Phosphatidylinositol 3-Kinase-dependent Membrane Recruitment of Rac-1 and p47phox Is Critical for α-Platelet-derived Growth Factor Receptor-induced Production of Reactive Oxygen Species*

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Platelet-derived growth factor (PDGF) plays a critical role in the pathogenesis of proliferative diseases. NAD(P)H oxidase (Nox)-derived reactive oxygen species (ROS) are essential for signal transduction by growth factor receptors. Here we investigated the dependence of PDGF-AA-induced ROS production on the cytosolic Nox subunits Rac-1 and p47phox, and we systematically evaluated the signal relay mechanisms by which the αPDGFR receptor (αPDGFR) induces ROS liberation. Stimulation of the αPDGFR led to a time-dependent increase of intracellular ROS levels in fibroblasts. Pharmacological inhibitor experiments and enzyme activity assays disclosed Nox as the source of ROS. αPDGFR activation is rapidly followed by the translocation of p47phox and Rac-1 from the cytosol to the cell membrane. Experiments performed in p47phox−/− cells and inhibition of Rac-1 or overexpression of dominant-negative Rac revealed that these Nox subunits are required for PDGF-dependent Nox activation and ROS liberation. To evaluate the signaling pathway mediating PDGF-AA-dependent ROS production, we investigated Ptk cells expressing mutant αPDGFRs that lack specific binding sites for αPDGFR-associated signaling molecules (Src, phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ, and SHP-2). Lack of P13K signaling (but not Src, phospholipase Cγ, or SHP-2) completely abolished PDGF-dependent p47phox and Rac-1 translocation, increase of Nox activity, and ROS production. Conversely, a mutant αPDGFR able to activate only P13K was sufficient to mediate these subcellular events. Furthermore, the catalytic P13K subunit p110α (but not p110β) was identified as the crucial isoform that elicits αPDGFR-mediated production of ROS. Finally, bromodeoxyuridine incorporation and chemotaxis assays revealed that the lack of ROS liberation blunted PDGF-AA-dependent chemotaxis but not cell cycle progression. We conclude that P13K/p110α mediates growth factor-dependent ROS production by recruiting p47phox and Rac-1 to the cell membrane, thereby assembling the active Nox complex. ROS are required for PDGF-AA-dependent chemotaxis but not proliferation.

Several growth factors and cytokines induce the production of reactive oxygen species (ROS) in nonphagocytic cells such as endothelial cells, vascular smooth muscle cells (VSMC), and fibroblasts (1, 2). This process is physiologically relevant, e.g. for the maintenance of vascular tone, but also bears pathophysiological significance for diseases like hypertension, atherosclerosis, and malignomas (3, 4). Various types of membrane-bound receptors, including G-protein-coupled receptors (e.g. angiotensin II type 1 receptor) and receptor tyrosine kinases (RTK), activate signaling cascades that lead to activation of a membrane-bound NAD(P)H oxidase, the major source of ROS in both phagocytic and nonphagocytic cells (1, 5, 6). Among ligands of RTKs, epidermal growth factor (7), transforming growth factor-β (8), and platelet-derived growth factor (PDGF) (9, 10) lead to increased superoxide production in various cell types, including VSMC and fibroblasts, and these ROS act as second messengers required for multiple cellular functions such as cell proliferation and adhesion (1, 2).

The mechanism of superoxide production by a membrane-bound NAD(P)H oxidase has been extensively studied in phagocytic cells (11). A similar enzyme with distinct characteristics has been described in a wide variety of nonphagocytic cells, e.g. the vascular system (6), thyroid gland (12), and cartilage (13). It generally consists of two membranous components, the flavocytochrome b protein Nox (nonphagocytes) or gp91phox (phagocytes) and p22phox and the two cytosolic subunits

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3 The abbreviations used are: ROS, reactive oxygen species; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PLCγ, phospholipase Cγ; P13K, phosphatidylinositol 3-kinase; RTK, receptor tyrosine kinase; DCF, 2’,7’-dichlorodihydrofluorescein; H2DCF, 2’,7’-dichlorodihydrofluorescein; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; DPI, diphenylene iodonium; NBT, nitro blue tetrazolium; RT, reverse transcription; MEF, mouse embryonic fibroblast; WT, wild type; PBS, phosphate-buffered saline; VSMC, vascular smooth muscle cells; BrdUrd, bromodeoxyuridine; GEF, guanine nucleotide exchange factor; PEG, polyethylene glycol.

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p47phox and p67phox (14). Although the cytosolic subunit p40phox known from phagocytic cells is usually not found in nonphagocytic cells, the small GTP-binding protein Rac (Rac-1/Rac-2) has regulatory function for the enzyme activity and migrates from the cytosol to the membrane during the activation process (1, 2).

Recent studies have shown that peptide growth factors are able to induce ROS production in nonphagocytic cells. PDGF-AB led to increased H$_2$O$_2$ formation in VSMC (9), and PDGF-dependent ROS liberation was shown to require PI3K activity in HepG2 cells (10). However, the contribution of each of the PDGF receptor (PDGFR) subtypes and the mechanism how PI3K is involved in activation of NAD(P)H oxidase by PDGF remain unknown. Furthermore, the exact signaling mechanisms and the role of the various NAD(P)H oxidase subunits for PDGF-dependent ROS formation have not been studied. Biologically active PDGF is a dimeric protein consisting of two of the four known homologous chains (A, B, C, and D) that are linked by disulfide bonds (15–18). It exists as homodimers (AA, BB, CC, and DD) or heterodimers (AB). Binding of PDGF induces the dimerization of two receptor subunits that leads to an increase of its intrinsic tyrosine kinase activity and subsequent autophosphorylation of the dimeric receptor. The PDGF B-chain associates with both PDGFR subtypes (α and β), whereas the A-chain only interacts with the αPDGFR. Therefore, PDGF-AA selectively assembles αα-homodimers (17, 18).

Upon stimulation with PDGF-AA, the activated αPDGFR recruits and activates several Src homology 2 domain containing signaling molecules, which interact with the activated receptor at specific tyrosine residues once they are phosphorylated as follows: Src family kinases (Src) bind to tyrosines 572 and 574 in the juxtamembrane region of the receptor; the phosphotyrosine phosphatase SHP-2 and phosphatidylinositol 3-kinase (PI3K) interact with tyrosines 720 and 731/42 within the kinase insert, respectively; and a thus far unidentified protein and phospholipase Cγ (PLCγ) associate with tyrosines 988 and 1018 in the C-terminal tail region, respectively.

