Phosphorylation of the Platelet-derived Growth Factor Receptor-β and Epidermal Growth Factor Receptor by G Protein-coupled Receptor Kinase-2

MECHANISMS FOR SELECTIVITY OF DESENSITIZATION*

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Accumulating evidence suggests that receptor protein-tyrosine kinases, like the platelet-derived growth factor receptor-β (PDGFRβ) and epidermal growth factor receptor (EGFR), may be desensitized by serine/threonine kinases. One such kinase, G protein-coupled receptor kinase-2 (GRK2), is known to mediate agonist-dependent phosphorylation and desensitization of multiple heptahelical receptors. In testing whether GRK2 could phosphorylate and desensitize the PDGFRβ, we first found by phosphoamino acid analysis that cells expressing GRK2 could serine-phosphorylate the PDGFRβ in an agonist-dependent manner. Augmentation or inhibition of GRK2 activity in cells, respectively, reduced or enhanced tyrosine phosphorylation of the PDGFRβ but not the EGFR. Either overexpressed in cells or as a purified protein, GRK2 demonstrated agonist-promoted serine phosphorylation of the PDGFRβ and, unexpectedly, the EGFR as well. Because GRK2 did not phosphorylate a kinase-dead (K634R) PDGFRβ mutant, GRK2-mediated PDGFRβ phosphorylation required receptor tyrosine kinase activity, as does PDGFRβ ubiquitination. Agonist-induced ubiquitination of the PDGFRβ, but not the EGFR, was enhanced in cells overexpressing GRK2. Nevertheless, GRK2 overexpression did not augment PDGFRβ down-regulation. Like the vast majority of GRK2 substrates, the PDGFRβ, but not the EGFR, activated heterotrimeric G proteins allosterically in membranes from cells expressing physiologic protein levels. We conclude that GRK2 can phosphorylate and desensitize the PDGFRβ, perhaps through mechanisms related to receptor ubiquitination. Specificity of GRK2 for receptor protein-tyrosine kinases, expressed at physiologic levels, may be determined by the ability of these receptors to activate heterotrimeric G proteins, among other factors.

The platelet-derived growth factor receptor-β (PDGFRβ)1 mediates signaling important for the proliferation, migration, and survival of mesenchymal cells (1). Like most receptor protein-tyrosine kinases, the PDGFRβ is thought to exist as a quiescent monomeric protein that, upon binding agonist, dimerizes and consequently phosphorylates itself and other proteins on Tyr residues (1). The significance of regulating PDGFRβ Tyr kinase activity is highlighted by the receptor’s critical role in embryonic development and in the pathogenesis of vascular proliferative diseases like atherosclerosis and neoimal hyperplasia subsequent to vascular injury (1–3).

A number of mechanisms have been demonstrated to diminish PDGFRβ signaling, including Tyr dephosphorylation (4, 5), removal of receptors from the cell surface (6), degradation of receptors (6, 7), and, described most recently, phosphorylation of the PDGFRβ on serine residues (8). In cells, the PDGFRβ Tyr kinase activity desensitizes or warms with persistent exposure to PDGF (9). Desensitization commonly occurs consequent to serine phosphorylation of heptahelical receptors that couple to heterotrimeric G proteins (10). In addition, serine phosphorylation of both the insulin receptor (11) and EGFR (12) has been associated with receptor desensitization.

The serine/threonine kinase G protein-coupled receptor kinase-2 (GRK2) phosphorylates and desensitizes a large number of heptahelical receptors (10). We found recently that overexpression of GRK2 could attenuate phosphoinositide hydrolysis, [3H]thymidine incorporation, chemotaxis, and cellular proliferation evoked via the PDGFRβ in vascular SMCs (13, 14). Importantly, receptor-specific desensitization by GRK2 correlated with a reduction in PDGF-promoted Tyr phosphorylation of the PDGFRβ (14). The plausibility of GRK2-mediated regulation of the PDGFRβ in cells is suggested by the caveolar localization of PDGFRs in cells (15) and the ability of caveolin to bind to GRK2 (16). GRK2 also has been demonstrated to associate with phosphoinositide 3-kinase-α (17), which translocates to activated PDGFRβs (1) and is required for PDGFRβ internalization (18). Moreover, a Tyr kinase that is a principal effector of the PDGFRβ, c-Src, appears to be a critical activator of GRK2 activity in cells (19, 20). Thus, by activating c-Src, agonist-activated PDGFRβs could activate GRK2 indirectly. To address whether GRK2 affected PDGFRβ desensitization directly, by phosphorylating the PDGFRβ, or indirectly, by phosphorylating other regulatory proteins, we undertook the current study.

