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Activation of Protein Kinase C α Is Necessary for Sorting the PDGF β-Receptor to Rab4a-dependent Recycling

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Previous studies showed that loss of the T-cell protein tyrosine phosphatase (TC-PTP) induces Rab4a-dependent recycling of the platelet-derived growth factor (PDGF) β-receptor in mouse embryonic fibroblasts (MEFs). Here we identify protein kinase C (PKC) α as the critical signaling component that regulates the sorting of the PDGF β-receptor at the early endosomes. Down-regulation of PKC abrogated receptor recycling by preventing the sorting of the activated receptor into EGFP-Rab4a positive domains on the early endosomes. This effect was mimicked by inhibition of PKCα, using myristoylated inhibitory peptides or by knockdown of PKCα with shRNAi. In wt MEFs, short-term preactivation of PKC by PMA caused a ligand-induced PDGF β-receptor recycling that was dependent on Rab4a function. Together, these observations demonstrate that PKC activity is necessary for recycling of ligand-stimulated PDGF β-receptor to occur. The sorting also required Rab4a function as it was prevented by expression of EGFP-Rab4aS22N. Preventing receptor sorting into recycling endosomes increased the rate of receptor degradation, indicating that the sorting of activated receptors at early endosomes directly regulates the duration of receptor signaling. Activation of PKC through the LPA receptor also induced PDGF β-receptor recycling and potentiated the chemotactic response to PDGF-BB. Taken together, our present findings indicate that sorting of PDGF β-receptors on early endosomes is regulated by sequential activation of PKCα and Rab4a and that this sorting step could constitute a point of cross-talk with other receptors.

INTRODUCTION

Members of the platelet-derived growth factor (PDGF) family stimulate cell growth, survival and motility. PDGF isoforms act through two structurally related protein tyrosine kinase receptors termed PDGF α- and β-receptors (Heldin *et al.,* 1998). Binding of PDGF to its receptors results in receptor dimerization promoting phosphorylation in *trans* between the two receptors in the complex. After its activation, the receptors are internalized and sorted toward lysosomal degradation.

The internalization and intracellular sorting of receptors have been extensively studied (Maxfield and McGraw, 2004). After internalization, the receptor reaches the early endosomes where a first sorting step occurs. At this site, some receptors such as transferrin receptors may rapidly recycle back to the membranes through vesicular transport dependent on the small GTPase Rab4a (Maxfield and McGraw, 2004). Other receptors that recycle, such as the EGF receptor (Waterman and Yarden, 2001; Dikic, 2003), are more commonly sorted through the recycling compartment, which involves Rab11 (van Ijzendoorn, 2006). Receptor signaling is terminated by sorting through late endosomes to the lysosomes. The sorting of receptor tyrosine kinases toward lysosomal degradation depends on the interaction with proteins forming the ESCRT systems, and the mechanisms underlying sorting of receptors into multivesicular bodies of the late endosomes are relatively well understood (Raiborg *et al.,* 2003). Comparatively little is known about the mechanisms underlying the sorting of receptors in the early endosomes, where sorting toward Rab4a-dependent recycling is determined (Deneka *et al.,* 2003).

The T-cell protein tyrosine phosphatase (TC-PTP) regulates growth factor receptor signaling, both at the level of receptor tyrosine phosphorylation and in the regulation of downstream signaling events (Bourdeau *et al.,* 2005). TC-PTP associates with (Markova *et al.,* 2003) and dephosphorylates the PDGF β-receptor in vivo (Persson *et al.,* 2004), and loss of TC-PTP results in the hyperphosphorylation of Y1021 of the PDGF β-receptor. Depletion of TC-PTP specifically induced recycling of PDGF β-receptor, and in parallel with this decreased receptor degradation (Karlsson *et al.,* 2006). In the present study, we show that recycling of the PDGF β-receptor is critically dependent on the activation of protein kinase C α (PKCα) downstream of Y1021 of the PDGF β-receptor. Importantly, we found that Rab4a activity is required for the sorting of PDGF β-receptor into Rab4a-positive domains on the early endosomes. Inhibition of PKC increased the rate of receptor degradation demonstrating that this sorting in the early endosomes is rate-limiting for PDGF β-receptor degradation.

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Abbreviations used: BSA, bovine serum albumin; LPA, L-α-lysophosphatidic acid; MEF, mouse embryonic fibroblast; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TC-PTP, T-cell protein tyrosine phosphatase.

MATERIALS AND METHODS

Reagents

Ionomycin, rottlerin, and the TC-PTP mAb were from Calbiochem (Darmstadt, Germany). phorbol 12-myristate acetate (PMA), lysophosphatidic acid (LPA), and the vector containing shRNA targeting PKCα was obtained from Sigma-Aldrich (St. Louis, MO). Antibodies toward the PKC isoforms were

from BD Transduction Laboratories (San Jose, CA). The phospho-MARCKS (S152/156) antibody was from Cell Signaling Technologies, (Beverly, MA). Rabbit polyclonal antibodies raised against a glutathione *S*-transferase (GST) fusion protein containing the C-terminal amino acid residues of the PDGF β-receptor (CTβ) has been previously described (Karlsson *et al.*, 2006). The myristoylated inhibitory peptides (Souroujon and Mochly-Rosen, 1998) targeting PKCα (SLNPEYRQT) or PKCε (EAVSLKPT) translocation were synthesized by Fmoc chemistry on an Applied Biosystems 433A peptide synthesizer (Foster City, CA) and purified by reverse-phase chromatography, followed by MALDI-TOF-MS to confirm the expected molecular weights.

Cell Culture

Mouse embryonic fibroblast (MEF) cell lines derived from TC-PTP ko (clone EFM4) and littermate wild-type (wt) mice have been described previously (You-Ten *et al.*, 1997). TC-PTP knockout (ko) cells where stably transfected with short hairpin RNA (shRNA) targeting PKCα (PKCαkd). HeLa cells were stably transfected with a shRNA targeting TC-PTP (a gift from F.D. Böhmer, Friedrich Schiller University, Jena, Germany). The cells were grown in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, and 5 μg/ml plasmocin.