This study focuses on the signal relay mechanisms by which the αPDGFR mediates NAD(P)H oxidase activation and superoxide production in nonphagocytic cells. The role of p47phox and Rac-1 was investigated by targeted gene deletion and pharmacological inhibition. To systematically evaluate the role of each of the signaling enzymes that are recruited to the activated αPDGFR, we stably expressed tyrosine to phenylalanine αPDGFR mutants that fail to associate with one or more of the signaling enzymes (Src, PI3K, PLCγ, and SHP-2) in an αPDGFR-deficient cell line (Ph cells) (19) and compared their ability to mediate PDGF-dependent NAD(P)H oxidase activation and ROS production to that of the wild type (WT) αPDGFR. Furthermore, we sought to investigate the role of specific class IA PI3K isoforms and to evaluate the importance of ROS for PDGF-dependent cellular functions.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and αPDGFR Mutants**—Ph cells and their maintenance were described previously (20, 21). The human WT and mutant αPDGFRs were created by site-directed mutagenesis as described previously (22) and were stably expressed in Ph cells to ~1 × 10^5 receptors per cell, using the pLNCX² retroviral vector (20, 23). DNA constructs were transfected into 293GPG cells (24), and the viral supernatant was collected for 7 days and concentrated by centrifugation (25,000 × g, 90 min, 4 °C). The virus was titered, and equal amounts of colony-forming units were used to infect Ph cells. The infected cells were selected in the presence of 1 mg/ml G418, and mass populations of drug-resistant cells were used in all of the experiments.

Primary cultures of p47phox(−/−) embryonic fibroblasts were derived from female mice from breeding pairs of p47phox(+/−) mice (kindly provided by Stephen M. Holland) as described previously (25). The p47phox knock-out mouse, which was created as a murine model for chronic granulomatous disease by targeted disruption of the p47phox gene, was described previously (26). In brief, female mice were sacrificed on day 16.5 postconception, and embryos were dissected from the uterus. The genotype of the embryos was determined by using DNA extracted from removed embryonic tissues by PCR as described previously (27). Embryonic fibroblasts were isolated by trypsin digest of embryo bodies, suspended, and plated at a density of 10^5/cm² in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, and adherent cells were passaged according to the 3T3 protocol of Todaro and Green (28). Gene deletion of p47phox in embryonic fibroblasts isolated from p47phox(−/−) mice was verified by RT-PCR.

**Determination of Intracellular Reactive Oxygen Species**—ROS production was investigated by 2',7'-dichlorofluorescein (DCF) fluorescence using confocal laser scanning microscopy. Subconfluent cells plated on glass carriers were starved by serum deprivation for 24 h and stimulated as indicated. The cells were then washed once in buffer E1 (containing NaCl 135 mmol/liter, KCl 5.4 mmol/liter, CaCl$_2$ 1.8 mmol/liter, MgCl$_2$ 1 mmol/liter, glucose 10 mmol/liter, HEPES 10 µmol/liter, pH 7.5, 37 °C) and incubated in the dark for 30 min in the same buffer containing 10 mmol/liter 2',7'-dichlorodihydrofluorescein-diacetate (H$_2$DCF-DA, Molecular Probes, Eugene, OR). H$_2$DCF-DA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative H$_2$DCF and thereby trapped within the cell. In the presence of a proper oxidant, H$_2$DCF is oxidized to the highly fluorescent DCF (29). Culture dishes were transferred to a Zeiss Axiovert 135 inverted microscope (Carl Zeiss, Jena, Germany), equipped with a 25×, numerical aperture 0.8, oil immersion objective (Plan-Neofluar, Carl Zeiss), and Zeiss LSM 410 confocal attachment, and ROS generation was detected as a result of the oxidation of H$_2$DCF (excitation, 488 nm; emission long pass LP515-nm filter set). 512 × 512 pixel images were collected by single rapid scans and identical parameters, such as contrast and brightness, for all samples. In three separate experiments, five groups of 25 cells each were randomly selected from the image, and fluorescent intensity was measured. Relative fluorescence intensity is expressed as means ± S.D. from three independent experiments, and each value reflects measurements performed on a minimum of 100 cells.

**Nitro Blue Tetrazolium (NBT) Reduction Assay**—Cells were plated in 6-well plates, grown to subconfluence, starved by serum deprivation for 24 h, and stimulated as indicated. The medium was then removed, and the cells were washed with 1 ml
of Hanks’ solution (Sigma). Filtered 0.25% NBT (1 ml, 37 °C, Sigma) was added with or without 600 units/ml superoxide dismutase (Sigma), and the cells were incubated at 37 °C for 15 min. They were then harvested, and NBT was pelleted by low speed centrifugation. The NBT pellet was resuspended in 1 ml Hanks’ solution (Sigma). Filtered 0.25% NBT (1 ml, 37 °C, Sigma) was added with or without 600 units/ml superoxide dismutase (Sigma). Sequences for sense (S) and antisense (A) primers, PCR conditions, cycle counts, and amplification fragment lengths of the murine PCR products are shown.

### Table 1

**Detection of NAD(P)H oxidase subunits in Ph cells**

| Primer | Conditions | Product (bp) |
|--------|------------|-------------|
| Nox 1  | S, 5'-AAG TGG TCG TAC TGG TGG G0-3' | 60 min, 94 °C, 60 min, 55 °C, 90 min, 72 °C, 39 cycles | 411 |
|        | A, 5'--CCA CAT AAG AAG AAG CCC ACC G-3' | | |
| Nox 2  | S, 5'-CCA AAG TGG TGG TGG TGG G0-3' | 60 min, 94 °C, 60 min, 55 °C, 90 min, 72 °C, 39 cycles | 406 |
|        | A, 5'-AGG TGG TGC ACT TGC AAT GTC C-3' | | |
| Nox 4  | S, 5'-GAA GCC CAT TGG AGG AGT CA-3' | 60 min, 94 °C, 60 min, 55 °C, 90 min, 72 °C, 39 cycles | 409 |
|        | A, 5'-AGG TGG TGC ACT TGC AAT GTC C-3' | | |
| p22^phox | S, 5'-GAG GCC TGG AGC AGT GGT ACT-3' | 60 min, 94 °C, 60 min, 65 °C, 90 min, 72 °C, 30 cycles | 485 |
|         | A, 5'-GAC GCT TCA CGC AGT GGT ACT-3' | | |
| rac1   | S, 5'-GTA AAA CCT GCC TGC TCA TC-3' | 60 min, 94 °C, 60 min, 55 °C, 90 min, 72 °C, 25 cycles | 692 |
|         | A, 5'-GCT TGG TCA AAG CTG GTC TGG G-3' | | |
| p47^phox | S, 5'-GAA GCC CAT TTG AGG AGT CA-3' | 60 min, 94 °C, 60 min, 65 °C, 90 min, 72 °C, 30 cycles | 414 |
|         | A, 5'-GTC TCA TCA CAT CCA GCA GCT G-3' | | |
| p67^phox | S, 5'-AGA GAT GCT CTA CTA GTA CCA CGG-3' | 60 min, 94 °C, 60 min, 55 °C, 90 min, 72 °C, 30 cycles | 473 |
|         | A, 5'-GCT TGC TGC TGG TGT TGT CC-3' | | |
| p40^phox | S, 5'-CAT TCG TCC CAT GCA AAC TCT AC-3' | 60 min, 94 °C, 60 min, 55 °C, 90 min, 72 °C, 30 cycles | 498 |
|         | A, 5'-CTG CTA TGG TGG TGG TGG G0-3' | | |

