Serine Phosphorylation of the Ligand-activated \( \beta \)-Platelet-derived Growth Factor Receptor by Casein Kinase I-\( \gamma2 \) Inhibits the Receptor’s Autophosphorylating Activity*

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Platelet-derived growth factor (PDGF) receptors (PDGFRs) are membrane protein-tyrosine kinases that, upon activation, become tyrosine-phosphorylated and associate with numerous SH2 domain-containing molecules involved in mediating signal transduction. In Rat-2 fibroblasts, we have characterized the phosphorylation of the \( \beta \)-PDGFR following its activation by PDGF. In contrast to tyrosine phosphorylation, which was transient and returned to near basal levels by 30 min, PDGF-stimulated Ser/Thr phosphorylation of the \( \beta \)-PDGFR was increased by 5 min and remained elevated after 30 min. In vivo, after 5 min of PDGF stimulation, serine phosphorylation of the \( \beta \)-PDGFR was greatly reduced by CKI-\( \gamma2 \), a specific inhibitor of casein kinase I (CKI). In vitro, recombinant CKI-\( \gamma2 \) phosphorylated the ligand-activated \( \beta \)-PDGFR on serine residues in a CKI-\( \gamma2 \)-sensitive manner and resulted in a marked inhibition of the receptor’s autophosphorylating activity. Furthermore, in Rat-2 fibroblasts, expression of hemagglutinin epitope-tagged active CKI-\( \gamma2 \) resulted in a dramatic decrease in the tyrosine phosphorylation state of the \( \beta \)-PDGFR in response to PDGF, consistent with receptor inactivation. Our data suggest that upon PDGF stimulation, CKI-\( \gamma2 \) is activated and/or translocated in proximity to the \( \beta \)-PDGFR, whereby it phosphorylates the \( \beta \)-PDGFR on serine residues and negatively regulates its tyrosine kinase activity, leading to receptor inactivation.

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1 The abbreviations used are: PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor; CKI, casein kinase I; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; GAM, goat anti-mouse; GAR, goat anti-rabbit.

Platelet-derived growth factor (PDGF)1 and its receptors (PDGFRs) play an important role in the regulation of normal cell growth and differentiation (1) and are involved in a variety of pathological processes, including atherosclerosis, neoplasia, tissue repair, and inflammation (2, 3). PDGFRs, which consist of two isoforms (\( \alpha \) and \( \beta \)) (4), are membrane protein-tyrosine kinases that, upon binding to PDGF, autophosphorylate on several tyrosine residues, creating docking sites for SH2 domain-containing proteins (5). Several of these SH2 domain-containing proteins have been identified and include Src family members; phospholipase C\( \gamma4 \); GTPase-activating protein (p120\( \text{GAP} \)); phosphatidylinositol 3’-kinase; the phosphotyrosine phosphatase SHP-2; and adaptor proteins such as Shc, Grb2, Grb7, and Nck (5–8). Recruitment of these SH2 domain-containing molecules initiates distinct or overlapping signaling cascades, which coordinate cellular responses (9–11). For example, binding of phosphatidylinositol 3’-kinase and phosphotyrosine \( \gamma4 \) to the ligand-activated PDGFR mediates the expression of immediate-early genes (11) and promotes mitogenesis (12). These results suggest a fundamental role for PDGFR activation in growth regulation through autophosphorylation and subsequent activation of critical signaling molecules. In addition, the fact that the expression of a constitutively active PDGFR leads to cellular transformation (13) suggests that in normal cells, PDGFR activity must be tightly regulated to oppose continuous activation of its downstream effectors.

