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Dependence of Peroxisome Proliferator-activated Receptor Ligand-induced Mitogen-activated Protein Kinase Signaling on Epidermal Growth Factor Receptor Transactivation*

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Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that function as ligand-activated transcription factors regulating lipid metabolism and homeostasis. In addition to their ability to regulate PPAR-mediated gene transcription, PPARα and γ ligands have recently been shown to induce activation of mitogen-activated protein kinases (MAPKs), which in turn phosphorylate PPARs, thereby affecting transcriptional activity. However, the mechanism for PPAR ligand-dependent MAPK activation is unclear. In the current study, we demonstrate that various PPARα (nafenopin) and γ (ciglitazone and troglitazone) agonists rapidly induced extracellular signal-regulated kinase (Erk) and/or p38 phosphorylation in rat liver epithelial cells (GN4). The selective epidermal growth factor receptor (EGFR) kinase inhibitors, PD153035 and ZD1839 (Iressa), abolished PPARα and γ agonist-dependent Erk activation. Consistent with this, PPAR agonists increased tyrosine autophosphorylation of the EGFR as well as phosphorylation at a putative Src-specific site, Tyr^{845}. Experiments with the Src inhibitor, PP2, and the antioxidant *N*-acetyl-L-cysteine revealed critical roles for Src and reactive oxygen species as upstream mediators of EGFR transactivation in response to PPAR ligands. Moreover, PPARα and γ ligands increased Src autophosphorylation as well as kinase activity. EGFR phosphorylation, in turn, led to Ras-dependent Erk activation. In contrast, p38 activation by PPARα and γ ligands occurred independently of Src, oxidative stress, the EGFR, and Ras. Interestingly, PPARα and γ agonists caused rapid activation of proline-rich tyrosine kinase or Pyk2; Pyk2 as well as p38 phosphorylation was reduced by intracellular Ca^{2+} chelation without an observable effect on EGFR and Erk activation, suggesting a possible role for Pyk2 as an upstream activator of p38. In summary, PPARα and γ ligands activate two distinct signaling cascades in GN4 cells leading to MAPK activation.

Peroxisome proliferator-activated receptors (PPARs)[1] α, γ, and δ are members of the nuclear hormone receptor family and function as ligand-activated transcription factors (1). Genes regulated by PPARs have crucial roles in lipid metabolism, cellular differentiation, glucose homeostasis, eicosanoid signaling, and inflammation (2, 3). These receptors have thus become an attractive target in the treatment of hyperlipidemia, noninsulin-dependent diabetes, coronary artery disease, inflammation, and possibly cancer prompting the development of synthetic PPAR agonists by the pharmaceutical industry.

The efficacy of PPAR ligands in modulating lipid homeostasis has been largely attributed to their ability to modulate gene transcription in a PPAR-dependent manner. For example, PPARα ligands are unable to up-regulate the expression of genes involved in the cellular uptake and β-oxidation of fatty acids in PPARα knockout mice (4). PPARγ was shown to be necessary and sufficient to promote adipocyte differentiation (5, 6). Furthermore, studies revealed that targeted activation of PPARδ in mice resulted in complete resistance to both high fat diet-induced and genetically predisposed obesity (7). Thus, activation of PPARs is clearly a critical mechanism by which PPAR agonists function.

Recently, both PPARα and γ ligands were shown to activate members of the mitogen-activated protein kinase (MAPK) family (8–11). This event occurred at times too rapid to account for new protein synthesis, suggesting that these agonists also exert PPAR-independent effects. Although these "nongenomic" effects of PPAR ligands are contradictory to the classical mechanism of steroid hormone action, additional studies have illustrated that a variety of such compounds (*e.g.* progesterone, estrogen, and vitamin D) evoke similar rapid changes in kinase-mediated signal transduction pathways that contribute to

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[1] The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; EGF, epidermal growth factor; EGFR, EGF receptor; nafenopin, 2-methyl-2-[p-(1,2,3,4-tetrahydro-1-napthyl)-phenoxyl]proionic acid; ciglitazone, (±)-5-[4-(1-methylcyclohexylmethoxy)-benzyl]thiazolidine-2,4-dione; troglitazone, (±)-5-[4-[(6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)methoxy]-benzyl]-2,4-thiazolidinedione; Wy-14,643, pirinixic acid; PD153035, 4-[(3-bromophenyl)amino]-6,7-dimethoxyquinazoline; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine; ZD1839, 4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholinopropoxy)-quinazoline; PP3, 4-amino-7-phenylpyrazol[3,4-d]pyrimidine; U0126, 1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene; BAPTA-AM, 1,2 bis-(*o*-aminophenyoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester; ROS, reactive oxygen species; PKC, protein kinase C; ER, estrogen receptor.

their biological mechanism of action (12). Moreover, *in vitro* and *in vivo* experiments have revealed that PPARα and γ can be phosphorylated by some (α) or all three (γ) of the well known mammalian MAPKs (extracellular signal-regulated kinase (Erk), p38, and c-Jun N-terminal kinase) leading to modulation of transcriptional activity (13–16). Transcriptional changes induced by PPARα and γ agonists can also be dissociated into distinct MAPK- and PPAR-dependent pathways (17), suggesting that MAPKs alone mediate some of the cellular effects of PPAR ligands. Thus, kinase activation by PPAR agonists appears to play an important role in the mechanism of action of these compounds; yet few studies have investigated how PPAR agonists stimulate MAPKs.

A classical mechanism for Erk activation is dependent upon the epidermal growth factor receptor (EGFR) (18). Binding of an extracellular ligand (*i.e.* EGF) to the EGFR increases receptor autophosphorylation on multiple tyrosine residues, which is followed by activation of Ras as well as Ras-dependent downstream kinase signaling cascades. Interestingly, recent reports showed that an EGFR kinase inhibitor blocked Erk activation by the PPARα agonist Wy-14,643 (19), suggesting possible cross-talk between EGFR and PPAR ligand-induced signaling. In addition to its role in relaying EGF-dependent signals to the cytosol, the EGFR has recently emerged as a critical transducer of intracellular signals in the absence of physiological ligands (20–22). This ligand-independent EGFR "transactivation" has been implicated as a central integrator by which multiple endogenous and synthetic compounds activate intracellular kinases leading to a variety of cellular responses.

In the present study, we provide evidence that PPARα and γ ligands induce ligand-independent EGFR phosphorylation or transactivation in a liver epithelial cell line. EGFR phosphorylation appears to require the nonreceptor tyrosine kinase Src, involves reactive oxygen species, and leads to downstream activation of Erk but not p38 MAPK. Collectively, these data depict a novel mechanism by which PPARα and γ agonists activate MAPKs and identify the EGFR as a key initiator of the mechanism of action of these compounds.

