Phosphorylation of the Platelet-derived Growth Factor Receptor-β by G Protein-coupled Receptor Kinase-2 Reduces Receptor Signaling and Interaction with the Na+/H+ Exchanger Regulatory Factor

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G protein-coupled receptor kinase-2 (GRK2) can phosphorylate and desensitize the platelet-derived growth factor receptor-β (PDGFRβ) in heterologous cellular systems. To determine whether GRK2 regulates the PDGFRβ in physiologic systems, we examined PDGFRβ signaling in mouse embryonic fibroblasts from GRK2-null and cognate wild type mice. To discern a mechanism by which GRK2-mediated phosphorylation can desensitize the PDGFRβ, but not the epidermal growth factor receptor (EGFR), we investigated effects of GRK2-mediated phosphorylation on the association of the PDGFRβ with the Na+/H+ exchanger regulatory factor (NHERF), a protein shown to potentiate dimerization of the PDGFRβ, but not the EGFR. Physiologic expression of GRK2 diminished (a) phosphoinositide hydrolysis elicited through the PDGFRβ but not heterotrimeric G proteins; (b) Akt activation evoked by the PDGFRβ but not the EGFR; and (c) PDGF-induced tyrosyl phosphorylation of the PDGFRβ itself. PDGFRβ desensitization by physiologically expressed GRK2 correlated with a 2.5-fold increase in PDGF-promoted PDGFRβ seryl phosphorylation. In 293 cells, GRK2 overexpression reduced PDGFRβ/NHERF association by 60%. This effect was reproduced by S1104D mutation of the PDGFRβ, which also diminished PDGFRβ activation and signaling (like the S1104A mutation) to an extent equivalent to that achieved by GRK2-mediated PDGFRβ phosphorylation. GRK2 overexpression desensitized only the wild type but not the S1104A PDGFRβ. We conclude that GRK2-mediated PDGFRβ seryl phosphorylation plays an important role in desensitizing the PDGFRβ in physiologic systems. Furthermore, this desensitization appears to involve GRK2-mediated phosphorylation of PDGFRβ Ser1104, with consequent dissociation of the PDGFRβ from NHERF.

The platelet-derived growth factor receptor-β (PDGFRβ) plays a pivotal role in development, in the growth and maintenance of mesenchymal cells, and in pathologic proliferative processes like malignant neoplasia (1, 2) and atherosclerosis (3–5). Maintaining cellular homeostasis and bridling neoplasia therefore requires precise regulation of signaling through this receptor protein tyrosine kinase. Mechanisms described thus far for long and short term regulation of the PDGFRβ include degradation and down-regulation of cellular PDGFRβs (6, 7), tyrosyl dephosphorylation (8, 9), and phosphorylation of the PDGFRβ on serine residues (10, 11).

We recently reported that PDGFRβ desensitization can be effected by GRK2-mediated phosphorylation of the PDGFRβ on serine residues (10). GRK2 is a ubiquitously expressed member of the GRK family of serine/threonine kinases and has previously been characterized by its ability to phosphorylate and desensitize a vast array of heptahelical receptors (12). As an allosteric enzyme, GRK2 is activated by agonist-occupied receptors (12). GRK2 phosphorylates the PDGFRβ in an agonist-dependent manner (10), just as it phosphorylates heptahelical receptors. GRK2-mediated PDGFRβ desensitization manifests in short term assays for phosphoinositide hydrolysis (13) and phosphatidylinositol 3-kinase activation (14) and in long term assays for [3H]thymidine incorporation, proliferation (13), and cellular migration (14). Moreover, GRK2-mediated PDGFRβ seryl phosphorylation reduces the activation of the PDGFRβ itself, as assessed by receptor tyrosyl phosphorylation (10, 13). In contrast, GRK2 overexpression does not desensitize the EGFR, even though GRK2 phosphorylates EGFR serine(s) in purified protein and cellular overexpression systems (10, 13).

To discern the mechanisms by which GRK2-mediated RPTK phosphorylation could desensitize the PDGFRβ but not the EGFR, we have focused this investigation on the interaction between the PDGFRβ and the Na+/H+ exchanger regulatory factor (NHERF), a PDZ domain-containing protein known to bind to the PDGFRβ, but not the EGFR, via the C-terminal tetrapeptide Asp-Ser1104-Leu-Leu of the receptor (15). The association of the PDGFRβ with NHERF (also known as EBP50) has been shown to potentiate PDGFRβ dimerization and signaling (15). NHERF also binds to the C-terminal tetrapeptide Asp-Ser411-Leu-Leu of the EGFR, even though GRK2 phosphorylates EGFR serine(s) in the EGFR, even though GRK2 phosphorylates EGFR serine(s) in purified protein and cellular overexpression systems (10, 13).