The regulatory mechanisms involved in terminating signals initiated by activated PDGFRs remain poorly defined. One such regulatory mechanism may involve the action of phosphatases, which can potentially dephosphorylate tyrosine residues associated with kinase activation and/or recruitment of signaling molecules. For example, the protein-tyrosine phosphatase Syp (SHP-2) associates with the activated PDGFR and mediates PDGFR signaling by recruiting other downstream signaling molecules. However, Syp also participates in signal termination by dephosphorylating key tyrosines on PDGFR (14). Another mechanism may involve Ser/Thr phosphorylation of the receptors. In fact, it is well documented that Ser/Thr phosphorylation of the insulin receptor by cAMP-dependent protein kinase (15), protein kinase C (16) and casein kinase II (17) inhibits insulin receptor autophosphorylation and kinase activity. The EGFR was shown to be negatively regulated by Ser/Thr phosphorylation in epidermal growth factor-stimulated A431 cells (18, 19); and prolactin dramatically decreases tyrosine phosphorylation of the EGFR and its kinase activity toward exogenous substrates, subsequent to an increase in threonine phosphorylation of the EGFR (20). To date, no such effect of Ser/Thr phosphorylation has been described for the PDGFR.

Recently, we have identified casein kinase I-\( \gamma2 \) (CKI-\( \gamma2 \)) as a protein kinase constitutively associated with the SH3 domains of Nck (21). CKI-\( \gamma2 \) is a member of the large CKI family of Ser/Thr protein kinases (22). In vitro, CKI members phosphorylate the \( \beta \)-subunit of the insulin receptor and inhibit its tyrosine kinase activity (23). In vivo, it has also been reported that CKI is constitutively associated with p75\( \text{TNFR} \) and phosphorylates this receptor in a ligand-dependent manner to neg-
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...regulate tumor necrosis factor signaling to apoptosis (24). Most recently, CKI-α was found to enhance the agonist-dependent serine phosphorylation of the m3-muscarinic G-protein-coupled receptor (25). Given that Nck binds to the activated PDGFR and that CKI-γ2 is constitutively associated with Nck, we investigated whether, upon PDGFR stimulation, CKI-γ2 could phosphorylate the PDGFR and negatively regulate its activity. Interestingly, eight potential CKI phosphorylation sites (XS[XXX][SX][X],[XX][XX][SX]) (26) lie within the intracellular moiety of the β-isofrom of the PDGFR amino acid sequence, suggesting that the β-PDGFR might be a direct target for CKI phosphorylation. In this report, we demonstrate that in vivo, the β-PDGFR is highly serine-phosphorylated upon ligand activation. In contrast to the transient state of tyrosine phosphorylation, phosphorylation of the β-PDGFR on serine residues is sustained and occurs at least in part in a CKI-dependent manner. Furthermore, we show that in vitro, recombinant CKI-γ2 phosphorylates the PDGFR-activated β-PDGFR on serine residues, leading to inhibition of the receptor's autophosphorylation activity. Finally, in Rat-2 fibroblasts expressing HA-tagged active CKI-γ2, tyrosine phosphorylation of the β-PDGFR in response to PDGFR stimulation is dramatically attenuated, indicating that CKI-γ2 impairs activation of the receptor by its ligand.

MATERIALS AND METHODS

Cell Culture—Rat-2 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc.) supplemented with 2 mM l-glutamine, 45 mM sodium bicarbonate, and 10% fetal bovine serum (Life Technologies, Inc.) and maintained in a humidified atmosphere of 95% air and 5% CO2 at 37 °C. Rat-2 fibroblasts stably transfected with the HPRT-neo cassette were obtained from Sigma. N-(2-aminoethyl)-5-chloroquinolin-8-sulfamid (CKI-7) was obtained from Seikagaku Corp. All other reagents were from commercial sources.

Antibodies—PDGFR antibodies were from two different sources. α-PDGFR, a rabbit polyclonal antibody, was raised against a GST fusion protein containing the carboxyl-terminal region of the human β-PDGFR (amino acids 953–1106). β-PDGFR, an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 958–1106 of the carboxyl terminus of the human β-PDGFR, was purchased from Santa Cruz Biotechnology. Anti-phosphotyrosine antibodies were a mixture of the mouse monoclonal antibodies 4G10 (1:3000; Upstate Biotechnology, Inc.) and PY20 (1:1000; Santa Cruz Biotechnology).

