Role of Tyrosine Kinase Activity of Epidermal Growth Factor Receptor in the Lysophosphatidic Acid-stimulated Mitogen-activated Protein Kinase Pathway*

(Received for publication, June 17, 1997, and in revised form, March 23, 1998)

Jess M. Cunnick‡§§, Jay F. Dorsey‡§, Todd Standley¶, James Turkson‡, Alan J. Kraker‡‡, David W. Fry‡‡, Richard Jove‡‡, and Jie Wu‡‡§§

From the §Molecular Oncology Program, H. Lee Moffitt Cancer Center and Research Institute, ¶Department of Medical Microbiology and Immunology, ‡Department of Biochemistry and Molecular Biology, University of South Florida College of Medicine, Tampa, Florida 33612 and §§Parke-Davis Pharmaceutical Research, Ann Arbor, Michigan 48105

Recent evidence indicates that the epidermal growth factor (EGF) receptor mediates a branch of lysophosphatidic acid (LPA)-induced signal transduction pathways that activate mitogen-activated protein (MAP) kinase. However, it is unclear whether the intrinsic tyrosine kinase activity of EGF receptor is involved. We previously showed that reactive oxygen species (ROS) were involved in the LPA-stimulated MAP kinase pathway. Here, we identify tyrosine phosphorylation of EGF receptor as an LPA signaling step that requires ROS. To evaluate the role of the tyrosine kinase activity of EGF receptor in the LPA-stimulated MAP kinase pathway, we examined the effects of an EGF receptor-specific tyrosine kinase inhibitor, PD158780. PD158780 potently inhibited the LPA-stimulated MAP kinase 1/2 (MKK1/2) activation and EGF receptor tyrosine phosphorylation in HeLa cells, while it had no detectable effect on c-Src kinase activity. PD158780 also inhibited LPA-induced MKK1/2 activation and DNA synthesis in NIH 3T3 cells. Furthermore, we compared LPA-stimulated MKK1/2 and MAP kinase activation, transcriptional activity of the c-fos promoter, and DNA synthesis in B82L cells, which lack endogenous EGF receptor, and B82L cells expressing kinase-defective or wild-type human EGF receptor. Results obtained from analysis of these cell lines suggest that the EGF receptor tyrosine kinase contributes to the LPA-stimulated MAP kinase activation, c-fos transcription, and mitogenesis.

Lysophosphatidic acid (LPA)1 is a bioactive phospholipid present in serum. LPA concentrations in serum are normally in the range of 2–20 μM (1), but higher concentrations have been reported (2). LPA induces cellular responses by binding to a specific cell surface receptor(s) that is coupled to G16, G20, and G12/13 subfamilies of heterotrimeric G-proteins (1, 3). LPA rapidly activates the mitogen-activated protein (MAP) kinase cascade, consisting of Ras, Raf-1, MAP kinase kinase (MKK or MEK) 1 and MKK 2 (MKK1/2), and MAP kinases (also called ERKs) (4–9). It has been observed that LPA, as well as other agonists of G protein-coupled receptors, induces tyrosine phosphorylation of several signaling proteins in diverse cell types (6, 10–15). Activation of protein tyrosine kinases, such as Src and PYK2, by agonists of G protein-coupled receptors have been reported in certain cell types (15, 16), but not others (17). The LPA-stimulated MAP kinase pathway is sensitive to certain protein tyrosine kinase inhibitors such as genistein (6).2

These observations suggest that regulation of protein tyrosine phosphorylation is an important signaling mechanism of LPA and other agonists of G protein-coupled receptors.

Recently, it was reported that LPA and some other agonists of G protein-coupled receptors stimulate tyrosine phosphorylation of the epidermal growth factor (EGF) receptor (14, 15). Expression of a truncated human EGF receptor lacking the cytoplasmic domain in Rat1 cells abrogated LPA-stimulated MAP kinase activation, suggesting that the EGF receptor mediates at least a branch of the LPA-stimulated MAP kinase activation pathway (14). However, it is unclear whether intrinsic tyrosine kinase activity of EGF receptor is involved in G protein-coupled receptor-stimulated tyrosine phosphorylation of the EGF receptor, MAP kinase activation, and downstream cellular responses such as gene transcription and DNA synthesis.

EGF stimulates tyrosine phosphorylation of its receptor by inducing homodimerization of EGF receptor and heterodimerization of EGF receptor with ErbB-2 or ErbB-4, and activation of these receptor tyrosine kinases (18–21). However, an increase in EGF receptor tyrosine phosphorylation can also occur without activation of the receptor tyrosine kinase. First, EGF receptor is basally active in the absence of EGF. The basal phosphorylating activity of EGF receptor is balanced by dephosphorylating activity (22). Thus, suppression of the dephosphorylating activity, such as incubation of cells with a protein tyrosine phosphatase inhibitor, will increase tyrosine phosphorylation of EGF receptor.2 Second, other cellular kinases such as c-Src or putative Src-activated tyrosine kinases may also cause tyrosine phosphorylation of EGF receptor (15, 23). By using transient expression of the Src-inactivating kinase, CSK, or a dominant negative c-Src mutant, it was reported that c-Src mediates G12/13 and LPA-induced tyrosine phosphorylation of the EGF receptor in COS cells (15). However, similar experi-

1 The abbreviations used are: LPA, lysophosphatidic acid; MAP kinase, mitogen-activated protein kinase; MKK1/2, MAP kinase kinase 1 and MAP kinase kinase 2; ROS, reactive oxygen species; EGF, epidermal growth factor; SRE, serum response element; PDGF, platelet-derived growth factor; NAC, N-acetylcysteine.

