THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 14, Issue of April 7, pp. 10527–10531, 2000
Printed in U.S.A.

Platelet-derived Growth Factor-induced H_2O_2 Production Requires the Activation of Phosphatidylinositol 3-Kinase*

(Received for publication, November 15, 1999, and in revised form, December 30, 1999)

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Autophosphorylation of the platelet-derived growth factor (PDGF) receptor triggers intracellular signaling cascades as a result of recruitment of Src homology 2 domain-containing enzymes, including phosphatidylinositol 3-kinase (PI3K), the GTPase-activating protein of Ras (GAP), the protein-tyrosine phosphatase SHP-2, and phospholipase C-γ1 (PLC-γ1), to specific phosphotyrosine residues. The roles of these various effectors in PDGF-induced generation of H_2O_2 have now been investigated in HepG2 cells expressing various PDGF receptor mutants. These mutants included a kinase-deficient receptor and receptors in which various combinations of the tyrosine residues required for the binding of PI3K (Tyr^{740} and Tyr^{751}), GAP (Tyr^{771}), SHP-2 (Tyr^{1009}), or PLC-γ1 (Tyr^{1021}) were mutated to Phe. PDGF failed to increase H_2O_2 production in cells expressing either the kinase-deficient mutant or a receptor in which the two Tyr residues required for the binding of PI3K were replaced by Phe. In contrast, PDGF-induced H_2O_2 production in cells expressing a receptor in which the binding sites for GAP, SHP-2, and PLC-γ1 were all mutated was slightly greater than that in cells expressing the wild-type receptor. Only the PI3K binding site was alone sufficient for PDGF-induced H_2O_2 production. The effect of PDGF on H_2O_2 generation was blocked by the PI3K inhibitors LY294002 and wortmannin or by overexpression of a dominant negative mutant of Rac1. These results suggest that a product of PI3K is required for PDGF-induced production of H_2O_2 in nonphagocytic cells, and that Rac1 mediates signaling between the PI3K product and the putative NADPH oxidase.

Many cell types produce hydrogen peroxide (H_2O_2) in response to a variety of extracellular stimuli that include cytokines, such as transforming growth factor-β1 (1, 2), interleukin-1 (3–5), interferon-γ (4), and tumor necrosis factor-α (3, 6); peptide growth factors, such as platelet-derived growth factor (PDGF)[1] (7), epidermal growth factor (8), basic fibroblast growth factor (6), insulin (9), and granulocyte-macrophage colony-stimulating factor (5); and agonists of receptors with seven transmembrane domains, such as angiotensin II (10), thrombin (11), thyrotropin (4, 12), parathyroid hormone (4), lysophosphatidic acid (13), sphingosine 1-phosphate (14), mechanical shear strain (15), phorbol esters (16), and phosphatidic acid (17) also induce H_2O_2 production in various cell types. Receptor-mediated generation of H_2O_2 has been linked to the activation of transcription factors such as nuclear factor κB (18) and AP1 (6, 19), mitogen-activated protein kinases (7, 13), phospholipase A_2 (20), protein kinase C (21), and phospholipase D (22, 23); it has also been linked to an increase in the cytosolic Ca^{2+} concentration (24), to the triggering of apoptosis (25), to the inhibition of protein-tyrosine phosphatases (26), and to modulation of ion transport (27). Because H_2O_2 is a small, diffusible, and ubiquitous molecule that can be synthesized, as well as destroyed, rapidly in response to external stimuli, it fulfills the important criteria for an intracellular messenger.

The mechanism of receptor-mediated generation of H_2O_2 has been studied extensively in phagocytic cells, in which O_2^- is produced via the one-electron reduction of O_2 by a multicomponent NADPH oxidase system (28). The O_2^- is then spontaneously or enzymatically dismutated to H_2O_2. The NADPH oxidase complex includes two cytosolic components ($p47^{phox}$ and $p67^{phox}$) and two transmembrane flavocytochrome b components ($gp91^{phox}$ and $p22^{phox}$). In addition, the small GTP-binding protein Rac (either Rac1 or Rac2) is required for activation of NADPH oxidase. In contrast, the mechanism of H_2O_2 generation in nonphagocytic cells remains unclear. Evidence suggests that the system responsible for H_2O_2 (O_2^-) production in nonphagocytic cells is structurally and genetically distinct from, but functionally similar to, the NADPH oxidase system of phagocytes (29, 30). Consistent with this notion, overproduction of Rac1 in fibroblasts was associated with increased production of H_2O_2 (31, 32) and a homolog of gp91-phox was found in several nonphagocytic cells (33).