Cell Labeling, Cell Stimulation, and PDGFR Immunoprecipitation—Rat-2 fibroblasts (75% confluence) were starved in low-serum (0.2%) DMEM for 24 h, rinsed three times with phosphate-free DMEM, and incubated for 4 h at 37 °C in phosphate-free DMEM containing 0.2% dialyzed fetal bovine serum and 0.2 mM iodonitrotetrazolium (INT). For PDGFR stimulation, at the end of the 4-h labeling period, human PDGFR-BB (50 ng/ml) was added to the culture medium for different times. For inhibitors studies, at the end of the 4-h labeling period, the cells were incubated for 30 min in the absence or presence of CKI-7 (300 µM) or wortmannin (100 nM), prior to stimulation with 50 ng/ml PDGFR-BB, for 5 min. Cells were washed three times with cold phosphate-buffered saline and lysed in lysis buffer (50 mM Hepes (pH 7.5), 150 mM sodium chloride, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1.5 mM magnesium chloride, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 200 µM sodium orthovanadate). When CKI-7 was used to treat the cells, 100 µM CKI-7 was maintained throughout all the subsequent procedures. Clarified cell lysates (1 mg of proteins) were incubated with α-PDGFR antibody and protein A on Sepharose beads for 4 h at 4 °C. Immunoprecipitated proteins were washed three times with HNTG buffer (20 mM Hepes (pH 7.5), 150 mM sodium chloride, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol, 200 µM sodium orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The final bead pellets were resuspended in Laemmli buffer, boiled, centrifuged, and resolved by SDS-PAGE, followed by transfer onto PVDF membranes.

Western Blots—The amount and degree of tyrosine phosphorylation of the β-PDGFR were assessed on PDGFR immunoprecipitates following SDS-PAGE, transfer onto nitrocellulose membranes, and immunoblotting with appropriate antibodies. For β-PDGFR immunoblots, membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% dry milk for 30 min at room temperature or overnight at 4 °C, prior to incubation with α-PDGFR antibody. For anti-phosphotyrosine immunoblots, membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% dry milk for 30 min at room temperature or overnight at 4 °C, prior to incubation with α-PDGFR antibody. For anti-phosphotyrosine immunoblots, membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% dry milk for 30 min at room temperature or overnight at 4 °C, prior to incubation with α-PDGFR antibody. For anti-phosphotyrosine immunoblots, membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% dry milk for 30 min at room temperature or overnight at 4 °C, prior to incubation with α-PDGFR antibody. For anti-phosphotyrosine immunoblots, membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% dry milk for 30 min at room temperature or overnight at 4 °C, prior to incubation with α-PDGFR antibody. For anti-phosphotyrosine immunoblots, membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% dry milk for 30 min at room temperature or overnight at 4 °C, prior to incubation with α-PDGFR antibody. For anti-phosphotyrosine immunoblots, membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% dry milk for 30 min at room temperature or overnight at 4 °C, prior to incubation with α-PDGFR antibody.

RESULTS

Rat-2 Fibroblasts Express Predominantly the β-PDGFR—Rat-2 fibroblasts were treated for 5 min with PDGFR-AA (50 ng/ml) or PDGFR-BB (25 and 50 ng/ml) to determine which isofrom of the PDGFR becomes activated in these cells. Total cell lysates from PDGFR-stimulated and unstimulated Rat-2 fibroblasts were analyzed by immunoblotting with anti-phosphotyrosine antibody. As shown in Fig. 1 (upper panel), PDGFR-AA did not induce tyrosine phosphorylation of proteins present in total cell lysates. In contrast, cell stimulation with...
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PDGFB increased tyrosine phosphorylation of several proteins. These results suggest that in Rat-2 fibroblasts, the α-isofom of the PDGFR is weakly expressed, if not absent, and that in response to PDGF-BB, the β-isofom is the major PDGFR activated. To confirm that the PDGFR immunoprecipitated by both PDGFR antibodies was the β-PDGFR, Rat-2 fibroblasts were stimulated with PDGF-AA or PDGF-BB, and proteins from cell lysates were immunoprecipitated with both PDGFR antibodies. PDGFRs immunoprecipitated by both PDGFR antibodies were tyrosine-phosphorylated only upon cell stimulation with PDGF-BB (Fig. 1, middle and lower panels), demonstrating that both PDGFR antibodies that we used in this study did not cross-react with the α-PDGFR if present.