EXPERIMENTAL PROCEDURES

Materials—Nafenopin (Ciba-Geigy) is a nonfibrate hypolipidemic PPARα agonist. The thiazolidinediones ciglitazone and troglitazone (Biomol) are PPARγ ligands. These compounds were prepared as stock solutions in dimethyl sulfoxide. Human recombinant EGF was purchased from Invitrogen. PD153035, PP2, and PP3 were purchased from Calbiochem. ZD1839, synthesized as described previously (23), was provided by David Rusnak of GlaxoSmithKline. U0126 was purchased from Promega, and BAPTA-AM was from Molecular Probes. MK886 and GM6001 were from Biomol, and 2-chloro-5-nitrobenzanilide was purchased from Cayman Chemical. Dexamethasone, *N*-acetyl-L-cysteine, glutathione, and 12-*o*-tetradecanoylphorbol-13-acetate were purchased from Sigma. Anti-phospho-Erk monoclonal antibody (E-4), anti-Erk polyclonal antibody (C-14), anti-p38 polyclonal antibody (C-20-G), anti-Src monoclonal antibody (B-12), and anti-phosphotyrosine (pan) (PY99) monoclonal antibody were all purchased from Santa Cruz Biotechnology. Anti-phospho-p38, anti-phospho-EGFR (Tyr^{845}), anti-EGFR, anti-phospho-Src (Tyr^{416}), anti-phospho-PKCα/βII, anti-phospho-PKCδ, anti-phospho-PKCζ/λ, and anti-phospho-PKC (pan) polyclonal antibodies were all purchased from Cell Signaling. Anti-Pyk2 monoclonal antibody was from Transduction Laboratories. Anti-EGFR C-terminal polyclonal antibody (#22) and anti-Pyk2 C-terminal polyclonal antibody (#72) were generated as previously described (24, 25). [γ-^{32}P]ATP was purchased from PerkinElmer Life Sciences.

Cell Culture—Rat liver epithelial cells, GN4, were grown in Richter's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin/amphotericin B as described previously (26). Ras(N17) stable integrated GN4 cell lines were established as detailed elsewhere (24). Prior to experiments, cells at 70–80% confluency were serum-starved overnight in Richter's minimum essential medium containing 0.1% fetal bovine serum. In certain experiments, Ras(N17) expression was induced at the time of serum starvation with 1 μM dexamethasone. Similarly, depletion of PKC was achieved by overnight treatment of cells with 5 μM 12-*o*-tetradecanoylphorbol-13-acetate.

Cell Lysate Preparation—Following stimulation for the times indicated, media was aspirated, and the cells were rinsed twice with ice-cold PBS. The cells were then scraped into ice-cold RIPA buffer (150 mM NaCl, 9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS, pH 7.4) with freshly added 200 μM Na_3VO_4, 250 μM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 10 nm microcysteine. The cell lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4 °C. Protein concentration of the supernatant was determined using the Coomassie protein assay reagent (Pierce).

Immunoblotting—In a typical experiment, 10 μg of cell lysate was resuspended in SDS-PAGE sample buffer (0.5 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.1% bromphenol blue) and heated at 95 °C for 5 min to denature proteins. The lysates were then resolved by SDS-PAGE on Novex precast 10% Tris-glycine gels (Invitrogen) and transferred to polyvinylidene fluoride (Immobilon-P; Millipore). The immunoblots were incubated with the appropriate primary antibody overnight at 4 °C, washed three times with TBST, and probed with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoblots were then developed with ECL (Amersham Biosciences) according to the manufacturer's instructions and visualized by autoradiography (Kodak X-Omat Blue film). In certain instances, the membranes were stripped in buffer (62.5 mM Tris, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol) at 55 °C for 30 min and reprobed with another antibody.

Immunoprecipitation—Following stimulation, the cells were rinsed as described above and scraped into ice-cold RIPA buffer without SDS. The lysates were cleared by centrifugation. 500 μg of cell lysate was immunoprecipitated by incubation with the antibody overnight at 4 °C under slight agitation. Twenty μl of protein A-agarose beads (Santa Cruz Biotechnology) were added to each sample, which were then incubated an additional hour at 4 °C. Immune complexes were collected by brief centrifugation in a microcentrifuge and washed four times in ice-cold lysis buffer. The remaining wash buffer was carefully removed with a Hamilton syringe; the immune complexes were then resuspended in SDS-PAGE sample buffer and resolved by SDS-PAGE as described above.

In Vitro Src Kinase Assay—Src kinase activity in stimulated GN4 cells was measured using a commercial Src assay kit (Upstate Biotechnology, Inc.) according to the manufacturer's instructions with slight modifications. Briefly, Src was immunoprecipitated as described above from 200 μg of cell lysate by overnight incubation with an anti-Src antibody (B-12; Santa Cruz Biotechnology). Src activity present in immune complexes was assessed by measuring the transfer of the γ-phosphate of [γ-^{32}P]ATP to a specific Src substrate peptide for 10 min at 30 °C. Phosphorylated substrate was then separated from residual [γ-^{32}P]ATP using P-81 phosphocellulose paper (Whatman) and quantified with a scintillation counter.

RESULTS

PPAR Ligands Activate Erk and p38 MAPK in GN4 Cells: Role of the EGFR—A rat liver epithelial cell line (GN4) was used to investigate the effects of PPAR ligands on MAPK activation. This cell line was used previously by our laboratory to examine the role of the EGFR and other kinases (*e.g.* proline-rich tyrosine kinase or Pyk2, also known as calcium-dependent tyrosine kinase/related adhesion focal tyrosine kinase/cell adhesion kinase β) in mediating angiotensin II-induced cell signaling (24, 25). In addition, the hepato-specific effects of PPARα ligands are well documented, and the liver is considered to be a target organ for the insulin-sensitizing actions of thiazolidinediones (4, 27); thus, GN4 cells provided a model cell line for use in the current study. Treatment of GN4 cells with either the PPARα agonist nafenopin (Fig. 1*A*) or the PPARγ agonist ciglitazone (Fig. 1*B*) caused transient stimulation of Erk that reached a peak at 10 min and declined thereafter to basal levels over 45 min (Fig. 2, *A* and *B*). Both nafenopin and ciglitazone also transiently activated p38; however, the time course for p38 activation differed from that of Erk with maximal phosphorylation in response to nafenopin appearing 10–30 min after stimulation, whereas ciglitazone-induced p38 activation peaked between 5 and 10 min. MAPK activation by