The binding of growth factors to their receptors results in receptor autophosphorylation on specific tyrosine residues. These phosphotyrosine residues initiate cellular signaling by acting as high affinity binding sites for the Src homology 2 domains of various effector proteins. In the PDGF β receptor

* This work was supported in part by Center of Excellence Grant 1998G0202 and International Joint Research Laboratory Grant 1999L0001 from the Korean Science and Engineering Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PDGF, platelet-derived growth factor; PDGFβR, PDGF β receptor; PI3K, phosphatidylinositol 3-kinase; GAP, GTPase-activating protein of Ras; SHP-2, Src homology 2 domain-containing protein-tyrosine phosphatase-2; PLC-γ1, phospholipase C-γ1; MEM, modified Eagle's medium; DCF, 2′,7′-dichlorofluorescein; GEF, guanine nucleotide exchange factor; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; p110-CAAX, membrane-targeted p110 subunit of PI3K.

(PDGFβR), seven autophosphorylation sites have been identified as specific binding sites for Src family tyrosine kinases (Tyr^{579} and Tyr^{581}), phosphatidylinositol 3-kinase (PI3K) (Tyr^{740} and Tyr^{751}), the GTPase-activating protein of Ras (GAP) (Tyr^{771}), Src homology 2 domain-containing protein-tyrosine phosphatase-2 (SHP-2) (Tyr^{1009}), and phospholipase C-γ1 (PLC-γ1) (Tyr^{1021}). A series of PDGFβR mutants was previously constructed that includes a kinase-deficient [kinase(−)] mutant and receptors in which the binding sites for PI3K, GAP, SHP-2, and PLC-γ1 were changed individually or in various combinations to phenylalanine (34).

To characterize the mechanism of H_2O_2 production in nonphagocytic cells, we have now measured PDGF-dependent H_2O_2 generation in human hepatoma HepG2 cells expressing these various PDGFβR mutants. Our data indicate that PDGFβR-dependent H_2O_2 production requires both the intrinsic kinase activity of the receptor as well as the activation of PI3K. Experiments with a dominant negative mutant of Rac1 (N17Rac1) also suggest that Rac1 participates in the PDGFβR-induced production of H_2O_2 in HepG2 cells.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium, modified Eagle's medium (MEM) without phenol red, Opti-MEM, fetal bovine serum, antibiotic-antimycotic mixture, and LipofectAMINE were obtained from Life Technologies, Inc.; expression plasmids encoding dominant negative Akt (Akt K179M) and constitutively activated Akt (Myr-Akt), PDGF-BB, and rabbit antibodies to Rac1 were from Upstate Biotechnology; LY294002 was from Bio-Mol, and 2′,7′-dichlorofluorescin diacetate was from Molecular Probes. Adenovirus encoding a dominant negative Rac1 (Ad.N17Rac1) was kindly provided by T. Finkel (NHLBI, NIH, Bethesda, MD). Expression plasmids encoding dominant negative mutants of Rho family proteins N17Cdc42 (pEXV-Cdc42N17) and N19RhoA (pEXV-RhoAN19) were kindly provided by J. H. Kim (KJIST, Kwhangju, Korea), and the expression plasmid encoding p110-CAAX (pCMVp110-CAAX) was provided by J. Downward (Cambridge University, UK).

