopurified proteins were then washed three times with EB buffer and two times with kinase buffer (25 mM HEPES, pH 7.1, 5 mM MgCl2, 100 mM NaCl). For preactivation, 10 ng/ml PDGF-BB was added on ice for 30 min. The phosphorylation reaction was performed in kinase buffer with [γ-32P]ATP (5 μCi/sample) and 10 μl unlabeled ATP by incubating at room temperature for 6 min. The reaction was stopped by the addition of boiling SDS-sample buffer. The samples were analyzed by 4–12% gradient SDS-PAGE and Western immunoblotting with anti-PDGFRβ antibodies, followed by quantification of radioactivity using a phosphor imager (Fuji).

Exogenous Substrate Phosphorylation Assay—The phosphorylation reaction was performed in kinase buffer in the presence of increasing concentrations of myelin basic protein, 10 μg/ml, 100 μg/ml, and 500 μg/ml. The [γ-32P]ATP (5 μCi/sample) for 15 min at 4 °C. The reaction was stopped by the addition of EDTA. The samples were separated on an 8–12% gradient SDS-PAGE and then subjected to immunoblotting with anti-PDGFRβ antibodies and analysis by PhosphorImager analysis.

Mitogenesis Assay—MEFs were seeded in 12-well plates and grown for 1 day. Then the medium was replaced with 0.1% FCS supplemented with 10% FCS and 500 μg/ml of PDGFRβ (10 μM). Cell viability was determined by a live/dead assay (Invitrogen). Adherent cells were treated with 0.5% Triton X-100 and 10% FCS, and after 2 h, cell viability was measured using a CellCounter fluorometric assay (Invitrogen). Data analysis was performed with the GraphPad Prism software.

Fluorescence Microscopy—Confocal MEF cultures were grown on glass coverslips coated with 0.1% gelatin and starved for 2 days in medium containing 0.05% serum. “Wounds” (three/glass) were introduced by scratching the coverslips with a 200-μl pipette tip. The assay was performed in the presence of 10 μg of mitomycin C/ml to inhibit proliferation. Phase-contrast microscopic pictures were taken at the indicated time points.

Flow Cytometry Analysis of Apoptosis Induction—Flow cytometry measurements of several independent apoptotic changes were simultaneously performed by a single tube analysis, as described (20). Briefly, after induction of apoptosis, cells were resuspended in HEPES buffer (10 mM HEPES, 135 mM NaCl, 5 mM CaCl2) and incubated for 15 min at 37 °C in fluorescein isothiocyanate-conjugated annexin V, tetramethylrhodamine methyl ester (200 nM), and propidium iodide (1 mg/ml), to detect phosphatidylserine exposure on the cell surface, mitochondrial inner membrane electrochemical gradient (ΔΨm), and plasma membrane integrity, respectively. Cell morphology changes were analyzed following variations of the forward and side light scatter. Samples were analyzed on a FACScalibur flow cytometer (BD Biosciences). Data acquisition was performed using CellQuest software, and data analysis was performed with WinMDI software.
An Activation Loop Mutation in the Human PDGFRβ Gives the Receptor Transforming Properties in the Presence of Ligand—It was recently shown that mutations of a highly conserved aspartic acid residue in the activation loop of Kit and Met receptors caused up-regulation of their enzymatic activity, resulting in increased transforming potential (7, 21).

To investigate the effect of this type of mutation in the human PDGFR, we generated an expression vector for human PDGFRβ harboring an aspartic acid to asparagine mutation at amino acid position 850 (D850N; corresponding to position 849 in the mouse receptor) in the activation loop of the kinase domain. Overexpression of the D850N mutant human PDGFRβ in COS cells resulted in significantly increased ligand-independent autophosphorylation (data not shown), as has been described for other tyrosine kinase receptors carrying similar mutations (22). Next, we employed NIH3T3 cells in a focus formation assay in order to compare the transforming potential of the wild-type and the mutant PDGFRβ. Each receptor type plasmid was transfected alone or in combination with a plasmid encoding the PDGF-B ligand, thus creating an autocrine stimulation loop. Cotransfection of wild-type PDGFRβ with PDGF-B gave rise to a moderate number of foci (Fig. 1). In the absence of ligand, the number of foci generated from D850N mutant cells was comparable with that from wild-type cells, whereas the coexpression of ligand significantly increased the number of foci from D850N mutant PDGFRβ cells.