2 J. M. Cunnick, J. F. Dorsey, Q. Chen, and J. Wu, unpublished data.
ments that c-Src is not involved in LPA signaling in Rat1 cells (3, 17).

The main objective of this study is to evaluate whether the intrinsic EGF receptor tyrosine kinase activity contributes to the LPA-induced tyrosine phosphorylation of the EGF receptor and MAP kinase pathway. We previously found that reactive oxygen species (ROS) are involved in the LPA-stimulated MAP kinase activation pathway (25). We report here that LPA-induced tyrosine phosphorylation of the EGF receptor requires ROS. Importantly, using a newly developed specific inhibitor of EGF receptor tyrosine kinase (24) and B82L cells that lack EGF receptor, we have found that the intrinsic tyrosine kinase activity of the EGF receptor is involved in the LPA-stimulated MAP kinase pathway.

**EXPERIMENTAL PROCEDURES**

**Materials—**Lysophosphatidic acid (1-oleoyl) and enolase were from Sigma. Anti-phosphotyrosine antibody PY20 was from Transduction Laboratories. Anti-EGF receptor antibody LA22 and anti-Src antibody GD11 were from UBI. Polyclonal antibody to EGF receptor was from Santa Cruz Biotech. Heregulin and anti-ErbB-2 antibody were from NeoMarkers. Anti-Active MAPK antibody and the luciferase assay system were from Promega. Anti-MAP kinase antibody TR12 was kindly provided by Dr. Michael J. Weber (University of Virginia). Enhanced chemiluminescence SuperSignal 3M substrate kit was from Pierce. Radiation was from Calbiochem. Monoclonal antibody against amino acid residues 2–17 of Src (N2–17) was from the National Cancer Institute Repository. [met-12]Thymidine (85.5 Ci/mmol) was from NEN Life Science Products. PD158780 (4-[3-bromophenyl]amino)-6-(methylamino)pyridine (3,4-dipryrimidine) is a specific inhibitor of the EGF receptor tyrosine kinase recently developed at Parke-Davis Pharmaceutical Research (25).

**Cell Culture—**HeLa cells and NIH 3T3 cells were grown and serum-starved as described previously (24). B82L cell lines were grown as described (26).

**Immunoprecipitation and Immunoblotting—**For immunoblotting analyses of EGF receptor or ErbB-2 following immunoprecipitation, cells were lysed in 1 ml/10 cm plate lysis buffer (50 mM Tris-HCl, pH 7.3, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 0.5 mM Na3VO4, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 100 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of aprotinin, leupeptin, and pepstatin). Precleared supernatants were incubated with anti-EGF receptor antibody LA22. Immunoprecipitates were collected with protein G-agarose. Immunoprecipitated proteins were separated on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose filters in some experiments, Immobilon-P filters were used. For immunoblotting analyses using anti-EGF receptor antibody LA22 or anti-ErbB-2 antibodies, blots were probed with antibodies in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Tween 20 (TBST) containing 5% dry milk. For immunoblotting analyses using anti-phosphotyrosine antibody (PY20), blots were blocked with ovalbumin in TBST. Blots were developed by the enhanced chemiluminescence method following incubation with horseradish peroxidase-conjugated secondary antibodies.

**Kinase Assays—**Total MKK1/2 activity was determined using a kinase defective p42MAPK mutant (KR) as substrate as described previously (24). Total MKK1/2 activity is used as a measure of activation of the MAP kinase pathway (24, 27). Activation of MAP kinase, p42MAPK and p44MAPK, was assayed by immunoblotting using the anti-Active MAPK antibody (Promega), which only reacts with the activated dual threonine and tyrosine-phosphorylated p42MAPK and p44MAPK. For Src kinase activity assays, c-Src was immunoprecipitated from cell lysates using the anti-Src antibody N2–17 (in some experiments, monoclonal antibody GD11 was used). Src immune complexes were mixed with Src-kinase buffer (25 mM Hepes, 50 mM NaCl, 0.01% Brij-35, 10 mM MgCl2, 25 mM γ-[32P]ATP (1,000 cpm/pmol) containing 75 μM Ray tide in a total volume of 30 μl. After incubating at 30 °C for 12 min, the reaction was terminated by addition of 50 μl of 10% phosphoric acid. Following a brief centrifugation, 60 μl of each supernatant was spotted onto P81 filters. The filters were washed 5 times with 2% phosphoric acid and radioactivity remaining on the filters was determined. In experiments using enolase as Src substrate, enolase (5 μg/reaction) was denatured with 25 μM acetic acid at 30 °C for 5 min and added to 1/10 of the total kinase reaction volume. Phosphorylation of acid-denatured enolase was analyzed by a PhosphorImager following SDS-polyacrylamide gel electrophoresis.

**Fig. 1. LPA induces tyrosine phosphorylation of the EGF receptor.** A, serum-starved HeLa cells were left untreated, or treated with LPA or EGF as indicated. The EGF receptor was immunoprecipitated from cell lysates, and analyzed by immunoblotting with the anti-phosphotyrosine antibody PY20. B, serum-starved HeLa cells were treated with LPA (40 μM, 2.5 min), H2O2 (5 mM, 5 min), hergulin (50 nm, 4 min), or EGF (3.4 nm, 3 min). Cell lysates were incubated with anti-phosphotyrosine antibody PY20 to immunoprecipitate tyrosine-phosphorylated proteins. Immunoprecipitated proteins were probed with an anti-ErbB-2 antibody.