Cell Lysis and Immunoblotting Analysis

Cells were starved overnight in medium supplemented with 0.1 mg/ml bovine serum albumin (BSA), and stimulated with 10 ng/ml or 50 ng/ml PDGF for the indicated time periods. After stimulation, the cells were rinsed twice in ice-cold PBS and lysed in 20 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 0.5% deoxycholate, 150 mM NaCl, 10 mM EDTA, 0.5 mM Na_3VO_4 and 1% Trasylol (Bayer HealthCare, Berlin, Germany), for 15 min on ice. The lysates were cleared by centrifugation at 13,000 rpm for 15 min at 4°C. Total cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were incubated with the indicated antibodies. Bound antibodies were visualized by enhanced chemiluminescence.

Down-Regulation of Cell Surface Receptors

Where indicated, cells were transiently transfected with enhanced green fluorescent protein (EGFP)-Rab4aS22N or EGFP-Rab4a (a gift from M. Zerial, Max Planck Institute, Dresden, Germany; Sonnichsen *et al.*, 2000) using Lipofectamine plus (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Before stimulation, the cells were preincubated with the indicated inhibitor or vehicle as stated in the figure legends. After stimulation with 10 ng/ml platelet-derived growth factor BB (PDGF-BB), cells were placed on ice to prevent membrane transport. Surface proteins were biotinylated by incubation with 0.2 mg/ml sulfo-NHS-SS-biotin (Pierce, Rockford, IL) in PBS with the pH adjusted to 8.0 for 1 h, and unbound biotin was inactivated by incubating the cells in 50 mM Tris, pH 8.0, for 5 min. The cells were lysed, as described above, and cell surface proteins were precipitated by incubating the lysates for 1 h with streptavidin agarose (GE Healthcare, Waukesha, WI). Cell surface receptors were visualized with the indicated antibodies using a Bio-Rad CCD camera (Richmond, CA). Where indicated, the cell surface proteins were quantified by densitometry using the Quantity One software supplied by Bio-Rad, and the data are presented as mean ± SEM of four separate experiments.

Confocal Analysis of PDGF β-Receptor Subcellular Localization

Cells were transiently transfected with either EGFP-Rab4a or EGFP-Rab4aS22N using Lipofectamine plus standard protocol (Invitrogen) for MEFs or Jet Pei standard protocol (PolyPlus, Illkirch, France) for HeLa cells. For the experiments in HeLa cells, wt PDGF β-receptor or the Y1009/1021F double mutant (Mori *et al.*, 1993) was cotransfected with EGFP-Rab4a. After stimulation with 50 ng/ml PDGF-BB, the cells were rinsed twice in PBS and fixed in 4% paraformaldehyde in PBS, pH 7.3, and permeabilized using 0.5% saponin, as previously described (Burden-Gulley and Brady-Kalnay, 1999). The PDGF receptors were detected by incubating the cells with 5 μg/ml CTβ, followed by a TRITC-conjugated secondary antibody. After mounting in Fluoromount G (Southern Biotechnology Associates, Birmingham, AL), the subcellular localization of the PDGF β-receptor was examined using a Zeiss Axiovert 200 M microscope equipped with an LSM 510 laser (Thornwood, NY). Images were captured using an Apochromat 63× oil objective with NA 1.4. The scans were 3D reconstructed using the LSM5 image examiner (Zeiss).

Cell Migration Assay

Cell migration was determined using a 96-well ChemoTX (Neuroprobe, Gaithersburg, MD) cell migration microplate with a 3.2-μm pore size. The filters were coated with 50 μg/ml fibronectin (BD Biochemicals, Erembodegem, Belgium) in PBS for 1 h at room temperature, rinsed twice in distilled water, and air dried. Serum-starved cells that were treated with DMSO or 1 μM PMA over night were trypsinized into single cells, and the trypsinization was terminated by the addition of 150 μl Trasylol/ml cell suspension. The cells were pelleted and diluted to a final concentration of 2.5×10^6 cells/ml in DMEM supplemented with 1 mg/ml BSA (DMEM/BSA). The wells of the ChemoTX microplate were filled with the indicated chemoattractant. The filters were placed on the wells, allowing contact with the medium, and 50,000 cells were added on top of each filter. The chamber was incubated for 4 h at 37°C, 5% CO_2. Cells adhering to the top of the filter were then removed, and cells adhering to the bottom of the filter were fixed by a 3-min incubation in 96% ethanol. The filters were washed three times in distilled water, and