Generation of Mutant Mice—The observation that the mutation in the activation loop of the PDGFRβ increased the receptor’s transforming ability prompted us to generate a potential mouse tumor model by introducing this point mutation via a knock-in approach.

The genomic arms necessary for homologous recombination in ES cells were cloned into a double selection targeting vector. The wild-type exon 17, containing aspartic acid 849 (the mouse PDGFRβ is one amino acid residue shorter; thus, Asp849 corresponds to Asp850 in the human receptor) was replaced with a mutated version with the aspartic acid replaced by an asparagine residue (D849N) (Fig. 2). After electroporation of R1-ES cells (15), G418-resistant clones were screened for homologous recombination by PCR. Positive clones were subsequently verified by Southern blot analysis, and DNA sequencing of the mutant allele was performed to ensure the presence of the point mutation (data not shown). One positive ES cell clone was used to generate germ line transmitters. Heterozygous offspring showed no obvious phenotype and were intercrossed to generate homozygous offspring, which were recovered in expected Mendelian proportions (data not shown), indicating that the mutation introduced was compatible with embryonic development. We have not found any signs of tumors or fibrotic developments in mice 1 year of age, independent of the genotype.

The D849N Mutant PDGFRβ Displays an Increased Phosphorylation and Is More Sensitive to Lower Ligand Concentrations—Based on the significantly increased transforming potential of the D849N mutation in the focus formation assay, the lack of an overt phenotype even in homozygous mutant mice came as a surprise. We therefore decided to compare the characteristics of the wild-type and the D849N mutant PDGFRβ from MEFs, a cell type that normally expresses the PDGFRβ.

We first examined the ligand-dependent phosphorylation kinetics of wild-type receptor and D849N mutant PDGFRβ in a physiological context. We subjected wild-type and mutant MEFs to increasing time periods of ligand stimulation. Receptors were immunopurified, separated by SDS-PAGE, and analyzed with antibodies against phosphotyrosine or against PDGFRβ (Fig. 3A, upper and lower panels, respectively). Analysis of the Western blot in Fig. 3A revealed that the wild-type receptor was maximally autophosphorylated after 5 min of stimulation, which then quickly decreased until 60 min. The D849N mutant receptor, however, displayed not only an increased basal tyrosine phosphorylation in the absence of ligand stimulation but also an increased and prolonged ligand-stimulated tyrosine phosphorylation compared with wild-type cells. Since we did not detect any significant difference in receptor amounts, we excluded the possibility that the increased phosphorylation of the D849N mutant was due to decreased receptor degradation. In addition, we found that the D849N mutant receptor was more sensitive to lower ligand concentrations compared with the wild-type receptor (Fig. 3B). These results indicated that the D849N point mutation in the PDGFRβ activation loop gave rise to basal constitutive receptor activation in absence of ligand, a higher sensitivity toward lower ligand concentrations and to a more robust ligand-dependent receptor phosphorylation.

The D849N Mutation Increases the Threshold of Kinase Activation of the Receptor—We next analyzed the effects of this mutation on the autoactivation and catalytic properties of the PDGFRβ. To this end, we performed in vitro autophosphorylation assays on immunopurified receptors from lysates of wild-type and mutant MEFs in the presence of [γ-32P]ATP. As illustrated in Fig. 4A, the unstimulated receptor harboring the D849N mutation was autophosphorylated at a high rate and reached full activation after 20 min of reaction at 20 °C, whereas the wild-type PDGFRβ was phosphorylated more slowly (Fig. 4A). The increased autoactivation velocity of the mutant receptor was observed only in the absence of the ligand. Ligand-dimerized wild-type and mutant receptors, had comparable kinetics of autoactivation (Fig. 4B). These data are compatible with a scenario in which mutant PDGFRβs, in the absence of ligand-induced dimerization, display higher kinase activity than wild-type PDGFRβ.