**Transcriptional Activity of c-fos Promoter—**Plasmid pLucSRE (28) contains a luciferase reporter gene controlled by two tandem copies of the c-fos promoter. To determine transcriptional activity of the c-fos promoter, cells were seeded in 35-mm plates in triplicate 20–24 h prior to transfection. A mixture of pLucSRE (1.5 μg) and the β-galactosidase expression vector pCMVβgal (0.075 μg) was used to transfect each plate of cells using the Superfect transfection reagent (Qiagen) according to the supplier’s instruction. Twenty-two h after transfection, cells were serum-starved for 24 h and then stimulated as indicated in the legend of Fig. 8. Luciferase activity was determined using the Luciferase Assay System from Promega according to supplier’s protocol and a Barthold luminometer. β-Galactosidase activity was determined by a colorimetric assay as described (28). Luciferase activity was normalized to β-galactosidase activity as an internal control for transfection efficiency.

**DNA Synthesis—**DNA synthesis was determined by [3H]thymidine incorporation assay essentially as described (29). Statistical analyses were performed using the nonparametric Wilcoxon Rank Sum test as well as two sample t test (assuming unequal variances). Differences in means were considered statistically significant when both tests indicated p < 0.05.

**RESULTS**

**Stimulation of Tyrosine Phosphorylation of EGF Receptor by LPA—**To determine whether LPA stimulates tyrosine phosphorylation of EGF receptor in HeLa cells, confluent, serum-starved cells were treated with LPA or EGF and lysed in detergents. EGF receptor was immunoprecipitated from cell lysates and analyzed with an antibody against phosphorylating EGF receptor. Fig. IA shows that LPA induced tyrosine phosphorylation of EGF receptor in HeLa cells. Slightly higher levels of EGF receptor tyrosine phosphorylation were consistently detected in cells treated with LPA for 2.5 min than for 4 min, suggesting a transient nature of the LPA-stimulated tyrosine phosphorylation of EGF receptor. Quantification of the EGF receptor bands by a densitometer indicated that a 2.5-min stimulation of HeLa cells with 10 μM LPA (a near-saturation concentration for HeLa cells) increased tyrosine phosphorylation of the EGF receptor approximately 3-fold (3.1 ± 0.5 in four experiments), which is similar to that induced by 0.17 nm EGF in HeLa cells. Other experiments showed that 10 μM LPA and 0.17–0.34 nm EGF activate MKK1 and MKK2 (MKK1/2) to a similar extent in HeLa cells.
To assess whether LPA also induces tyrosine phosphorylation of other growth factor receptors, we analyzed tyrosine phosphorylation of ErbB-2 in HeLa cells. While both heregulin and EGF markedly induced tyrosine phosphorylation of ErbB-2 in HeLa cells, LPA did not appear to affect ErbB-2 in HeLa cells (Fig. 1B). In other experiments, we found no detectable tyrosine phosphorylation of the PDGF receptor in LPA-stimulated NIH 3T3 and Rat1 cells (HeLa cells have no PDGF receptor), while control experiments showed that LPA-stimulated tyrosine phosphorylation of EGF receptor in NIH 3T3 cells. Thus, LPA appears to preferentially induce tyrosine phosphorylation of EGF receptor.

Role of ROS in LPA-induced Tyrosine Phosphorylation of the EGF Receptor—We reported previously that LPA rapidly increased intracellular levels of ROS and that ROS were involved in LPA-stimulated MKK1/2 activation (24). Similarly, LPA-stimulated Raf-1 activation is also mediated by ROS. Other evidence suggests that ROS modulate the MAP kinase pathway at signaling steps upstream of the Ras protein (30). To assess whether ROS have a role in LPA-induced tyrosine phosphorylation of the EGF receptor, we analyzed the effect of the antioxidant N-acetylcysteine (NAC) on LPA-induced tyrosine phosphorylation of the EGF receptor. Our previous studies showed that NAC attenuated the LPA-stimulated MKK1/2 activity in HeLa cells (24). As illustrated in Fig. 2A, NAC effectively blocked the LPA-induced tyrosine phosphorylation of the EGF receptor in HeLa cells. Consistent with these results, direct exposure of HeLa cells to H2O2 induced tyrosine phosphorylation of EGF receptor (Fig. 2A). The increase in tyrosine phosphorylation of EGF receptor resulted in increased binding affinity of EGF receptor to the signaling adaptor protein Grb2, as demonstrated by binding to a GST-Grb2 fusion protein in vitro (Fig. 2C), confirming a previous observation (14).

To verify that the inhibitory effect of NAC was attributable to its ROS scavenger/antioxidant activity, we determined whether other antioxidants could similarly block the LPA-induced tyrosine phosphorylation of EGF receptor. L-Histidine and dimethyl sulfoxide are hydroxyl radical scavengers (31) that inhibit the LPA-induced MKK1/2 activation in HeLa and NIH 3T3 cells (24). Fig. 2D shows that both L-histidine and dimethyl sulfoxide attenuated the LPA-induced tyrosine phosphorylation of EGF receptor. By contrast, the catalase inhibitor aminotriazole, which augments the LPA stimulated MKK1/2 activity (24), slightly enhanced the LPA-induced tyrosine phosphorylation of EGF receptor in HeLa cells. These data indicate that ROS are required for LPA-stimulated tyrosine phosphorylation of the EGF receptor.