1 The abbreviations used are: PDGFR, platelet-derived growth factor receptor; PDGF, platelet-derived growth factor; EGFR, epidermal growth factor; EGFR, EGF receptor; GRK, G protein-coupled receptor kinase; SMC, smooth muscle cell; IP, immunoprecipitation or immunoprecipitate; PVDF, polyvinylidene fluoride; LPA, lysophosphatidic acid; ROS, reactive oxygen species; PTX, pertussis toxin; ERK, extracellular signal-regulated kinase; IB, immunoblot; HEK, human embryonic kidney; GTPγS, guanosine 5’-3-O-(thio)triphosphate.
Materials—All cell culture products were from Invitrogen. Human PDGF-BB was from Upstate Biotechnology, and LPA, human EGF, and nudeulin were from Sigma. All radionuclides were from PerkinElmer Life Sciences. Sources for antibodies are provided below. Plasmid and Adenovirus Constructs—Plasmids encoding bovine GRK2, dominant-negative (K220R) mutant GRK2, and the GRK2 C-terminal peptide were described previously (21), as were the empty vector and GRK2-encoding recombinant adenosiviruses (14). Plasmids encoding EGFR, human PDGF receptor, and kinase-dead (K220R) mutant constructs were described previously (22) and the human EGFR (23) were the generous gifts of Andrés Kuzalaukas and Axel Ullrich, respectively. Cassette PCR was employed to replace the endogenous signal sequence of each cDNA with an influenza virus hemagglutinin signal sequence followed by the FLAG epitope (24). The 5′ (mutagenic) primer for the EGFR was 5′-ccctgggagacgtcctgattctgtgtctgctgtcctgtc-3′. Tol2 indicates a NotI site, parentheses surround the signal sequence, brackets surround the epitope sequence, boldface type highlights the initiator methionine, and underlined type denotes nucleotides 259 to 279 of the native EGFR sequence. The mutagenic primer for the PDGFRβ was similar, except that EcoRI replaced NotI, and nucleotides 452 to 473 of the PDGFRβ (25) were used. To create the FLAG-tagged EGFR (F-EGFR) construct, a 770-bp NotI/XmaI-cut PCR fragment was subcloned into the native EGFR cDNA, which had been subcloned previously into pCDNA 1 (Invitrogen). For the F-PDGFRβ construct, a 108-bp EcoRI/XmaI-cut PCR fragment was subcloned into the native PDGFRβ, subcloned previously into pCDNA1. Sequence fidelity was confirmed by dideoxy sequencing. To create the F-PDGFRβ (S634AR) mutant, a 506-bp FspI/BstEI fragment of the mutant construct was subcloned into the cognate site in the F-PDGFRβ. Throughout the text, the F-PDGFRβ and F-EGFR constructs are referred to as PDGFRβ and EGFβ, respectively.

Cell Culture, Transfection, Adenoviral Infection, and Gene Expression—HEK 293 cells (21) (from the American Type Culture Collection) were used. Human F-PDGFRβ, human PDGF receptor and kinase-dead (K220R) mutant constructs were described previously (22) and the human EGFR (23) were the generous gifts of Andrés Kuzalaukas and Axel Ullrich, respectively, was typically 35% in 293 cells (assessed by cell surface receptor expression levels) within 30% of that measured in empty vector-co-transfected 293 cells (challenged or not with agonist) were washed three times in IP buffer 1, aspirated dry, and resuspended in 100 μl of counting buffer (50 mM Tris-Cl, pH 7.4, 20 mM MgCl2, 150 mM NaCl), 50 μl of which was added to scintillation fluid and counted. Specific cpm from [35S]GTPγS (which constituted 75% of total cpm) were calculated as (total cpm) − (nonspecific cpm).

To determine the effect of pertussis toxin (PTX) on agonist-stimulated ERK activation, serum-starved SMCs were pretreated with PTX (100 ng/ml; List Laboratories) or vehicle for 16 h (26), challenged with the indicated agonist for 10 min, and then lysed in Laemmli buffer (24). Multiple sample aliquots were subjected to replicate SDS-PAGE and immunoblotting (14), and replicate blots were probed for either phospho-ERK1/2 or total ERK1/2, with antibodies from New England Biolabs. Graded amounts of sample were loaded, so as to create standard curves from which to interpret relative signal intensities and densitometry. Phospho-ERK signals were normalized to cognate total ERK signals.

Receptor Immunoprecipitations—To assess the effects of GRK2 on PDGFRβ and EGFR total and Tyr phosphorylation, and to determine the association of GRK2 with the PDGFRβ and EGFR, cells were serum-starved overnight, exposed to vehicle- or agonist-containing medium for the indicated times, and subjected to IP as described previously for the FLAG-tagged endothelin B receptor (24), except that M2-agarose beads (Sigma) were used. For co-immunoprecipitation of GRK2 with the PDGFRβ or EGFR, the cell-permeant cross-linker di-thiobis(succinimidyl propionate) (Pierce) was used as described (24). For the complex of immunoprecipitated kinase assays (see Figs. 7-9), IP was performed similarly, except that we used IP buffer II (50 mM Hepes, pH 7.5, 10% (v/v) glycerol, 1% (v/v) Triton-X-100, 150 mM NaCl, 1.5 mM MgCl2, 1 mM sodium orthovanadate, and protease inhibitors).