In Vivo Phosphorylation of the β-PDGFR—To investigate in vivo the phosphorylation of the β-PDGFR, Rat-2 fibroblasts were metabolically labeled with [32P]orthophosphate and treated with PDGF-BB for different times, and the β-PDGFRs were immunoprecipitated using α-PDGFR antibody (Fig. 2A). β-PDGFRs from unstimulated cells showed substantial constitutive phosphorylation, whereas phosphorylation of β-PDGFRs from cells treated with PDGF for 5 min was significantly increased (Fig. 2B). However, the extent of PDGF-stimulated phosphorylation of the β-PDGFR was transient, and although still significantly higher than the basal level, it decreased after 30 min compared with 5 min of stimulation (Fig. 2B). To determine the nature of the amino acids residues that are phosphorylated upon PDGF stimulation, immunoprecipitated β-PDGFRs from metabolically labeled Rat-2 fibroblasts stimulated or not with PDGF were subjected to phosphoamino acid analysis (Fig. 2, C and D). The unstimulated β-PDGFR was phosphorylated almost exclusively on serine residues (Fig. 2D). Upon 5 min of PDGF treatment, serine phosphorylation of the β-PDGFR increased and remained elevated at 30 min (Fig. 2C). Phosphorylation of the β-PDGFR on threonine residues, almost undetected under basal conditions, was weakly detected at 5 min of PDGF stimulation, but was clearly present at 30 min (Fig. 2C). In contrast, tyrosine phosphorylation of the β-PDGFR, almost absent under basal conditions, transiently increased following 5 min of PDGF stimulation and then decreased at 30 min (Fig. 2C). Tyrosine phosphorylation of the PDGFR and its role in mediating activation of diverse signaling cascades are largely documented (8). In contrast, Ser/Thr phosphorylation of the PDGFR has not been studied.

In Vivo CKI-dependent Serine Phosphorylation of the Li-
gand-activated β-PDGFR—Upon activation, the β-PDGFR recruits several SH2 domain-containing proteins, which themselves complex with other effector proteins. For example, the p85 regulatory subunit of phosphatidylinositol 3′-kinase is constitutively associated with p110, the catalytic subunit of phosphatidylinositol 3′-kinase, and Nck is constitutively associated
with p21 (Cdc42/Rac1)-activated protein kinase (28), Nck-associated kinase (29), protein kinase C-related kinase 2 (30), and CKI-γ2 (21), all of which are protein kinases with Ser/Thr kinase specificity. Among these Ser/Thr protein kinases, we attempted to determine whether phosphatidylinositol 3'-kinase or CKI-γ2 participates in serine phosphorylation of the β-PDGFR upon its activation. We studied in vivo the effects of wortmannin and CKI-7 on Ser/Thr phosphorylation of the β-PDGFR upon PDGF stimulation. We measured in vivo the effects of wortmannin and CKI-7 on Ser/Thr phosphorylation of the β-PDGFR upon PDGF stimulation (Fig. 3, A and C). At the concentrations used in this study, wortmannin (100 nM) and CKI-7 (300 μM) are specific inhibitors of phosphatidylinositol 3'-kinase and CKI enzymes, respectively (24, 31, 32). Rat-2 fibroblasts pretreated with wortmannin showed the same overall level of PDGF-induced β-PDGFR phosphorylation that was observed in the absence of wortmannin (Fig. 3, A and C), indicating that phosphatidylinositol 3'-kinase is not the Ser/Thr protein kinase mediating the agonist-dependent serine phosphorylation of the β-PDGFR. Moreover, wortmannin did not apparently affect the phosphorylation of the unstimulated β-PDGFR. In contrast, CKI-7 strongly inhibited β-PDGFR phosphorylation upon PDGF stimulation (Fig. 3, A and C) and may also weakly inhibit its phosphorylation in unstimulated cells. To determine that equal amounts of receptors were immunoprecipitated under each condition, similar experiments were performed on unlabeled Rat-2 fibroblasts, and the amount of β-PDGFR immunoprecipitated was detected by immunoblotting with α-PDGFR antibody (Fig. 3B). In unstimulated and PDGF-stimulated cell lysates, prior cell treatment with wortmannin or CKI-7 did not affect the amounts of β-PDGFR immunoprecipitated. Phosphoamino acid analysis of the β-PDGFR from labeled cells treated with CKI-7 revealed that serine phosphorylation of the PDGF-stimulated β-PDGFR was decreased (Fig. 3D). Indeed, in cells exposed only to DMSO (3%), phosphorylation of the β-PDGFR on serine residues represented 74.6% (S.E. = 6.1, n = 5) of the total 32P incorporated into the three phosphoamino acids, whereas in cells treated with CKI-7 (3% MeSO4), it significantly decreased to 40.8% (S.E. = 4.5, n = 3). These observations provide strong evidence that upon its activation, the β-PDGFR is a substrate at least for CKI Ser/Thr protein kinases since its serine phosphorylation is strongly inhibited by CKI-7, a specific inhibitor of CKI. Interestingly, in lysates of Rat-2 fibroblasts treated with CKI-7, a significant decrease in phosphorylation of other proteins that normally co-immunoprecipitate with the activated β-PDGFR was also observed (Fig. 3A). This suggests that fewer of these proteins are associated or less efficiently phosphorylated by the β-PDGFR in the presence of CKI-7. Alternatively, these proteins could be substrates of CKI, which are inhibited by CKI-7.