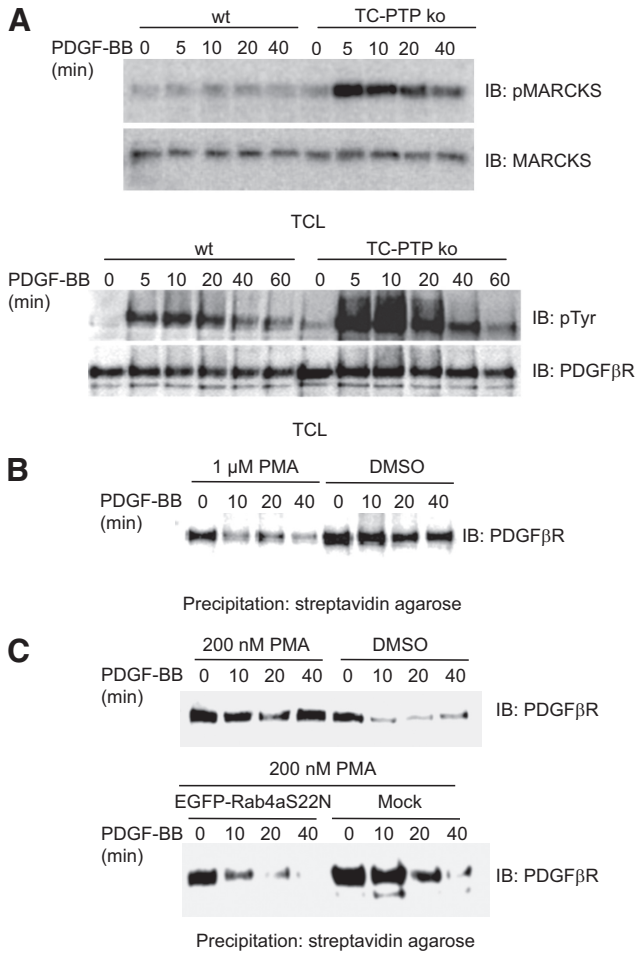

Figure 1. PKC activation affects PDGF β-receptor trafficking. (A) TC-PTP ko and wt MEFs were stimulated with 50 ng/ml PDGF-BB for the time periods indicated. After cell lysis, equal amounts of total cell lysates were separated by SDS-PAGE, and the phosphorylation of MARCKS was detected by immunoblotting with a phospho-MARCKS (Ser152/156) antibody followed by stripping and reprobing with a MARCKS antibody (top panels). The activation of the PDGF β-receptor was detected by immunoblotting with phosphotyrosine antibody followed by stripping and reprobing with a PDGF β-receptor antibody (bottom panels). (B) TC-PTP ko MEFs were treated with 1 μM PMA or vehicle (0.01% DMSO) overnight. After stimulation with 10 ng/ml PDGF-BB, cell surface proteins were subsequently biotinylated using sulfo-NHS-SS-biotin. Biotinylated proteins, containing the cell surface receptor pool, were precipitated using streptavidin agarose. Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose filters, and the amount of cell surface PDGF β-receptors was detected by immunoblotting with PDGF β-receptor antibodies. (C) Wt MEFs were treated with 200 nM PMA and 3 μM ionomycin for 15 min to activate PKC or with vehicle (0.04% DMSO), before stimulation with 10 ng/ml PDGF-BB, as indicated (top panel). Alternatively, wt MEFs were mock-transfected or transfected with EGFP-Rab4aS22N. After pretreatment with 200 nM PMA and 3 μM ionomycin, cells were stimulated with 10 ng/ml PDGF-BB (bottom panel). Cell surface PDGF β-receptors were detected as described above.

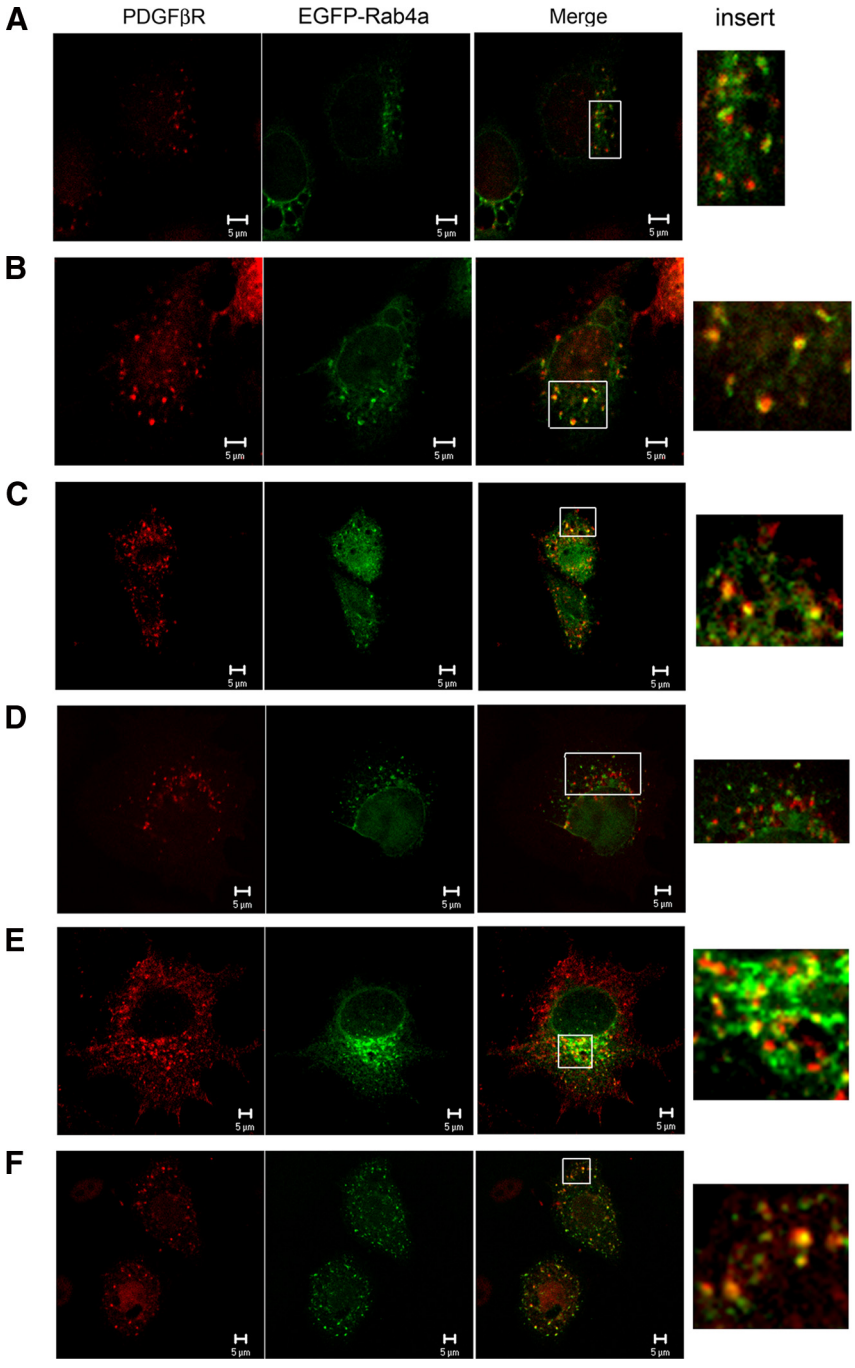

Figure 2. Recycling of PDGF β-receptors in HeLa cells. HeLa cells were transfected with PDGF β-receptor and EGFP-Rab4a. The cells were stimulated with 50 ng/ml PDGF-BB for 20 min (A). (B) The cells were also preincubated with 200 nM PMA for 15 min before stimulation with 50 ng/ml PDGF-BB for 20 min. The cells were then fixed and stained for PDGF β-receptor. (C) HeLa/TC-PTPkd cells expressing PDGF β-receptor and EGFP-Rab4a were stimulated for 20 min with 50 ng/ml PDGF-BB. (D) HeLa/TC-PTPkd cells were transfected with the PDGF β-receptor Y1009/1021F double mutant and EGFP-Rab4a. (E) The HeLa/TC-PTPkd cells expressing the Y1009/1021F mutant were preincubated with 200 nM PMA for 15 min before stimulation with 50 ng/ml PDGF-BB. (F) HeLa cells expressing wt PDGF β-receptors were pretreated with 10 μM LPA for 15 min before stimulation with 50 ng/ml PDGF-BB for 20 min. The cells were fixed and stained for PDGF β-receptor. The subcellular localizations of the PDGF β-receptor and EGFP-Rab4a were determined using an LSM-510 confocal microscope. Scale bars, 5 μm.