To further characterize the mutant receptors, their in vitro kinase activities were measured in the presence of the exogenous substrate myelin basic protein. Fig. 5A shows that the extent of myelin basic protein phosphorylation was higher by the unstimulated D849N mutant receptor compared with the wild-type receptor. Again, after PDGF-BB stimulation, there
was no difference between the wild-type and D849N mutant receptors in the phosphorylation rate (Fig. 5B).

Tyr^{856} in the Activation Loop of the D849N Mutant PDGFRβ Is Phosphorylated in the Absence of Ligand—Activation loop trans-phosphorylation is a general mechanism of catalytic enhancement in tyrosine kinase receptors. This event induces a conformational change of the activation loop, which moves from a conformation not optimal for phosphoryl transfer to a catalytically competent configuration. Phosphorylation of Tyr^{856} in the putative kinase activation loop has been shown to be required for full activation of PDGFRβ (23). Introduction of a mutation homologous to D849N in the activation loop of the Met receptor has been found to decrease the threshold of kinase activation, perhaps by promoting the active conformation of the activation loop (13).

To study the effects of the D849N mutation on the phosphorylation of Tyr^{856}, we developed antibodies specific for the phosphorylated form of the activation loop of the PDGFRβ. The
specificity of affinity-purified antibodies was analyzed by Western blotting using COS cells transiently expressing human wild-type PDGFR/H9252 as well as mutant receptors lacking the tyrosine corresponding to mouse Tyr 856 (Y857F) or devoid of kinase activity (K634A) as controls. As shown in Fig. 6A, the antibody recognized phosphorylated wild-type PDGFR/H9252 but did not react with Y857F or K634A mutant receptors, demonstrating that the antibody specifically recognized PDGFR/H9252 phosphorylated on Tyr857.

Wild-type and D849N mutant PDGFR/H9252 immunoprecipitated from unstimulated or ligand-stimulated MEFs were analyzed by Western blotting using the specific antibody against the phosphorylated Tyr856 (Fig. 6B, pTyr856). The activation loop tyrosine of the D849N mutant receptor was found to be substantially phosphorylated even in absence of ligand, whereas the wild-type receptor was phosphorylated only following ligand stimulation.

Phosphospecific Antibodies Revealed Deregulated Phosphorylation of Several Individual Autophosphorylation Sites in the D849N Mutant PDGFR/H9252—After we had detected significant differences not only in the total tyrosine phosphorylation, but also in the activation loop tyrosine phosphorylation, we were interested to investigate whether the D849N mutation also influences other individual tyrosine phosphorylation sites, using different site-specific, phosphotyrosine antibodies.

MEFs were subjected to prolonged starvation (48 h) in order to decrease the ligand-independent phosphorylation detected in the D849N mutant PDGFR/H9252 and treated for different time periods with PDGF-BB. Immunopurified wild-type and mutant receptors were analyzed using specific antibodies directed against phosphorylated tyrosine residues 578, 770, 762/1008, 750, and 1020 (i.e. binding sites for the tyrosine kinase Src, RasGAP, the tyrosine phosphatase Shp-2, PI3K, and phospholipase C-γ, respectively). The phosphorylation kinetics of Tyr 578, Tyr 1008, Tyr 750, and Tyr 1020 were comparable in both wild-type and D849N mutant receptor with the phosphorylation maximum at 5 min of stimulation, followed by a gradual decrease (Fig. 7A, C, and D). However, despite a 48-h starvation, the D849N mutant PDGFR/H9252, but not the wild-type receptor, still displayed a basal, ligand-independent phosphorylation at all tyrosine residues tested, except for Tyr1008 and Tyr 762 (Fig. 7A). In addition, the phosphorylation kinetics of Tyr 770 and of Tyr 762 were markedly changed in the D849N mutant PDGFR/H9252 (Fig. 7A and B). In the wild-type receptor, both Tyr 770 and Tyr 762 followed the global tyrosine phosphorylation pattern; conversely, in the mutant receptor, Tyr 770 was maximally phosphorylated at 60 min (longest stimulation tested), whereas Tyr 762 reached the maximal phosphorylation at 30 min instead of 5 min, as observed in the wild-type receptor. These data, summarized in Fig. 8, are consistent with our observations using phosphopeptide mapping (data not shown), indicating that the D849N mutation led to deregulated phosphorylation kinetics of certain tyrosine residues representing crucial binding sites for downstream molecular components of the PDGFR/H9252 signaling.