Immunoprecipitation—After transfected 293 cells were serum-starved overnight, these assays were performed and quantitated with a PhosphoImager™ (Molecular Dynamics) as described previously (24).

Immunoblotting—Receptor expression levels and the protein concentration of cell lysates were used to load equivalent amounts of receptor per lane for SDS-PAGE as described (24). Proteins were transferred to either nitrocellulose or PVDF membranes, immunoblotted, stripped, and reprobed as described (24), with the following IgGs: for phospho-EGFR or EGFR C-terminal domains, respectively, rabbit IgGs sc-432 and sc-03 (Santa Cruz Biotechnology); for ubiquitin, monoclonal Ub(P4D1) (Santa Cruz); and for equivalent identification of GRK2 and the GRK2 C-terminal peptide (GRK2ct), monoclonal E23/6 (14). Horseradish peroxidase-conjugated anti-IgGs for rabbit and mouse came from Jackson ImmunoResearch. For anti-ubiquitin blots, peroxidase-conjugated anti-mouse IgG came from Amersham Biosciences. For sequential immunoblotting, anti-phospho-Tyr blotting always followed anti-receptor blotting (because our nitrocellulose stripping procedure could not remove primary antibody, not shown).

ImmuneComplex Kinase Assays—Bovine GRK2 was produced in Sf9 cells by recombinant baculovirus-mediated expression and purified as described (21). Receptors immunoprecipitated from 100-mm dishes of 293 cells (challenged or not with agonist) were washed three times in IP buffer II (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton-X-100, 1 mM sodium orthovanadate) and three times with kinase buffer (20 mM Tris-Cl, pH 8.0 (25° C), 2 mM EDTA, 10 mM MgCl2, 1 mM dithiothreitol). Immune complex beads were aspirated to dryness with a 28-gauge needle and resuspended in 15 μl of kinase buffer. Reactions were performed (35° C, 30 min) in a total volume of 30 μl of kinase buffer with 200 nm GRK2 or vehicle and 0.1 mM ATP containing 10 μCi of [γ-32P]ATP. Reactions were terminated by the addition of 500 μl cold IP buffer 1, and samples were heated to 65° C for 10 min to dissociate immune complexes. Samples of equivalent receptor mass (determined from protein assay of solubilized cells) were loaded onto each lane of 6% SDS-polyacrylamide gels. For each sample, 90% was subjected to SDS-PAGE for subsequent phosphoamino acid analysis, and 10% was subjected to SDS-PAGE for subsequent immunoblotting with phospho-Tyr Receptor. Receptor phosphorylation was quantitated with a PhosphorImager™ (Molecular Dynamics).

Phosphoamino Acid Analysis—32P-Labeled receptor bands were excised from PVDF membranes after autoradiography, subjected to acid hydrolysis in 5.7 N HCl (110° C, 70 min), and dried as described (27). Phosphoamino acids were then dissolved in FAA buffer, pH 3.5 (5% (v/v) pyridine, 100 mM citric acid, 0.25% (v/v) pyridine, 0.5% (v/v) EDTA) and subjected to one-dimensional thin-layer electrophoresis followed by ninhydrin staining as described (28). Radioactivity in phosphoamino acids was quantitated with a PhosphorImager™ analysis as described (24).
PDGFRβ Down-regulation—SMCs infected with either the empty vector or GRK2-encoding adenovirus were serum-starved overnight and then exposed to serum-free medium containing either 2 nM PDGF-BB or vehicle for the indicated times. To terminate incubations, SMCs were transferred to ice, washed twice with cold phosphate-buffered saline, and lysed for membrane preparation, as described above. Membrane pellets were then resuspended in solubilization buffer (50 mM Hepes, pH 7.4, 4% (v/v) Nonidet P-40, 250 mM NaCl, 10% (v/v) glycerol, 2 mM EDTA, 10 mM NaF, with protease inhibitors) and mixed gently for 45 min (4 °C). Insoluble debris was pelleted at 20,000 × g for 10 min (4 °C), and the supernatant was subjected to protein assay (24). For each sample, 35 μg of membrane protein were loaded in duplicate lanes of a 4–12% gradient SDS-polyacrylamide gel and subjected to immunoblotting for PDGFRβ.

Data Analysis—A paired t test was used to compare GRK2-overexpressing or PTX-treated with cognate control cells, and two-sided p values were calculated with Excel™ software (Microsoft). With Prism™ software (Graphpad, Inc.), repeated measures one-way analysis of variance with a post-hoc Tukey’s test was used to compare GTP loading among SMC membrane preparations treated with various agonists. Data in the text are mean ± S.D., whereas means ± S.E. are depicted in figures.