HepG2 Cell Lines Expressing Wild-type or Mutant PDGFβRs—Construction of the various PDGFβR mutants shown in Fig. 1 and of the HepG2 cells expressing these mutants has been described previously (34). The cells were maintained at 37 °C under an atmosphere of 5% CO_2 in culture dishes containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Assay of Intracellular H_2O_2—Intracellular production of H_2O_2 was assayed as described previously (8). In brief, at various times after stimulation with PDGF-BB (100 ng/ml), dishes of confluent cells were washed with MEM lacking phenol red and then incubated in the dark for 5 min in Krebs-Ringer solution containing 5 μM 2′,7′-dichlorofluorescin diacetate. This compound is converted by intracellular esterases to 2′,7′-dichlorofluorescin, which is then oxidized by H_2O_2 to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Culture dishes were transferred to a Zeiss Axiovert 135 inverted microscope that was equipped with a x20 Neofluor objective and Zeiss LSM 410 confocal attachment, and DCF fluorescence was measured with an excitation wavelength of 488 nm and emission at 515–540 nm. To avoid photooxidation of 2′,7′-dichlorofluorescin, the fluorescence image was collected by a single rapid scan (4-line average, total scan time of 4.33 s), with identical parameters (such as contrast and brightness) for all samples. After collection of the fluorescence image, the cells were imaged by digital interference contrast. Five groups of 10–20 cells each were randomly selected from each sample, and the mean relative fluorescence intensity for each group of cells was then measured by Karl Zeiss vision system (KS400, version 3.0) and averaged. All experiments were repeated at least five times.

Expression of N17Rac1 in HepG2 Cells—HepG2 cells were plated at a density of 2×10^5/well in culture dishes and allowed to recover for 24 h. Adenovrus infection was performed at an multiplicity of infection of 200. After adenovirus infection for 72 h, the cells were then washed with MEM without phenol red, exposed for 30 min to PDGF-BB (100 ng/ml), and subjected to assay of H_2O_2 production. Western blot analysis of Myc-tagged form of N17Rac was performed using an antibody to rac1 (35).

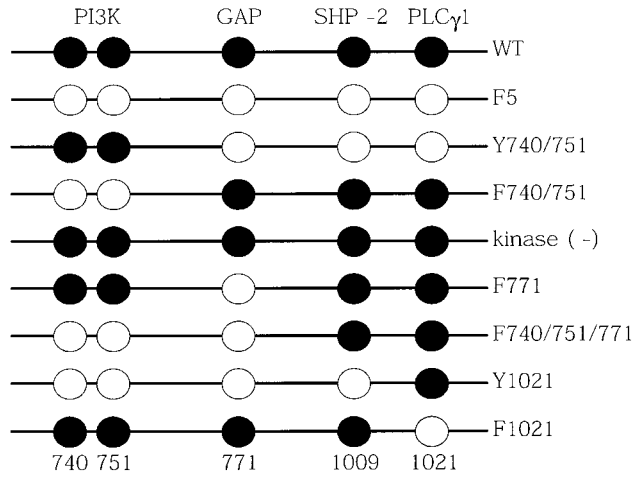

FIG. 1. **Schematic representation of PDGFβR mutants.** The *closed* and *open circles* represent tyrosine (Y) and phenylalanine (F) residues, respectively, at the indicated amino acid positions. The proteins (PI3K, GAP, SHP-2, and PLC-γ1) that bind to the various phosphorylated tyrosine residues are indicated at the *top* of the figure. *WT*, wild type.

RESULTS

Vallius and Kazlauskas (34) previously constructed the series of PDGFβR mutants shown in Fig. 1. The kinase(−) mutant was generated by mutating Lys^{634} to Arg; the F740/751 mutant by replacing the residues that constitute the PI3K binding site (Tyr^{740} and Tyr^{751}) with Phe; the F771 mutant by replacing the GAP binding site (Tyr^{771}) with Phe; the F1021 mutant by replacing the PLC-γ1 binding site (Tyr^{1021}) with Phe; the F740/751/771 mutant by replacing Tyr^{740}, Tyr^{751}, and Tyr^{771} with Phe; and the F5 mutant by replacing the five tyrosine residues Tyr^{740}, Tyr^{751}, Tyr^{771}, Tyr^{1009} (the SHP-2 binding site), and Tyr^{1021} with Phe (Fig. 1). In addition, the ability of F5 to signal via PI3K was selectively restored by changing Phe^{740} and Phe^{751} in this mutant back to Tyr, thereby generating Y740/751 (Fig. 1). Similarly, the ability of F5 to signal via PLC-γ1 was restored by changing Phe^{1021} in this mutant back to Tyr, generating Y1021 (Fig. 1). The wild-type and mutant PDGFβR proteins were expressed, with the use of a retroviral expression vector, in HepG2 cells, which do not express detectable amounts of endogenous PDGFβ receptors. The level of PDGFβR expression was approximately 5×10^5 receptors/cell for both wild-type and mutant receptors (34).