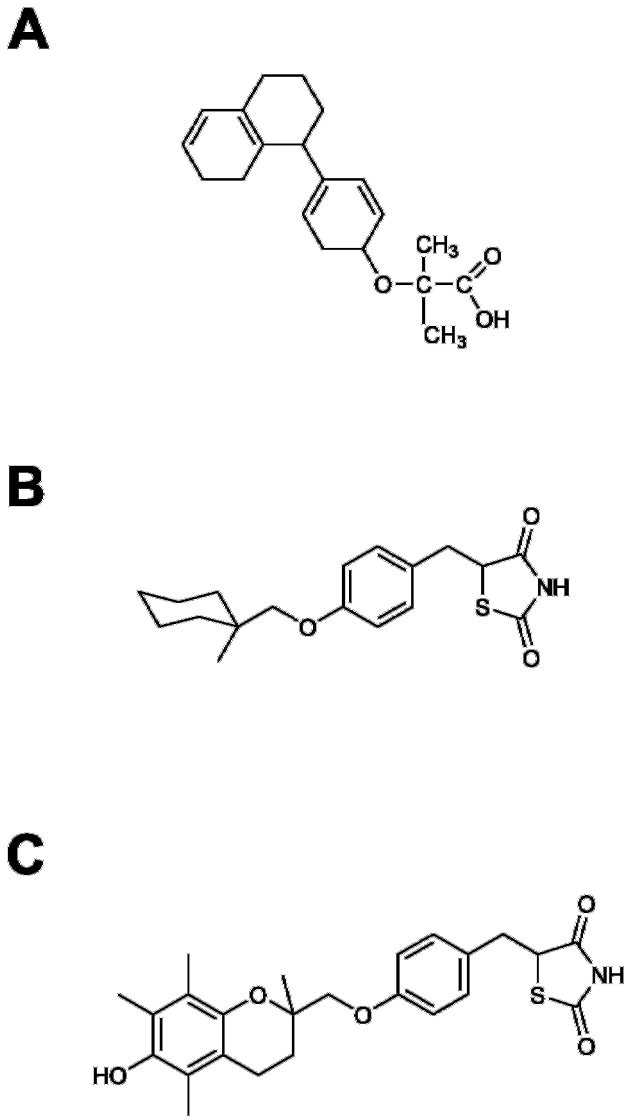

FIG. 1. **Chemical structures of PPARα and γ ligands.** *A*, nafenopin. *B*, ciglitazone. *C*, troglitazone.

nafenopin and ciglitazone was also concentration-dependent over a 20–100 μM range (data not shown). Similar to ciglitazone, the PPARγ agonist troglitazone (Fig. 1*C*) induced p38 phosphorylation; in contrast, however, troglitazone was a weaker activator of Erk (Fig. 2*C*). Thus, these data demonstrate that both PPARα and γ agonists are capable of activating Erk and p38 in GN4 cells.

Erk activation by the PPARα ligand Wy-14,643 was previously shown to be blocked by a nonspecific EGFR kinase inhibitor (19). To examine the involvement of the EGFR in MAPK activation by PPARα and γ ligands in GN4 cells, the ability of these compounds to activate Erk and p38 in the presence of specific EGFR kinase inhibitors, PD153035 and ZD1839, was evaluated. Pretreatment with either PD153035 or ZD1839 blocked EGF-induced Erk phosphorylation as expected (Fig. 3). Interestingly, inhibition of EGFR kinase activity also abolished nafenopin and ciglitazone-induced Erk activation. In contrast, EGFR kinase inhibition had no effect on p38 activation by these ligands. Taken together, these findings suggest that MAPK phosphorylation by PPAR ligands occurs via EGFR kinase-dependent as well as EGFR kinase-independent pathways.

PPAR Agonists Induce EGFR Phosphorylation—The ability of a specific EGFR kinase inhibitor to prevent Erk activation by PPARα and γ ligands suggested that these compounds directly

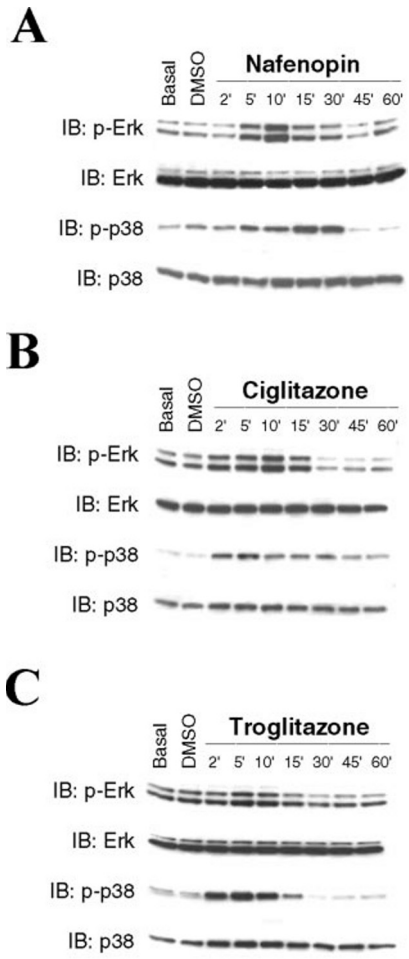

FIG. 2. **PPARα and γ ligands rapidly activate Erk and p38 MAPKs in GN4 cells.** Rat liver epithelial cells (GN4) were grown to near confluency and starved overnight in medium containing 0.1% fetal bovine serum. The cells were stimulated with 50 μM nafenopin (*A*), 50 μM ciglitazone (*B*), 50 μM troglitazone (*C*), or dimethyl sulfoxide (0.1%) as a vehicle control for the indicated times. The cell lysates were prepared and subjected to 10% SDS-PAGE. Activated Erk and p38 were detected by immunoblotting (*IB*) using anti-phospho-Erk and anti-phospho-p38 antibodies. The blots were then stripped and reprobed using antibodies directed against total Erk and p38, respectively. *DMSO*, dimethyl sulfoxide.

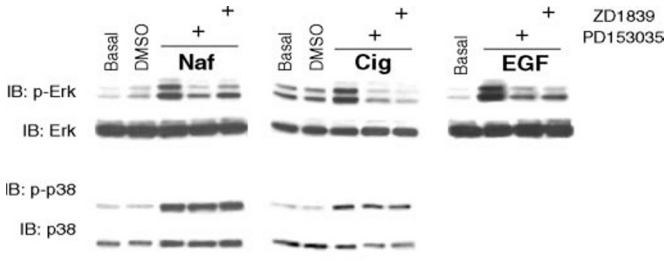

FIG. 3. **PPAR ligand-induced Erk but not p38 activation is sensitive to EGFR kinase inhibition.** GN4 cells cultured as described in the legend to Fig. 2 were preincubated with or without the EGFR kinase inhibitors PD153035 (1 μM for 2 h) or ZD1839 (10 μM for 1 h), followed by acute stimulation with nafenopin (*Naf*, 50 μM), ciglitazone (*Cig*, 50 μM), or EGF (100 ng/ml) for 10 min. Immunoblotting (*IB*) was performed as described under "Experimental Procedures" to detect changes in Erk and p38 phosphorylation. *DMSO*, dimethyl sulfoxide.

affect EGFR phosphorylation status and kinase activity. To test this hypothesis, EGFR phosphorylation in response to PPAR agonists was assessed. Immunoprecipitation of the EGFR followed by immunoblotting with a pan anti-phosphoty-