RESULTS

Coupling of the PDGFRβ to Goi—We found recently that signaling through the PDGFRβ was desensitized, in a receptor-specific manner, in SMCs overexpressing GRK2 (13, 14). Unlike the PDGFRβ, all other receptors known to be desensitized by GRK2 are heptahelical receptors that activate heterotrimeric G proteins allosterically (10). We therefore asked whether the PDGFRβ, like heptahelical receptors, could also activate G proteins allosterically. To answer this question, we tested the ability of the PDGFRβ to promote GTP turnover in Goi subunits in rabbit SMC membranes (which express only the PDGFR) (29). We could find no evidence that PDGF-stimulated GTPγS binding to Goi, whereas such binding was stimulated by endothelin-1 (data not shown). However, PDGF stimulated a 1.7-fold increase in GTPγS binding to Goi (Fig. 1A). LPA, which is known to activate Gi-coupled, heptahelical endothelial differentiation gene (Edg) receptors (30), stimulated a 2.8-fold increase in GTPγS binding to Goi (31) (Fig. 1A). Thus, the PDGFRβ appeared to activate Goi isoforms in SMC membranes, but the allosteric nature of the PDGFRβ/Goi interaction remained to be established. PDGFRβs may induce the production of reactive oxygen species (ROS) (31), and ROS may activate Goi isoforms (32). We therefore sought to ascertain whether the PDGFRβ activated Goi isoforms allosterically, as heptahelical receptors do (33), or only indirectly via the production of ROS.

To demonstrate that the PDGFRβ activated Goi via a mechanism(s) different from ROS, we employed two approaches. First, in SMC membrane preparations (Fig. 1A) we found no activation of Goi upon stimulation of the EGFR, which, like the PDGFRβ, is also known to promote production of ROS (32). Second, in intact SMCs, we used PTX to inhibit Gαi3-mediated activation of ERK1/2, because H2O2-induced ERK activation via Gαi3 has been shown to be PTX-insensitive (32). We found that PDGF-induced ERK activation in SMCs was reduced 30 ± 15% (p < 0.02) by PTX (Fig. 1B), consistent with Gαi3 activation that is not mediated by ROS. By comparison, Gαi1-3-mediated ERK activation induced by LPA was abolished by PTX treatment, and EGF-induced ERK activation of comparable magnitude was not affected significantly by PTX (Fig. 1B). Thus, at least a fraction of PDGFRβ-dependent ERK activation in vascular SMCs can proceed via PTX-insensitive G proteins. Moreover, the PTX sensitivity of the PDGF-promoted ERK activation suggests that the PDGFRβ can couple to Gi directly, as heptahelical receptors do, rather than just indirectly via the production of ROS. Interestingly, the selectivity of GRK2 for desensitizing the PDGFRβ and not the EGFR in SMCs (14) is mirrored by the selectivity of PTX-sensitive G protein coupling to the PDGFRβ, and not the EGFR, in these cells.

Functional Effects of GRK2 on PDGFRβ Activation—To inhibit the possible effects of GRK2 on activation of the PDGFRβ (14), we employed a kinase-dead mutant of GRK2 (K220R), which we (21, 24) and others (36) have used extensively to inhibit GRK-mediated serine phosphorylation of heptahelical receptors. In Fig. 2, reciprocal effects on PDGFRβ activation were observed with overexpression of either K220R-GRK2 or wild type GRK2. K220R-GRK2 overexpression effected a 69% increase in PDGFRβ Tyr phosphorylation (p < 0.02), whereas GRK2 overexpression effected a 28% decrease in agonist-promoted PDGFRβ Tyr phosphorylation (p < 0.02). In contrast, neither K220R-GRK2 nor GRK2 itself had a significant effect on agonist-promoted Tyr phosphorylation of the EGFR. In this heterologous overexpression system, PDGFRβ-mediated G protein activation (Fig. 1) did not appear to be important for GRK2 membrane recruitment (10) and activity on the PDGFRβ. Expression of a Gβγ-sequestering GRK2 C-terminal polypeptide (14), at levels equivalent to those of the co-overexpressed GRK2, did not alter GRK2-mediated PDGFRβ desensitization (data not shown). These findings in 293 cells confirm and

![Fig. 1. The PDGFRβ signals via Goi in vascular SMCs. A. agonist-stimulated GTPγS loading of Goi in SMC membranes. Membrane aliquots from quiescent SMCs were incubated with 10 μM LPA, 2 nM PDGF-BB, 1.7 nM EGF or vehicle (basal) in the presence of [35S]GTPγS, and Goi was immunoprecipitated subsequently. [35S]GTPγS co-immunoprecipitated with Goi, was quantitated as described under “Experimental Procedures” and normalized to values from unstimulated samples as % above basal, 100 × [(stimulated)/basal]) – 1. Shown are mean ± S.E. from four experiments performed in triplicate. Inset, immunoblots of total SMC protein resolved on a 10% polyacrylamide SDS gel, detected with either non-immune rabbit IgG (Rabbit) or the rabbit anti-Goi, IgG (Go) used for IP. B. PDGF-promoted ERK signaling in SMCs; inhibition by pertussis toxin. Quiescent SMCs were pretreated without (-) or with (+) PTX and then stimulated without (None) or with 10 μM LPA, 0.2 nM PDGF-BB, or 20 μM EGF for 5 min at 37 °C. Cell lysates were subjected to SDS-PAGE. Duplicate immunoblots were scored for either phospho-ERK1/2 or total ERK1/2. Results are from one experiment, representative of four experiments performed in duplicate.](image-url)
extend our findings with GRK2 overexpression in SMCs (14). Because potentiating and inhibiting GRK2 activity in cells affected PDGFRβ activation reciprocally, and in a receptor-specific fashion, a direct interaction between GRK2 and the PDGFRβ seemed likely.