To discern the mechanisms by which GRK2-mediated RPTK phosphorylation could desensitize the PDGFRβ but not the EGFR, we have focused this investigation on the interaction between the PDGFRβ and the Na+/H+ exchanger regulatory factor (NHERF), a PDZ domain-containing protein known to bind to the PDGFRβ, but not the EGFR, via the C-terminal tetrapeptide Asp-Ser1104-Leu-Leu of the receptor (15). The association of the PDGFRβ with NHERF (also known as EBP50) has been shown to potentiate PDGFRβ dimerization and signaling (15). NHERF also binds to the C-terminal tetrapeptide Asp-Ser411-Leu-Leu of the β2-adrenergic receptor, in an agonist-dependent manner (16). Association between NHERF and the β2-adrenergic receptor affects receptor-mediated regulation of PDGFRβ, PDGF receptor-β, GRK2, G protein-coupled receptor kinase-2; NHERF, Na+/H+ exchanger regulatory factor; MEFs, mouse embryonic fibroblasts; PLC, phospholipase C; IP, immunoprecipitate, immunoprecipitation; WT, wild type; ERK, extracellular signal-regulated kinase; RPTK, receptor protein-tyrosine kinase; EGFR, epidermal growth factor receptor.
Na\(^+\)/H\(^-\) exchange (16) as well as receptor recycling (17). This \(\beta\)-adrenergic receptor/NHERF association appears to be disrupted by GRK5-mediated phosphorylation of the \(\beta\)-adrenergic receptor on Ser\(^{111}\) (17) (a site not phosphorylated by GRK2 (18)). By analogy with the action of GRK5 on the \(\beta\)-adrenergic receptor, we hypothesized that GRK2 could phosphorylate the PDGFR\(\beta\) on Ser\(^{1104}\) and thereby decrease the affinity of NHERF/PDGFR\(\beta\) interaction, diminish PDGFR\(\beta\) dimerization and activation, and reduce downstream signaling. To test this hypothesis, we examined the effect of GRK2-mediated PDGFR\(\beta\) phosphorylation on NHERF/PDGFR\(\beta\) association. In addition, to determine whether GRK2-mediated PDGFR\(\beta\) phosphorylation and desensitization occur in physiologic systems, we examined PDGFR\(\beta\) regulation in MEFs derived from GRK2\(^{-/-}\) and cognate WT mice.

MATERIALS AND METHODS

Plasmid Constructs—Plasmids encoding the N-terminal FLAG-tagged human PDGFR\(\beta\) and bovine GRK2, each in pcDNA1 (Invitrogen), have been described (10, 19). The plasmid encoding an N-terminal hemagglutinin-tagged rabbit NHERF\(\beta\) was the kind gift of Randi Hall (15). The bovine GRK2 cDNA was subcloned into pcDNA3 3.5 hygro (Invitrogen) by using HindIII sites that flanked the insert (19). To create S1104D and S1104A mutations in the PDGFR\(\beta\), we employed casette PCR with the following mutagenic primers: 5'-ggagggccgcggcctcctgcttccgcccgaggcgc-3' (S1104D) and 5'-ggagggccgcggcctcctgcttccgcccgaggcgc-3' (S1104A) (the underscore indicates the mutation to Asp or to Ala, italics indicate the 3' NotI site, and bold type denotes the stop codon). The 5' (nonmutagenic) primer comprised nucleotides 3061–3080 of the native PDGFR\(\beta\) sequence. After PCR, a BstII/NotI fragment was subcloned into the FLAG-tagged PDGFR\(\beta\) construct, and the fidelity of mutagenesis was confirmed by dideoxy sequencing.

Cell Culture—MEFs were derived from embryonic day 10 embryos by the method of outgrowth and 3T3 approaches (20) and propagated in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin (medium A). GRK2\(^{-/-}\) and WT littermate embryos were obtained from C57Bl/6J/129 hybrid mice and were genotyped bySouthern blotting of genomic DNA, as described (21). All of the animal protocols were approved by the Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals.

To increase the number of cell lines expressing physiologic levels of GRK2 and the number of GRK2\(^{-/-}\) cell lines available for genetic analysis, we stably transfected our two GKR2 clones with the GRK2/pcDNA3.5 hygro plasmid (using LipofectAMINE 2000; Invitrogen) according to the manufacturer’s protocol. The colonies were selected in medium A containing hygromycin at 250 \(\mu\)g/ml. HEK 293 cells were propagated and transfected as previously reported (10). For each transfection, cell surface PDGFR\(\beta\) expression was measured by immunofluorescence and flow cytometry, as described (22). Co-transfected and mutant PDGFR\(\beta\) cell lines were used only when cell surface PDGFR\(\beta\) density was within 30% of that measured in control cells. GRK2 overexpression levels ranged from 20- to 40-fold over endogenous levels, assessed by immunoblotting serially diluted specimens, and was equivalent for all GRK2-overexpressing cells used within a single experiment.