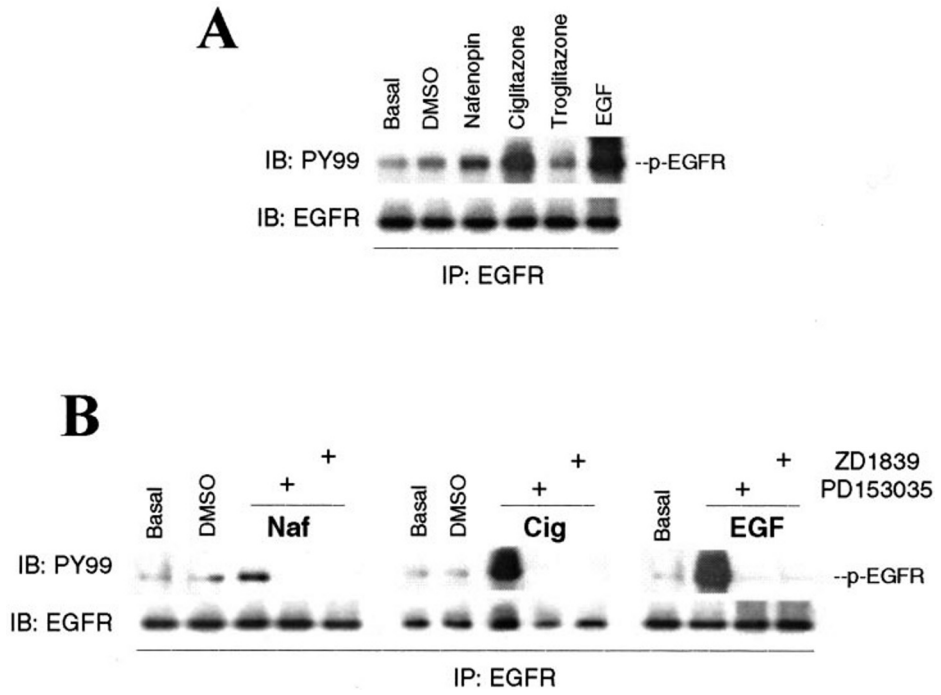

FIG. 4. **PPARα and γ agonists induce EGFR phosphorylation in an EGFR kinase-dependent manner.** Serum-starved GN4 cells were treated with nafenopin (*Naf*, 50 μM), ciglitazone (*Cig*, 50 μM), troglitzone (50 μM), or EGF (100 ng/ml) for 10 min (*A*). Prior to stimulation, some cells were preincubated with PD153035 or ZD1839 as described in Fig. 3 (*B*). EGFR was immunoprecipitated from cell lysates, and samples from each lysate were resolved by SDS-PAGE. The immunoblots (*IB*) were probed with a pan anti-phosphotyrosine antibody, PY99, or an anti-EGFR antibody. *DMSO*, dimethyl sulfoxide.

rosine antibody showed that treatment of GN4 cells with nafenopin and ciglitazone induced EGFR phosphorylation (Fig. 4*A*). Interestingly, these compounds displayed different potencies for activating the EGFR, with ciglitazone being much stronger than nafenopin. The time course for EGFR phosphorylation was rapid (~5–10 min; data not shown) and closely resembled that observed for Erk activation, supporting our data that activation of Erk requires the EGFR. Indeed, pretreatment of GN4 cells with PD153035 or ZD1839 abolished receptor phosphorylation in response to nafenopin and ciglitazone (Fig. 4*B*). In comparison, troglitazone failed to significantly increase EGFR activation. This finding is consistent with its weaker effects on Erk activation.

Ras Acts Downstream of EGFR to Mediate Erk but Not p38 Activation—Erk is known to be activated by both Ras-dependent and Ras-independent pathways in GN4 cells (24). In support of Ras-dependent Erk activation, PPAR agonists increased EGFR phosphorylation at Tyr^{1068} (data not shown), the major Grb2-binding site within the EGFR (28). To further examine whether Ras is required for PPARα and γ ligand-induced Erk activation, GN4 cells (S19) expressing a dominant negative form of Ha-Ras, Ras(N17), under the control of an inducible promoter, were used as described previously (24). To verify that induction of Ras(N17) in GN4.S19 cells was sufficient to interfere with endogenous Ras signaling, EGF-dependent Erk activation was assessed. As expected, EGF caused robust Erk phosphorylation in cells in the absence of Ras(N17) induction (Fig. 5*A*). In contrast, induction of Ras(N17) via dexamethasone pretreatment blocked EGF-dependent Erk activation. To determine whether Ras is required for MAPK activation by PPARα and γ ligands, MAPK phosphorylation was examined in GN4.S19 cells pretreated with or without dexamethasone. Although EGFR activation was unaffected (data not shown), induction of Ras(N17) prevented Erk phosphorylation by nafenopin and ciglitazone (Fig. 5*A*). This result suggests that PPAR ligands activate Erk through a Ras-dependent pathway

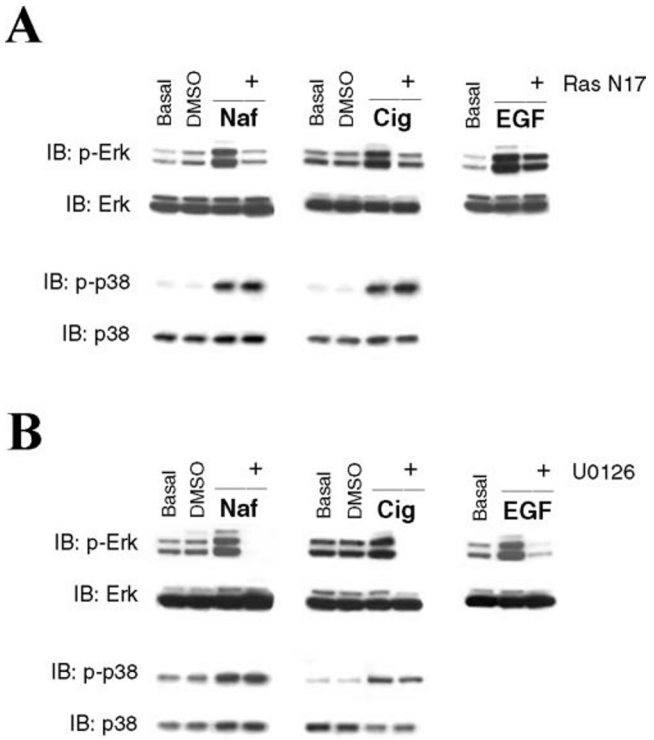

FIG. 5. **Ras acts upstream of Erk but not p38 to facilitate MAPK phosphorylation by PPARα and γ ligands.** Ras(N17) transfected GN4 cells (GN4.S19) (*A*) and wild-type GN4 cells (*B*) were pretreated with or without dexamethasone (1 μM, 24 h) or U0126 (10 μM, 1 h), respectively, followed by exposure to nafenopin (*Naf*, 50 μM), ciglitazone (*Cig*, 50 μM), or EGF (100 ng/ml) for 10 min. The cell lysates were prepared and then resolved by SDS-PAGE as described under "Experimental Procedures." Activated Erk and p38 were detected by immunoblotting (*IB*) using anti-phospho-Erk and anti-phospho-p38 antibodies. The blots were stripped and reprobed for total Erk and p38, respectively. *DMSO*, dimethyl sulfoxide.

