PDGFRαα Signaling Is Regulated through the Primary Cilium in Fibroblasts

Linda Schneider, Christian A. Clement, Stefan C. Teilmann, Gregory J. Pazour, Else K. Hoffmann, Peter Satir, and Søren T. Christensen

Supplemental Experimental Procedures

Cell Cultures

Swiss NIH3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum and 10 ml l^{-1} penicillin-streptomycin, at 37°C, 5% CO_2, 95% humidity. Mouse embryonic fibroblasts (MEF) were isolated after Freshney protocols 11.1 and 11.5 [S1]. Primary cultures of MEFs from wild-type and *Tg737*^{orpk} mice were grown in 45% DMEM and 45% F12 + L-glutamine (InVitrogen, Taastrup, Denmark) supplemented with 10% heat-inactivated fetal calf serum and 10 ml l^{-1} penicillin-streptomycin at 37°C, 5% CO_2, and 95% humidity. Cells were examined at either 70%–80% confluency (interphase, nonarrested cells) or at 90%, followed by serum starvation for 0, 6, 12, 24, and 48 hr to induce growth arrest. In some experiments, cells were stimulated with 50 ng/ml PDGF-AA (Oncogene; VWR International Aps, Albertslund, Denmark) or PDGF-BB (Calbiochem; VWR International Aps). Cell cultures were passaged every 3–4 days by trypsination (0.5%) and only passages 10–30 for NIH3T3 cells and passages 10–20 for MEF cells were used for experiments.

Antibodies and Blocking Peptides

Monoclonal mouse antibodies from SigmaAldrich, Saint Louis, MI were mouse anti-acetylated α-tubulin (T6793) and mouse monoclonal anti-β-actin (A5441). Polyclonal rabbit antibodies from SigmaAldrich were anti-phospho-PDGFRα Y^{742} (P8246). Polyclonal goat antibodies from R&D Systems Europe, Ltd., Abingdon, UK were goat anti-PDGFRα (AF1062). Polyclonal rabbit antibodies from Santa Cruz Biotechnology, Inc., Santa Cruz, CA were anti-γ-tubulin (sc-10732), anti-PDGFRα (sc-338), anti-phospho-PDGFRα Y^{720} (sc-12910); and anti-PDGFRβ (sc-432), anti-PDGFRβ Y^{857} (sc-12907-R), anti-IRα (sc-710) and anti-phosphotyrosine (sc-18182). Polyclonal rabbit antibodies from Cell Signalling Technology (Medinova Scientific A/S, Glostrup, Denmark) were anti-MEK1/2 (9122), anti-phospho-Mek1/2 (9121), anti-Erk1/2 (9102), anti-phospho-Erk1/2 (9101), anti-Akt (9272), and anti-phospho-Akt (587F11). Blocking peptide for rabbit anti-PDGFRα (Santa Cruz Biotechnology, Inc., sc-338 P). Mouse anti-pericentrin was a generous gift from Prof. J.L. Salisbury, Mayo Clinic College of Medicine.

Immunofluorescence Microscopy Analysis

Cells grown on glass coverslips in six-well test plates (NUNC A/S, Roskilde, Denmark) were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, quenched in PBS with 2% BSA, and incubated with primary antibodies at room temperature for 2 hr (mouse anti-acetylated α-tubulin [1:2,000–1:50,000], rabbit anti-γ-tubulin [1:2000], rabbit anti-pericentrin [1:5000], goat anti-PDGFRα, rabbit anti-PDGFRα, rabbit anti-PDGFRβ, rabbit anti-IRα, rabbit anti-phosphotyrosine, and rabbit phospho-Mek1/2 [1:300]). Cells were washed in PBS (137 mM NaCl, 2.6 mM KCl, 6.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) and incubated with DAPI and Alexa Fluor$^{488/568}$-conjugated goat anti-mouse IgG or IgG F(ab́)$_2$, Alexa Fluor$^{488/568}$-conjugated goat anti-rabbit IgG and Alexa Fluor^{488}-conjugated chicken anti-mouse IgG (all 1:600, Molecular Probes, Eugene, OR) for 1 hr. Fluorescence was visualized on either a Microphot-FXA and Eclipse E600 microscopes (Nikon, Tokyo, Japan) with EPI-FL3 filters and MagnaFire cooled CCD camera (Optronics, Goleta, CA) or an IX70 confocal laser scanning microscope (Olympus, Tokyo, Japan) with a Kr/Ar laser by which special care was taken to avoid