Phosphoinositide Hydrolysis—MEFs were labeled in medium B (medium A without fetal bovine serum and with 0.1% (w/v) bovine serum albumin) with \([\text{H}]\)myoinositol (PerkinElmer Life Sciences) for 18 h, as described (13), except that PDGF-AA (Calbiochem) was added to the labeling medium at 10 ng/ml to down-regulate the PDGFR\(\beta\)-receptors (20) expressed by the MEFs. MEFs were then challenged with the indicated agonist for 15 min (37 \(^\circ\)C) before being lysed and processed for isolation of total inositol phosphates by anion exchange chromatography, as described (22). HEK 293 cells were also challenged with the indicated stimuli for 15 min (37 \(^\circ\)C); they were metabolically labeled in medium C (minimal essential medium, 0.1% (w/v) bovine serum albumin, penicillin/streptomycin, 1% (v/v) nonessential amino acids, and 1% pyruvate) and assayed as described (13, 22). Data processing involved normalizing total inositol phosphates to the total amount of \([\text{H}]\)myoinositol taken up by the cells to obtain the percent conversion of \(\text{H}\) to inositol phosphates, as described (22).

Immunoprecipitations and Immunoblotting—These procedures were performed as described previously (10, 13, 14). Cells were serum-starved for 16 h in medium B (MEFs) or C (293 cells) before assays. For co-IP experiments, the cell proteins were cross-linked with diethio-bis(succinimidyl)propionate (Pierce) as described (10, 22). Immunological reagents were those described previously (10, 13, 24), with the addition of the following IgGs: rabbit anti-EPB50 (NHERF) (Calbiochem, Inc.), mouse anti-FLC-1 and rabbit anti-GRK2 (Santa Cruz Biotechnology, Inc.), and rabbit anti-phosphoserine (Chemicon, Inc.). To confirm antibody specificity for endogenous proteins, parallel blots were performed with nonimmune IgG of species and isotype identical to that used for identifying proteins of interest. For sequential immunoblots, anti-PDGFR\(\beta\) IgG was always used before anti-phosphoserine or anti-phosphotyrosine IgG, because the latter two IgGs failed to desorb from PDGF-stimulated bands (data not shown). To quantitate PDGFR\(\beta\) tyrosyl and seryl phosphorylation, we performed densitometry on chemiluminescence-exposed BioMax MR\(\text{TM}\) film (Kodak). Anti-phosphotyrosine- or anti-phosphoserine-scored PDGFR\(\beta\) band densities were normalized to cognate PDGFR\(\beta\) band densities. (Because phosphotyrosine and phosphoserine signals were negligible in the absence of agonist, only PDGFR\(\beta\) bands from PDGF-stimulated cells were used for comparisons among cell lines.) To quantitate NHERF/PDGFR\(\beta\) co-IP, NHERF band densities from co-immunoprecipitations were normalized to cognate PDGFR\(\beta\) band densities. Phospho-Akt and phoso-ERK1/2 band densities were normalized to cognate actin band densities.

Statistical Analyses—Independent means were compared with unpaired t tests and one-way analysis of variance (with Tukey’s post-hoc test for multiple comparisons) was used for comparisons among more than two groups, with Prism 2 software (GraphPad, Inc.). The data are presented as the means \(\pm\) S.D. in the text and as the means \(\pm\) S.E. in the figures.

RESULTS

Phosphorylation and Desensitization of the PDGFR\(\beta\) by Physiologic Levels of GRK2—Although we have found that GRK2 phosphorylates and desensitizes the PDGFR\(\beta\) both in cellular overexpression and in purified protein systems, we have yet to determine whether physiologically expressed GRK2 regulates the PDGFR\(\beta\). To address this question, we began by comparing endogenous PDGFR\(\beta\) signaling in GRK2\(^{-/-}\) MEFs with that in cognate WT MEFs. Physiologic expression of GRK2 in WT MEFs reduced PDGFR\(\beta\)-evoked phosphoinositide hydrolysis by 2.3-fold, compared with that observed in GRK2\(^{-/-}\) MEFs (Fig. 1A). This effect of GRK2 was receptor-specific, because physiologic GRK2 expression had no effect on phosphoinositide hydrolysis elicited in these cells through the protease-activated receptor-1 (thrombin receptor) (Fig. 1A), which we have previously shown is a relatively poor GRK2 substrate (25). To determine whether random genetic variation could be confounding our comparisons among WT and GRK2\(^{-/-}\) MEF lines, we phenotypically characterized each cell line by immunoblotting cell extracts for key proteins in the PDGFR\(\beta\)-evoked phosphoinositide hydrolysis signaling pathway (Fig. 1B). Although distinctly different with regard to GRK2 expression, both of the WT and both of the GRK2\(^{-/-}\) cell lines expressed comparable (or functionally equivalent) levels of PDGFR\(\beta\), PLC\(\gamma\)-1, and NHERF. Thus, our inferences about the role of GRK2 on PDGFR\(\beta\) signaling were facilitated.