NAD(P)H Oxidase Activity—NADH or NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescence in a 50 mmol/liter phosphate buffer (buffer A), pH 7.0, containing 1 mmol/liter EGTA, protease inhibitors (Complete®, Roche Applied Science), 150 mmol/liter sucrose, 5 μmol/liter lucigenin (Sigma), and either 100 μmol/liter NADH or NADPH as substrate (5). Quiescent cells were starved by serum deprivation for 24 h and treated as indicated, washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.4, and harvested. After low spin centrifugation, the pellet was resuspended in ice-cold buffer A, lacking lucigenin and substrate. The cells were then mechanically lysed by using a glass/Teflon potter on ice. The total protein concentration was determined using a Bradford assay (Bio-Rad, Heidelberg, Germany) and adjusted to 1 mg/ml. 100-μl aliquots of the protein sample were measured over 10 min in quadruplicate using NADH or NADPH as substrate in a scintillation counter (Berthold Lumat LB 9501). Data were collected at 1-min intervals to measure relative changes in NAD(P)H oxidase activity.

**RT-PCR**—Total RNA was isolated by gandolinium. RNA (3 μg) was converted to cDNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen). Single-stranded cDNA was amplified by PCR amplification using RedTaq® DNA polymerase (Sigma). Sequences for sense and antisense primers, PCR conditions, cycle counts, and amplification fragment lengths of the murine PCR products are given in Table 1.

**Membrane Preparation and Western Blotting**—For preparation of membrane and cytosolic proteins, cells were washed with PBS, pelleted, and dissolved in 1× reporter lysis buffer (Promega; shaken), frozen three times at −80 °C, and sonicated. After centrifugation (10 min, 3000 rpm, 4 °C), the supernatant was transferred into ultracentrifuge tubes (Beckman Quickseal) and spun for 45 min at 25,000 rpm, 4 °C, in a Beckman NTV 65.2 rotor. The resulting pellet represented the membrane proteins, and the supernatant represented the cytosolic fraction. Proteins were separated on SDS-PAGE, and immunoblotting was performed using antibodies as indicated. Immunodetection was accomplished using appropriate secondary antibodies as indicated and an enhanced chemiluminescence kit (Amersham Biosciences).

**Overexpression of Dominant-negative Rac-1 (rac-N17)—Ph cells were harvested and resuspended in electroporation medium (Amaxa, Cologne, Germany; MEF NucleofectorTM kit) at a concentration of 5 × 10^6 cells/ml. The following constructs were transfected: insertless vector (pcDNA3) as control and pRK5-myc-rac1-N17 (dominant-negative Rac-1 mutant) (31). 5 μg of plasmid DNA and 100 μl of cell suspension were placed in a 0.2-cm cuvette and mixed, and transfection was performed according to the manufacturer’s instructions. The cells were plated and cultured for 24 h prior to treatment with PDGF-AA or vehicle.

**Quantitative Immunocytochemistry**—Antibody staining was performed on cells mounted on glass slides. Monoclonal human Rac-1 and polyclonal anti-p47^phox were each used at a final concentration of 1:50. Cells were washed in sterile PBS, fixed in 4% paraformaldehyde (4 °C), and permeabilized in PBS supplemented with 1% Triton X-100 (PBST) (Sigma). Subsequently, they were incubated for 1 h in 0.01% PBST containing 10% fat-free milk powder to reduce nonspecific binding and further incubated for 2 h with primary antibody. After washing three times in PBST (0.1%), cells were incubated for 60 min in PBST (0.01%) supplemented with 10% milk powder and either a Cy5-conjugated F(ab')2 fragment of goat anti-rabbit IgG (1:100) or Cy5-conjugated F(ab')2 rabbit anti-mouse IgG (1:100). Excitation was performed using 633-nm helium-neon laser of the confocal setup. Emission was recorded using a long pass LP 655-nm filter set. For quantification of the optical signal, and the aim to measure signals in a mainly membrane-containing cellular volume, confocal images were recorded from cells grown in monolayers by tangential scans through the cell “caps.” The pinhole settings of the confocal setup were
adjusted to yield optical slices of 2.5 μm. After subtraction from background fluorescence measured by cells stained with secondary antibody alone, the signal was counted by image analysis software of the confocal setup of the area of interest (“membrane fraction”) and exported for data processing to the Sigma Plot graphical software. At least three independent experiments were performed for each condition, and 25 randomly selected cells were recorded and analyzed in each experiment. Thus, each value reflects measurements performed on a minimum of 75 cells.

BrdUrd Incorporation Assay—DNA synthesis was measured by a 5-bromodeoxyuridine (BrdUrd) incorporation assay. Cells were cultured in 96-well plates to about 90% confluence, washed, fed with Dulbecco’s modified Eagle’s medium, and starved for 24 h. PDGF-AA was added to cells for 18 h at the indicated concentrations. The BrdUrd incorporation assay was carried out according to the manufacturer’s specifications (Roche Applied Science) with an incorporation time of 5 h.