adherent cells were stained with 0.04% crystal violet (wt/vol) in 4% ethanol (vol/vol) and detected spectrophotometrically in a Wallac Victor2 1420 multilabel counter (Perkin Elmer-Cetus, Norwalk, CT) using a 600-nm filter. All experiments were performed in quadruplicate, and the data from three separate experiments ± SEM are shown.

RESULTS

Loss of TC-PTP has been shown to result in PDGF β-receptor hyperphosphorylation (Persson *et al.*, 2004) and the induction of Rab4a-dependent receptor recycling (Karlsson *et al.*, 2006). In this study, we investigated the signaling pathways involved in the sorting of the PDGF β-receptor toward recycling in the early endosomes. The hyperphosphorylation of the PDGF β-receptor observed in TC-PTP ko MEFs, which occurs primarily on Y1021, results in hyperactivation of PLCγ (Persson *et al.*, 2004). Because PKC is activated downstream of PLCγ, we investigated the phosphorylation of MARCKS, a known substrate for PKC. As expected, the PKC activity was increased in TC-PTP ko MEFs compared with wt MEFs after stimulation with PDGF-BB (Figure 1A).

To investigate if the observed increase in PKC activity is important for the sorting of the PDGF β-receptor in the early endosomes, conventional and novel PKC isoforms were down-regulated by incubating TC-PTP ko MEFs overnight with PMA (Supplemental Figure S1). This treatment increased the rate of clearance of receptors from the cell sur-

face, indicating that PKC affects receptor sorting (Figure 1B). Activation of PKC by short-term pretreatment of wt MEFs with PMA and ionomycin decreased the rate of clearance of the receptor from the cell surface (Figure 1C, top panels). The observed delay in receptor clearance was due to recycling rather than to decreased internalization rate, because transient expression of dominant negative Rab4a reverted this effect (Figure 1C, bottom panels). We previously showed that loss of TC-PTP did not affect the rate of receptor internalization (Karlsson *et al.*, 2006). In accordance with this finding, neither down-regulation of PKC by PMA in TC-TP ko MEFs, nor short-term activation of PKC in wt MEFs affected the rate of ligand internalization (Supplemental Figure S2), further consolidating the conclusion that PKC act on receptor sorting rather than altering receptor internalization rate.

To verify the importance of the TC-PTP-regulated phosphorylation of Y1021 of the PDGF β-receptor for its sorting, we used HeLa cells, which do not express endogenous PDGF β-receptors (data not shown). Confocal analysis of HeLa cells coexpressing PDGF β-receptors and EGFP-Rab4a after transfection revealed no colocalization, consistent with the notion that the receptors do not recycle after ligand activation (Figure 2A). As was the case for fibroblasts, preincubation with PMA for 15 min to activate PKC, induced sorting of the PDGF β-receptors into Rab4a-positive endosomes, suggesting that PKC activation leads to the induction of PDGF β-receptor recycling also in HeLa cells (Figure 2B). We did not observe any significant changes in Rab4a subcellular localization after treatment with PMA. In line with our previous finding (Karlsson *et al.*, 2006), the PDGF α-receptor did not recycle in HeLa cells even after PKC activation by PMA (data not shown). To investigate whether TC-PTP regulates PDGF β-receptor trafficking also in HeLa cells, these cells were stably transfected with shRNA targeting TC-PTP (HeLa/TC-PTPkd; Supplemental Figure S3). Confocal analysis revealed that a decreased level of TC-PTP–induced colocalization of PDGF β-receptors and Rab4a (Figure 2C). When expressing a PDGF β-receptor containing Y1009/1021F double-point mutations, which does not bind PLCγ, no colocalization between the mutant and EGFP-Rab4a was observed in HeLa/TC-PTPkd cells (Figure 2D). Again, this phenotype was partially overcome by preactivation of PKC by PMA (Figure 2E), further demonstrating the requirement for signaling through the PLCγ/PKC pathways for the sorting of the PDGF β-receptor into Rab4a-positive endosomes.

MEFs express the α, δ, and ε isoforms of the conventional and novel PKC subfamilies, which are the subfamilies responding to PMA (Supplemental Figure S1). To identify the isoform required for the sorting of PDGF β-receptors into Rab4a-positive endosomes, TC-PTP ko MEFs were pretreated with myristoylated inhibitory peptides targeting PKCα or PKCε (Souroujon and Mochly-Rosen, 1998). The inhibitory peptide toward PKCα abrogated recycling, whereas the PKCε-selective peptide had no effect on the rate of clearance from the cell surface (Figure 3A). Rottlerin, which inhibits PKCδ, but not other PKC isoforms, did not affect the clearance of receptors from the cell surface of TC-PTP ko MEFs (Figure 3B). To confirm a role for PKCα in PDGF β-receptor sorting, TC-PTP ko MEFs was stably transfected with a vector containing shRNA targeting PKCα. A clone where the expression of PKCα was decreased by ~80% showed an increased rate of clearance of cell surface PDGF β-receptors (Figure 3C), further strengthening the notion that activation of PKCα induces receptor recycling in the TC-PTP ko MEFs.