To minimize the effect of undetected genetic drift on our comparisons among GRK2\(^{-/-}\) and WT MEFs, we used our two GRK2\(^{-/-}\) MEF lines to create MEF clones stably transfected to express either GRK2\((n = 4)\) or no protein (GRK2\(^{+/+}\) cells, \(n = 4\)). We selected GRK2-transfected clones that expressed GRK2 at levels comparable with those observed in WT MEFs (Fig. 2A). In addition, clones were selected on the basis of expressing comparable levels of proteins that could affect PDGFR\(\beta\) signaling, including the PDGFR\(\beta\) itself, PLC\(\gamma\)-1, GRK5, GRK6, and NHERF (Fig. 2A). In results averaged from these four GRK2-expressing and 4 GRK2\(^{+/+}\) clones, physiologic levels of GRK2 expression reduced PDGFR\(\beta\)-evoked phosphoinositide hydrolysis by 38% (\(p < 0.01\)) but had no effect on phosphoinositide hydrolysis evoked through direct activation of heterotrimeric G

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From each of four MEF lines, 50 nM agonist peptide (SFLLRN, phosphoinositide hydrolysis. The indicated MEFs were exposed to either phosphoinositide hydrolysis assays (A) or phenotypic profiling by immunoblotting (B). A, phosphoinositide hydrolysis. The indicated MEFs were exposed (37°C for 15 min) to vehicle (basal), PDGF-BB (2 nM), or thrombin agonist peptide (SFLLRN, 10 μM). Inositol phosphates were isolated as described under “Materials and Methods.” Plotted are the means ± S.E. from two MEF lines of each genotype, from at least three experiments performed in triplicate. Basal inositol phosphates were 0.7 ± 0.4 and 0.9 ± 0.4 percent conversion units for WT and knockout (KO) cells, respectively. *, p < 0.05 compared with WT. B, MEF protein expression. From each of four MEF lines, 50 μg of protein was subjected to SDS-PAGE and immunoblot (IB), with the indicated IgGs. Rabbit nonimmune IgG was used as a negative control for rabbit anti-NHERF and anti-PDGFRβ antibodies; nonimmune mouse IgG (not shown) demonstrated no bands co-migrating with either GRK2 or PLCγ1 bands (immunoblotted with mouse IgGs). Blots are from a single experiment, representative of three performed. Std, standard (purified) bovine GRK2.

Fig. 1. Endogenous cellular GRK2 desensitizes PDGFRβ signaling in a receptor-specific manner. MEFs derived from GRK2+/+ (WT) and GRK2−/− (KO) mice were processed for either phosphoinositide hydrolysis assays (A) or phenotypic profiling by immunoblotting (B). A, phosphoinositide hydrolysis. The indicated MEFs were exposed (37°C for 15 min) to vehicle (basal), PDGF-BB (2 nM), or thrombin agonist peptide (SFLLRN, 10 μM). Inositol phosphates were isolated as described under “Materials and Methods.” Plotted are the means ± S.E. from two MEF lines of each genotype, from at least three experiments performed in triplicate. Basal inositol phosphates were 0.7 ± 0.4 and 0.9 ± 0.4 percent conversion units for WT and knockout (KO) cells, respectively. *, p < 0.05 compared with WT. B, MEF protein expression. From each of four MEF lines, 50 μg of protein was subjected to SDS-PAGE and immunoblot (IB), with the indicated IgGs. Rabbit nonimmune IgG was used as a negative control for rabbit anti-NHERF and anti-PDGFRβ antibodies; nonimmune mouse IgG (not shown) demonstrated no bands co-migrating with either GRK2 or PLCγ1 bands (immunoblotted with mouse IgGs). Blots are from a single experiment, representative of three performed. Std, standard (purified) bovine GRK2.

ERK1/2 was not (data not shown). Lastly, physiologic levels of GRK2 expression reduced activation of the PDGFRβ itself. Tyrosyl phosphorylation of the PDGFRβ was 32% less in GRK2-expressing cells compared to GRK2 null cells (p < 0.05; Fig. 3B). Thus, by comparing cells expressing physiologic GRK2 levels with cells expressing no GRK2, we found GRK2-mediated PDGFRβ desensitization to be remarkably similar to that observed by comparing GRK2-overexpressing with endogenous GRK2-expressing cells (10, 13, 14).