To determine whether GRK2 interacted with the PDGFRβ itself, we performed co-immunoprecipitation experiments, using the EGFR as a negative control (because its activation (Fig. 2) and signaling (14) were not altered by changes in GRK2 activity). In cells expressing both the PDGFRβ and GRK2 (Fig. 3), GRK2 associated with the PDGFRβ in an agonist-dependent manner, much like that we observed previously for heptahelical receptors desensitized by GRK2 (24). Surprisingly, we obtained similar results with the EGFR (data not shown). This agonist-promoted association between GRK2 and either the PDGFRβ or the EGFR suggested that GRK2 could phosphorylate both of these receptors but with functionally distinct consequences for each receptor.

**GRK2-mediated Phosphorylation of the PDGFRβ and EGFR—**If endogenous GRK2 could phosphorylate the PDGFRβ in 293 cells, we would expect to observe agonist-dependent phosphorylation of the PDGFRβ on serine residues. In 293 cells labeled metabolically with $^{32}$P, we found the PDGFRβ to be phosphorylated tonically on serine residues and to increase its degree of serine phosphorylation substantially with PDGF challenge (Fig. 4A). These findings accorded with those of Bioukar et al. (8) in Rat2 fibroblasts.

As we found in Fig. 2, overexpression of GRK2 in these experiments diminished agonist-promoted PDGFRβ Tyr phosphorylation, by $37 \pm 8\%$, as assessed by phospho-Tyr immunoblotting (Fig. 4B). However, GRK2 overexpression had no effect on the total level of PDGFRβ phosphorylation, assessed by receptor immunoprecipitation and autoradiography (Fig. 4B). Thus, a GRK2-mediated increase in PDGFRβ serine phosphorylation could have counterbalanced the GRK2-engendered decrease in PDGFRβ Tyr phosphorylation, yielding no net change in total PDGFRβ $^{32}$P incorporation. Data supporting GRK2-mediated Ser/Thr phosphorylation of the EGFR in intact cells were more straightforward. Overexpression of GRK2 in 293 cells increased the agonist-induced incorporation of $^{32}$P into the EGFR without altering the agonist-induced Tyr phosphorylation of the EGFR (Fig. 4C).

To confirm that GRK2 overexpression could increase serine phosphorylation of the agonist-stimulated PDGFRβ or EGFR, we adapted a system (8) that allowed us to preserve agonist-mediated activation of the receptors but enabled us to minimize the effect of endogenous 293 cellular GRK2 (21, 37) on our phosphorylation reactions. With purified GRK2, we phosphorylated receptors immunoprecipitated from cells that had been exposed to vehicle- or agonist-containing medium. Receptor phosphorylation was evaluated after resolution of immune complexes by SDS-PAGE, and proteins were transferred to PVDF membrane to facilitate subsequent phosphoamino acid analysis. As depicted in Fig. 5A and Fig. 6A, incubation with GRK2 augmented agonist-induced phosphorylation of the PDGFRβ and EGFR, compared with vehicle controls. As is characteristic of GRK2-mediated heptahelical receptor phosphorylation, GRK2-mediated phosphorylation of the PDGFRβ and EGFR was agonist-promoted. Furthermore, GRK2 catalyzed the phosphorylation of the PDGFRβ and EGFR on serine(s), as revealed by hydrolysis and phosphoamino acid analysis of the receptor (see Fig. 5C and Fig. 6B). Phospho-serine was detected only in PDGFRβs and EGFRs incubated with GRK2. Interestingly, GRK2 also demonstrated some serine phosphorylation of PDGFRβs and EGFRs not challenged with agonist, to the extent that receptor Tyr phosphorylation was induced by IgG-mediated receptor cross-linking during IP (22). These findings suggest that GRK2-mediated serine phosphorylation of these receptor protein-tyrosine kinases depended upon the extent of receptor activation, as has been demonstrated with the β$_2$-adrenergic (heptahelical) receptor (38). Taken together, these results demonstrate the PDGFRβ and EGFR to be novel receptor substrates for GRK2.