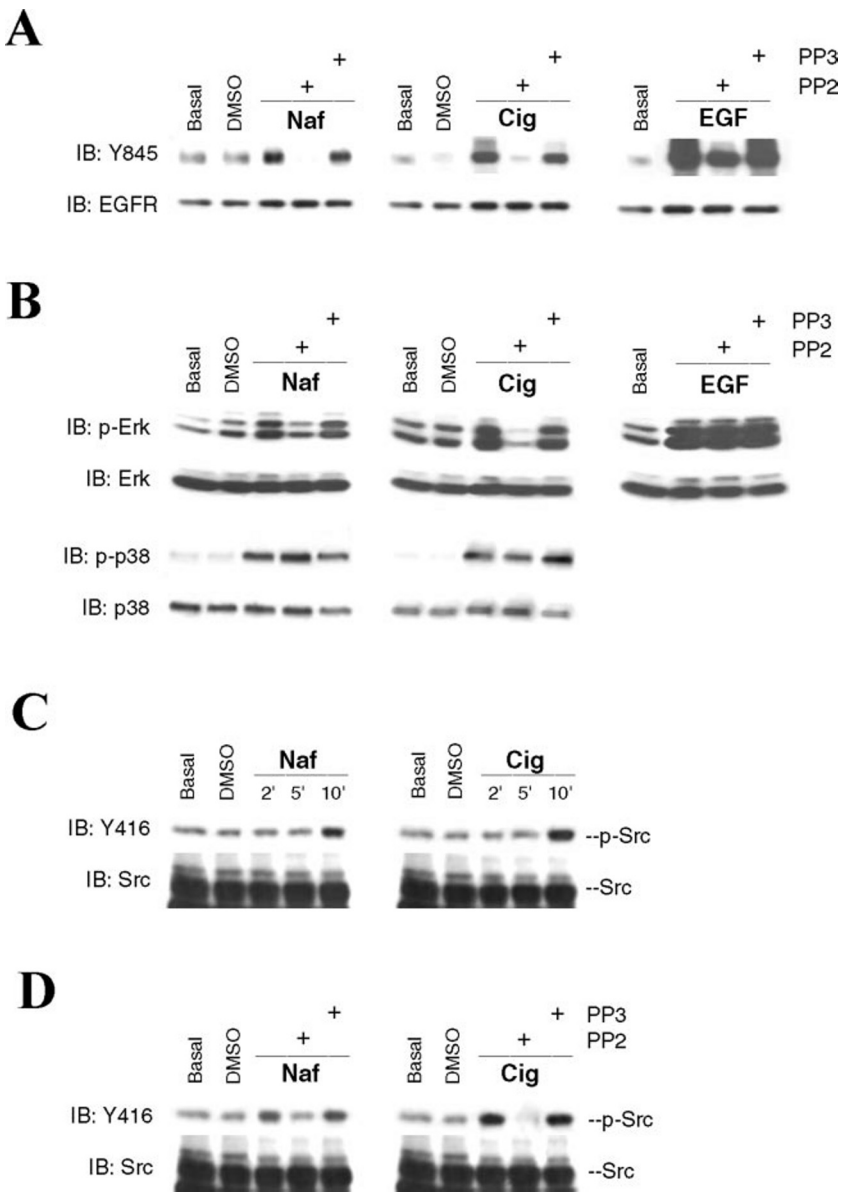

FIG. 6. **PPAR agonist-induced EGFR and Erk but not p38 phosphorylation are sensitive to PP2 and require Src.** *A*, GN4 cells were preincubated with or without PP2 or PP3 (10 μM, 1 h), followed by stimulation with nafenopin (*Naf*, 50 μM), ciglitazone (*Cig*, 50 μM), or EGF (100 ng/ml) for 10 min as indicated. Changes in EGFR phosphorylation at Tyr^{845} were assessed using an anti-phospho-EGFR antibody (Y845). Total EGFR was determined by immunoblotting (*IB*) with an anti-EGFR antibody. *B*, cells were treated as discussed for *A*. The effect of PP2/PP3 pretreatment on PPAR ligand-induced Erk and p38 activation was determined by immunoblotting using anti-phospho-Erk and anti-phospho-p38 antibodies. The blots were stripped and reprobed for total Erk and p38, respectively. *C*, serum-starved GN4 cells were treated with nafenopin or ciglitazone (50 μM) for the times indicated. The cell lysates were resolved by SDS-PAGE, and changes in Src phosphorylation at Tyr^{416} were determined by immunoblotting with anti-phospho-Src antibody (Y416). Total Src was assessed by immunoblotting with an anti-Src antibody. *D*, GN4 cells were treated with nafenopin and ciglitazone as discussed for *A*. The effect of PP2/PP3 pretreatment on PPAR agonist-induced Src phosphorylation was examined by immunoblotting as described for *C*. *DMSO*, dimethyl sulfoxide.

in GN4 cells. In contrast, Ras(N17) had no effect on p38 phosphorylation by these compounds.

To provide additional evidence that PPARα and γ agonist-induced Erk and p38 activation are triggered via Ras-dependent and independent pathways, respectively, MAPK phosphorylation in response to these ligands was determined in the presence of U0126, a MAPK kinase inhibitor. In agreement with the Ras(N17) data, U0126 inhibited Erk activation by nafenopin and ciglitazone (Fig. 5*B*). Furthermore, p38 phosphorylation was unaffected by U0126. Although PKC-dependent, Ras-independent signals have been implicated in Erk activation in GN4 cells (24), we were unable to detect PKC activation in response to PPARα and γ agonists. In addition, PKC down-regulation had no effect on Erk phosphorylation (data not shown). These data suggest that Erk activation by PPAR ligands is PKC-independent and supports our finding that Erk phosphorylation requires Ras.

Role of Src in EGFR Activation by PPAR Ligands—Recent studies have revealed a central role for the EGFR in mediating MAPK activation in response to a variety of nonligands through a mechanism known as transactivation (21, 29, 30). Multiple mechanisms have been shown to mediate EGFR transactivation, and they are largely dependent upon the particular stimulus. One potential mechanism responsible for EGFR transactivation involves the nonreceptor tyrosine kinase Src. Specifically, Src has been shown to directly phosphorylate the EGFR at Tyr^{845} and Tyr^{1101}, leading to receptor activation (31). Using an antibody that recognizes the EGFR when phosphorylated at Tyr^{845}, we observed that both nafenopin and ciglitazone induced phosphorylation of the EGFR at this site, whereas treatment with troglitazone did not (Fig. 6*A* and data not shown).

To further determine the role of Src in PPARα and γ agonist-induced EGFR phosphorylation, the ability of these compounds to activate the EGFR was evaluated in the presence of PP2, a selective Src kinase inhibitor. As shown in Fig. 6*A*, PP2 completely blocked EGFR phosphorylation at Tyr^{845} by both nafenopin and ciglitazone. Pretreatment of GN4 cells with PP3, a pharmacologically inactive analog of PP2, did not prevent receptor phosphorylation, suggesting that these inhibitory effects were specific for PP2. Consistent with the finding that a PP2-sensitive kinase, such as Src, is important for EGFR phosphorylation by PPAR ligands, PP2 but not PP3 also blunted Erk activation by nafenopin and ciglitazone (Fig. 6*B*). Importantly, PP2 failed to significantly effect EGFR and Erk phosphorylation in response to EGF. This finding provides compel-