In cellular overexpression and purified protein systems, we previously found that GRK2 phosphorylated the PDGFRβ on serine(s), in the short time frame (minutes) congruent with GRK2-mediated PDGFRβ desensitization (10, 13, 14). In this regard, GRK2-mediated PDGFRβ desensitization paralleled GRK2-mediated heptahelical receptor desensitization. To determine whether GRK2 phosphorylates the PDGFRβ
serine(s) when GRK2 is expressed at physiologic levels, we examined PDGF-induced PDGFRβ seryl phosphorylation in GRK2-expressing and GRK2null MEFs (Fig. 4). Within 10 min of PDGFRβ activation, seryl phosphorylation of the PDGFRβ was 2.5 ± 0.6-fold greater in MEFs expressing physiologic GRK2 levels than it was in GRK2null MEFs (Fig. 4; p < 0.05). Thus, GRK2 serine-phosphorylated the PDGFRβ in cells expressing physiologic levels of both proteins, in a manner that correlated temporally with GRK2-mediated PDGFRβ desensitization. Moreover, the magnitude of PDGFRβ seryl phosphorylation in GRK2-expressing and -null fibroblasts suggested that GRK2 mediates the majority of agonist-dependent PDGFRβ seryl phosphorylation in these cells.

**GRK2-mediated PDGFRβ Phosphorylation Attenuates NHERF/PDGFRβ Association**—Could GRK2 phosphorylate the PDGFRβ on serine 1104 and thereby diminish the affinity of PDGFRβ/NHERF association? If so, such a mechanism could help to explain why GRK2-mediated phosphorylation reduces tyrosyl phosphorylation of the PDGFRβ but not the EGFR (10). Although the PDGFRβ is cross-linked by NHERF, the EGFR is not (15). To explore this possibility, we first determined that NHERF overexpression augmented PDGFRβ tyrosyl phosphorylation in our transfected cell system (2.7 ± 0.3-fold (p < 0.02); Fig. 5), as first demonstrated by Maudsley et al. (15). Maudsley et al. also demonstrated with mutant PDGFRβs that reducing PDGFRβ/NHERF interaction reduces PDGFRβ tyrosyl phosphorylation (15), as GRK2-mediated PDGFRβ phosphorylation does (Fig. 3B). To determine whether GRK2-mediated PDGFRβ phosphorylation attenuated the association of
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Fig. 6. GRK2 overexpression augments PDGFRβ seryl phosphorylation and reduces PDGFRβ/NHERF association. HER 293 cells were transfected (or not, −) with plasmids encoding the PDGFRβ, GRK2, or no protein (Vector, “control”), as indicated. Serum-starved cells were exposed to vehicle (−) or 2 nM PDGF-BB for 5 min (37 °C) and then subjected to PDGFRβ IP. A, immunoprecipitates were immunoblotted (IB) sequentially for PDGFRβ and then phosphoserine (pSer). Shown are immunoblots from a single experiment, representative of three performed. B, band densities for pSer were divided by cognate band densities for the PDGFRβ and then endogenous NHERF. The cell lysates were immunoblotted for GRK2 (20 μg of protein from control cells and 2 μg from GRK2-overexpressing cells) and endogenous NHERF (20 μg of protein

NHERF with the PDGFRβ, we co-immunoprecipitated NHERF with the PDGFRβ from cells that either overexpressed GRK2 or expressed endogenous GRK2. With GRK2 overexpression, seryl phosphorylation of the PDGFRβ increased by 2.1 ± 0.6-fold (Fig. 6, A and B; p < 0.05), much as we found by comparing MEFs expressing physiologic levels of GRK2 with GRK2− MEFs (Fig. 4). Correspondingly, there was a 60 ± 30% reduction in the amount of NHERF associated with the PDGFRβ (Fig. 6, C and D; p < 0.05). This correlation of reduced NHERF/PDGFRβ association with GRK2-mediated PDGFRβ seryl phosphorylation suggested that serine 1104 of the PDGFRβ was indeed a GRK2 phosphorylation site.