Up-regulation of the Ras Pathway Downstream of the D849N Mutant PDGFR/H9252—PDGF is known to be a powerful mitogen for mesenchymal cells. An important pathway driving PDGFR/H9252-induced mitogenesis includes the Ras protein and the MAP kinase cascade. Upon ligand stimulation, Ras becomes activated via direct or indirect binding of the adaptor Grb2 in complex with the nucleotide exchange factor Sos to the activated PDGFR/H9252. This pathway is negatively controlled by RasGAP, which binds to phosphorylated Tyr770 in the kinase insert.
of the receptor. Lack of interaction with RasGAP is correlated with an increased Ras activation and mitogenicity (24). In order to investigate the functional consequences of the changed phosphorylation kinetics in Tyr770 of the mutant receptor, we studied the physical association of RasGAP to wild-type and mutant receptor. We found that RasGAP associated with the wild-type receptor only very transiently following ligand stimulation (Fig. 9A), whereas in the case of mutant receptor, the association was increasing successively with stimulation time. These data were consistent with our previous finding of a delayed phosphorylation of the Tyr770 in ligand-induced D849N mutant PDGFR/H9252 (Fig. 7B). In the next step, we wanted to investigate whether the changed interaction behavior between the mutant receptor and RasGAP had any functional consequences on Ras signaling. To monitor Ras activity, we examined whether the changed interaction behavior between the mutant receptor and RasGAP had any functional consequences on Ras signaling. To monitor Ras activity, we examined the phosphorylation state of a downstream effector p44/p42 MAP kinase (ERK1/2). MAP kinase activation was analyzed by immunoblotting whole cell lysates from MEFs untreated or stimulated with PDGF-BB with antibodies against phosphorylated ERK1/2, followed by immunoblotting with anti-ERK1/2 antibodies to confirm an equal amount of protein (Fig. 9B). In the mutant cells, ERK1/2 was found to be phosphorylated at a low but significant level in the absence of ligand. In addition, upon ligand stimulation, ERK1/2 phosphorylation was more sustained in mutant cells compared with wild-type cells.

Finally, we tested the mitogenic response of wild-type and mutant MEFs toward PDGF-BB stimulation. Cells were stimulated with increasing amounts of PDGF-BB in the presence of [3H]thymidine. The diagram in Fig. 9C shows that MEFs expressing D849N mutant PDGFRβ displayed an increased mitogenic response, compared with wild-type MEFs.

**D849N Mutant PDGFRβ Constitutively Binds Phosphatidylinositol 3′-Kinase**—Recent findings indicated that the PDGFRβ activates multiple signaling pathways that are involved in cellular transformation and that PI3K might be a key component by protecting cells from apoptosis (25). In addition, PI3K plays a central role in actin reorganization necessary for cellular migration and contraction (26).

Using site-selective, phosphospecific antibodies, we had already found that Tyr 750, one of the two PI3K binding sites on the PDGFR/H9252, was phosphorylated without ligand stimulation (Fig. 7C). We were interested to see whether this would also translate into changes in the interaction between PI3K and the mutant receptor. In immunoprecipitation experiments, we found a ligand-dependent association of the p85 regulatory subunit with the wild-type receptor, as described earlier (27). The mutant receptor, however, interacted constitutively with p85 (Fig. 10A) and thus could possibly initiate PI3K signaling even in the absence of ligand stimulation.