The production of H_2O_2 by HepG2 cells was measured with a fluorescence-based assay with 2′,7′-dichlorofluorescin diacetate and laser-scanning confocal microscopy. Stimulation of HepG2 cells expressing wild-type PDGFβR with PDGF resulted in a time-dependent increase in the intensity of DCF fluorescence, with the maximal, 3.5-fold increase apparent 10–30 min after stimulation; fluorescence had returned to the baseline value after 60 min (Fig. 2*A*). In contrast, cells expressing the kinase(−) or F5 mutant receptors failed to produce H_2O_2 in response to PDGF (Fig. 2, *B* and *C*). These results suggest that PDGF-induced H_2O_2 production requires the kinase activity of PDGFβR as well as at least one of the five tyrosine phosphorylation sites mutated in F5.

We next measured PDGF-induced H_2O_2 production in cells expressing other PDGFβR mutants (Fig. 3). Whereas PDGF had no effect on H_2O_2 production in cells expressing F740/751 or F740/751/771 mutants, it increased H_2O_2 production in cells expressing the F771 or F1021 mutants to an extent similar to that observed in cells expressing the wild-type receptor. Cells expressing Y740/751 (equivalent to F5 in which the PI3K binding site has been restored) showed a response to PDGF that

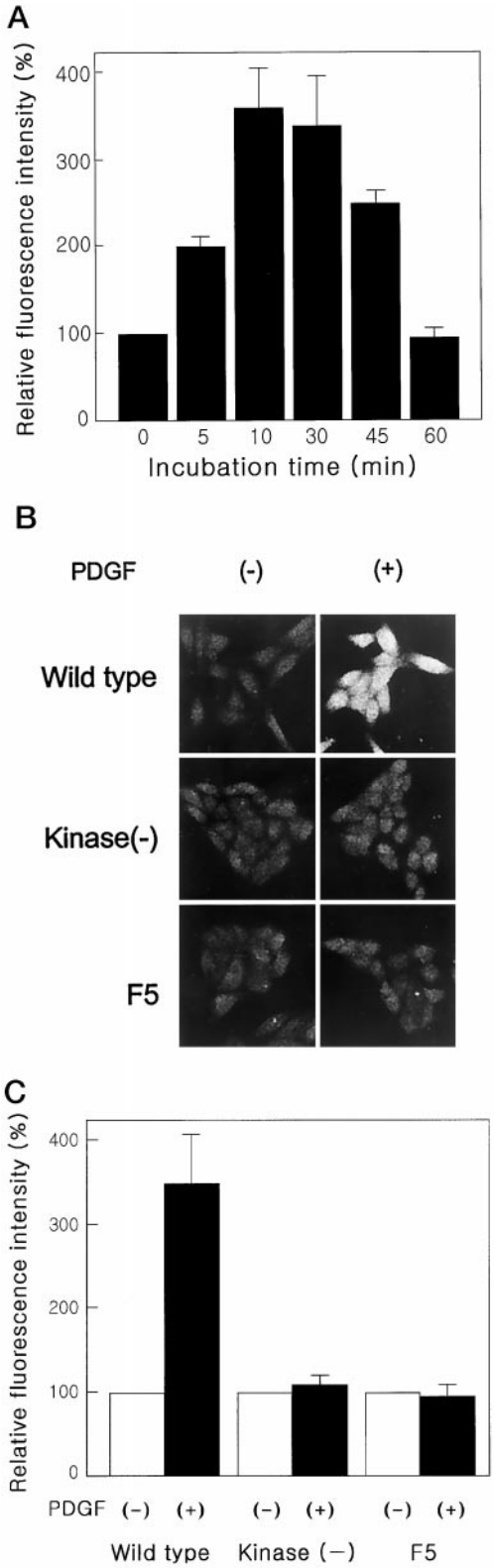

FIG. 2. **Effects of PDGF on H_2O_2 production in HepG2 cells expressing wild-type PDGFβR or the kinase(−) or F5 mutants.** *A,* time course of H_2O_2 generation induced by PDGF-BB (100 ng/ml) in cells expressing the wild-type PDGFβR. The generation of H_2O_2 was assayed on the basis of DCF fluorescence as described under "Experimental Procedures." Data are expressed relative to the value for nonstimulated cells. *B* and *C,* DCF fluorescence of HepG2 cells expressing wild-type PDGFβR or the kinase(−) or F5 mutants after incubation for 30 min in the absence or presence of PDGF-BB (100 ng/ml). Representative microscopic fields are shown in (*B*) and relative fluorescence intensity relative to nonstimulated cells is shown in *C*. Data in *A* and *C* are mean ± S.E. of five independent experiments.

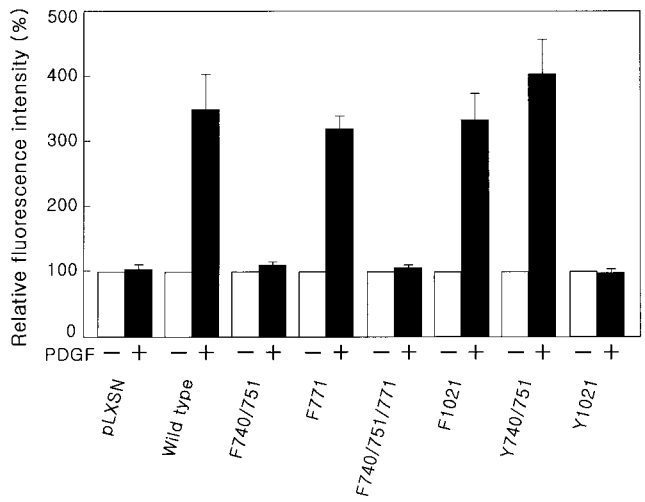