GRK2-mediated phosphorylation of heptahelical receptors requires agonist-mediated stabilization of the active conformation of the receptor (10). For the PDGFRβ, agonist dimerizes receptor monomers, with consequent activation of receptor ty-
Regulation of the PDGFRβ by GRK2-mediated Phosphorylation

Agonist-induced PDGFRβ phosphorylation in 293 cells effects of GRK2 overexpression. HEK 293 cells transfected or not (−) with the indicated plasmids were labeled metabolically with 32P, exposed to medium-containing vehicle, 2 nM PDGF-BB, or 1.7 nM EGF for 5 min, and subjected to receptor IP. IPs resolved by SDS-PAGE (6% gels) were transferred to PVDF membrane and processed for autoradiography, followed by either phosphoamino acid analysis or immunoblotting. A, agonist-induced serine phosphorylation of the PDGFRβ in 293 cells expressing endogenous levels of GRK2. Shown is a thin-layer electrophoresis of phosphoamino acids derived from PDGFRβ bands. The positions of ninhydrin-stained phosphoamino acid standards (pS, pT, and pY) are indicated with circles. Similar results were obtained in three independent experiments. B, GRK2 overexpression reduces PDGFRβ Tyr but not total phosphorylation induced by agonist. A single PVDF membrane is imaged either by PhosphorImager™ (32P) or after serial immunoblotting for the EGFR and phospho-Tyr.

RESULTS

Several lines of evidence from this study support the inference that GRK2 phosphorylates and desensitizes the PDGFRβ, the only receptor protein-tyrosine kinase yet demonstrated to be regulated by a GRK. First, as would be expected of any GRK2 substrate, the PDGFRβ demonstrated agonist-promoted serine phosphorylation both in cells and in the presence of purified GRK2. Second, GRK2 associated with the PDGFRβ in cells in an agonist-dependent manner. Moreover, Tyr phosphorylation is a late event in the PDGFRβ phosphorylation and activation process, which suggests that Tyr phosphorylation is not the primary determinant of GRK2 association. Third, in 293 cells, agonist-induced Tyr phosphorylation of the PDGFRβ is reduced by GRK2 overexpression (Fig. 2) and correlates with receptor desensitization (i.e. down-regulation) and degradation (Fig. 8). Fourth, the PDGFRβ of the wild type, but not the Tyr kinase-dead mutant, is recognized as a substrate by GRK2. Finally, similar levels of PDGFRβ phosphorylation are observed in GRK2-overexpressing cells (data not shown), PDGFRβ down-regulation proceeded at a rate indistinguishable from that observed in control cells (Fig. 9).

DISCUSSION

Several lines of evidence from this study support the inference that GRK2 phosphorylates and desensitizes the PDGFRβ, the only receptor protein-tyrosine kinase yet demonstrated to be regulated by a GRK. First, as would be expected of any GRK2 substrate, the PDGFRβ demonstrated agonist-promoted serine phosphorylation both in cells and in the presence of purified GRK2. Second, GRK2 associated with the PDGFRβ in cells in an agonist-dependent manner. Moreover, Tyr phosphorylation is a late event in the PDGFRβ phosphorylation and activation process, which suggests that Tyr phosphorylation is not the primary determinant of GRK2 association. Third, in 293 cells, agonist-induced Tyr phosphorylation of the PDGFRβ is reduced by GRK2 overexpression (Fig. 2) and correlates with receptor desensitization (i.e. down-regulation) and degradation (Fig. 8). Fourth, the PDGFRβ of the wild type, but not the Tyr kinase-dead mutant, is recognized as a substrate by GRK2. Finally, similar levels of PDGFRβ phosphorylation are observed in GRK2-overexpressing cells (data not shown), PDGFRβ down-regulation proceeded at a rate indistinguishable from that observed in control cells (Fig. 9).
Regulation of the PDGFRβ by GRK2-mediated Phosphorylation

Fig. 5. GRK2 mediates agonist-promoted serine phosphorylation of the PDGFRβ. HEK 293 cells transfected or not (None) with the PDGFRβ plasmid were exposed to medium-containing vehicle or 2 nM PDGF-BB for 10 min at 37 °C, solubilized, and subjected to PDGFRβ IP. Immunoprecipitated PDGFRs were used to perform immune complex kinase assays with [γ-32P]ATP in the absence (−, Control) or presence (+) of purified GRK2. SDS-PAGE and blotting to PVDF membrane followed. Parallel PVDF membranes were processed either for phosphoamino acid analysis or for immunoblotting total PDGFRβ or phospho-Tyr (not shown). A, autoradiogram from a single representative PVDF membrane. With parallel immunoblots (not shown), GRK2 and control samples showed equivalent levels of PDGFRβ phospho-Tyr. B, receptor phosphorylation data (mean ± S.E.) from five independent experiments are summarized. Radioactivity in PDGFRβ bands was first normalized to the relative amount of PDGFRβ quantitated by immunoblotting; receptor-normalized 32P counts were then normalized to those obtained from IPs of unstimulated cells incubated without GRK2 (control basal). *, p < 0.05 compared with cognate control value. C, receptor bands from panel A were hydrolyzed and subjected to phosphoamino acid analysis as in Fig. 4. Shown are results from a single experiment representative of five experiments performed. The positions of ninhydrin-stained phosphoamino acid standards (pS, pT, and pY) are indicated with arrows.