**D849N Mutant PDGFRβ Constitutively Activates Protein Kinase B/Akt**—In order to characterize the extent of PI3K signaling by the mutant PDGFRβ, we tested the phosphorylation status of protein kinase B/Akt, an important and well characterized mediator downstream of PI3K. As illustrated in Fig. 10B, phosphospecific antibodies able to recognize active Akt showed a ligand-inducible phosphorylation of this molecule in wild-type MEFs. In D849N mutant PDGFRβ MEFs, Akt was found to be constitutively phosphorylated, and the amount of phosphorylation could only be slightly increased by ligand...
stabilizes an intermediate state, which is partially inhibited by the Akt inhibitor, FK506. In the presence of serum, the PI3K inhibitor LY294002 could not induce apoptosis, per se, but it enhanced the effect of staurosporine (STS), in this way abolishing the differences recorded between D849N and wild-type MEFs (Fig. 10C).

The D849N Mutant PDGFRβ Mediates Cellular Motility in the Absence of Ligand—PI3K plays a pivotal role in mediating actin reorganization and motility responses (26). In order to investigate whether the constitutive activation of PI3K by the D849N mutant PDGFRβ causes an increased motility of the cells, we performed an in vitro “wound healing” assay. In this experiment, a “wound” was introduced into a confluent layer of MEFs that had been seeded on collagen-coated glass coverslips and starved overnight. Concomitant with wounding, the cells were either left unstimulated or stimulated with PDGF-BB or 10% fetal calf serum. In the presence of stimulation, both wild-type and mutant MEFs showed a strong tendency to close the wounds by migrating into the void space (Fig. 11). In the absence of stimulation, the wild-type cells showed no, or little, motility indicated by the clear and sharp wound edges even after 36 h. On the contrary, under these conditions, D849N mutant fibroblasts showed a strong tendency to migrate into the wounded area (Fig. 11). The difference in motility was also evident by the examination of filamentous actin in the cells. In the absence of ligand, mutant cells showed vivid edge ruffling activity, a prerequisite of migration, already after 2 h (Fig. 12); this biological effect was abolished in the presence of the PDGFRβ inhibitor (data not shown). In wild-type cells, edge ruffling was only observed after ligand or serum stimulation (Fig. 12).

DISCUSSION

Perturbation of RTK signaling by gain of function mutations results in deregulated kinase activity and is frequently associated with malignant transformation (22). In this paper, we have analyzed the biochemical mechanism and effects of an aspartic acid to asparagine mutation of codon 849 in the murine PDGFRβ. We found that mice homozygous for the D849N mutation are fertile and do not display any neoplastic phenotype for up to 12 months of age, whereas NIH3T3 fibroblasts expressing the D849N mutant PDGFRβ have transformed properties in vitro provided that stimulating ligand is present. In order to find possible explanations of why the D849N mutant PDGFRβ displays transforming ability in vitro but not in vivo, we have compared the wild-type and the mutant PDGFRβ's with respect to biochemical and signaling properties as well as cellular responses.