Chemotaxis Assay—PDGF-dependent chemotaxis was assayed utilizing a 48-well modified Boyden chemotaxis chamber (NeuroProbe Inc., Baltimore) and polyvinyl pyridine-free polycarbonate filters (8-μm pore size) (Poretics Corp., Livermore, CA) as described (32). Briefly, the lower wells of the chamber were filled with Dulbecco’s modified Eagle’s medium + 0.1% calf serum supplemented with 10 ng/ml PDGF-AA or vehicle. The filters were coated with 50 μg/ml rat type I collagen (Collaborative Biomedical Products, Bedford, MA) and fixed atop the bottom wells. Cells were trypsinized, washed, and diluted in Dulbecco’s modified Eagle’s medium containing 0.1% calf serum to a final concentration of 4 × 10^5 cells per ml, and 50 μl of this cell suspension was placed into the top wells of the chamber. In each experiment, at least 6 of the 48 wells of the chamber were used for each condition examined. The chamber was incubated for 5 h at 37 °C in a 5% CO₂ atmosphere.

Antibodies and Inhibitors—Clostridium sordellii lethal toxin was kindly provided by K. Aktories (University of Freiburg, Germany). Diphenylene iodonium (DPI), 4-(2-amino-ethyl)-benzenesulfonyl fluoride (AEBSF), wortmannin, N-acetylcysteine, PEG catalase, and 4-hydroxy-3-methoxyacetophenone (apocynin) were purchased from Sigma. LY294002 was from Merck, and TGX-221 was from Cayman (Ann Arbor, MI). PIK75 was a kind gift from Peter R. Shepherd (University of Auckland, New Zealand). For immunoblotting and immunocytochemistry, the following antibodies were used: monoclonal anti-human Rac-1 (catalog number 05-389, Upstate Biotechnology, Inc., Lake Placid, NY); anti-p47phox (sc-7660, Santa Cruz Biotechnology). The RasGAP antibody (69.3) was kindly provided by Andrius Kazlauskas (Harvard Medical School, Boston).

Statistical Analysis—Data are given as means ± S.D. Student’s t test for unpaired data was applied as appropriate. A p value <0.05 was considered significant.

RESULTS

The αPDGF Induces Intracellular ROS Production via NAD(P)H Oxidase—To investigate whether αPDGF activation induces the liberation of ROS in fibroblasts, intracellular ROS levels were determined by DCF fluorescence. Selective stimulation of the αPDGF with PDGF-AA (50 ng/ml) in WT αPDGF expressing Ph cells led to a time-dependent increase of intracellular ROS, which was maximally 189 ± 24% at 4 h (Fig. 1, A–C).
Recent research focused on the molecular and biochemical characterization of growth factor-induced ROS production in nonphagocytic cells. Several authors proved the role of a phagocyte like NAD(P)H oxidase in this context. To identify the source of PDGF-AA-dependent ROS formation in our cells, we used common inhibitors of enzymes capable of ROS production. As shown in Fig. 1, the flavoprotein inhibitor DPI (10 μmol/liter), a rather nonspecific but highly effective inhibitor of FAD-containing proteins like NAD(P)H oxidase, completely abolished ROS production induced by PDGF-AA. Other more specific inhibitors of NAD(P)H oxidase such as the serine protease AEBSF (10 μmol/liter) (33) and 4-hydroxy-3-methoxyacetophenone (apocynin; 0.3 mmol/liter) (34, 35), which inhibits the translocation of cytosolic subunits of NAD(P)H oxidase to the cell membrane during its activation process, also suppressed PDGF-AA-induced ROS production to basal levels (each p < 0.05 versus PDGF-AA, see Fig. 1, E and F). Thus, αPDGFR-induced intracellular ROS production appears to be mediated via NAD(P)H oxidase in Ph cells, and translocation of NAD(P)H oxidase subunits seems important. Pharmacological inhibition of PI3K with wortmannin (0.1 μmol/liter) also completely abolished PDGF-dependent intracellular ROS production, indicating that this signaling enzyme contributes to NAD(P)H oxidase activation/ROS production (Fig. 1G).

To differentiate between intracellular and extracellular ROS production in intact cells, we also measured superoxide dismutase-inhibitable NBT reduction, which reflects mainly extracellularly produced ROS. After stimulation of Ph cells with PDGF-AA (50 ng/ml), no superoxide production could be measured at various time points (Fig. 2). Similar results were obtained by the use of two alternative redox-sensitive chemiluminescent dyes for monitoring extracellular ROS formation, lucigenin (5 μmol/liter), and L-012 (500 μmol/liter) (36) (not shown). Thus, aPDGFR activation leads to intracellular (but not extracellular) ROS production, and this response involves NAD(P)H oxidase.

The above findings have led us to the hypothesis that PI3K is required for subcellular translocation of NAD(P)H oxidase subunits and thereby mediates PDGF-dependent NAD(P)H oxidase activation and ROS production. To test this hypothesis, we used two different approaches as follows: (i) identification and specific inhibition of NAD(P)H oxidase subunits by targeted gene deletion or pharmacological inhibition, and (ii) characterization of PDGFR signaling by the use of aPDGFR mutants or isoform-selective PI3K inhibitors.

**p47phox and Rac-1 Are Critical for PDGF-induced ROS Production**—In nonphagocytes, NAD(P)H oxidase consists of several cytosolic subunits that translocate from the cytosol to the cell membrane during the activation process to form the active complex (6). Each cell type expresses a specific pattern of membrane-bound and cytosolic subunits (or their isoforms) leading to distinct properties of the enzyme. We used qualitative RT-PCR to detect the expression of known subunits in our cellular model. In contrast to murine tissues (e.g. spleen), no expression of p40phox and p67phox could be detected (Fig. 3A). However, other NAD(P)H oxidase subunits, including the
membrane-bound gp91phox (Nox) and p22phox as well as the cytosolic subunits p47phox and Rac-1, were readily detected in Ph cells. To further discriminate between the recently identified Nox isoforms, RT-PCR was performed to detect Nox 1–4. As demonstrated in Fig. 3B, Nox 2 and Nox 4 are expressed in Ph cells, whereas Nox 1 was not found.

To investigate the dependence of PDGF-AA-induced ROS liberation and NAD(P)H oxidase activation on p47phox and Rac-1, these NAD(P)H oxidase subunits were individually inhibited. In mouse embryonic fibroblasts (MEF) isolated from p47phox(−/−) mice, no p47phox was detected by RT-PCR (Fig. 4A). Compared with control cells expressing normal levels of p47phox, p47phox−/− MEFs were not able to liberate significant levels of ROS upon stimulation with PDGF-AA (Fig. 4B). Because there is no Rac-1 knock-out model available (37), inhibition of Rac-1 was established by overexpression of dominant-negative Rac-1 (rac1-N17) in Ph cells, and by pharmacological inhibition with C. sordellii lethal toxin. In the absence of significant Rac-1 activity, PDGF-AA was unable to activate NAD(P)H oxidase and induce ROS production (Fig. 4, C and D). These data indicate that both Rac-1 and p47phox are absolutely required for PDGF-AA-dependent NAD(P)H oxidase activation and ROS production in fibroblasts.