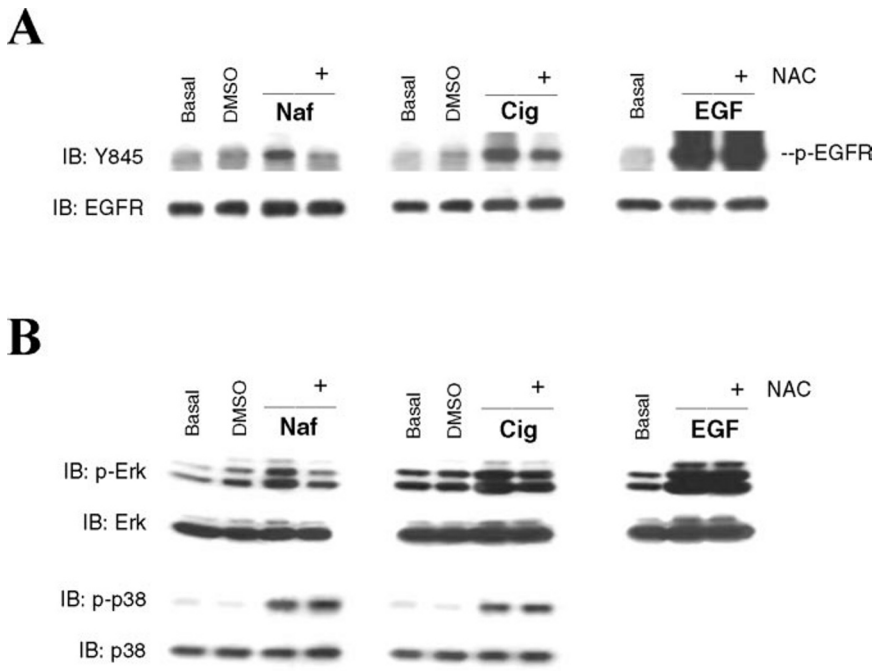

FIG. 7. **PPARα and γ ligand-dependent EGFR and Erk but not p38 activation involves reactive oxygen species.** GN4 cells were pretreated with or without *N*-acetyl-L-cysteine (*NAC*, 10 mM, pH adjusted to 7.5, for 30 min), followed by stimulation with nafenopin (*Naf*, 50 μM), ciglitazone (*Cig*, 50 μM), or EGF (100 ng/ml) for 10 min. Changes in EGFR phosphorylation at Tyr^{845} as well as changes in MAPK activation were assessed by immunoblotting (*IB*) with an anti-phospho-EGFR antibody (Y845) (*A*), an anti-phospho-Erk antibody, and an anti-phospho-p38 antibody (*B*). Total EGFR, Erk and p38 are shown in the *lower panels*. *DMSO*, dimethyl sulfoxide.

ling evidence that PPAR agonists transactivate the EGFR by a mechanism distinct from that of the natural ligand EGF. Interestingly, PP2 did not affect the ability of PPARα and γ agonists to activate p38 (Fig. 6*B*). Because Src appears to play an important role in EGFR transactivation by PPAR ligands, this finding in is agreement with our earlier observations that induction of p38 by these compounds is independent of the EGFR.

Further, the ability of nafenopin and ciglitazone to stimulate Src activity was examined. Using an antibody that recognizes Src when phosphorylated at Tyr^{416}, the major autophosphorylation site (32), we observed that both nafenopin and ciglitazone increased Tyr^{416} phosphorylation. Src was maximally activated within 10 min after exposure to these compounds, a time frame that coincides with EGFR and Erk phosphorylation (Fig. 6*C*). Similar to their effect on the EGFR, ciglitazone was a more potent Src activator than nafenopin. In addition, nafenopin and ciglitazone-induced Src activation was reduced by PP2 but not PP3 (Fig. 6*D*).

Oxidative Stress-dependent Effects of PPAR Agonists—Changes in intracellular oxygen tensions have been shown to induce activation of multiple protein kinases including Src, the EGFR, Erk, and p38 (33–35). To elucidate the involvement of ROS in kinase signaling by PPARα and γ ligands, GN4 cells were treated with the glutathione precursor *N*-acetyl-L-cysteine prior to stimulation with these compounds. *N*-Acetyl-L-cysteine attenuated EGFR phosphorylation at Tyr^{845} by both nafenopin and ciglitazone but had no effect on the EGF-induced response (Fig. 7*A*). Similar results were obtained when cells were preincubated with reduced glutathione (data not shown). In addition to its effect on EGFR phosphorylation, *N*-acetyl-L-cysteine also reduced PPAR ligand-induced Erk activation (Fig. 7*B*). Consistent with our observation that Erk and p38 activation are mediated by separate kinase signaling pathways, p38 phosphorylation in response to nafenopin and ciglitazone was not affected by antioxidants.

Pyk2 Is Activated by PPAR Ligands—In addition to Src, another nonreceptor tyrosine kinase, the calcium-dependent, proline-rich tyrosine kinase or Pyk2 has been implicated in ligand-independent EGFR phosphorylation (36) as well as activation of Erk (37), p38 (38), and c-Jun N-terminal kinase (39). Interestingly, Pyk2 is highly expressed in GN4 cells (25), suggesting that it could play a role in PPAR ligand-induced EGFR transactivation. To test this hypothesis, Pyk2 was immunoprecipitated from GN4 cells following stimulation with PPARα and γ agonists. Immunoblotting with a pan anti-phosphotyrosine antibody revealed that nafenopin, ciglitazone, and troglitazone rapidly induced Pyk2 phosphorylation with activation occurring as early as 90 s after stimulation (Fig. 8*A* and data not shown). Pretreatment of GN4 cells with the intracellular calcium chelator BAPTA-AM, which has been previously shown to inhibit Pyk2 activation by angiotensin II (40, 41), blocked Pyk2 phosphorylation by PPAR ligands. Although BAPTA-AM had minimal effects on EGFR and Erk activation by these compounds (data not shown), p38 phosphorylation in response to ciglitazone and troglitazone was reduced (Fig. 8*B*), suggesting that Pyk2 is an upstream activator of p38.

DISCUSSION

The hypolipidemic and insulin-sensitizing actions of PPARα and γ agonists, respectively, have been well documented. In addition to these PPAR-mediated, transcription-dependent effects, PPAR ligands also rapidly activate MAPKs most likely via nongenomic signaling. This effect on MAPK signaling has been demonstrated in multiple cell types in response to a variety of PPAR agonists (8–11); yet the mechanism responsible for MAPK activation has not been clearly defined. Here, we show that PPAR ligands cause rapid, transient activation of Erk and/or p38 in liver epithelial cells. MAPK phosphorylation is mediated by two independent kinase signaling pathways: 1) Src-dependent EGFR transactivation leading to Erk activation and 2) EGFR-independent p38 phosphorylation that correlates with Pyk2 activation (Fig. 9).