ROS Negatively Regulate c-Src Activity—A protein tyrosine kinase that could potentially contribute to tyrosine phosphorylation of the EGF receptor is c-Src. It was observed previously that LPA marginally activated c-Src kinase in neuroblastoma cells, but not in fibroblasts (32). In addition, a recent report suggests that c-Src mediates Gp130 and LPA-induced tyrosine phosphorylation of the EGF receptor (15). Despite many attempts, we have not been able to detect activation of c-Src tyrosine kinase activity by LPA in HeLa cells (for an example, see Fig. 3B) or NIH 3T3 cells. Because ROS positively regulate tyrosine phosphorylation of EGF receptor and the MAP kinase pathway in HeLa cells, we examined whether H2O2 could activate c-Src kinase in HeLa cells. Serum-starved HeLa cells were treated with H2O2 or LPA and c-Src was immunoprecipitated. c-Src kinase activity was determined in the immune complexes using either acid-denatured enolase (Fig. 3A) or Raytide (Fig. 3B) as substrate. As shown in Fig. 3, basal activity of c-Src kinase was detectable in serum-starved HeLa cells. H2O2 treatment reduced c-Src kinase activity in a concentration-dependent manner. These data indicate that ROS negatively regulate c-Src kinase activity, which is opposite to the effects of ROS on LPA-stimulated tyrosine phosphorylation of EGF receptor and the MAP kinase pathway (Ref. 24, Fig. 2).
Inhibition of LPA-stimulated MKK1/2 activation and Tyrsoine Phosphorylation of the EGF Receptor by PD158780—The compound PD158780 is a newly developed, specific inhibitor of the EGF receptor tyrosine kinase (25). PD158780 is more potent than another previously reported specific inhibitor of the EGF receptor tyrosine kinase, PD153035 (33). In human epidermoid carcinoma cells and in mouse fibroblasts, PD158780 inhibits EGF-stimulated autophosphorylation of its receptor with an IC₅₀ of approximately 13 nM (34). To evaluate whether the intrinsically EGF receptor tyrosine kinase activity is required for the LPA-stimulated MAP kinase pathway, we determined whether inhibition of the EGF receptor tyrosine kinase activity by PD158780 affected LPA-stimulated MKK1/2 activation. Serum-starved HeLa cells were preincubated with 0–100 nM PD158780 for 120 min and c-Src kinase activity was assayed. Fig. 4A shows that PD158780 inhibited LPA-stimulated MKK1/2 activity in a concentration-dependent manner in HeLa cells. The IC₅₀ value for the effect of PD158780 on LPA-stimulated MKK1/2 activation is approximately 20 nM, which is very similar to that for inhibition of EGF receptor autophosphorylation. Fig. 4B illustrates the inhibitory effects of 50 nM PD158780 on LPA- and EGF-stimulated MKK1/2 activity in HeLa. In other experiments, we examined whether PD158780 affected PDGF-stimulated MKK1/2 activity in NIH 3T3 cells. Although control experiments showed that PD158780 (50 nM) inhibited LPA- and EGF-stimulated MKK1/2 activation (Fig. 4, C and D), preincubation of NIH 3T3 cells with PD158780 (50 nM) had little effect on the PDGF-stimulated MKK1/2 activity in NIH 3T3 cells (Fig. 4D). To confirm that the inhibitory effect of PD158780 on LPA-stimulated MKK1/2 activation is due to suppression of LPA-induced tyrosine phosphorylation of the EGF receptor, we examined LPA-induced tyrosine phosphorylation of the EGF receptor in HeLa cells with or without PD158780 pretreatment. Confluent, serum-starved HeLa cells were pretreated with PD158780 (50 nM) or mock-treated, followed by LPA stimulation. The EGF receptor was immunoprecipitated and analyzed by immunoblotting with an anti-phosphotyrosine antibody. As shown in Fig. 5, preincubation of HeLa cells with PD158780 (50 nM) completely blocked LPA-induced tyrosine phosphorylation of the EGF receptor.

To assess the possibility that PD158780 may nonspecifically inhibit c-Src kinase activity, we immunoprecipitated c-Src from serum-starved HeLa cells and determined c-Src kinase activity in the presence or absence of PD158780 (50 nM) in the kinase reaction. Alternatively, serum-starved HeLa cells were treated with PD158780 (50 nM) and the c-Src kinase activity was subsequently determined following immunoprecipitation. Even though our assay is able to detect not only the basal kinase activity of c-Src but also decreases in c-Src kinase activity below the basal level (Fig. 3), no inhibitory effect of PD158780 on c-Src kinase activity was detected either in vitro or in vivo (Fig. 6). These data indicate that the inhibitory effect of PD158780 on LPA-stimulated MKK1/2 activation is not attributed to the nonspecific effect of PD158780 on c-Src kinase activity.

Comparison of LPA-stimulated MKK1/2 and MAP Kinase Activation in Mouse B82L Fibroblasts and B82L Cell Lines Expressing the Wild-type and Kinase-defective Human EGF Receptor—B82L is a mouse L fibroblast cell line that has no detectable EGF receptor mRNA and protein (Refs. 35 and 36; Fig. 7A). To further evaluate the role of intrinsic kinase activity of EGF receptor in the LPA-stimulated MAP kinase pathway, we compared the LPA-stimulated MKK1/2 activity in parental B82L cells, and transfected B82L cell lines expressing the wild-type or kinase-defective EGF receptor (Fig. 7B).
wild-type human EGF receptor (B82L/hER\(^{\ast}\)) or the kinase-defective human EGF receptor (B82L/hER\(^{-}\)) (27). Immunoblotting analysis confirmed that EGF receptor was expressed in B82L/hER\(^{-}\) and B82L/hER\(^{\ast}\) cells (Fig. 7A). The level of EGF receptor expression in B82L/hER\(^{\ast}\) cells appears to be higher than that in B82L/hER\(^{-}\) cells (Fig. 7A).