FIG. 3. **Effects of mutation of specific effector binding sites in PDGFβR on PDGF-induced H_2O_2 production.** HepG2 cells transfected with the empty vector (pLXSN) or with vectors encoding either wild-type PDGFβR or the indicated receptor mutants were incubated for 30 min in the absence or presence of PDGF-BB (100 ng/ml). The production of H_2O_2 was then measured on the basis of DCF fluorescence. Data are mean ± S.E. of values from five independent experiments.

was slightly greater than that of cells expressing the wild-type receptor. However, cells expressing Y1021 (equivalent to F5 in which the PLC-γ1 binding site has been restored) did not generate H_2O_2 in response to PDGF. These results thus indicate that activation of PI3K, but not of GAP, SHP-2, or PLC-γ1, is necessary for H_2O_2 production in response to PDGF.

The essential role of PI3K was further supported by the observation that prior incubation with 10 μM LY294002, a specific inhibitor of PI3K, completely blocked the PDGF-induced increase in H_2O_2 production in HepG2 cells expressing wild-type or Y740/751 PDGF receptors (Fig. 4). Similar inhibition was also observed with wortmannin, another inhibitor of PI3K (data not shown). Prior incubation with the inhibitors had no effect on the basal level of H_2O_2 production. PDGF also induced a transient increase in H_2O_2 production, with the maximal, 6-fold increase apparent 5–10 min after stimulation, in NIH 3T3 cells, and this effect was also abolished by 10 μM LY294002 (data not shown).

We further examined whether activation of PI3K is sufficient to produce H_2O_2 by transiently expressing in HepG2 cells the catalytic p110 subunit of PI3K with c-Myc epitope and farnesylation signal (CAAX) sequences at the NH_2 and COOH termini, respectively. It was previously shown that even modest expression of the membrane-targeted p110 (p110-CAAX) is sufficient to trigger activation of downstream events (36, 37). Expression of p110-CAAX in HepG2 cells expressing F5 mutant receptor resulted in a 3-fold increase in H_2O_2 production, and the effect was blocked by prior incubation of cells with LY294002 or wortmannin (Fig. 5).