P13K Is Required for Subcellular Translocation of Rac-1 and p47phox—Pharmacological inhibitor experiments disclosed NAD(P)H oxidase as the source of ROS produced upon αPDGFR stimulation. Direct measurement of NAD(P)H oxidase activity in cellular homogenates of WT receptor-expressing cells showed a 62 ± 12.2% increase upon PDGF-AA stimulation compared with nonstimulated cells, which was maximal after 3 h (Fig. 5). In phagocytes, translocation of cytosolic NAD(P)H oxidase subunits to the cell membrane is necessary for the assembly of the active NAD(P)H oxidase complex. Therefore, we investigated whether PDGF-AA induces translocation of Rac-1 and p47phox in Ph cells, and which of the αPDGFR-associated signaling molecules is critical for this response. To evaluate the role of each of the signal relay enzymes that are recruited to the activated αPDGFR for PDGF-AA-dependent translocation of Rac-1 and p47phox, NAD(P)H oxidase activation, and ROS liberation, we stably expressed various αPDGFR mutants, in which the tyrosine residues required for the association of individual signaling molecules were substituted by phenylalanine, in an αPDGFR-deficient cell line (Ph cells). The “subtraction panel” contains mutant receptors, in which one specific binding site was deleted from the WT receptor (Fig. 6A). The “add-back panel” includes the “F7” receptor, in which all seven tyrosine phosphorylation sites were mutated (21) (Fig. 6B).
To measure the PDGF-dependent translocation of single subunits, immunofluorescence staining was performed during early activation of the enzyme complex. The cells were stimulated with PDGF-AA (50 ng/ml) for 30 min, and immunocytochemical stainings were obtained by single rapid scans using laser scanning microscopy. When the various mutant receptors were compared for their ability to mediate PDGF-dependent translocation of Rac-1 and p47phox, PI3K was identified as the critical signaling enzyme for this response. As demonstrated in Fig. 7A, activation of the WT αPDGFR was rapidly followed by the translocation of Rac-1 from the cytosol to the cell membrane. In contrast, the F7 mutant was not capable of mediating the PDGF-dependent translocation of NAD(P)H oxidase subunits, indicating that at least one of the αPDGFR-associated signaling enzymes mediates this effect. The inability of the αPDGFR to associate with or activate PI3K (F31/42) prevented PDGF-dependent Rac-1 translocation, whereas restoration of the PI3K-binding site (Y31/42) showed similar staining compared with WT receptor-expressing cells (Fig. 7A). Similar observations were made with staining the cells for p47phox (not shown). As determined by Western blot analysis of total cell lysates, the expression of Rac-1 was not altered upon PDGF stimulation during this time interval (Fig. 7B). However, Rac-1 Western blots performed on cytosolic or membrane fractions isolated from buffer- or PDGF-stimulated Ph cells confirmed the importance of PI3K for PDGF-dependent subcellular trafficking, as stimulation with PDGF-AA led to translocation of Rac-1 from the cytosol to the membrane in WT and Y31/42 but not in F7 and F31/42 receptor-expressing cells (Fig. 7, C and D).

To quantify and compare the amount of membrane-localized p47phox and Rac-1 upon PDGF stimulation in the various cell types, we chose a confocal setup obtaining quantitative signals from a membrane-focused cellular volume. Therefore, tangential single rapid scans were obtained. Consistent with the findings demonstrated in Fig. 7, quantitative analysis of membrane-associated Rac-1 and p47phox revealed that the F31/42 receptor (which does not bind or activate PI3K) was unable to mediate the translocation of p47phox and Rac-1 upon PDGF stimulation (Fig. 8, A and B). Conversely, restoration of the PI3K-binding sites to the F7 receptor (Y31/42) rescued the PDGF-dependent translocation of p47phox and Rac-1 (Fig. 8, C and D).

### α-PDGF Receptor-induced ROS Production

**A Subtraction Panel:**

| Name: | Properties: |
|-------|-------------|
| WT | binds / activates all signaling molecules |
| F72/74 | does not bind or activate Src |
| F720 | does not bind or activate SHP-2 |
| F31/42 | does not bind or activate PI3K |
| F988 | does not bind or activate ?? |
| F1018 | does not bind or activate PLCγ |

**B "Add-back" Panel:**

| Name: | Properties: |
|-------|-------------|
| F7 | binds no signaling molecules |
| Y72/74 | binds / activates only Src |
| Y720 | binds / activates only SHP-2 |
| Y31/42 | binds / activates only PI3K |
| Y988 | binds / activates only ?? |
| Y1018 | binds / activates only PLCγ |

**FIGURE 6. Schematic diagram illustrating the series of αPDGFR mutants.** Shown is the cytoplasmic domain of the αPDGFR in which the tyrosine phosphorylation sites (P), which act as specific binding sites for receptor-associated signaling molecules identified at the top of the schemes, have been individually deleted by Tyr to Phe substitution (black squares). The nomenclature of the Subtraction panel (A) and Add-back panel (B) of αPDGFR mutants is indicated to the right of each receptor representation. In the subtraction panel, the names indicate which of the tyrosine residues have been replaced with phenylalanine (F), and in the add-back panel the name of each mutant denotes which of the mutations in the F7 construct has been repaired (Y). Tyr-572 and -574 are located in the juxtamembrane domain (JM) of the receptor and are required for Src binding to the receptor; Tyr-720, -731, and -742 are in the kinase insert (KI) of the receptor and are responsible for SHP-2 and PI3K binding, respectively; Tyr-988 and -1018 are located in the C-terminal tail of the receptor and are involved in the binding of a yet unidentified protein and PLCγ, respectively.