In Vitro, Recombinant GST-CKI-γ2 Phosphorylates the Activated β-PDGFR on Serine Residues—To confirm whether the β-PDGFR could be phosphorylated by a CKI-7-dependent protein kinase, we submitted the immunoprecipitated β-PDGFR from PDGF-stimulated or unstimulated Rat-2 fibroblasts to an in vitro phosphorylation assay with active recombinant GST-CKI-γ2, the CKI isoform that we have previously found constitutively associated with Nck (21). Recombinant CKI-γ2 efficiently phosphorylated the PDGF-activated β-PDGFR in a CKI-γ2-sensitive manner, but failed to phosphorylate the β-PDGFR immunoprecipitated from unstimulated cells (Fig. 4A). As expected, phosphoamino acid analysis confirmed that recombinant CKI-γ2 phosphorylates the activated β-PDGFR on serine residues (Fig. 4B). These results clearly demonstrate that the β-PDGFR is phosphorylated by CKI-γ2, however, only once the receptor is activated by its ligand, suggesting that an agonist-induced conformational change or prephosphorylation of the β-PDGFR is a necessary event for CKI-γ2 phosphorylation.

Serine Phosphorylation of the Activated β-PDGFR by CKI-γ2 Inhibits Its Autophosphorylating Activity—To investigate whether CKI-γ2 serine phosphorylation of the ligand-activated β-PDGFR affects the receptor’s kinase activity, we examined the phosphorylation of the activated β-PDGFR by CKI-γ2 using immunoprecipitation followed by phosphoamino acid analysis. As expected, CKI-γ2 2-phosphorylated the activated β-PDGFR in the presence of CKI-7. Alternatively, these proteins could be substrates of CKI, which are inhibited by CKI-7.
the autophosphorylation of the immunoprecipitated β-PDGFR from PDGF-stimulated Rat-2 fibroblasts after in vitro phosphorylation by GST-CKI-γ2. In the presence of unlabeled ATP, autophosphorylation of the β-PDGFR, evaluated by immunoblotting with anti-phosphotyrosine antibody, was dramatically reduced when GST-CKI-γ2 was added to the assay compared with autophosphorylation of an equal amount of immunoprecipitated β-PDGFR not exposed to GST-CKI-γ2 (Fig. 5). These results demonstrate that serine phosphorylation of the β-PDGFR by CKI-γ2 results in inhibition of the ability of PDGF to autophosphorylate. To test whether the CKI-γ2-dependent phosphorylation of the activated β-PDGFR resulting in decreased receptor kinase activity is restricted to its autophosphorylation, we attempted to study the ability of the CKI-γ2-phosphorylated PDGF-activated β-PDGFR to phosphorylate the exogenous substrates poly(Glu,Tyr) and enolase in vitro. Unfortunately, these substrates were efficiently phosphorylated by GST-CKI-γ2, which remained in our samples even after several washes. CKI enzymes were described as dual specific kinases in yeast (33). Although the mammalian counterparts are not known to possess this dual activity, GST-CKI-γ2 efficiently phosphorylates poly(Glu,Tyr) and enolase in an artificial in vitro system, creating a high background signal that does not allow specific detection of the receptor activity toward these exogenous substrates (data not shown).