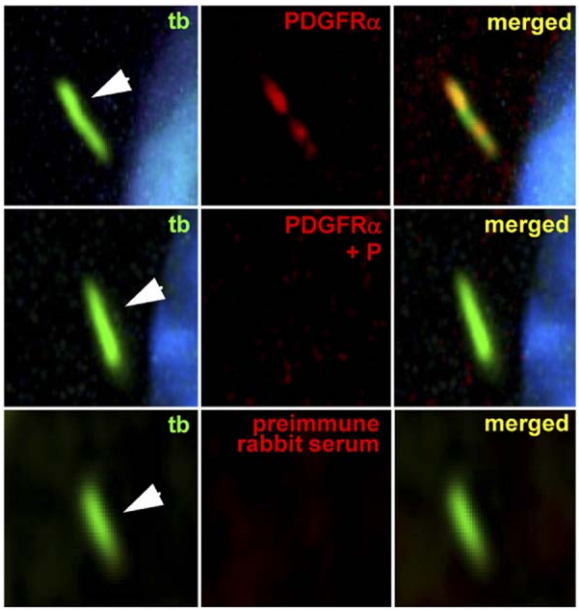

Figure S1. PDGFRα Specifically Localizes to the Primary Cilium of NIH3T3 Fibroblasts

Ciliary localization (Tb, green, arrows) of rabbit anti-PDGFRα in the absence (red, upper panel row) and in the presence of blocking peptide, P (red; middle panel row), in 24 hr serum-starved cells. Control, substitution of rabbit anti-PDFGRα with preimmune rabbit serum (red, lower panel row).

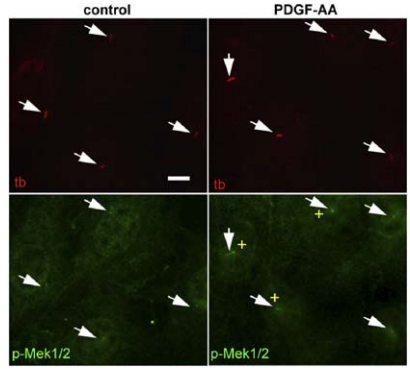

Figure S2. PDGF-AA Activates Mek1/2 in the Primary Cilium and at the Ciliary Basal Body of NIH3T3 Fibroblasts

Immunofluorescence microscopy analysis showing that PDGF-AA (10 min stimulation) increases the level of phospho-Mek1/2 (green; c-Raf-dependent Mek1/2 phosphorylation in the activation loop on serines in positions 217 and 221 that activates Erk1/2) in the mother centriole and along the primary cilium (red, tb, arrows, and marked by a plus sign). Scale bar: 10 μm.

bleed through between channels using different filters. Differential interference contrast (DIC) images were obtained separately after fluorescence image acquisition, and digital images were processed by Adobe Photoshop 6.0.

SDS-PAGE, Immunoprecipitation, and Western Blotting

Cells grown in Petri dishes were quickly washed in ice-cold PBS, treated with 100 μl boiling lysis buffer with 1% SDS, scraped off with a rubber policeman, and processed ten times through a 27 gauge needle. The lysates were centrifuged at 16,000 × g to precipitate nonsoluble material. The protein concentrations were estimated with a BCA protein kit (Pierce Biotechnology, Rockford, IL) so that equal concentrations of protein could be loaded on the gels. Rabbit anti-PDGFRα was added to lysates for immunoprecipitation in RIPA buffer with no SDS and incubated overnight at 4°C. Protein A- and G-conjugated sepharose (1:1) equilibrated in RIPA was then added and incubated for 2 hr at room temperature. The beads were washed with ice-cold RIPA and the precipitate was dissolved in sample buffer. Proteins from whole-cell lysates and immunoprecipitates were resolved by gel electrophoresis by SDS-PAGE under denaturing and reducing conditions and electrophoretically transferred to nitrocellulose membranes as previously described [S2] using the NOVEX XCell I system (Invitrogen A/S, Taastrup, Denmark). The membranes were incubated either with primary antibodies (1:300) overnight at 4°C or at room temperature for 2 hr. Crossreactivity of the antibodies was identified with species-specific alkaline phosphatase-coupled secondary antibodies (1:1200, Jackson Laboratory) followed by developing with BCIP/NBT (KPL, Gaithersburg, MD). Mark 12 (Invitrogen A/S, Taastrup , Denmark) was used as molecular mass standard in SDS-PAGE. Band intensities of proteins in Western blot analysis were measured using UN-SCAN-IT Version 5.1 (Silk Scientific, Inc., Orem, Utah, USA). Data are presented either as individual experiments representative of at least three independent experiments, or as mean values ± SEM from a minimum of three individual experiments.

Supplemental References

S1. Freshney, R.I. Culture of Animal Cells. (New York: Wiley-Liss, Inc.), pp. 150–163.

S2. Christensen, S.T., Guerra, C.F., Wada, Y., Valentin, T., Angeletti, R.H., Satir, P., and Hamasaki, T. (2001). A regulatory light chain of outer arm dynein in *Tetrahymena thermophila*. J. Biol. Chem. *276*, 20048–20054.