A role for the EGFR in mediating the carcinogenic effects of peroxisome-proliferating PPARα agonists has been suggested by previous studies. For example, Wy-14,643-dependent Erk activation as well as increases in immediate early genes were prevented by a nonspecific EGFR kinase inhibitor (19). In addition, EGF and PPARα ligands were shown to act synergistically to promote the clonal expansion of hepatocytes (42), suggesting possible cross-talk between these signaling pathways. The peroxisome proliferator ciprofibrate was in fact

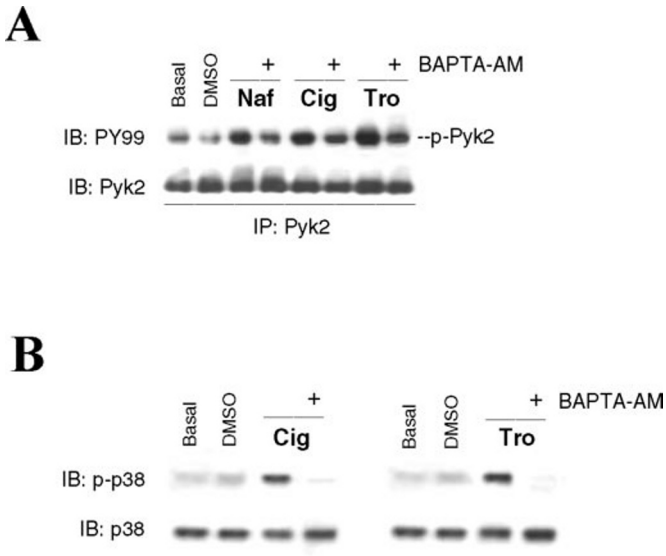

FIG. 8. **Pyk2 is activated by PPARα and γ ligands: correlation with p38 phosphorylation.** *A*, serum-starved GN4 cells were treated with the PPAR ligands nafenopin (*Naf*, 50 μM), ciglitazone (*Cig*, 50 μM), or troglitazone (*Tro*, 50 μM) for 1.5 min. Pyk2 was immunoprecipitated from cell lysates, and the immune complexes were resolved by SDS-PAGE. Immunoblots (*IB*) were probed with a pan anti-phosphotyrosine, PY99, antibody or an anti-Pyk2 antibody. *B*, cells were treated with ciglitazone and troglitazone as described for *A* for 10 min. The cell lysates were subjected to SDS-PAGE; immunoblots were probed with anti-phospho-p38 antibodies. The blots were stripped and reprobed for total p38. Prior to stimulation with PPARα and γ agonists, some cells were preincubated with BAPTA-AM (50 μM, 20 min). *DMSO*, dimethyl sulfoxide.

shown to increase EGFR phosphorylation in isolated rat hepatocytes (43). Our data support these earlier findings and, importantly, depict a mechanism identifying the EGFR and Ras as critical upstream regulators of Erk phosphorylation in response to PPARα agonists. Activation of this kinase-driven signaling pathway in liver epithelial cells was not specific to PPARα ligands because the PPARγ agonist ciglitazone also activated Erk in an EGFR kinase-dependent manner. To our knowledge, this is the first evidence that PPARγ ligands influence MAPK activation through specific phosphorylation of the EGFR.

The mechanism for EGFR transactivation by nonligands is variable depending on the particular stimulus and cell type; proteolytic cleavage of EGF-like ligands (*e.g.* heparin-binding EGF) from the cell surface by matrix metalloproteinases (44), nonreceptor tyrosine kinases (*i.e.* Src) (36), oxidative stress (34), cell adhesion (45), G-protein-coupled receptors (30), and cytokine receptors (46) have all been associated with EGFR transactivation. The data presented here support the hypothesis that EGFR phosphorylation by PPARα and γ agonists in liver epithelial cells is dependent on Src. PPARα and γ ligands increase Src autophosphorylation at times consistent with their effects on the EGFR and MAPKs. These compounds cause selective phosphorylation of the EGFR at Tyr^{845}, a target site for Src action (31). Ciglitazone, a stronger activator of Src than nafenopin, also induces more significant EGFR phosphorylation. Similar results were obtained using an *in vitro* Src kinase assay; in these studies, nafenopin and ciglitazone activated Src 1.94 ± 0.17- and 5.20 ± 0.48-fold, respectively. Moreover, experiments using the selective Src kinase inhibitor PP2 and the inactive analog PP3 demonstrate a necessary role for Src in EGFR and downstream Erk activation by nafenopin and ciglitazone. In addition, PP2 but not PP3 prevented Src autophosphorylation by these ligands. Collectively, these data suggest that Src is a critical upstream mediator of PPAR agonist-induced kinase signaling in GN4 cells.

The data presented here also depict a role for ROS in EGFR transactivation by PPARα and γ ligands as the antioxidant *N*-acetyl-L-cysteine attenuated Tyr^{845}-specific EGFR as well as Erk phosphorylation. Inhibition of Tyr^{845} phosphorylation suggests that ROS may be involved in Src activation by nafenopin and ciglitazone. Interestingly, Src is known to be activated by oxidative stress (33). Whether nafenopin and ciglitazone induce oxidative stress in GN4 cells and whether this leads to Src phosphorylation requires further investigation; however, previous studies have shown that exposure to certain PPARα and γ ligands is associated with reactive oxygen species production (47, 48). Moreover, ciglitazone-induced superoxide generation was necessary for Erk activation in muscle cells and astrocytes (10, 48), supporting the present data that a ROS-dependent pathway is important for kinase activation by PPAR ligands. Collectively, our findings suggest a mechanism for EGFR transactivation whereby PPARα and γ ligands induce oxidative stress triggering Src, which then phosphorylates the EGFR leading to downstream Ras and ultimately Erk activation (Fig. 9).

Similar to what has been described for other nuclear hormone receptor agonists (12), evidence from our studies suggests that rapid MAPK activation by PPAR ligands occurs independently of PPAR-mediated transcription. Although ciglitazone strongly activated EGFR and Erk, another structurally related thiazolidinedione, troglitazone, failed to induce similar phosphorylation of these kinases. In addition, pretreatment of GN4 cells with the protein synthesis inhibitor cyclohexamide had no effect on PPAR ligand-induced kinase activation (data not shown). Recent studies focusing on another nuclear hormone receptor, the estrogen receptor (ER), support the existence of a membrane-associated ER that is functionally distinct from the classical ER (49). In contrast to being a ligand-activated transcription factor, this membrane-associated ER is thought to mediate the rapid nongenomic effects of estrogen. Specifically, the interaction of ligand-bound ER with a certain cofactor, modulator of nongenomic activity of estrogen receptor or MNAR, promotes Src activation (50), leading to cleavage of heparin-binding-EGF from the cell surface by MMPs, EGFR transactivation, and ultimately Erk phosphorylation (51). Progesterone receptor contains a polyproline motif that can directly and ligand-dependently interact with the SH3 domain of Src, also leading to an increase in kinase activity (52). Although these signaling events are similar to what is reported here for PPAR ligands, there is no precedence for a membrane-associated, transcription-independent PPAR, nor is there evidence that PPAR either directly or indirectly interacts with Src. In support of our hypothesis that PPAR ligand-induced kinase signaling is PPAR-independent, the PPARα antagonist MK886 and the PPARγ antagonist 2-chloro-5-nitrobenzanilide were unable to prevent Src activation by nafenopin and ciglitazone, respectively (data not shown). Moreover, the MMP inhibitor GM6001 did not significantly inhibit PPAR ligand-dependent EGFR phosphorylation (data not shown).