LPA weakly activated MKK1/2 in B82L cells (2.9 ± 0.01-fold), indicating that one or more EGF receptor-independent pathways exists for MKK1/2 activation in this cell line (Fig. 7, B and C). This observation is consistent with the notion that multiple signaling pathways mediate LPA signal transduction, including the LPA-stimulated MAP kinase pathway, and that EGF receptor mediates one of these pathways. While expression of kinase-defective human EGF receptor increased LPA-stimulated MKK1/2 activation by 96% (5.7 ± 0.13-fold activation), expression of wild-type human EGF receptor increased LPA-stimulated MKK1/2 activation by 196% (8.6 ± 0.31-fold activation) (Fig. 7, B and C). These observations suggest that EGF receptor is involved in one of the LPA signal transduction pathways that activate the MAP kinase cascade, and that another cellular kinase activity as well as the intrinsic kinase activity of EGF receptor both contribute to the function of EGF receptor in these cells.

To confirm that similar changes in MAP kinase activation also occur in B82L, B82L/hER\(^{\ast}\), B82L/hER\(^{-}\) cells, LPA- and EGF-stimulated p42\(^{MAPK}\)/p44\(^{MAPK}\) activation was analyzed. As shown in Fig. 7D, LPA- and EGF-stimulated activation of p42\(^{MAPK}\)/p44\(^{MAPK}\) in these cells correlated well with that of MKK1/2. Additional experiments were also performed to analyze tyrosine phosphorylation of Shc proteins. Shc has been implicated in mediating signal transduction of receptor tyrosine kinases (37). The 52- and 46-kDa Shc proteins were detected in B82L and the two B82L-derived cell lines. The 52-kDa Shc is basally phosphorylated on tyrosine in B82L cells. While LPA did not increase tyrosine phosphorylation of Shc in B82L or B82L/hER\(^{-}\) cells, it stimulated tyrosine phosphorylation of the 52-kDa Shc in B82L/hER\(^{\ast}\) cells. However, a much higher level of 52-kDa Shc tyrosine phosphorylation was observed in EGF-stimulated B82L/hER\(^{\ast}\) cells (most likely due to heterodimerization of EGF receptor with ErbB-2) than that detected in LPA-stimulated B82L/hER\(^{-}\) cells and B82L/hER\(^{\ast}\) cells. Therefore, the level of Shc tyrosine phosphorylation did not appear to correlate well with activation of the MAP kinase pathway (Fig. 7) or transcriptional activity of the c-fos promoter described below (Fig. 8).

c-fos Promoter Activation and DNA Synthesis—LPA is
known to induce transcription of the c-fos gene (38, 39) and have mitogenic activity in some cells (1, 2). Previous studies in NIH 3T3 cells suggest that induction of c-fos transcription by LPA is mediated primarily via the serum response element (SRE), which is controlled by both the MAP kinase-regulated ternary complex factor as well as the MAP kinase-independent, Rho-mediated serum response factor (39). To further assess the role of EGF receptor in LPA signaling, we compared transcriptional activity of the c-fos promoter in B82L, B82L/hER<sup>−</sup>, and B82L/hER<sup>+</sup> cells. pLucSRE, which contains a luciferase reporter gene controlled by the c-fos promoter, was transfected into B82L, B82L/hER<sup>−</sup>, and B82L/hER<sup>+</sup> cells. Luciferase activity in the cell lysates were measured after stimulation of cells with LPA or EGF. As shown in Fig. 5A, low, but detectable, LPA-stimulated luciferase activity was observed in B82L cells. On the other hand, EGF did not activate the c-fos promoter in B82L cells. While expression of the kinase-defective EGF receptor increased c-fos promoter activity in LPA-stimulated B82L/hER<sup>−</sup> cells to 2.2 ± 0.07-fold of that observed in LPA-stimulated B82L cells, expression of the wild-type EGF receptor further increased c-fos promoter activity in LPA-stimulated B82L/hER<sup>+</sup> cells to 6.6 ± 0.26-fold of that detected in LPA-stimulated B82L cells (Fig. 8A). Increases in c-fos promoter activity were also observed in EGF-stimulated B82L/hER<sup>−</sup> cells and B82L/hER<sup>+</sup> cells (Fig. 8A). These changes in c-fos promoter activity were similar to the changes observed above in MKK1/2 and MAP kinase activation in these three cell lines, suggesting that EGF receptor contributes to the LPA-induced c-fos transcriptional activity.

c-fos promoter activity was also analyzed in HeLa cells. Fig. 8B shows that the EGF receptor inhibitor PD158780 reduced the LPA-stimulated c-fos promoter activity by 62% and the EGF-stimulated c-fos promoter activity by 54%. Thus, inhibition of the EGF receptor tyrosine kinase activity decreased the LPA- and EGF-stimulated c-fos promoter activity, indicating that the intrinsic EGF receptor tyrosine kinase contributes to the LPA-induced c-fos transcription in HeLa cells.

Analysis of DNA synthesis by the [³H]thymidine incorporation assay in three independent triplicate experiments indicated that LPA did not induce DNA synthesis in B82L cells (Table I). DNA synthesis in LPA-treated B82L/hER<sup>−</sup> and B82L/hER<sup>+</sup> cells was 1.16 ± 0.27-fold and 1.49 ± 0.24-fold of the controls, respectively (Table I). While the 16% increase in DNA synthesis in LPA-stimulated B82L/hER<sup>−</sup> cells is not statistically significant (p > 0.05 in both the Wilcoxon Rank Sum test and the t test), the 49% increase in DNA synthesis induced by LPA in B82L/hER<sup>+</sup> cells is statistically significant (p < 0.001 in both tests). Thus, the DNA synthesis analysis indicates that EGF receptor tyrosine kinase activity contributes to the LPA-induced mitogenic response.