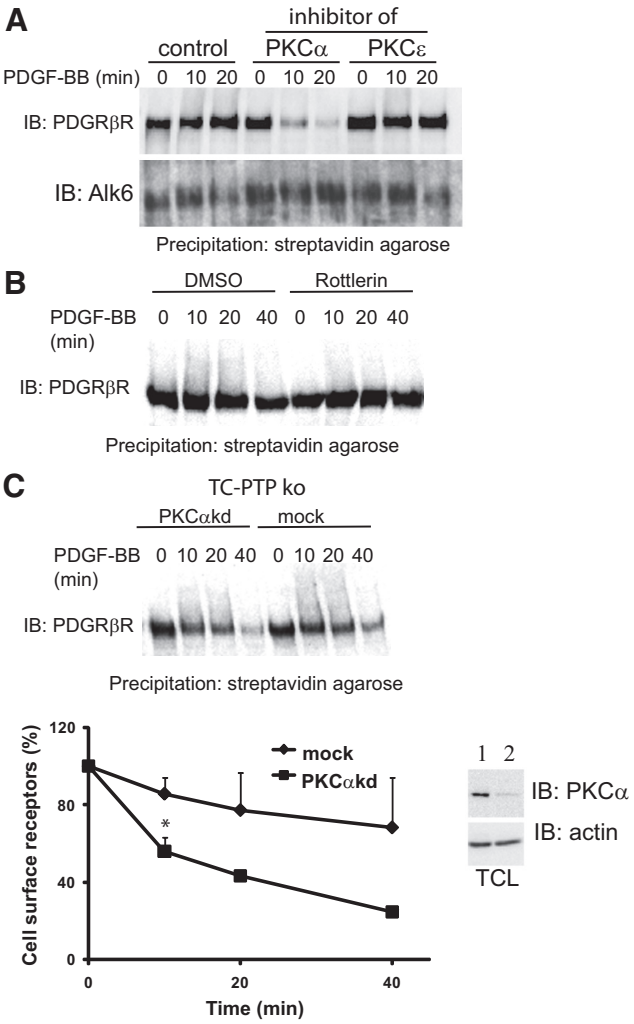

Figure 3. PKCα is required for PDGF β-receptor recycling. (A) TC-PTP ko MEFs were preincubated for 30 min with or without 10 μM inhibitory peptide followed by stimulation with 10 ng/ml PDGF-BB, as indicated. The cells were transferred to ice and cell surface proteins were labeled using sulfo-NHS-SS-biotin. After lysis, cell surface proteins were precipitated using streptavidin agarose. Precipitated PDGF β-receptors were detected by immunoblotting. The membrane was stripped and reprobed with Alk 6 as a loading control. (B) TC-PTP ko MEFs were preincubated with 10 μM rottlerin, a PKCδ inhibitor, and stimulated with 10 ng/ml PDGF-BB, as indicated. Cell surface proteins were isolated and detected as described above. (C) TC-PTP ko MEFs were transfected with shRNA to stably reduce PKCα expression (PKCαkd, inset). TC-PTP ko MEFs and PKCαkd MEFs were stimulated with 10 ng/ml PDGF-BB, and cell surface proteins were isolated and detected as described above (top panel). The relative rate of receptor clearance was determined, and the relative amount of cell surface receptors ± SEM (n = 4) was plotted (bottom panel). Statistical significant differences (Student's *t* test) are indicated by *$p < 0.05$.

We previously showed that in TC-PTP ko MEFs the internalized PDGF β-receptor sorts through Rab5-positive and EEA1-positive endosomes and that it is found also in Rab4a-positive endosomal domains after 10 min of stimulation (Karlsson *et al.*, 2006; Figure 4A). Confocal analysis of TC-PTP ko MEFs, where PKC was down-regulated, revealed that after 10 min of stimulation the PDGF β-receptor is localized on early endosomal compartments adjacent to Rab4a-positive domains with little or no overlap (Figure

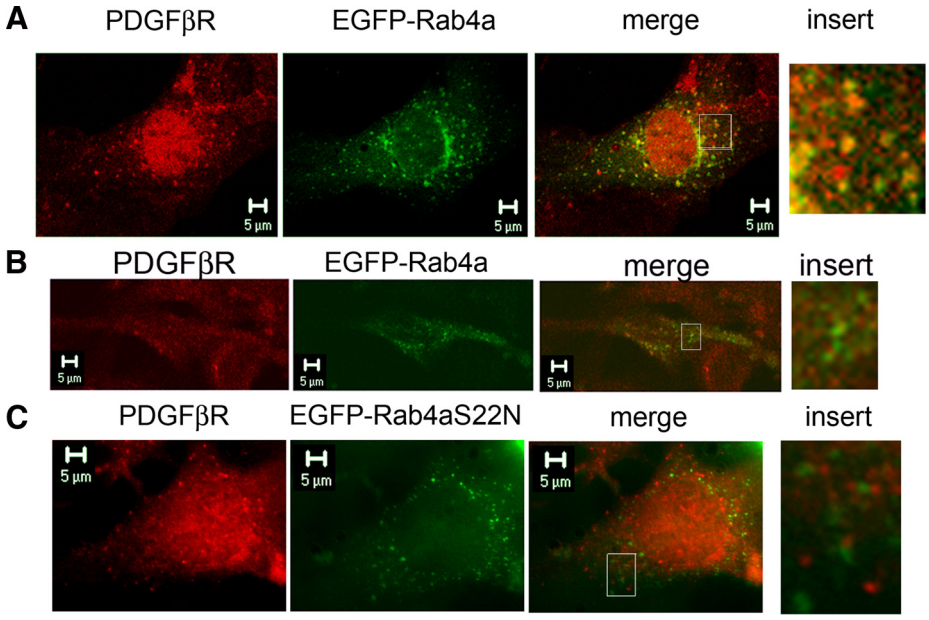

Figure 4. Confocal analysis of the subcellular localization of the PDGF β-receptor. (A) TC-PTP ko MEFs transiently expressing EGFP-Rab4a, (B) TC-PTP ko MEFs transiently expressing EGFP-Rab4a that were incubated over night with 1 μM PMA to down-regulate PKC, or (C) TC-PTP ko MEFs transiently transfected with a dominant negative EGFP-Rab4a (Rab4aS22N) were stimulated for 10 min with 50 ng/ml PDGF-BB, fixed, and stained with antibodies against the PDGF β-receptor. The subcellular localizations of the PDGF β-receptor and EGFP-Rab4a were determined using an LSM-510 confocal microscope. The insert represents a magnification of the region inside the white square. Scale bars, 5 μm.