In addition to Erk activation, PPARα and γ agonists induce rapid phosphorylation of p38 in GN4 cells. Interestingly, the data presented here suggest that Erk and p38 activation occur through two independent signaling mechanisms. Although nafenopin, ciglitazone, and troglitazone potently activated p38, only nafenopin and ciglitazone induced significant EGFR and Erk phosphorylation. Inhibition of EGFR kinase activity completely blocks EGFR as well as Erk activation in response to nafenopin and ciglitazone, clearly demonstrating that Erk is a downstream target of the EGFR. In contrast, p38 activation is not sensitive to EGFR kinase inhibition. Furthermore, inhibi-

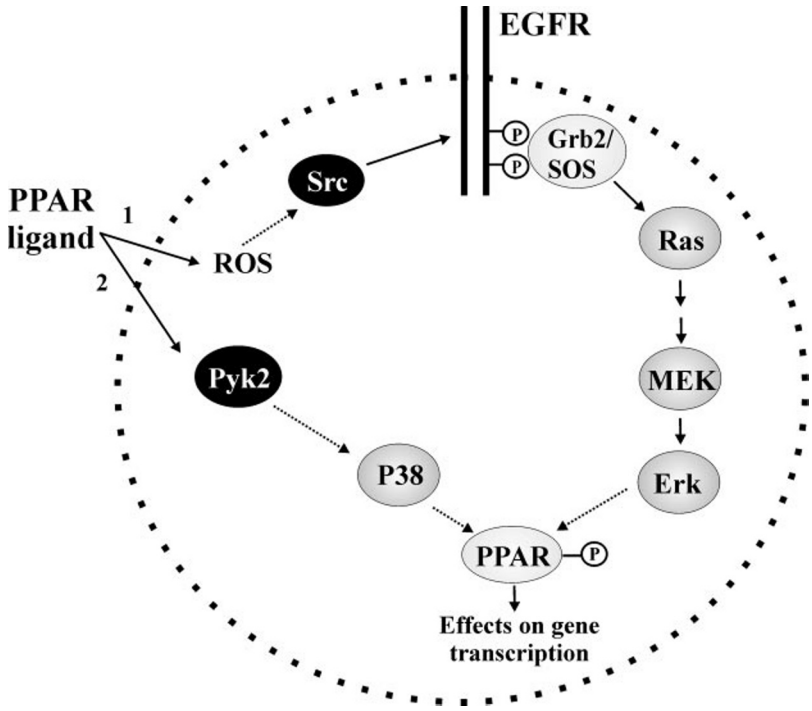

FIG. 9. **Schematic representation of the signaling pathways leading to MAPK activation by PPARα and γ ligands in GN4 cells.** Stimulation of rat liver epithelial cells with PPARα and γ agonists initiates two independent kinase-driven signaling cascades resulting in MAPK phosphorylation. *1*, PPAR ligands increase Src activity possibly by a mechanism involving ROS. Phosphorylated Src transactivates the EGFR, leading to the recruitment of adaptor proteins such as Grb2/SOS, which in turn promote Ras activation and subsequent Erk phosphorylation. *2*, simultaneously, p38 MAPK is activated by a Src/EGFR/Ras-independent pathway that could be mediated by Pyk2.

tion of Src, ROS generation, and Ras, which we show to be key mediators of EGFR and Erk phosphorylation, have no effect on p38 activation by PPAR ligands. This observation is in agreement with an earlier study showing that Wy-14,643-dependent p38 phosphorylation was not blocked by tyrphostin (19). Additionally, p38, in contrast to Erk, is classically activated by proinflammatory cytokines and/or environmental stresses rather than receptor tyrosine kinases (53).

In the current study, we demonstrate that Pyk2, a member of the focal adhesion kinase family, is strongly activated by PPARα and γ agonists in GN4 cells. Activation of Pyk2 was blocked by the calcium chelator BAPTA-AM, suggesting that PPAR ligands modulate intracellular calcium flux. Indeed, both troglitazone and ciglitazone (54) as well as the PPARα agonist Wy-14,643 have been shown to rapidly increase intracellular calcium.[2] Although calcium-dependent Pyk2 activation in GN4 cells has been well documented (25, 40, 41), the overall mechanism for Pyk2 phosphorylation remains unclear. Thus, further study is needed to determine how PPARα and γ agonists stimulate Pyk2. Based on our findings, it does not appear that Pyk2 plays a role in EGFR-dependent Erk activation by PPAR ligands. Although Pyk2 is activated at times prior to the EGFR, inhibition of Pyk2 is not correlated with reduced EGFR and Erk phosphorylation. Moreover, whereas troglitazone activated Pyk2, it failed to significantly induce EGFR phosphorylation. These data are consistent with previous studies in GN4 cells where Erk activation was shown to be Pyk2-independent (25). Interestingly, Pyk2 has been shown to act upstream of p38 in several cell types (38, 55). Here, inhibition of Pyk2 by intracellular Ca^{2+} chelation blunts p38 phosphorylation by ciglitazone and troglitazone, supporting these previous findings. Despite the observed correlation between Pyk2 and p38, a Pyk2-specific inhibitor is not available, and thus further work is needed to clearly define a connection between Pyk2 and p38 in GN4 cells.

In addition to the suggested role of Pyk2 as an upstream activator of p38, our observation that PPARγ ligands induce Pyk2 phosphorylation may also be relevant to their insulin-sensitizing actions. Many studies have found that thiazolidinediones increase the expression and membrane translocation of glucose transporters in adipocytes as well as muscle cells (56–58). The mechanism for this effect remains unclear, because evidence has shown these changes in glucose uptake to be both PPARγ-dependent and -independent (57, 58). Interestingly, sorbitol (59), endothelin-1 (60), and glucose (61) increase membrane localization of glucose transporters in a Pyk2-dependent manner. Whether Pyk2 activation by PPARγ ligands contributes to alterations in glucose in GN4 cells uptake remains to be determined.

Activation of MAPKs has previously been shown to increase phosphorylation of PPARα and γ, resulting in altered transcriptional activity depending on the isoform (13–16). Whereas the transcriptional capacity of PPARγ is inhibited by MAPK phosphorylation, phosphorylation of PPARα results in enhanced activity. The current study along with others (8–11) provides evidence that PPARα and γ ligands themselves activate MAPKs. This suggests that these compounds are not only agonists for PPARs but also influence PPAR transcriptional activity independent of receptor binding. In support of this, the PPARα agonist ciprofibrate was previously shown to increase both EGFR (43) as well as PPARα phosphorylation (62). Activation of PPARα is required for the hypolipidemic and carcinogenic effects of peroxisome-proliferating PPARα ligands (4, 63). The role of PPARγ in mediating glucose homeostasis is less clear; although it remains controversial, genetic studies in mice and humans suggest that decreased PPARγ activity is associated with increased insulin sensitivity (64–66). The ability of MAPKs to regulate PPAR activity gives them a critical role in mediating the effects of PPAR agonists. Here, we provide novel mechanistic evidence detailing independent kinase signaling pathways leading to MAPK activation by PPAR ligands in GN4 cells. The role of Src, the EGFR, and Pyk2 in modulating the transcriptional activity of PPARα and γ remains to be determined; yet identification of these kinases as well as an understanding of their roles in MAPK activation provide further insight into the molecular mechanism of action of these pharmaceutical agents.

[2] R. G. Thurman, unpublished observation.

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