Surprisingly, EGF, which clearly activates MAP kinase and the c-fos promoter in B82L/hER<sup>−</sup> cells to higher extents than that induced by LPA, did not stimulate DNA synthesis in B82L/hER<sup>+</sup> cells. In other experiments, we stimulated serum-starved B82L/hER<sup>−</sup> cells with various concentrations of EGF (0.05–20 ng/ml) and detected no EGF-stimulated DNA synthesis at all concentrations tested. In addition, we also detected no EGF-stimulated DNA synthesis in B82L/hER<sup>+</sup> cells in the presence of insulin. This observation suggests that while the EGF receptor-mediated MAP kinase-c-fos pathway is necessary for the LPA-induced DNA synthesis in B82L cell lines, activation of the MAP kinase-c-fos pathway alone is insufficient to induce DNA synthesis in these cells. Signaling pathways besides MAP kinase and c-fos transcription must also be activated for induction of DNA synthesis in B82L cell lines. Our data suggest that LPA is capable of activating these additional signaling pathways.

To further evaluate the role of EGF receptor tyrosine kinase activity in the LPA-stimulated mitogenic response, we analyzed LPA- and EGF-stimulated DNA synthesis in NIH 3T3 cells. Fig. 9 shows that LPA (50 μM) and EGF (10 ng/ml) induced DNA synthesis in NIH 3T3 cells approximately 16- and 24-fold, respectively. The 16-fold increase in DNA synthesis induced by LPA at all concentrations was statistically significant (p < 0.001 in both Wilcoxon Rank Sum test and the t test). The 24-fold increase in DNA synthesis induced by EGF was also statistically significant (p < 0.001 in both experiments).

**DISCUSSION**

We previously reported that LPA rapidly raises intracellular concentrations of ROS in HeLa cells and that ROS participate in the LPA-stimulated MAP kinase pathway (24). Evidence presented in Fig. 2 indicates that induction of tyrosine phosphorylation of EGF receptor by LPA requires ROS. Involvement of ROS in EGF-elicited tyrosine phosphorylation of its receptor and signaling pathways was also reported recently by other investigators (40). Although the mechanisms by which ROS mediate tyrosine phosphorylation of EGF receptor and
Serum-starved NIH 3T3 cells were treated with PD158780 (50 nM, 1 h) or mock-treated, and then stimulated with LPA (50 μM) or EGF (10 nM). Eighteen h after stimulation, [methyl-3H]thymidine (3 μCi/ml) was added and incubation continued for 6 h. After which, cells were processed for determination of [3H]thymidine incorporation into DNA (29). Data shown are the mean and standard deviations of two independent experiments performed in triplicate.

other growth factor receptors remains to be delineated, a possible mechanism is by inhibiting a specific protein tyrosine phosphatase activity rather than directly increasing the EGF receptor kinase activity (22). Protein tyrosine phosphatases contain a catalytic cysteine residue and are sensitive to oxidative inhibition (41–44). Inhibition of protein tyrosine phosphatase activity would shift the balance of tyrosine phosphorylation and dephosphorylation of EGF receptor toward the phosphorylation direction. Because of differences in sensitivity to oxidative inhibition and in cellular location, inhibition of a specific protein tyrosine phosphatase activity may be achieved under physiological conditions in which only low levels of ROS are produced. This protein tyrosine phosphatase inhibition hypothesis predicts that basal phosphorylation of EGF receptor occurs in the absence of EGF, and that inhibition of protein tyrosine phosphatase activity will increase tyrosine phosphorylation of EGF receptor. Indeed, basal phosphorylation of the EGF receptor in serum-starved cells was detectable in HeLa cells (Figs. 1, 2, and 5). Moreover, treatment of HeLa cells with the protein tyrosine phosphatase inhibitor Na3VO4 resulted in increased tyrosine phosphorylation of EGF receptor.2

Tyrosine phosphorylation has been recognized as an important signaling mechanism of LPA and other G protein-coupled receptors (6, 10–15). Recent evidence indicates that tyrosine phosphorylation of EGF receptor mediates a branch of the LPA signaling pathways that leads to MAP kinase activation (14, 15). While conflicting evidence has been reported regarding the role of c-Src in signal transduction by LPA and G protein-coupled receptors (14, 17), the role of intrinsic kinase activity of EGF has not been critically evaluated. In the present investigation, experiments were carried out to assess the involvement of intrinsic kinase activity of EGF receptor in LPA-stimulated tyrosine phosphorylation of EGF receptor and the MAP kinase activation pathway. Our results show that in HeLa cells: (i) while LPA-stimulated MAP kinase pathway and tyrosine phosphorylation of EGF receptor are positively regulated by ROS, ROS negatively regulate c-Src kinase activity; (ii) PD158780, a specific inhibitor for the EGF receptor tyrosine kinase, is very potent in inhibiting LPA- and EGF-induced MKK1/2 activation. PD158780 also inhibits LPA-induced c-fos promoter activity in HeLa cells. Furthermore, PD158780 inhibits LPA-induced tyrosine phosphorylation of the EGF receptor, whereas it has no inhibitory effects on c-Src tyrosine kinase both in vitro and in vivo. These observations suggest that the intrinsic EGF receptor tyrosine kinase activity contributes to the LPA-stimulated MAP kinase pathway in HeLa cells and that c-Src is likely not involved. Other evidence has recently been obtained by Boyer et al. (45) that indicates c-Src is not involved in the EGF-stimulated MAP kinase pathway in NBT-II carcinoma cells.