4B), indicating that PKC activity is required for the sorting of PDGF β-receptors into Rab4a-dependent recycling endosomes. Note that the nuclear staining detected with the PDGF β-receptor antibody is unspecific, as weak nuclear staining also occurs in untransfected HeLa cells. The sorting into Rab4a-positive domains on the early endosomes also required Rab4a activity downstream of PKC activation, because the PDGF β-receptor did not sort into EGFP-Rab4aS22N–positive domains (Figure 4C) in TC-PTP ko MEFs. After this sorting step in the early endosomes, the PDGF β-receptor is degraded, a process that is delayed in TC-PTP ko MEFs compared with wt MEFs (Karlsson *et al.*, 2006).

Inhibition of PKC signaling by down-regulation of PKC isoforms using long-term treatment with PMA (Figure 5A) significantly increased the rate of receptor degradation, demonstrating that the PKC-induced sorting into Rab4a-positive endosomes and the subsequent recycling of the receptors observed in TC-PTP ko MEFs directly leads to a decreased rate of receptor degradation. Inhibition of receptor recycling, by partial knockdown of PKCα using shRNA (Figure 5B) or by transient expression of dominant negative Rab4a (Figure 5C), also showed tendencies toward increased rate of receptor degradation, whereas expression of wt Rab4a did not affect the degradation rate (Figure 5C).

To investigate if the activation of PKC by other receptor types would also induce recycling of the PDGF β-receptor, we pretreated HeLa cells transiently expressing PDGF β-receptor and EGFP-Rab4a with 10 μM LPA, which acts via a G protein–coupled receptor and induces activation of PLCβ and subsequently PKCα (Mahanivong *et al.*, 2008). Subsequent stimulation with PDGF-BB induced colocalization between the PDGF β-receptor and EGFP-Rab4a (Figure 2F), which was not seen in the absence of LPA (Figure 2A), indicating that the sorting of PDGF β-receptors at early endosomes could constitute a point of receptor cross-talk. The TC-PTP ko MEFs display increased chemotaxis toward PDGF-BB compared with wt MEFs (Persson *et al.*, 2004), and the rate of chemotaxis is at least partly dependent on PLCγ activation (Rönnstrand *et al.*, 1999). We therefore investigated the effect of LPA on PDGF-BB–induced chemotaxis. At the dose used, LPA in itself was only mildly chemotactic, but it synergistically increased the chemotactic response of wt MEFs to low doses of PDGF-BB (Figure 6A). At low concentrations of PDGF-BB, this effect could be entirely blocked by down-regulating PKC by PMA. For the highest PDGF-BB concentration, the LPA-induced potentiation was only partly dependent on PKC, an effect that might be dependent on other signaling pathways such as phosphatidylinositol 3′-kinase (PI3K) activation, which also regulates chemotaxis. In accordance with these findings, LPA also potentiated the PDGF β-receptor phosphorylation after stimulation with low concentrations of PDGF-BB. Although LPA alone did not affect the PDGF β-receptor phosphorylation or expression (data not shown), cells stimulated with 1 ng/ml PDGF-BB displayed an increased PDGF β-receptor phosphorylation when the ligand was given in combination with 10 μM LPA (Figure 6B), whereas this effect was not observed when 10 μM LPA was given in combination with 10 ng/ml PDGF-BB (Figure 6C).

DISCUSSION

We previously showed that TC-PTP dephosphorylates the PDGF β-receptor (Persson *et al.*, 2004) and that depletion of TC-PTP induced recycling and delayed the degradation of the PDGF β-receptor (Karlsson *et al.*, 2006). Here we show that the sorting of PDGF β-receptors at early endosomes is controlled by the sequential activation of PKCα (Figures 3 and 4) and Rab4a (Figure 4). The delay in receptor degradation observed in TC-PTP ko MEFs was reverted by inhibiting PKCα (Figure 5, A and B) or by expressing a dominant negative Rab4a (Figure 5C), indicating that this could constitute a checkpoint that is a critical rate-limiting step in the regulation of PDGF β-receptor degradation. Activation of PKC induced by LPA could also redirect the PDGF β-receptor to Rab4a-positive endosomes in HeLa cells (Figure 2F), which correlated with an increase in the chemotaxis of wt MEFs toward PDGF-BB (Figure 6).

After internalization, vesicles fuse with early endosomes in a Rab5-dependent process. At the early endosomes, receptors may be sorted into Rab4a-positive domains for transport back to the plasma membrane via a short recycling