Identification of PDGFRβ Ser1104 as a GRK2 Phosphorylation Site—From the foregoing observations, we reasoned that GRK2 could phosphorylate PDGFRβ Ser1104 (of the C-terminal Asp-Ser1104-Phe-Leu) and thereby diminish both the affinity of NHERF/PDGFRβ interaction and, consequently, the degree of PDGF-induced receptor activation. To test this possibility, we sought to determine whether phosphorylation of PDGFRβ Ser1104 could effect at least some of the changes in PDGFRβ behavior we observed consequent to GRK2-mediated PDGFRβ phosphorylation. To that end, we created S1104D and S1104A PDGFRβ mutants. Although we expected the S1104D mutant to model the Ser1104-phosphorylated WT PDGFRβ, we expected that both the S1104A and S1104D mutants would demonstrate diminished binding affinity for NHERF, because each of the analogous mutations in the β2-adrenergic receptor C-terminal tetrapeptide severely attenuated NHERF binding in blot overlay assays (26). To test these expectations, we first assessed the association of NHERF with the S1104D PDGFRβ. As with the GRK2-phosphorylated PDGFRβ, the S1104D PDGFRβ demonstrated a 50 ± 10% decrease in NHERF association, compared with the WT PDGFRβ (Fig. 7A; p < 0.05). Moreover, this reduction in NHERF association correlated with a 20 ± 5% reduction in tyrosyl phosphorylation of the S1104D PDGFRβ (Fig. 7A; p < 0.05). In a similar fashion, the S1104A PDGFRβ also demonstrated reduced tyrosyl phosphorylation (50 ± 20%, p < 0.05), compared with the WT PDGFRβ (Fig. 7B).

If Ser1104 of the PDGFRβ is a GRK2 target, then we should expect the S1104A PDGFRβ mutant to demonstrate less seryl phosphorylation than the WT PDGFRβ. Indeed, this expectation was borne out (Fig. 7C). The S1104A PDGFRβ demonstrated only 60 ± 10% of the agonist-induced seryl phosphorylation observed with the WT PDGFRβ (Fig. 7C; p < 0.05). This result was consonant with our earlier estimate of stoichiometry for GRK2-mediated PDGFRβ seryl phosphorylation (~2 mol seryl phosphate/mol PDGFRβ (10)), and our hypothesis that GRK2 phosphorylates PDGFRβ Ser1104.

Like GRK2-mediated PDGFRβ phosphorylation, mutation of PDGFRβ Ser1104 reduced PDGFRβ/NHERF association and PDGFRβ activation. For this reason, we expected that Ser1104 mutation would also reduce signaling downstream of the PDGFRβ, as GRK2-mediated PDGFRβ desensitization does (Figs. 1–3 and Refs. 13 and 14). Indeed, the S1104A and the S1104D PDGFRβs each demonstrated impairment of PDGF-induced phosphoinositide hydrolysis in cells expressing endogenous NHERF (Fig. 8A), and the magnitude of impairment was similar to that observed with GRK2-phosphorylated WT PDGFRβs (Fig. 2). Moreover, impairment of signal transduc-
ser1104 mutant PDGFR

The results are from a single experiment, representative of three performed. B, cells transfected with either the WT PDGFRβ (S at residue 1104) or the S1104A PDGFRβ served as a control to determine phosphoinositide hydrolysis elicited by the WT and S1104A PDGFRβ. A, cells were exposed to medium containing vehicle (−) or 0.16 nM PDGF-BB for 5 min (37°C) and then solubilized and subjected to PDGFRβ IP. Immunoprecipitates were immunoblotted as in Fig. 3B. The results are from a single experiment, representative of three performed. C, cells were stimulated and subjected to PDGFRβ IP as in Fig. 6A. The immunoprecipitates were immunoblotted sequentially for PDGFRβ and then phosphoserine (pSer). The results are from a single experiment, representative of three performed.

If GRK2 does desensitize the PDGFRβ by phosphorylating PDGFRβ Ser1104, then we would expect the S1104A PDGFRβ construct to mimic the pattern we observed previously with GRK2-mediated desensitization, at least in part by Ser1104 phosphorylation. In Fig. 3 and Ref. 14, whereas Ser1104 mutant PDGFRβs failed to activate PLCγ1 as much as the WT PDGFRβ did (Fig. 3A), the S1104D PDGFRβ activated ERK just as much as the WT PDGFRβ (Fig. 8B). Thus, Ser1104 mutant PDGFRβs resemble GRK2-phosphorylated WT PDGFRβ with regard to NHERF binding, PDGFRβ activation, and downstream signaling.

If GRK2 does desensitize the PDGFRβ by phosphorylating PDGFRβ Ser1104, then we would expect the S1104A PDGFRβ to resist desensitization by GRK2. To test this prediction, we examined phosphoinositide hydrolysis elicited by the WT and S1104A PDGFRβs, in cells expressing either endogenous or high levels of GRK2. With the WT PDGFRβ, PDGFinduced phosphoinositide hydrolysis was reduced by 40% when GRK2 was overexpressed. Interestingly, this reduction in phosphoinositide hydrolysis was equivalent to that observed with the S1104A mutation of the PDGFRβ (Fig. 8C). However, overexpression of GRK2 failed to desensitize S1104A PDGFRβ signaling, even though the levels of GRK2 overexpression tested were equivalent to those achieved in cells expressing WT PDGFRβs (Fig. 8C). These results further support a model in which GRK2 phosphorylates Ser1104 of the PDGFRβ and thereby engenders PDGFRβ desensitization, at least in part by reducing PDGFRβ/NHERF association.