In Vivo, CKI-γ2 Attenuates PDGF-dependent Tyrosine Phosphorylation of the β-PDGFR—We measured the relative contribution of CKI-γ2 to act as a negative regulator of the β-PDGFR activity in vivo by examining tyrosine phosphorylation of the β-PDGFR in Rat-2 fibroblasts stably expressing an HA epitope-tagged mouse CKI-γ2. Three clones expressing different levels of active HA-CKI-γ2 (Fig. 6, A and B) were stimulated for 5 min with PDGF, and the β-PDGFR tyrosine phosphorylation state was determined by anti-phosphotyrosine immunoblotting of the immunoprecipitated β-PDGFR. Increased expression of CKI-γ2 dramatically reduced the ability of the β-PDGFR to become tyrosine-phosphorylated upon PDGF stimulation (Fig. 6C), and this was not attributed to differential amounts of β-PDGFR immunoprecipitated (Fig. 6D). These results strongly support that in vivo, CKI-γ2 negatively regulates the autophosphorylating activity of the β-PDGFR.

DISCUSSION

Receptor tyrosine kinases are ligand-regulated transmembrane enzymes for which the tyrosine kinase activity of their intracellular domain is subject to diverse regulatory mechanisms. Such mechanisms may consist of receptor internalization and degradation processes (34), specific Ser/Thr phosphorylation within their cytoplasmic domain (35), and dephosphorylation by phosphatases (14). Among these regulatory mechanisms, serine phosphorylation has been shown to impair insulin- and epidermal growth factor-induced activation of their cognate receptors (18, 36–38). For the EGFR, mutagenesis of these serine phosphorylation sites results in enhanced EGFR activity and increased EGFR signal transduction (18).

Similarly, binding of the brain-derived neurotrophic factor to TrkA receptor cytoplasmic domain and results in a strong attenuation of TrkA signaling (39).