GRK2-mediated PDGFRβ Phosphorylation and Desensitization—Potential regulation of the PDGFRβ by serine phosphorylation was first demonstrated with casein kinase I-γ2 (8), which, like GRK2, can phosphorylate peptides more efficiently when they contain serines located C-terminal to acidic amino acids (10), and ≥14 such serines exist in the cytoplasmic domain of the PDGFRβ (40). As with GRK2 in our study, overexpression of casein kinase I-γ2 in cells reduced agonist-promoted PDGFRβ Tyr phosphorylation. However, inhibition of cellular casein kinase I-γ2 also reduced the overall level of PDGFRβ phosphorylation and perhaps even reduced PDGFRβ Tyr phosphorylation, as suggested by a decrease in the number of phosphorylated proteins co-immunoprecipitated with the PDGFRβ (8). In contrast to inhibiting cellular casein kinase I-γ2, inhibiting cellular GRK2 activity increased PDGFRβ activation, assessed by receptor Tyr phosphorylation. Thus, whereas in-
Regulation of the PDGFRβ by GRK2-mediated Phosphorylation

**Fig. 8.** GRK2 overexpression augments agonist-induced ubiquitination of the PDGFRβ but not the EGFR. HEK 293 cells were transfected with N-terminal FLAG-tagged constructs of either the PDGFRβ or the EGFR and either vector (Empty) or GRK2-encoding plasmids. Exposed to medium containing vehicle (basal) or agonist for 30 min at 37°C, cells were then solubilized, and lysates were subjected to receptor IP/immunoblotting, as in Fig. 2. Serial immunoblotting was performed first for ubiquitin (top panels) and then for either the PDGFRβ or EGFR (bottom panels). A, immunoblots from single experiments, representative of three performed in duplicate for each receptor. B, ubiquitinated receptor band densities were first normalized to cognate total receptor band densities. Next, agonist-stimulated ubiquitination signals from each cell line were divided by the corresponding values obtained for the appropriate unstimulated, vector-co-transfected (control) cells to obtain -fold/control basal. Data are summarized (mean ± S.E.) graphically from three experiments performed in duplicate. *p < 0.04 compared with control.

Inhibition of cellular GRK2 activity abrogated PDGFRβ desensitization assessed by receptor Tyr phosphorylation, inhibition of cellular casein kinase I activity did not.

Consequences of PDGFRβ phosphorylation by casein kinase I-γ2 and GRK2 differed, as well, in immune complex kinase assays. When immunoprecipitated PDGFRβ was serine-phosphorylated by purified GST-casein kinase I-γ2, Tyr phosphorylation of the PDGFRβ was reduced (8). Casein kinase I-γ2-mediated phosphorylation might therefore have reduced PDGFRβ Tyr kinase activity, as Biovar et al. (8) propose. By contrast, no decrease in PDGFRβ Tyr phosphorylation obtained when immunoprecipitated PDGFRβ was serine-phosphorylated by purified GRK2 (see legend to Fig. 5). How then might GRK2-mediated phosphorylation diminish PDGFRβ Tyr phosphorylation in cells? Likely molecular mechanisms would seem to involve accessory cellular proteins, whose binding to the PDGFRβ could be affected by GRK2-mediated serine phosphorylation of the receptor. Such accessory proteins might include β-arrestin isofoms, important to GRK-mediated desensitization of heptahelial receptors (10), or SHP-2, important in dephosphorylating the PDGFRβ (4), among other possibilities.

Because PDGFRβ Tyr phosphorylation is not altered by GRK2-mediated receptor serine phosphorylation in immune complex kinase assays (Fig. 5), we can make reasonable inferences about the stoichiometry of GRK2-mediated PDGFRβ serine phosphorylation in these assays. GRK2-mediated serine phosphorylation increases total PDGFRβ phosphorylation by ~20% in the immune complex kinase assay (Fig. 5B). Because the expected stoichiometry of agonist-induced PDGFRβ Tyr phosphorylation is ~9 (9), an incremental 20% phosphorylation from GRK2 would correspond to ~2 mol of phosphate (on serines) per mol of PDGFRβ. This analysis assumes equal probability of phosphate exchange with 32P, of course, for PDGFRβ Tyr and serine residues phosphorylated in 293 cells before IP.) Thus, the magnitude of GRK2-mediated PDGFRβ serine phosphorylation seems congruent with that observed for heptahelial receptors in cells (21, 24) and in purified protein systems validated by saturation binding with β-arrestins (41).