Mutation of PDGFRβ Ser1104 selectively desensitizes PDGFβ signaling and abrogates GRK2-mediated desensitization. A, phosphoinositide hydrolysis. HEK 293 cells transfected with the indicated PDGFRβ construct were processed for phosphoinositide hydrolysis as in Fig. 2 except no PDGFRβs down-regulation was necessary. Total inositol phosphates produced in response to 2 nM PDGF-BB or fluoroaluminate were normalized to those measured in PDGF-challenged cells expressing WT PDGFRβs (‘control’). Plotted are the means ± S.E. from three experiments performed in triplicate. Basal inositol phosphates were 1.3 ± 0.5, 1.5 ± 0.5, and 1.6 ± 0.8 (percent conversion units) for WT, S1104A- and S1104D-PDGFRβ cells, respectively. Cell surface expression of the S1104D- and S1104A-PDGFRβ, respectively, were 120 ± 30 and 110 ± 25% of WT PDGFRβ levels, * p < 0.05 compared with WT. B, PDGFRβ S1104D mutation fails to alter ERK signaling. The cells from A were exposed to medium containing either vehicle (−) or 2 nM PDGF-BB for 10 min (37°C) and then solubilized; lysate proteins resolved by SDS-PAGE were immunoblotted (IB) sequentially with IgG against phospho-ERK1/2 (pERK1/2) and endogenous NHERF or in parallel with nonimmune rabbit IgG (bottom gel). Shown are the results of a single experiment, representative of three performed. C, GRK2 fails to desensitize the S1104A PDGFRβ. HEK 293 cells were transfected with plasmids encoding either the WT or the S1104A PDGFRβ, and either GRK2 (GRK2 level, Natloc), or no protein (GRK2 level, Vector). Control cells were those transfected with the WT PDGFRβ and empty vector plasmids. Left panel, cells were assayed for PDGF-induced phosphoinositide hydrolysis, as in A. The values for PDGF-induced total inositol phosphates (percent conversion units) were averaged across three experiments and plotted as the means ± S.E. * p < 0.05 compared with control cells. Basal values for inositol phosphates (percent conversion units) were 1.9 ± 0.1, 1.7 ± 0.1, 1.7 ± 0.1, and 1.7 ± 0.3 for cells transfected with WT PDGFRβ/vector, WT PDGFRβ/GRK2, S1104A PDGFRβ/vector, and S1104A PDGFRβ/GRK2, respectively. Right panel, 40 μg of “native GRK2” cells and 2 μg of “high GRK2” cells were immunoblotted for GRK2. The results are from a single experiment, representative of three performed.
DISCUSSION

This study provides the first evidence that physiologically expressed GRK2 regulates PDGFRβ activation and downstream signaling. In fibroblasts, we have demonstrated that GRK2 engenders the preponderance of agonist-promoted PDGFRβ seryl phosphorylation, which we have correlated with PDGFRβ desensitization. Furthermore, this study reveals a novel mechanism that explains how GRK2-mediated phosphorylation can engender PDGFRβ desensitization without engineering EGFR desensitization (10, 13, 14). By promoting dissociation of the PDGFRβ from the PDZ domain-containing NHERF, a protein that potentiates PDGFRβ dimerization and activation (15). Our data link PDGFRβ/NHERF association mechanistically with GRK2-mediated phosphorylation of PDGFRβ Ser1104, and with consequent PDGFRβ desensitization, with two complementary approaches. First, effects of PDGFRβ seryl phosphorylation by GRK2 correlated with effects of PDGFRβ Ser1104 mutation; second, GRK2 failed to desensitize a S1104A PDGFRβ mutant deficient in NHERF binding.

GRK-mediated receptor phosphorylation has previously been shown to effect dissociation of PDZ domain-containing proteins only from two heptahelical receptors. Overexpression of GRK5 (but not GRK2) altered recycling of the β2-adrenergic receptor in transfected 293 cells, as did mutation of the β2-adrenergic receptor C-terminal DSLL to DDDL; the DDDL mutation also promoted dissociation of NHERF from the β2-adrenergic receptor (17). Similarly, overexpression of GRK5 (but not GRK2) with the β1-adrenergic receptor diminished association of that receptor with PSD-95 (27), a protein shown to diminish β1-adrenergic receptor internalization without affecting receptor signaling (28). GRK-mediated seryl phosphorylation disrupts receptor/PDZ domain-containing protein association because it disrupts hydrogen bonding between PDZ domains and the free hydroxyl at position –2 (Ser) of the class I PDZ domain-interacting tripeptide (STX/XL/IV) (29). Our study demonstrates that GRK2, and not just GRK5, can mediate receptor phosphorylation that promotes dissociation of a PDZ domain-containing protein from an RPTK (and not just a heptahelial receptor). Thus, this work substantially extends the generalizability of an increasingly important paradigm: that agonist-dependent, GRK-mediated seryl phosphorylation regulates PDZ domain-containing protein/receptor interactions.