**PI3K-dependent Translocation of p47phox and Rac-1 Is Critical for PDGF-AA-induced ROS Production/NAD(P)H Oxidase Activation**—To systematically investigate the role of each of the signaling molecules for PDGF-AA-induced ROS production, we compared the response of Ph cells expressing the WT αPDGFR to that mediated by Ph cells expressing the various mutant receptors (see Fig. 6). Quiescent cells were stimulated with PDGF-AA for up to 8 h, and DCF fluorescence was measured. Stimulation of WT receptor-expressing cells with PDGF-AA resulted in a significant increase of ROS production (Fig. 9A; see also Fig. 1A). The inability of the αPDGFR to interact with either Src, SHP-2, or PLCγ, as well as mutation of tyrosine 988, did not affect the ability of the receptor to mediate ligand-induced ROS production (Fig. 9A). In contrast, mutation of the binding sites for PI3K fully abolished PDGF-dependent ROS production, as F31/42 receptor-expressing cells showed no increase in DCF fluorescence upon stimulation with PDGF-AA (Fig. 9A, p < 0.05 versus WT). Consistent with these observations, the restoration of the PI3K-binding sites to the F7 mutant (in which all of the binding sites are deleted) was sufficient to fully salvage the ability of the...
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FIGURE 7. PDGF-AA-dependent subcellular translocation of NAD(P)H oxidase subunit Rac-1. Immunocytochemical staining of Rac-1 with specific antibodies was performed on intact cells mounted on glass slides. The cells were stimulated with 50 ng/ml PDGF-AA for 30 min, and translocation was measured by confocal laser scanning microscopy. A, PDGF-AA-dependent translocation of Rac-1 in Ph cells expressing either the WT αPDGF or the F31/42-, F7-, or Y31/42 mutants. Shown are representative transverse scans through the cells. B, effects of PDGF-AA on Rac-1 expression in Ph cells. Cells were stimulated with PDGF-AA (50 ng/ml) for times indicated, lysed, and total cell lysates were subjected to Western blot analysis using antisera against RasGAP (control) or Rac-1. C, subcellular localization of Rac-1 in −/−PDGF-stimulated Ph cells expressing the WT αPDGF or the F31/42, F7, or Y31/42 mutants. Quiescent Ph cells were left resting or stimulated with PDGF-AA (50 ng/ml) for 1 h, and cytosolic and membrane fractions were separated as described under “Experimental Procedures.” The proteins were separated by SDS-PAGE and subjected to Western blot analysis using antisera which recognize RasGAP (control) or Rac-1. D, densitometric analysis of the Western blots as shown in C. Data represent means ± S.D. from three independent experiments. *, p < 0.05 versus buffer.

receptor also partly rescued PDGF-dependent ROS production (Fig. 9B, p < 0.05 versus F7). In contrast, restoration of the binding sites for Src, SHP-2, or PLCγ did not rescue αPDGF-mediated ROS production. The fact that restoration of tyrosine 988 to the F7 receptor partly rescued PDGF-dependent ROS production indicates that a yet unidentified enzyme binding to this site may also contribute to this response, although the inability of the αPDGFR to interact with this molecule (F988 mutant) did not affect PDGF-AA-induced ROS production (Fig. 9A). We also examined the role of PI3K for PDGF-dependent NAD(P)H oxidase activation. As expected, PI3K was also identified as the critical signaling enzyme for this response. The inability of the αPDGFR to associate with or activate PI3K (F31/42) completely abolished the activation of NAD(P)H oxidase induced by the WT receptor, whereas restoration of the PI3K-binding sites to the F7 receptor (Y31/42) completely salvaged PDGF-dependent NAD(P)H oxidase activation (Fig. 9C). These findings indicate that of the receptor-associated proteins tested, PI3K is the major contributor to PDGF-AA-dependent NAD(P)H oxidase activation and ROS production.

The Catalytic PI3K Subunit p110α Is Crucial for αPDGF-medi- ated ROS Production—To further investigate which of the class 1A PI3K isoforms that signal downstream of RTKs are involved in PDGF-dependent ROS production, we focused on the catalytic subunits p110α and p110β, as these two subunits are ubiquitously expressed, whereas the expression of p110δ and p110γ is restricted to hematopoietic cells and the heart, respectively (38). Consistent with the data obtained by the use of αPDGFR mutants, pharmacological inhibition with two broad PI3K inhibitors (wortmannin and LY294002) completely inhibited αPDGF-dependent ROS liberation and thus confirmed the importance of PI3K activity (Fig. 9D). To dissect the role of the above isoforms, we applied isoform-selective inhibitors against p110α (PIK75) and p110β (TGX-221). These compounds were recently shown to effectively and specifically inhibit each isoform in cells (39, 40). As demonstrated in Fig. 9E, inhibition of p110α with PIK75 (1 μM) completely blunted PDGF-AA-dependent ROS production, whereas inhibition of
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**FIGURE 8.** Quantification of PDGF-dependent subcellular translocation of p47phox and Rac-1 in Ph cells expressing mutant αPDGFRs. Immunocytological staining of Rac-1 and p47phox with specific antibodies was performed on intact cells mounted on glass slides. The cells were stimulated with 50 ng/ml PDGF-AA for 30 min, and translocation was measured by confocal laser scanning microscopy. To obtain immunofluorescent signals of the membranous fraction of p47phox and Rac-1, a confocal setup obtaining quantitative signals from a membrane-focused cellular volume was chosen, and tangential single rapid scans were recorded. Data represent the percent increase of the membranous fluorescent signal over buffer stimulation (100%) and are expressed as means ± S.D. For each condition, a total of at least three independent experiments was analyzed. *p < 0.05 versus buffer.

p110β with TGX-221 (1 μM) did not affect this response. TGX-221 (0.1 μM) did, however, potently inhibit p110β activity and the production of PI3K lipid products such as phosphatidylinositol 3,4-bisphosphate, as shown in shear-activated platelets (40). Hence, the catalytic activity of p110α, but not p110β, appears to be crucial for αPDGFR-mediated ROS production in fibroblasts.

**ROS Are Required for PDGF-AA-dependent Chemotaxis but Not DNA Synthesis**—Finally, we aimed to characterize the role of ROS for PDGF-AA-dependent cellular responses, namely chemotaxis and proliferation. DNA synthesis was measured by a BrdUrd incorporation assay in MEF isolated from p47phox(+/-) or p47phox(-/-) mice. Stimulation of either cell type with PDGF-AA led to a concentration-dependent increase of BrdUrd incorporation to ~400% compared with unstimulated cells, indicating that ROS are not required for αPDGFR-mediated cell cycle progression (Fig. 10A). PDGF-dependent chemotaxis was measured by utilizing a Boyden chemotaxis chamber. As demonstrated in Fig. 10B, PDGF-AA (10 ng/μl) led to an ~4-fold increase of chemotaxis in p47phox(+/-) cells, and this response was prevented by preincubation with the NAD(P)H oxidase inhibitor DPI as well as the ROS scavengers PEG catalase and N-acetylcysteine. In line with this observation, PDGF-AA did not induce a chemotactic response in p47phox(-/-) cells (Fig. 10B), indicating that αPDGFR-mediated chemotaxis requires NAD(P)H oxidase activity and ROS.