GRK2-mediated Phosphorylation of the EGFR without EGFR Desensitization—Data from this study also demonstrate for the first time that GRK2 can phosphorylate the EGFR, both in cells and in purified protein preparations. This finding was unexpected, because neither augmentation nor inhibition of cellular GRK2 activity affected EGFR activation (see Fig. 2 and Ref. 14), and GRK2 overexpression failed to affect EGFR ubiquitination (Fig. 8), SMC [3H]thymidine incorporation (14), or SMC chemotaxis (13) promoted by EGFR. Thus, there is an apparent paradox between GRK2-mediated phosphorylation of EGFRs and a lack of demonstrable GRK2-mediated EGFR desensitization in certain cells. Of course, it is possible that GRK2-mediated EGFR phosphorylation does desensitize as yet unexplored signaling mechanisms downstream of the EGFR. To explain our current findings, however, it remains to be determined whether GRK2-phosphorylated EGFRs fail to undergo subsequent desensitizing modifications, such as receptor Tyr dephosphorylation, in the cell models we have examined.

Although GRK2 can phosphorylate the EGFR in a 293 cell overexpression model, the ability of GRK2 to phosphorylate the EGFR under more physiologic conditions remains uncertain. At physiologic levels of expression, GRK2 appears to require G protein βγ subunits to facilitate its translocation from the cytosol to plasma membrane receptors (10), and the EGFR,
unlike the PDGFRβ, does not appear to activate heterotrimeric G proteins (at least not in mesenchymal cells like SMCs) (Fig. 1). Moreover, the action of GRK2 is inhibited by calcium/calmodulin, in a manner that is relieved by protein kinase C-mediated phosphorylation of GRK2 (42). Because EGFR stimulation is known to increase cytosolic calcium without inducing significant protein kinase C activation in mesenchymal cells (12), net inhibition of GRK2 by calcium/calmodulin might be significant protein kinase C activation in mesenchymal cells mediated phosphorylation of GRK2 (42). Because EGFR stimulation, in a manner that is relieved by protein kinase C-1). Moreover, the action of GRK2 is inhibited by calcium/calmodulin (Fig. 48268 (7), remains an open question.

Conjugating (E2) enzyme CDC34 (49) and thereby potentially

Pol/ty kinase activity (7), much as GRK2-mediated serine phosphorylation seems more plausible under physiologic conditions in cells like rat hepatocytes, in which the EGFR does activate heterotrimeric G proteins (44).

**PDGFRβ Coupling to Heterotrimeric G Proteins**—Whether assessed by PDGFR-promoted guanine nucleotide turnover in Go,,-3 or by PTX-mediated inhibition of signaling (Fig. 1), the PDGFRβ in SMCs demonstrated coupling to heterotrimeric G proteins. Alderton et al. (45) also found PTX to inhibit PDGFRβ, but not EGFR-evoked ERK phosphorylation in 293 cells; however, the [EGF] used in their experiments (100 nM) was far greater than in ours. In Fig. 1B, although PTX inhibited ERK activation by PDGFβ, it had no effect on comparable levels of ERK activation evoked by EGF. In contrast, using PDGF concentrations ≈2-fold higher than ours, Lutfrell et al. (35) found that PTX inhibited neither PDGF- nor EGF-induced ERK activity in Rat1 fibroblasts. With higher PDGF concentrations, we too found that PTX failed to reduce PDGF-promoted ERK activation (data not shown). This result is to be expected, because most PDGFRβ-mediated ERK phosphorylation proceeds through Tyr kinase-dependent pathways producing Ras activation (1). At higher PDGF concentrations, high levels of PDGFRβ-promoted, Ras-mediated (and saturable) ERK activation could easily obscure the effect of abrogating G/G,-mediated ERK activation. It should be noted that the PDGFRβ is not unique among receptor Tyr kinases in signaling through heterotrimeric G proteins. Similar findings have been described for the insulin receptor (26), insulin-like growth factor-1 receptor (35), fibroblast growth factor receptors-1 and -2 (46, 47), and even the EGFR in hepatocytes (48).

**Effect of GRK2 on PDGFRβ Ubiquitination**—Agonist-induced ubiquitination of the PDGFRβ requires PDGFRβ Tyr kinase activity (7), much as GRK2-mediated serine phosphorylation of the receptor does. How GRK2-mediated phosphorylation of the PDGFRβ might lead to enhanced receptor ubiquitination remains obscure. However, serine or threonine phosphorylation of proteins is known to trigger protein ubiquitination (44, 49, 50). It is therefore possible that GRK2-mediated serine phosphorylation of the PDGFRβ (but not the EGFR) could alter the interactions of the receptor with other proteins so as to promote the binding of c-Cbl (51) or other potential ubiquitin E3 ligases to the receptor. Alternatively, because allosterically activated GRK2 can phosphorylate non-receptor substrates (10), it could, after PDGFRβ activation, act like casein kinase 2 in serine-phosphorylating the ubiquitin-conjugating (E2) enzyme CDC34 (49) and thereby potentially alter receptor ubiquitination. These and other possibilities remain to be explored. Likewise, whether receptor ubiquitination can contribute to PDGFRβ desensitization, because ubiquitination has only modest effects on PDGFRβ down-regulation (7), remains an open question.

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