To investigate the role of Rac1 in PI3K-dependent H_2O_2 production, we infected HepG2 cells expressing the Y740/751 receptor either with recombinant adenovirus encoding N17Rac1, a dominant negative mutant of Rac1 (Ad.N17rac), or control adenovirus (35). Expression of N17Rac1 in the infected HepG2 cells was confirmed by immunoblot analysis (Fig. 6*A*). Whereas HepG2 cells expressing the Y740/751 receptor infected control adenovirus showed an increase in H_2O_2 production in response to PDGF, those infected with recombinant adenovirus encoding N17Rac1 (Ad.N17rac) did not (Fig. 6*B*). Similar results were observed after infection of cells expressing wild-type PDGFβR with the recombinant adenovirus encoding

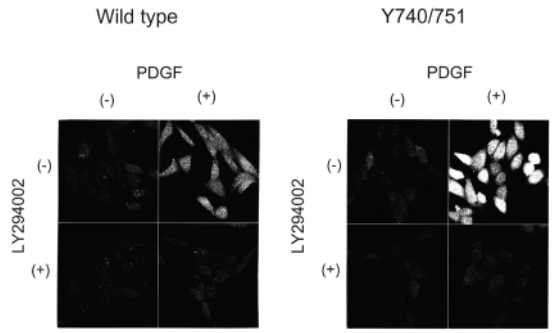

FIG. 4. **Effect of the PI3K inhibitor LY294002 on PDGF-induced H_2O_2 production.** HepG2 cells expressing wild-type (*left panels*) or Y740/751 PDGF receptors were incubated for 45 min in the absence or presence of 10 μM LY294002 and then for 30 min with or without PDGF-BB (100 ng/ml), after which DCF fluorescence was measured with a confocal laser-scanning microscope.

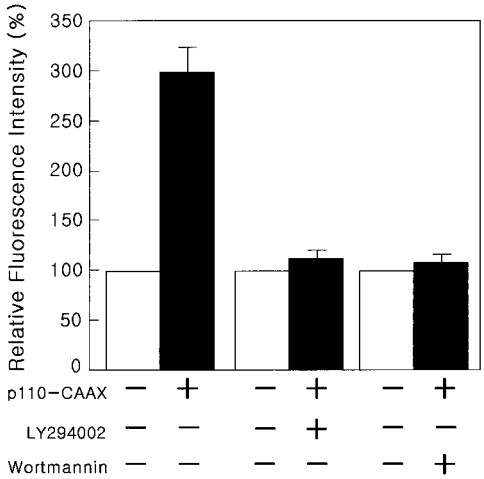

FIG. 5. **Effect of the expression of the membrane-targeted p110 subunit of PI3K (p110-CAAX) on H_2O_2 production.** HepG2 cells expressing the F5 mutant PDGF receptor were plated at a density of 3×10^5/well in 6-well plates and allowed to recover for 24 h. The cells were then incubated for 6 h with 1.3 μg of empty (pCMV) or MYC-p110-CAAX-encoding (pCMVp110-CAAX) plasmid and 6.7 μl of LipofectAMINE in 1 ml of opti-MEM medium (Life Technologies, Inc.), after which 1 ml of Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum was added to each well and the cells were incubated for an additional 24 h. The cells were then incubated in the absence or presence of 10 μM LY294002 or 10 nM wortmannin for 45 min. The production of H_2O_2 was assayed on the basis of DCF fluorescence as described under "Experimental Procedures." Data are expressed as a percentage of the values for pCMV-transfected cells in the absence of the PI3K inhibitors and are mean ± S.E. of three independent experiments.