The role of NHERF in potentiating PDGFRβ activation and signaling was first proposed by Maudsley et al. (15) on the basis of observations in Chinese hamster ovary and COS-7 cells. More recently, Demoulin et al. (30) have used porcine aortic endothelial cells, fibroblasts, and 293 cells to produce data that could be interpreted as contradicting the work of Maudsley et al. However, our results, obtained by cellular approaches distinct from both of these groups, support the model proposed by Maudsley et al. Indeed, we believe that technical factors can explain most, if not all of the significant discrepancies among results obtained by these two groups. To assess NHERF/PDGFRβ association in cells, Maudsley et al. immunoprecipitated an N-terminal epitope-tagged NHERF, whereas Demoulin et al. immunoprecipitated endogenous NHERF with IgG raised against a 14-mer peptide that falls within a secondary PDGFRβ-binding domain of NHERF (15). Thus, as compared with Maudsley et al., Demoulin et al. may have been able to co-IP only a subset of total cellular PDGFRβ/NHERF complexes: those complexes in which the PDGFRβ itself did not out-compete anti-NHERF IgG for binding to NHERF. This problem may underlie an assertion by Demoulin et al. that contradicts the work of both Maudsley and our own group: that NHERF/PDGFRβ association is PDGF-dependent and appears to correlate (temporally) with PDGFRβ internalization. In contrast to Demoulin et al., we co-immunoprecipitated the PDGFRβ and NHERF with an IgG specific for the N-terminal epitope tag of the PDGFRβ. In so doing, we avoided confounding our results by potential competition between NHERF/IgG and NHERF/PDGFRβ interactions. Both the Maudsley and Demoulin groups used a L1106A PDGFRβ mutant to diminish PDGFRβ/NHERF association in cells. However, whereas Maudsley’s group observed that abrogating PDGFRβ/NHERF interaction (with the L1106A mutant) reduced PDGFRβ signaling (15), Demoulin’s group observed the opposite (30). This discrepancy may derive from disparate expression levels of PDGFRβ constructs used in the work of Demoulin et al.: their L1106A-PDGFRβ was expressed at levels significantly higher than their WT PDGFRβ (30). Therefore, excess signaling engendered by higher (mutant) PDGFRβ density may have reversed the decrement in signaling expected from abolishing NHERF-dependent PDGFRβ cross-linking (15).

In using GRK2−/− cells to demonstrate a physiologic role for GRK2 in phosphorylation and desensitization of the PDGFRβ, this study corroborates findings obtained in a variety of GRK2 overexpression and purified protein systems (10, 13, 14). In the only other study of physiologic GRK2 expression published to date, GRK2−/− cardiomyocytes were used to demonstrate β1-adrenergic receptor desensitization by physiologic levels of GRK2 (31), and these cells corroborated findings obtained previously in GRK2 overexpression and purified protein systems (19). To what can we attribute the concordance between physiologic and GRK2 overexpression systems? One possibility is the requirement of GRK2 for allosteric activation by activated receptors. Even when overexpressed, GRK2 remains relatively quiescent until agonist activates one of its target receptors (12).

It is becoming increasingly clear that RPTK signaling persists for a considerable time after agonist-induced receptor activation and internalization (32, 33), perhaps until RPTKs are degraded in lysosomes. In this context, constraining PDGFRβ and other RPTKs signaling until RPTK proteolysis occurs attains substantial significance for cellular homeostasis. Judged by our work in GRK2-expressing and -null fibroblasts, GRK2-mediated phosphorylation appears to be an important mechanism for constraining signaling by the PDGFRβ. Whether GRK2 or other GRKs regulate other RPTKs remains to be established.

Acknowledgment—We are grateful to Susan Suter for genotyping MEFs.

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Phosphorylation of the Platelet-derived Growth Factor Receptor-β by G Protein-coupled Receptor Kinase-2 Reduces Receptor Signaling and Interaction with the Na⁺/H⁺ Exchanger Regulatory Factor

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J. Biol. Chem. 2004, 279:41775-41782.
doi: 10.1074/jbc.M403274200 originally published online July 22, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M403274200

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