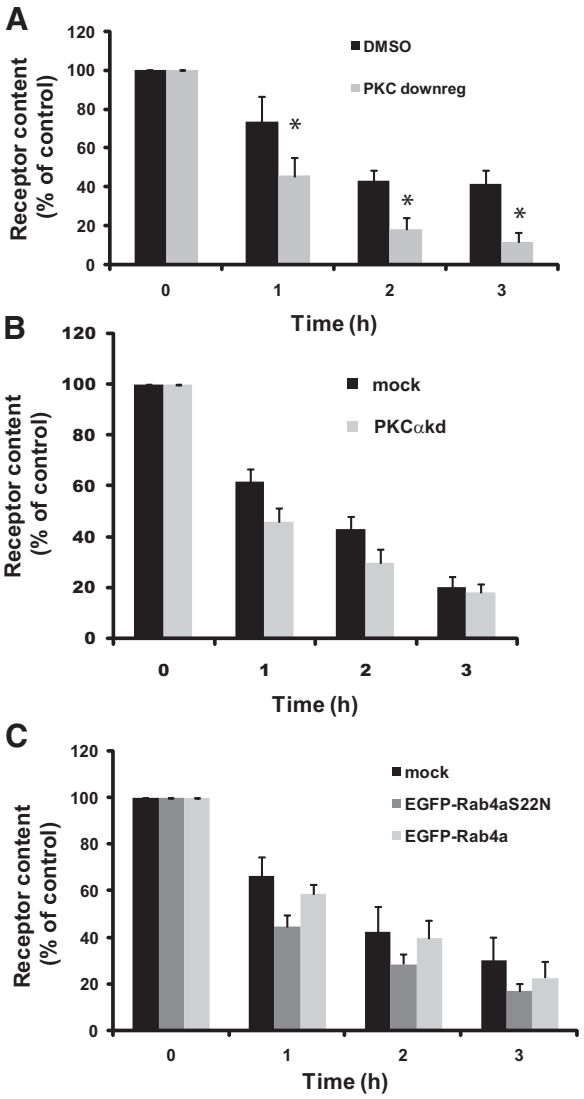

Figure 5. The rate of PDGF β-receptor degradation is regulated by sorting in early endosomes. (A) TC-PTP ko MEFs incubated over night with DMSO or 1 μM PMA were stimulated with 10 ng/ml PDGF-BB for the indicated time periods. Total cell lysates were separated by SDS-PAGE and the PDGF β-receptor was detected by immunoblotting. (B) TC-PTP ko MEFs or PKCαkd MEFs were stimulated with 10 ng/ml PDGF-BB for the indicated times. The PDGF β-receptor amounts were detected as described above. (C) TC-PTP ko MEFs mock-transfected, or transfected with EGFP-Rab4aS22N or EGFP-Rab4a. After stimulation with 10 ng/ml PDGF-BB for the indicated time periods, the PDGF β-receptor amounts were detected, as described above. After densitometric analysis, the relative rate of receptor content ± SEM (n = 4) was plotted. Statistically significant differences; Student's *t* test; *$p < 0.05$.

loop. Alternatively, receptors are sorted either through a Rab11-containing recycling compartment to enter a long recycling loop or through multivesicular endosomes and late endosomes to lysosomal degradation. Sorting of activated receptors into multivesicular endosomes have been extensively studied (Raiborg *et al.*, 2003), and many components of this machinery, notably the ESCRT systems (Williams and Urbe, 2007) have been identified. Despite the identification of many components in the sorting machinery, little is known about the signals regulating and fine-tuning the sorting of receptor tyrosine kinases. The epidermal growth factor (EGF) receptor has been shown to recycle (Sorkin *et al.*, 1989), and this appears to occur primarily through the endosomal Rab11-positive recycling compartment (Felder *et al.*, 1990; Waterman and Yarden, 2001).

Unlike the EGF receptor, the PDGF receptors do not normally recycle (Figure 2) (Karlsson *et al.*, 2006), and the current finding that activation of PKCα signaling–induced recycling of the PDGF β-receptor constitutes the first physiological regulatory mechanism identified for sorting of this receptor. PKC-mediated phosphorylation has been implicated in the trafficking of a number of receptors. For example, the EGF receptor can be directly phosphorylated on several residues by PKC (Cochet *et al.*, 1984; Hunter *et al.*, 1984). One of the sites, T654, has been linked to EGF receptor trafficking, and its phosphorylation by PKC-induced receptor recycling by preventing c-Cbl–mediated receptor ubiquitination (Bao *et al.*, 2000). Also the inhibitory killer Ig-like receptor is phosphorylated by conventional PKCs, and this is required for its internalization and subsequent recycling (Chwae *et al.*, 2008). We have been unable to detect a PKC-dependent phosphorylation of the PDGF β-receptor itself, both in vivo after metabolic labeling of cells and in in vitro kinase assays using full-length receptors or GST fusion proteins containing the entire tail of the receptor as substrate (data not shown). Furthermore, we previously found that the PDGF β-receptor ubiquitination is not affected by loss of TC-PTP (Karlsson *et al.*, 2006), arguing that the observed effect of PKCα is not through targeting the ubiquitination pathway. PKCα activation has been shown to enhance the trafficking of c-Met to the perinuclear region in HeLa cells after preactivation of PKC by PMA (Kermorgant *et al.*, 2004). Although it was not specifically investigated, the study by Kermorgant *et al.* did not report any recycling of c-Met after PMA treatment, and unlike the case for the PDGF β-receptor, inhibition of PKCα did not affect c-Met degradation. Interestingly, PKC activation does not either affect sorting of the PDGF α-receptor at the early endosomes despite its similarity to the β-receptor (Karlsson *et al.*, 2006).

Endocytosis and the correct subsequent intracellular sorting allows for modulation in the signaling of receptor tyrosine kinases as well as adhesion molecules, and defects in these processes can contribute to cell transformation (Mosesson *et al.*, 2008). The recent understanding of the ability of receptors to signal from the endosomal compartments (Hoeller *et al.*, 2005; von Zastrow and Sorkin, 2007), underscores the importance for the cell to tightly regulate the kinetics and routing of intracellular transport of activated receptors. The dephosphorylation of Y1021 of the PDGF β-receptor by TC-PTP provides an important regulation of PDGF β-receptor signaling, both by terminating PLCγ signaling and by increasing the rate of receptor degradation. The finding that TC-PTP is a direct regulator of the intracellular sorting of the PDGF β-receptor suggests that variations in the expression and/or activation of TC-PTP would have consequences for the intensity and duration of PDGF β-receptor signaling (Karlsson *et al.*, 2006). Further understanding of the regulation of TC-PTP expression and activation will be required to elucidate this possibility.

The recycling of the PDGF β-receptor observed in cells depleted of TC-PTP is transient, implying that other signals regulate the duration of the receptor recycling. It has long been recognized that PKC activation induces the down-regulation of PKC protein levels (Parker *et al.*, 1995) by ubiquitination and degraded by proteasomes (Junoy *et al.*, 2002; Leontieva and Black, 2004), a process in which the RINCK E3 ligase has been implicated (Chen *et al.*, 2007).