This study is the first to demonstrate that the activity of the β-PDGFR is negatively regulated by serine phosphorylation. Moreover, we have shown that this negative regulation by serine phosphorylation is initiated by the ligand. We have identified CKI-γ2 as a candidate Ser/Thr protein kinase mediating this effect. In vitro, we have shown that phosphorylation of the ligand-activated β-PDGFR by CKI-γ2 inhibits the recep-
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The amino acid sequence of the intracellular domain of the β-PDGFR harbors several consensus motifs for potential phosphorylation by CKI enzymes (X(S)PXXS*, EXXS*) (26, 42). Three of these motifs are of particular interest. The first two, which contain serines 573 and 574 (VIESVS*SG*DG) in the juxtamembrane domain, are in close proximity to tyrosines 579 and 581, for which autophosphorylation has been recently reported to be critical for maximal PDGFR activity (43). Mutant 579/581 of the β-PDGFR only modestly autophosphorylates in response to PDGF, and its kinase activity toward exogenous substrates was not increased by PDGF stimulation. Given that phosphorylation of tyrosines 579 and 581 is required to associate Src and phosphorylate the β-PDGFR (45), it has been suggested that Src might play a role in the receptor activation. Putative phosphorylation of serine 573 or 754 by CKI-γ2 could impair phosphorylation of tyrosines 579 and 581, affecting Src association, and thus result in incomplete activation of the receptor. Alternatively, serine phosphorylation of the β-PDGFR in the catalytic domain may directly affect its kinase activity. In favor of this hypothesis, the third consensus motif contains serine 862 (YIKSKGS*SG*TF) in the putative activation loop close to tyrosine 857. Phosphorylation of Tyr*857 has been shown to be required for maximal receptor activity (46, 47); thus, phosphorylation of serine 862 by CKI-γ2 could impair phosphorylation of tyrosine 857 and, consequentially, activation of the receptor. However, we cannot exclude the presence of another protein kinase(s) in the β-PDGFR immunoprecipitates, such that in vitro phosphorylation of the activated β-PDGFR by CKI-γ2 may be indirect. Nonetheless, the activity of these kinases, if present, is CKI-dependent since CKI-7 inhibited β-PDGFR serine phosphorylation in vitro. Experiments are in progress to identify the serine residues within the β-PDGFR intracellular domain that are phosphorylated upon ligand-mediated receptor activation. These studies will confirm whether these sites are direct substrates for CKI phosphorylation and will clarify the mechanism by which serine phosphorylation of the β-PDGFR leads to inhibition of its autophosphorylating activity.

The activity of receptor tyrosine kinases is determined by the balance of positive and negative regulatory events occurring with different kinetics in normal cells. Ligand binding to the β-PDGFR rapidly induces receptor autophosphorylation and increases receptor tyrosine kinase activity, which contribute to signal transduction by promoting recruitment and/or activation of signaling molecules. Simultaneously, ligand activation of the β-PDGFR initiates events leading to serine phosphorylation of the receptor (Fig. 2C), which could negatively regulate receptor's autophosphorylating activity. The finding that expression of HA-CKI-γ2 in Rat-2 fibroblasts dramatically reduced tyrosine phosphorylation of the PDGFR-activated β-PDGFR is in agreement with the in vitro observations. These data may be explained by the fact that the activated β-PDGFR binds the adaptor protein Nck (40), which is constitutively associated with the γ2-isoform of CKI (21). Upon PDGF stimulation, the Nck-CKI-γ2 complex could be translocated into close proximity to the activated β-PDGFR, allowing direct phosphorylation of the receptor by CKI-γ2. Serine phosphorylation of β-PDGFR could then effect the termination of the receptor's signaling activity through inhibition of its tyrosine kinase activity.

In the case of the former receptors, serine phosphorylation may directly impair association and activation of signal transduction molecules. Alternatively, by analogy to the β-adrenergic receptor, where binding of β-arrestin to the β-adrenergic receptor kinase serine-phosphorylated β-adrenergic receptor prevents its interaction with G-proteins and therefore signaling, (41), CKI-mediated phosphorylation of p75TNFR and m3-muscarinic receptor may promote association of molecules that prevent binding of critical transducing molecules, thereby abolishing signaling. For the β-PDGFR, a different mechanism can be proposed whereby CKI-dependent serine phosphorylation attenuates the β-PDGFR autophosphorylating activity, thus terminating signal transduction through direct negative regulation of the receptor's kinase activity.
its activity and hence oppose activation. What is interesting is that simultaneously to receptor activation, the inhibitory factors are already set in place, but are only manifest when they become sufficiently powerful to override the factors maintaining receptor activity. Thus, the inhibitory signals are recruited early, but the kinetics of their effect allow sufficient time for signal transmission. Herein, we demonstrated that this mechanism as its relates to the β-PDGFR involves CKI-γ2 at an early stage in signaling. This does not exclude that other serine protein kinases may also participate in regulating PDGFR activity, especially at later times after PDGFR activation. However, it is tempting to speculate that overexpression of CKI-γ2 could overcome cell transformation due to hyperactivation of the β-PDGFR. We are currently investigating this possibility.

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