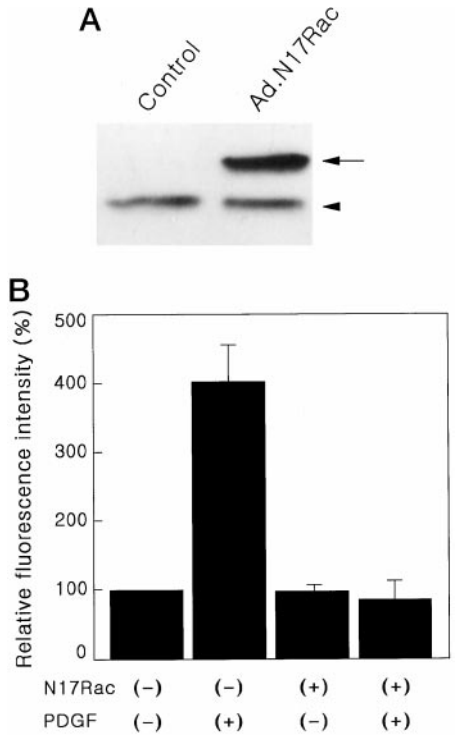

FIG. 6. **Effect of N17Rac1 on PDGF-induced H_2O_2 production in HepG2 cells expressing the Y740/751 PDGFβR.** *A,* immunoblot analysis with antibodies to Rac1 of lysates of HepG2 cells expressing Y740/751 that had been infected either with the recombinant adenovirus encoding Myc-tagged N17Rac1 (Ad.N17rac) or with control adenovirus. Endogenous Rac1 and Myc-tagged N17Rac1 are indicated by the *arrowhead* and *arrow*, respectively. *B,* DCF fluorescence of cells expressing the Y740/751 receptor after infection either with the recombinant adenovirus encoding N17Rac1 or with control virus and subsequent incubation for 30 min in the absence or presence of PDGF-BB (100 ng/ml). Data are mean ± S.E. of values from three independent experiments.

N17Rac1 (data not shown). In contrast, expression of dominant negative mutants of other Rho-family members (N19RhoA or N17Cdc42) did not affect the PDGF-induced increase in H_2O_2 production in HepG2 cells expressing wild-type PDGFβR (data not shown).

DISCUSSION

The binding of peptide growth factors to their specific receptors results in receptor autophosphorylation at several tyrosine residues, thereby triggering the recruitment of Src homology 2 domain-containing effector enzymes that include Src, PI3K, GAP, SHP-2, and PLC-γ1 (34, 38). The interaction of growth factors with their receptors also induces a transient increase in the intracellular concentration of H_2O_2 (5–9). In the present study, the role of the receptor-associated effectors in H_2O_2 production was investigated with HepG2 cells expressing various PDGFβR mutants. The PDGFβR was well suited for this study because the effector enzymes bind only when the receptor is phosphorylated at specific tyrosine residues (34). Mutation of these tyrosine residues individually prevents association of a specific effector without affecting the binding of other enzymes to the receptor mutant. In the case of other growth factor receptors, either the effector enzymes bind less specifically at multiple tyrosine residues or their binding sites are not well defined.

Our results suggest that, among the effector enzymes PI3K, GAP, SHP-2, and PLC-γ1, only the binding of PI3K to PDGFβR is necessary for PDGF-induced H_2O_2 production. The absence of the binding sites for GAP, SHP-2, and PLC-γ1 in the Y740/751 receptor mutant was actually associated with a slight, but reproducible, increase in the extent of PDGF-induced H_2O_2 production relative to that apparent with the wild-type receptor. This observation is consistent with the results of a previous study showing that PLC-γ1 and GAP negatively regulate the PDGF-induced activation of PI3K (38). Given that phosphorylation of Tyr^{740} and Tyr^{751} is required to generate the binding site for PI3K, it follows that the intrinsic kinase activity of PDGFβR is also required for PDGF-induced generation of H_2O_2. We previously also showed that an epidermal growth factor receptor mutant lacking kinase activity did not induce an increase in H_2O_2 production in response to epidermal growth factor (8). In contrast, the observation that a membrane-bound NADPH oxidase prepared from human fat cells produced H_2O_2 in the absence of ATP in response to insulin suggested that this growth factor is able to induce H_2O_2 production independently of the kinase activity of its receptor (39).