In summary, we show for the first time that P13K/p110α mediates PDGF-AA-dependent ROS production by translocating p47phox and Rac-1 from the cytosol to the cell membrane, thereby assembling the membranous NAD(P)H oxidase complex, a critical event for NAD(P)H oxidase activation. This process appears to be critical for PDGF-AA-induced chemotaxis but not proliferation.

**DISCUSSION**

In this study, we demonstrate that Rac-1 and p47phox are absolutely required for PDGF-dependent liberation of ROS in fibroblasts. Furthermore, we show for the first time that these cytosolic NAD(P)H oxidase subunits translocate to the cell membrane upon PDGF stimulation, and this response is exclusively mediated by P13K but not by other αPDGFR-associated signaling molecules, including Src family members, SHP-2, or PLCγ. P13K thereby assembles the active NAD(P)H oxidase complex and is thus required for PDGF-AA-dependent NAD(P)H oxidase activation and intracellular ROS production. More specifically, this response is mediated by the catalytic P13K subunit p110α but not p110β.

**Importance of Rac-1 and p47phox for NAD(P)H Oxidase Activation**—The current knowledge of superoxide production by a membrane-bound NAD(P)H oxidase is mainly based on findings obtained in phagocytic cells. Although the molecular mechanisms required for NAD(P)H oxidase activation are not fully understood, assembly of the membranous enzyme complex is essential for its activation in phagocytes (11).

The idea that translocation of the cytosolic subunits to the membrane is critical for NAD(P)H oxidase activation appears to also apply for nonphagocytes. Nonphagocytic cells, including endothelial cells, VSMC, and fibroblasts, express a specific pattern of NAD(P)H oxidase subunits. However, the expression of single subunits as well as the functional characteristics of this enzyme have not been systematically addressed in these cells. In this study, inhibitor experiments and an enzyme activity assay showed that NAD(P)H oxidase is the source of intracellular ROS upon αPDGFR activation. In Ph cells, RT-PCR identified significant expression of the major components of the NAD(P)H oxidase, the membrane-bound subunits Nox (formerly known as gp91phox) and p22phox, and the cytosolic subunits p47phox and Rac-1 but not p40phox and p67phox. Our study reveals that Rac-1 and p47phox are critically involved in PDGF-AA-dependent activation of NAD(P)H oxidase in fibroblasts. This is demonstrated in MEFs lacking the p47phox gene and by pharmacological inhibition of Rac-1 or overexpression of dominant-negative Rac (rac-N17) (Fig. 4). These data are consistent with other studies demonstrating that p47phox is required for phorbol 12-myristate 13-acetate- and growth factor-induced ROS production in VSMC (41), that p22phox is crucial for...
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αPDGF-induced liberation of ROS (42), and that Rac activity regulates NAD(P)H oxidase activation in phagocytes and VSMCs (43–45). Specific inhibition of Rac-1 or pretreatment with hydroxymethylglutaryl-CoA reductase inhibitors (statins), which also inhibit Rac, completely abolished angiotensin II- and peptide growth factor-induced ROS production in several cell types (10, 46–48). However, the signaling pathways and molecular mechanisms by which these cytosolic proteins contribute to NAD(P)H oxidase activation have not yet been determined. By using quantitative immunocytochemistry and Western blotting of membrane and cytosolic fractions of total cell lysates, we demonstrate that PDGF-AA rapidly recruits p47<sub>phox</sub> and Rac-1 to the cell membrane in WT receptor-expressing cells (Figs. 7 and 8). The fact that mutant receptors that fail to mediate PDGF-dependent subcellular translocation of these NAD(P)H oxidase subunits are also unable to induce NAD(P)H oxidase activation and ROS production indicates that p47<sub>phox</sub> and Rac-1 translocation to the cell membrane is a critical event for PDGF-induced ROS production by NAD(P)H oxidase. This is consistent with the observation that pharmacological inhibition of the translocation of cytosolic subunits to the cell membrane by apocynin also completely inhibits superoxide production by NAD(P)H oxidase, as shown by us (see Fig. 1F) and others (49, 50). In human endothelial cells expressing the membranous subunits gp91<sub>phox</sub> and p22<sub>phox</sub> and the cytosolic subunits p67<sub>phox</sub> and p47<sub>phox</sub>, NAD(P)H oxidase activation by phorbol 12-myristate 13-acetate, which is a potent activator of this enzyme in phagocytes, correlated with p47<sub>phox</sub> accumulation in the membrane fraction of cellular homogenates as determined by Western blot analyses (49). When viewed together, the above findings strongly indicate that recruitment of p47<sub>phox</sub> and Rac-1 to the cell membrane is critical for growth factor-induced ROS liberation in nonphagocytes.

PI3K Mediates PDGF-AA-dependent Translocation of Rac-1 and p47<sub>phox</sub>—Experiments utilizing mutant αPDGFRs and pharmacological inhibitors revealed that both NAD(P)H oxidase activation and translocation of its cytosolic subunits p47<sub>phox</sub> and Rac-1 depend on PI3K activity. This is shown by the fact that the F31/42 mutant, which does not bind or activate PI3K (22), was unable to mediate PDGF-dependent p47<sub>phox</sub>/Rac-1 translocation and NAD(P)H oxidase activation, whereas binding and activation of PI3K in the absence of all other αPDGF-associated signaling molecules, including Src, SHP-2, and PLCγ (Y31/42 mutant), were sufficient to mediate these responses as efficiently as the WT receptor (see Figs. 7, 8, and 9C). This leads to the question how PI3K, p47<sub>phox</sub>, and Rac-1 interact to mediate NAD(P)H oxidase activation.