Although our experiments suggest that c-Src is probably not involved in the LPA-stimulated MKK1/2 activation in HeLa cells, we do not exclude the possibility that other members of the Src family of protein kinases may be involved. Many protein tyrosine kinases are positively regulated by tyrosine phosphorylation. In contrast, c-Src is activated primarily by dephosphorylation of a tyrosine residue at its C-terminal regulatory region. This is consistent with our observation that exposure of cells to H2O2, which inhibits protein tyrosine phosphatase activities, decreases c-Src kinase activity (Fig. 3). On the other hand, some other members of the Src family kinases, such as Lck in T cells, are activated predominantly by autophosphorylation in the kinase domain (46, 47). It is possible that one of these Src family kinases or other cellular tyrosine kinases also contribute to LPA-induced tyrosine phosphorylation of the EGF receptor in HeLa cells. Furthermore, our data in no way argue against the possibility that c-Src and EGF receptor may cooperatively regulate certain signal transduction pathways that mediate cell transformation and tumorigenesis (48).

The role of EGF receptor in LPA-stimulated MAP kinase activation was further evaluated in mouse fibroblasts B82L, B82L/hER+, and B82L/hER− cells. LPA weakly activates MKK1/2 and MAP kinases in B82L cells, suggesting that at least one alternate, EGF receptor-independent signaling mechanism exists in this cell line. This finding is in agreement with the notion that more than one signaling pathway mediates LPA-stimulated MAP kinase activation. Expression of a kinase-defective human EGF receptor in B82L cells increases the level of MAP kinase activation in LPA-stimulated cells, implying that another cellular kinase activity contributes to the function of EGF receptor in mediating the LPA signaling pathway in B82L cell lines. Because cross-phosphorylation of the EGF receptor by other ErbB family members requirements EGF-induced heterodimerization, the cellular kinase that contributes to the function of the kinase-defective EGF receptor in LPA signaling may be a non-receptor tyrosine kinase. Importantly, expression of the wild-type human EGF receptor in B82L cells further increases LPA-stimulated MKK1/2 and MAP kinase activation to a level higher than that of B82L cells expressing the kinase-defective EGF receptor. These observations indicate that intrinsic tyrosine kinase activity of EGF receptor as well as another cellular kinase both contribute to the role of EGF receptor in the LPA-stimulated MAP kinase pathway in B82L cell lines.

To further assess the role of EGF receptor in LPA signaling, we analyzed the LPA-stimulated c-fos promoter activity and DNA synthesis. LPA weakly induces c-fos promoter activity in B82L cells, which may be attributed primarily to Rho-mediated serum response factor activity that is independent of MAP kinase (39). Higher levels of c-fos promoter activity were detected in LPA-treated B82L/hER+ and B82L/hER− cells, corresponding to higher levels of MKK1/2 and MAP kinase activation in these cells. Thus, these data again suggest that EGF receptor contributes to LPA signaling and that both the intrinsic EGF receptor kinase as well as another cellular kinase are involved. At the same time, LPA-stimulated c-fos promoter activity was inhibited by PD158780 in HeLa cells (Fig. 5B). This observation is consistent with the model that EGF receptor kinase has a role in LPA signaling.

Further support for a role of EGF receptor tyrosine kinase in LPA signaling was obtained from DNA synthesis analysis. LPA has no mitogenic activity in B82L cells that lack EGF receptor.
While the lower levels of increase in MAP kinase activation and c-fos promoter activity in B82L/hER cells did not result in a statistically significant increase in LPA-induced DNA synthesis in B82L/hER cells, expression of the wild-type EGF receptor in B82L/hER cells restores the mitogenic activity of LPA in this fibroblast line. Furthermore, the LPA-stimulated DNA synthesis is inhibited by a specific inhibitor of EGF receptor tyrosine kinase. Therefore, the role of EGF receptor tyrosine kinase in mediating LPA signaling is biologically relevant.

Acknowledgments—We thank Drs. Gordon Gill and Michael Weber for the B82L cell lines, Dr. Michael Weber for the TR12 antibody, Dr. Alan Cantor of the Moffitt Biostatistics Core for statistical analyses, and Drs. Doug Cress, Nancy Olashaw, Ed Seto, and Hua Yu for critical reading of the manuscript.