Activation of PI3K appears to be sufficient to produce H_2O_2 as indicated by the observation that expression of p110-CAAX

induced H_2O_2 production. One of the major events following PI3K activation is the phosphorylation and activation of Akt, a serine/threonine kinase (40). Therefore, we determined whether Akt is involved in PDGF-induced H_2O_2 production. Transient expression of either a dominant negative Akt mutant in HepG2 cells expressing Y740/751 receptor or a constitutively active Akt in HepG2 cells expressing F5 receptor had no effect on H_2O_2 production (data not shown). These results suggest that Akt is not involved in the PDGF-induced H_2O_2 production.

It was previously shown that the blockage of the growth factor-induced H_2O_2 increase by catalase in vascular smooth muscle and A431 cells resulted in a marked decrease in tyrosine phosphorylation of various proteins including the growth factor receptors (7, 8). At the present time, we do not know why PDGFRs (F740/751, F740/751/771, and F5) that fail to induce H_2O_2 production were fully autophosphorylated in HepG2 cells (34, 38). It is possible that in HepG2 cells, H_2O_2 production makes a smaller contribution to receptor tyrosine phosphorylation as compared with vascular smooth muscle and A431 cells.

An essential role for Rac in activation of the NADPH oxidase complex has been well established in phagocytes (28). Rac also appears important in this regard in nonphagocytic cells, as indicated by the observations that transient expression of an active form of Rac1 (V12Rac1) in NIH 3T3 cells itself resulted in an increase in H_2O_2 production and that expression of N17Rac1 inhibited the increase in H_2O_2 concentration induced by PDGF, epidermal growth factor, tumor necrosis factor-α, or interleukin-1 (31). Our observation that N17Rac1 blocked the Y740/751 receptor-induced generation of H_2O_2 indicates that Rac1 acts downstream of PI3K in the signaling pathway that leads to activation of NADPH oxidase. Moreover, signaling by this pathway appears independent of activation of GAP, SHP-2, and PLC-γ1.

Additional evidence suggests that Rac functions downstream of PI3K (41–44). Thus, the exchange of Rac-bound GDP for GTP catalyzed by guanine nucleotide exchange factors (GEFs) is stimulated by phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$), a product of the action of PI3K. A family of GEF proteins that mediate the activation of Rac-related proteins has been identified. All members of this family, including Vav, Sos, and Pix, contain a pleckstrin homology domain that binds inositol-containing phospholipids such as $PI(4,5)P_2$ and $PI(3,4,5)P_3$ (44–47). $PI(4,5)P_2$, when bound to the pleckstrin homology domain of Vav, inhibited activation of Vav GEF activity by the protein-tyrosine kinase Lck, whereas $PI(3,4,5)P_3$ enhanced phosphorylation and activation of Vav by Lck (42). Thus, the activation of PI3K might serve to convert a Vav inhibitor to an activator, resulting in a rapid transformation of inactive, GDP-bound Rac to its active, GTP-bound form. Sos possesses two distinct domains that allow it to function as a GEF for both Ras and Rac; the Rac GEF activity requires the binding of activated Ras to the Ras GEF domain of Sos as well as the binding of $PI(3,4,5)P_3$ to the pleckstrin homology domain (43). Although not yet demonstrated, the GEF activity of Pix is also likely dependent on a product of PI3K.

The mechanism by which GTP-bound Rac increases the production of superoxide (and thus H_2O_2) in nonphagocytic cells is not clear. In phagocytes, activated Rac1 associates directly with p67-phox to stimulate NADPH oxidase (48, 49). Although the NADPH oxidase system in nonphagocytic cells is not well characterized, a similar interaction might occur.

In summary, we have shown that the activation of PI3K is required for PDGF-induced H_2O_2 production. The essential role of PI3K is likely to provide $PI(3,4,5)P_3$ for the activation of Rac, a component of the H_2O_2-generating system in both phagocytes and nonphagocytic cells.

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J. Biol. Chem. 2000, 275:10527-10531.
doi: 10.1074/jbc.275.14.10527