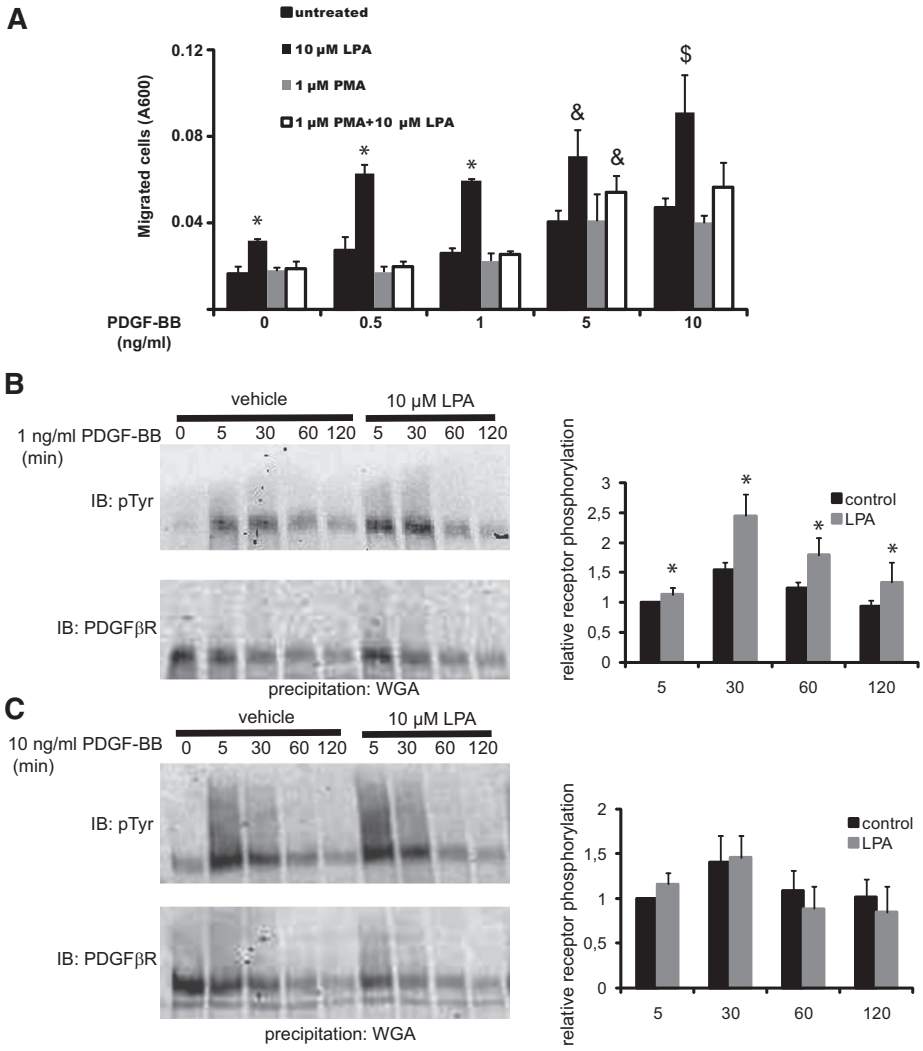

Figure 6. LPA potentiates fibroblast migration toward PDGF-BB. (A) Cell migration experiments were carried out in a 96-well ChemoTX cell migration microplate with a 3.2-μm pore size. The wells of the microplate were filled with medium containing the indicated combinations of PDGF-BB and 10 μM LPA, and the filters were placed on top of the wells. Wt MEFs, treated over night with 0.01% DMSO or 1 μM PMA as indicated, were placed on top of the filters and allowed to migrate across the filter for 4 h at 37°C. Migrated cells adhering to the filter were fixed, stained with crystal violet, and quantitated using a Wallac Victor plate reader. The amount of cells migrated are given as absorbance units ± SEM representing three experiments, each performed in quadruplicate. Statistically significant differences; Student's *t* test; *$p < 0.05$ compared with untreated, LPA, and PMA; $^{\&}p < 0.05$ compared with untreated; $^{\$}p < 0.05$ compared with untreated and PMA. Wt fibroblasts were stimulated with 1 ng/ml PDGF-BB (B) or 10 ng/ml PDGF-BB (C) in the absence or presence of 10 μM LPA as indicated. After lysis, PDGF β-receptors were precipitated using wheat germ agglutinin, and the precipitates were separated by SDS-PAGE. The activation of the PDGF β-receptor was detected by immunoblotting with phosphotyrosine antibody followed by stripping and reprobing with a PDGF β-receptor antibody (lower panels). The signals were quantified and, after correction for receptor content, the relative increase in phosphorylation shown ± SEM for four experiments. Statistically significant differences; Student's *t* test; *$p < 0.05$.

Alternatively, the priming phosphorylation site(s) can be dephosphorylated leading to proteasomal-independent degradation (Gao *et al.*, 2008). This process can be further regulated by the binding of dephosphorylated PKC to Hsp70, which allows for rephosphorylation of PKC, increasing its stability (Gao and Newton, 2006). It is therefore likely that the PDGF β-receptor sorting is regulated by the sequential activation and inactivation of PKCα and that the duration of the PKCα signaling regulates the duration of receptor recycling and thereby also the rate of receptor degradation. Other signaling pathways that activate PKCα could thus control PDGF β-receptor signaling. Our finding that LPA-induced activation of PKCα induces PDGF β-receptor recycling (Figure 2F) and enhanced signaling (Figure 6) supports this notion. The role for receptor recycling in the LPA-induced potentiation of the chemotactic response to PDGF-BB remains to be determined; however, dominant negative Rab constructs cannot be used in these analyses because integrin recycling, which is required during cell migration, is also controlled by Rab4a (Roberts *et al.*, 2001).

The family of monomeric Rab GTPases and their effector proteins are critical components of the intracellular machinery for membrane transport (Zerial and McBride, 2001; Deneka *et al.*, 2003). The Rab proteins regulate vesicle tethering, fusion, and fission, but are also important for the motility of vesicles along the actin and microtubule filaments. Proteins binding both Rab5 and Rab4a on the early endosomes have been identified (de Renzis *et al.*, 2002). These include Rabaptin-4, Rabaptin-5, rabenosyn-5, and the two splice variants of rabip4. Binding of these proteins to Rab5 and Rab4a simultaneously provide a physical link between the Rab protein–containing domains and are presumably important in the generation of subcompartments on the early endosomes (Sonnichsen *et al.*, 2000). The precise mechanism whereby receptor tyrosine kinases are sorted from the Rab5 subcompartment to the Rab4a subcompartment, which will subsequently bud off and recycle back to the plasma membrane, remains to be elucidated.

In summary, we have identified activation of PKCα as a critical step in the sorting of the PDGF β-receptor toward Rab4a-dependent recycling. Activation of PKC through the LPA receptor (Mahanivong *et al.*, 2008) also redirected PDGF β-receptors into Rab4a-positive endosomes, indicating that PKCα constitutes a point of cross-talk between receptor subclasses. It is an interesting possibility that induction of PDGF β-receptor recycling, and thereby stronger PDGF signaling, contributes to the well-known protumorigenic effects of PKC activation.

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