The kinetic parameters of the kinase activity are frequently found altered in receptors harboring point mutations in the activation loop. For instance, the homologous amino acid substitution (Asp → Asn) in the activation loop of the Met receptor lowers its threshold of activation, stabilizing an intermediate conformation close to the “open” one, characteristic of the fully active receptor. As a consequence, the mutant Met receptor becomes hyperphosphorylated and hyperactive (13). Similarly, our biochemical analysis has revealed that the amino acid substitution in the activation loop increases the overall tyrosine phosphorylation of the mutant PDGFRβ (Fig. 3A) as well as its sensitivity toward lower amounts of ligand. In vitro experiments showed that the introduction of the D849N mutation in the PDGFRβ does not cause full constitutive activation of the kinase per se but lowers the threshold for activation...
This finding might explain basal activity of the mutant receptor even after 2 days of starvation as well as the higher sensitivity of the mutant receptor. In agreement with the above observations, the major autophosphorylation site located in the activation loop of the PDGFRβ (Tyr856) is phosphorylated in the unstimulated mutant receptor (Fig. 6B). We assume that a conformational change is induced by the presence of the mutation, which increases the chance of Tyr856 to become a substrate of an adjacent protomer and to activate the receptor, thus overcoming the need for stable receptor dimerization. Hence, we speculate that, as in the case of Met, the mutant PDGFRβ has a higher velocity of activation, compared with the wild-type receptor, but still needs ligand to achieve full activation.

The transforming activity triggered by receptor tyrosine kinases harboring a point mutation in the activation loop depends on the altered activation of downstream signaling molecules (29–31). Here, we undertook a detailed analysis linking changes in the phosphorylation of single receptor tyrosine residues with the activation of specific effector molecules. We have found that, in the mutant PDGFRβ, the binding sites for Src, PI3K, RasGAP, and phospholipase C-γ (34) are phosphorylated in a ligand-independent fashion (Fig. 8). The Src binding sites are essential for the full activation of the PDGFRβ (23), since their phosphorylation is required for the removal of one of the inhibitory constraints controlling the PDGFRβ activity, located in the juxtamembrane domain of the receptor (32, 33).

Furthermore, we observed that changes in the phosphorylation pattern of the receptor tyrosines directly translated into a deregulated recruitment of the cognate signal transducers, as exemplified by RasGAP and PI3K. RasGAP, which negatively regulates the Ras mitogenic pathway, displayed a successively increasing association with the mutant receptor, whereas the association to the wild-type receptor was seen only shortly after stimulation and very transiently (Fig. 9A). Interestingly, RasGAP was found to be tyrosine-phosphorylated with the corresponding kinetics in the respective cell lines (Supplemental...
Fig. 2. Lack of the RasGAP binding site on the PDGFRβ results in increased mitogenic signaling and mitogen-activated protein kinase activation (24). Intriguingly, the ERK/mitogen-activated protein kinase downstream of the D849N mutant PDGFRβ showed a higher and prolonged phosphorylation, and MEFs with mutant receptors showed increased proliferation upon ligand stimulation (Fig. 9, B and C). We have no plausible explanation for this apparent discrepancy, except that RasGAP could be trapped by the activated mutant receptor, such that it becomes excluded from its substrate, membrane-bound Ras. Due to the deregulated phosphorylation of the receptor, it is also possible that other effectors dominate ERK/mitogen-activated protein kinase signaling via other phosphorylation sites. Remarkably, the corresponding mutation in the Met receptor (i.e. D1228N) has been described to be transforming by preferential activation of the Ras pathway (30).

PI3K plays a central role in transduction pathways mediating different cellular responses, such as apoptosis protection and actin cytoskeletal reorganization (26, 28, 35). As mentioned earlier, the PI3K binding site in the D849N mutant
receptor was constitutively phosphorylated (Fig. 7C). This phosphorylation resulted in constitutive association with PI3K (Fig. 10A). Moreover, we found that the serine/threonine kinase Akt, downstream of PI3K, was activated by the mutant receptor even in the absence of ligand (Fig. 10B). This ligand-independent activation translated into an increased antiapoptotic signaling of serum-starved MEFs and an increased motility in cellular wounding assays in the absence of ligand (Figs. 10C and 11). In a mutant form of the Kit receptor, carrying a substitution of valine for aspartic acid in the corresponding codon (D816V) in the activation loop, PI3K is also constitutively bound and contributes to cellular transformation (36). Interestingly, however, in this case, Akt is only phosphorylated following ligand stimulation. Our findings indicate that the ligand-independent phosphorylation of the PI3K binding site (Tyr<sup>750</sup>) activates the PI3K pathway and sustains its biological programs.