REFERENCES
1. Moolenaar, W. H. (1995) J. Biol. Chem. 270, 12949–12952
2. Tokumura, A., Iimori, M., Nishioka, Y., Kitahara, M., Sakashita, M., and Tanaka, S. (1994) Am. J. Physiol. 267, C204–C210
3. Moolenaar, W. H., Kranenburg, O., Postma, F. R., and Zondag, G. C. M. (1997) Curr. Opin. Cell Biol. 9, 166–173
4. Howie, L. R., and Marshall, C. J. (1993) J. Biol. Chem. 268, 20717–20720
5. Anan, N. G., Seger, R., and Krebs, E. G. (1992) Curr. Opin. Cell Biol. 4, 992–999
6. Hordijk, P. L., Verlaan, I., van Curen, E. J., and Moolenaar, W. H. (1994) J. Biol. Chem. 269, 645–651
7. Cobb, M. H., and Goldsmith, E. J. (1995) J. Biol. Chem. 270, 14843–14846
8. Jelinek, T., Catling, A. D., Reuter, C. W., Moodie, S. A., Wolfman, A., and Weber, M. J. (1994) Mol. Cell Biol. 14, 8212–8218
9. Davis, R. J. (1993) J. Biol. Chem. 268, 14553–14556
10. Ridley, A. J., and Hall, A. (1994) EMBO J. 13, 2600–2610
11. Seufferlein, T., and Reuzengart, E. (1994) J. Biol. Chem. 269, 9345–9351
12. Saville, M. K., Graham, A., Malarkey, K., Paterson, A., Gould, G. W., and Plevin, R. (1994) Biochem. J. 301, 407–414
13. van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Porfiri, E., Sakaue, M., Luttrell, L. M., and Lefkowitz, R. J. (1995) Nature 376, 781–784
14. Dush, H., Weiss, F. U., Wallasch, C., and Ulrich, A. (1996) Nature 379, 557–560
15. Luttrell, L. M., Roeca, G. J. D., van Biesen, T., Luttrell, D. K., and Lefkowitz, R. J. (1997) J. Biol. Chem. 272, 4637–4644
16. Levine, S., Moreno, H., Martinez, R., Cinalli, P., Pelus, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J. (1995) Nature 376, 737–745
17. Kranenburg, O., Verlaan, I., Hordijk, P. L., and Moolenaar, W. H. (1997) EMBO J. 16, 3097–3105
18. Pinkas-Kramarski, R., Sourourn, L., Waterman, H., Levkowitz, G., Alroy, I., Klapner, L., Lavi, S., Seger, R., Ratziun, B. J., Sela, M., and Yarden, Y. (1996) EMBO J. 15, 2452–2460
19. Riese, D. J., van Raaij, T. M., Plowman, G. D., Andrews, G. C., and Stern, D. F. (1995) Mol. Cell. Biol. 15, 5770–5776
20. Ullrich, A., and Schlessinger, J. (1990) Cell 61, 203–212
21. Carpenter, G., and Cohen, S. (1990) J. Biol. Chem. 265, 7709–7712
22. Weiss, F. U., Dush, H., and Ulrich, A. (1997) Curr. Opin. Genet. Dev. 7, 80–86
23. Watanen, S. J., Palmer, A. D., Thompson, A. M., Bridges, A. J., Cody, D. R., Zhou, H., Fry, D. W., McMichael, A., and Denny, W. A. (1996) J. Med. Chem. 39, 1823–1835
24. Wright, J. D., Reuter, C. W., and Weber, M. J. (1995) J. Biol. Chem. 270, 12095–12098
25. Wu, J., Spiegel, S., and Sturgill, T. W. (1995) J. Biol. Chem. 270, 11484–11488
26. Yamazuchi, K., Holt, K., and Pessin, J. E. (1993) J. Biol. Chem. 268, 14597–14600
27. Sekharam, M., Trott, A., Cunnick, J. M., and Wu, J. (1998) Toxicon. Appl. Pharmacol. 149, 210–216
28. Guyton, K. Z., Liu, Y., Gorsepe, M., Xu, Q., and Holbrook, N. (1996) J. Biol. Chem. 271, 4138–4142
29. Basu-Madak, S., and Tyrrell, R. M. (1993) Cancer Res. 53, 4505–4510
30. Jalink, K., Eichholzt, T., Postma, F. R., van Corven, E. J., and Moolenaar, W. H. (1993) Cell Growth & Differ. 4, 247–255
31. Fry, D. W., Kraker, A. J., McMichael, A., Ambrose, L. A., Nelson, J. M., Leopold, W. R., Connors, R. W., and Bridges, A. J. (1994) Science 263, 1093–1095
32. Fry, D. W., Nelson, J. M., Slintak, V., Keller, P. R., Reuel, G. W., Zhou, H., and Bridges, A. J. (1997) Biochem. Pharmacol. 54, 877–887
33. Lin, C. H., Chen, W. S., Lazar, C. S., Carpenter, C. D., Gill, G. N., Evans, R. M., and Rosenfeld, M. G. (1996) Cell 44, 839–848
34. Chen, W. S., Lazar, C. S., Pieniec, M., Tsets, R. Y., Gill, G. N., and Rosenfeld, M. G. (1997) Nature 328, 820–823
35. Pelici, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavollo, F., Fein, G., Nicot, I., Grignani, F., Pawson, T., and Pellici, P. G. (1992) Cell 70, 93–104
36. Chuprun, J. K., Raymond, J. R., and Blakeshear, P. J. (1997) J. Biol. Chem. 272, 773–781
37. Hill, C. S., Wynne, J., and Treisman, R. (1995) Cell 81, 1159–1170
38. Bae, S. Y., Kang, S. W., Seo, M. S., Baines, I. C., Tekke, E., Chock, B. P., and Rhe, S. G. (1997) J. Biol. Chem. 272, 217–221
39. Guan, K., and Dixon, J. E. (1991) J. Biol. Chem. 266, 17026–17030
40. Tonks, N. K., Diltz, C. D., and Fisher, E. H. (1988) J. Biol. Chem. 263, 6731–6737
41. Denu, J. M., Lohse, D. L., Vijayalakshmi, J., Saper, M. A., and Dixon, J. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2493–2498
42. Dixon, J. E. (1996) Recent Prog. Horm. Res. 51, 465–414
43. Bae, S. Y., Cho, H. J., and Thiery, J. P. (1997) EMBO J. 16, 5904–5913
44. Hardwick, J. S., and Selton, B. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4527–4531
45. Yurchak, L. K., Hardwick, J. S., Amrein, K., Pernio, R., and Sefton, B. M. (1996) J. Biol. Chem. 271, 12549–12554
46. Meier, M., Scholz, T., and Schatzman, R. C., and Parsons, S. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6981–6985