Several studies have shown that Rac-1 is readily activated by PI3K (51–53). Rho-GTPases such as Rac-1 cycle between GTP-
The interaction between PI3K and p47phox is less clear. In COS cells, the phox homology domain of p47phox is recruited to the cell membrane upon insulin-like growth factor-1 stimulation (58). Although this response requires PI3K activity, direct binding of phosphoinositide to the p47phox phox homology domain is not sufficient to mediate subcellular translocation. A recent study indicated that a downstream target of PI3K, the serine-threonine kinase Akt, directly interacts with p47phox and mediates its phosphorylation in response to formylmethionyl-leucylphenylalanine in neutrophils, thereby contributing to respiratory burst activity (59). In addition to direct involvement of PI3K/Akt in p47phox translocation, PI3K-dependent activation of Rac-1 may also contribute to this response. In COS cells stably expressing essential NAD(P)H oxidase components, expression of constitutively active Rac-1 (G12V, Q61L) or activation of endogenous Rac by expression of constitutively active GEFs (Vav, Tiam-1) induced phagocyte NAD(P)H oxidase activity and translocation of p47phox and p67phox (43). Membrane recruitment of p47phox was induced even in the absence of p67phox expression, but p47phox did not directly bind Rac. Vav-1 was identified as the most effective GEF activating ROS production in this model. Hence, in addition to directly participating in the oxidase complex, Rac may contribute to oxidase activation by promoting signaling events that lead to assembly of the active oxidase complex. Whether PI3K-dependent Rac activation is necessary for PDGF-induced membrane recruitment of p47phox still remains unclear. Further studies are required to identify how PI3K mediates translocation of p47phox and Rac-1 and thereby activates NAD(P)H oxidase.

**Dependence of PDGF-AA-induced ROS Production on PI3K**—Our finding that PDGF induces ROS production in nonphagocytic cells is consistent with other studies (9, 10, 42). Sundaresan et al. (9) reported that stimulation of rat VSMC with PDGF-AB led to a characteristic raise of intracellular ROS within 5 min. Because VSMC express both PDGFRs and PDGF-AB activates both subtypes, the contribution of the α- and βPDGFR for PDGF-dependent ROS production is not clear from this study. This may be important as the two PDGFRs differ from one another in both their signaling mechanisms and biological functions (15). A recent study by Bae et al. (10) demonstrated that the βPDGFR induces ROS production in a PI3K-dependent manner in HepG2 cells. In this study we demonstrate that the αPDGFR also induces ROS production via PI3K, as disruption of PI3K signaling in the F31/42 mutant and by pharmacological inhibition with wortmannin or LY294002 blunted this response, whereas PI3K activation alone (Y31/42 mutant) was sufficient to induce ROS. Furthermore, we show that p110α, but not p110β, is the critical catalytic PI3K subunit that mediates αPDGFR-induced ROS production in fibroblasts (see Fig. 9E). This is in line with recent observations demonstrating a unique role for p110α in other systems. Knight et al. (39) have shown that p110α is the primary insulin-responsive PI3K in cultured 3T3-L1 adipocytes, and compounds targeting p110α block the acute effects of insulin treatment in vivo, whereas a p110β inhibitor had no effect.

A recent study suggested that in addition to PI3K, Src family kinases are also required for ROS generation in response to angiotensin II in VSMCs, as assessed by pretreatment of cells...
with the Src inhibitor PP1 (60). In contrast, our data indicate that Src is not required for PDGF-dependent liberation of ROS in fibroblasts. This difference may be explained by the fact that the angiotensin II type 1 receptor uses Src to induce ROS formation, whereas the αPDGFR does not. In addition, cell type-specific responses may exist in VSMCs versus fibroblasts. A third explanation may include the lack of specificity of Src inhibitors. In our hands, Src inhibitors, including PP1, PP2, and SU6656, may also inhibit the tyrosine kinase activity of RTKs such as the αPDGFR at the same concentrations that inhibit Src. Particularly when incubation times are long (hours), some inhibitors appear to accumulate in cells and may thus act rather unspecific because intracellular concentrations are high. When using receptor mutants, our data clearly show that Src is not required for PDGF-AA-dependent ROS liberation, as the F72/74 receptor, which does bind or activate Src and does not phosphorylate Src substrates such as enolase (20), was fully capable of inducing ROS liberation in response to PDGF-AA.

It remains unclear why the Y988 mutant is able to induce ROS liberation to some extent. Our data indicate that there may exist an alternative weaker signaling pathway leading to ROS production that appears to be independent of PI3K. It is possible that a yet unidentified protein interacting with tyrosine residue 988 contributes to superoxide production, although the F988 mutant was able to fully mediate this response. Another possibility is that tyrosine 988 somehow helps to activate PI3K, as the corresponding tyrosine residue in the βPDGFR (tyrosine 1009) is able to stimulate the formation of PI3K lipid products in HepG2 cells. However, the Y988 mutant does not recruit active PI3K upon PDGF stimulation (22) and is also unable to induce phosphorylation of the PI3K target Akt (data not shown). Therefore, the involvement of PI3K in Y988-induced ROS production seems unlikely. Finally, it is possible that G proteins interact with tyrosine 988, as Gα was recently shown to be involved in αPDGFR-induced ROS production (61).

When compared with other RTKs such as the βPDGFR and the epidermal growth factor receptor (9), the αPDGFR induces a sustained increase of NAD(P)H oxidase activity and intracellular ROS for several hours, a characteristic that was also shown for the angiotensin II type 1 receptor (5). The distinct responses to different agonists may be explained by the disparities in oxidase composition between phagocytes and the various nonphagocytic cell types and by unique signaling mechanisms, which possibly determine their primary physiological role in the various cell types.

Importance of ROS for PDGF-AA-dependent Cellular Responses—Several studies imply that ROS act as signaling molecules for growth factor-induced cellular responses and thus may serve as essential second messengers in malignant and nonmalignant proliferative diseases. Indeed, regulation of the intracellular redox state by growth factor-induced changes of NAD(P)H oxidase activity is thought to have important impact on redox-sensitive signaling cascades. For example, the proliferative state of fibroblasts is tightly correlated with intracellular ROS levels, such that low ROS levels correlate with growth arrest induced by cell density (contact inhibition) (62). Some studies suggest that PDGF-induced cell cycle progression is dependent on ROS (9, 10). However, our results on p47phox-deficient cells indicate that ROS are not required for αPDGFR-mediated proliferation of fibroblasts, although PDGF-AA-dependent cellular proliferation is exclusively correlated with the ability of the αPDGFR to associate with PI3K (22). In contrast, the chemotactic response to PDGF-AA was shown to depend on the liberation of ROS (see Fig. 10B). These data are consistent with the recent observation that pharmacological inhibition of NAD(P)H oxidase blunts the βPDGFR chemotactic signals in VSMC, whereas the mitogenic response to PDGF–BB is unaffected (63).

In summary, our study provides a novel mechanism, how PI3K, and particularly its catalytic subunit p110α, contributes to growth factor-induced NAD(P)H oxidase activation and ROS production in nonphagocytes, as it mediates the translocation of cytosolic subunits Rac-1 and p47phox to the cell membrane and hence induces assembly of the active enzyme complex.

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