In summary, the D849N mutant PDGFR<sub>β</sub> does not represent a fully constitutive active kinase but rather a deregulated enzyme with a lower threshold for activation. Signaling via the

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**Fig. 10.** The PI3K signaling pathway is constitutively activated downstream of D849N mutant PDGFR<sub>β</sub>. A, constitutive binding of the p85 subunit of PI3K to the D849N mutant PDGFR<sub>β</sub>. Lysates from MEFs expressing wild-type or D849N mutant PDGFR<sub>β</sub>, stimulated with PDGF-BB for increasing time periods, were subjected to immunoprecipitation (IP) with anti-PDGFR<sub>β</sub> or anti-p85 antibodies. As a negative control, immunoprecipitation with anti-p85 was performed using cell lysates from MEFs derived from knock-in mice with PDGFR<sub>β</sub> unable to bind PI3K, due to mutation of the two autophosphorylation sites Tyr<sup>739</sup> and Tyr<sup>750</sup> (14). Immunoprecipitates were analyzed by immunoblotting (IB) using anti-PDGF<sub>β</sub> or anti-p85 antibodies. A representative experiment of three performed is shown. B, constitutive phosphorylation of Akt in D849N mutant MEFs. Lysates from MEFs expressing wild-type and D849N mutant PDGFR<sub>β</sub>, stimulated with PDGF-BB for different time periods, were either separated directly or first subjected to immunoprecipitation with anti-Akt followed by immunoblotting with anti-phospho-Akt, anti-Akt, and anti-actin antibodies. C, increased Akt-dependent resistance to apoptosis of MEFs expressing D849N mutant PDGFR<sub>β</sub>. Cells were exposed for 24 h to 25 ng/ml STS or were kept under starvation conditions (No FCS) to induce apoptosis. Where indicated, cells were incubated with the PI3K inhibitor LY294002 (LY; 20 μM). Apoptotic cells were scored using a fluorescence-activated cell sorter. Diagrams represent annexin V-fluorescein isothiocyanate (i.e. phosphatidylserine (PS) flipping across the plasma membrane) versus tetramethylrhodamine methyl ester staining (i.e. the measure of mitochondrial membrane polarity). Cells displaying mitochondrial depolarization, an early apoptotic hallmark, are shown in the lower part of each plot, whereas cells exposing PS on their surface, an intermediate apoptotic feature, are shown in the right part of the diagrams. Cells inside the selected area are viable cells, whose percentage is indicated by H (healthy). Shr, refers to shrunken cells, another intermediate apoptotic parameter. PI+, the percentage of propidium iodide-permeable (i.e. dead) cells. Both shrunk and propidium iodide-permeable cells were measured in the same experiment (plots not shown).
mutant PDGFRβ is differently modulated by changes in tyrosine phosphorylation level and kinetics. This deregulates the activity and possibly also the localization of downstream molecules, like RasGAP and PI3K. Constitutively active PI3K signaling from the mutant receptor results in increased antiapoptotic signaling, which contributes to cellular transformation. However, the D849N mutant PDGFRβ is able to display its oncogenic potential only upon stimulation with PDGF-BB, as we have shown in the focus formation assay (Fig. 1). In this scenario, we can speculate that the mutation in the activation loop of the PDGFRβ provides an oncogenic predisposition and that other "hits" are required to achieve a fully transformed phenotype. This may explain why mutant mice have not developed tumors at the age of 1 year. Such a second hit could, for example, be sustained ligand stimulation. In order to test this exciting possibility, we are currently crossing a PDGF-B-transgene into the D849N mutant mice.

In conclusion, we provide evidence that a D849N mutation in the PDGFRβ affects the regulatory mechanism, ensuring a tight regulation of the kinase, and gives rise to a mutant receptor with a lower threshold for activation. In the presence of activating ligand, the mutant PDGFRβ displays abnormal autophosphorylation kinetics and consequently deregulates downstream signaling pathways leading to oncogenic conversion.

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