Optimal Lysophosphatidic Acid-induced DNA Synthesis and Cell Migration but Not Survival Require Intact Autophosphorylation Sites of the Epidermal Growth Factor Receptor*

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Lysophosphatidic acid (LPA)-elicited transphosphorylation of receptor tyrosine kinases has been implicated in mediating extracellular signal-regulated kinase (ERK) 1/2 activation, which is necessary for LPA-induced cell proliferation, migration, and survival. B82L cells lack epidermal growth factor receptor (EGFR) but express LPA1–3, platelet-derived growth factor (PDGF), ErbB2, and insulin-like growth factor receptor transcripts, yet LPA caused no detectable transphosphorylation of these receptor tyrosine kinases. LPA equally protected B82L cells, or transfectants expressing EGFR, the kinase dead EGFRK721A, EGFRY5F receptor mutant, which lacks five autophosphorylation sites, or EGFRY845F, which lacks the Src phosphorylation site from tumor necrosis factor-α-induced apoptosis. In contrast, LPA-elicited DNA synthesis and migration were augmented in cells expressing EGFR, EGFRK721A, or EGFRY845F, but not EGFRY5F, although the PDGF responses were indistinguishable. LPA-elicited transphosphorylation of the EGFR, ErbB2, or PDGF receptor was not required for its antiapoptotic effect. EGFR with or without intrinsic kinase activity or without the Src-phosphorylation site augmented, but was not required for, LPA-elicited cell proliferation or migration. In B82L cells, augmentation of these two LPA responses required intact autophosphorylation sites because among the four EGFR mutants, only cells expressing the EGFRY5F mutant showed no enhancement. In EGFRY5F-expressing cells, LPA failed to elicit tyrosine phosphorylation of Src homologous and collagen protein (SHC) and caused only a modest increase in ERK1/2 phosphorylation similar to that in wild-type B82L cells. The present data pinpoint the lack of importance of the intrinsic kinase activity in contrast to the importance of autophosphorylation sites of the EGFR for SHC phosphorylation in the enhancement of select ERK1/2-dependent LPA responses.

The phospholipid growth factor, lysophosphatidic acid (1-acyl-2-hydroxy-sn-glycero-3-phosphate, LPA), a normal component of blood plasma, is a confirmed ligand of the LPA1, LPA2, LPA3, and putative agonist of the GPR23 G protein-coupled receptors (GPCR) (1, 2). Cell survival, proliferation, and migration are responses elicited by LPA GPCR and also by activation of polypeptide growth factor receptors tyrosine kinase (RTK) for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF). RTK activation results in ligand-induced autophosphorylation of the receptor, which recruits adapter and docking proteins to initiate the assembly and activation of a multimolecular signaling complex (3–9). It is interesting that inhibition of the heterotrimeric G protein αi/o by pertussis toxin (PTX) abolishes the LPA-elicited antiapoptotic, proliferative, and migratory effects (1, 2, 10–12). In some, but not all cell types (13, 14), activation of LPA GPCR is accompanied by phosphorylation of the epidermal growth factor receptor (EGFR) (15–20), the platelet-derived growth factor receptor (PDGFR) (21–23), or insulin-like growth factor receptor-1 (IGFR) (21). Several laboratories have proposed a cross-talk between the LPA GPCR and RTK in which ligand activation of the GPCR elicits either the ligand-independent transactivation of RTK mediated by intracellular kinases of the Src family (6, 17) and/or the GPCR-elicited cleavage of pro-EGF leading to a paracrine production of EGF at the cell surface, causing ligand-induced activation of the EGFR (4, 9, 24). Many of these studies noted that LPA-induced activation of ERK1/2 and phosphoinositide-3-kinase, was inhibited by blockers of the EGFR tyrosine kinase, including AG1478 and PD158780 (20, 23–25), and interpreted the diminished activation of these kinases as evidence for the functional requirement of transactivation EGFR in the cellular responses to LPA.

On the other hand, several groups noted that LPA was capable of eliciting one or another of its characteristic cellular or signaling responses in cells in which EGF and PDGF did not elicit such responses (e.g. migration (26)), or in cells that lacked EGFR (e.g. ERK1/2, cFOS (14, 23, 25)), or in cells derived from Src−/−, Src−/−Yes−/−Fyn−/−, and Pyk2−/−Src−/− knock

1 The abbreviations used are: LPA, lysophosphatidic acid 18:1; GPCR, G protein-coupled receptor; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; PDGF, platelet-derived growth factor BB; IGF, insulin-like growth factor; PTX, pertussis toxin; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; IGF, insulin-like growth factor receptor; ERK, extracellular signal-regulated kinase; SHC, Src homologous and collagen protein; TNF, tumor necrosis factor; C4, IGFR-positive F32 VIII C4 mouse hybridoma cells.
out animals (e.g. ERK1/2, Ras (14)). Andreew and colleagues (14) reported that although neither Pyk2 nor Src was necessary for ERK1/2 activation in response to LPA, a Src-dependent activation of Pyk2 mediated EGFR phosphorylation. We and others have shown that the antitumorigenic effect of LPA requires ERK1/2 activation (27–30) that is sensitive to PTX and to the EGFR kinase inhibitor AG1478 but not to AG1296 (29) in IEC-6 intestinal epithelial cells. In these cells, LPA-elicited phosphorylation of the EGFR was inhibited by AG1478 but not by PTX, raising the question of whether EGFR transphosphorylation is required for ERK1/2 activation. Despite the many reports on GPCR-induced phosphorylation of RTK, the role of such transregulation in the modulation of distinct cellular responses elicited by LPA remains unknown.

Rubio et al. (31) recently provided evidence for a parallel signaling network connecting LPA receptors to EGFR and ERK1/2 activation. These authors, using COS-7 cells, distinguished an EGFR-dependent, AG1478-inhibited basal nucleotide turnover on Ras in addition to an LPA-activated, PTX-sensitive component requiring Gsa proteins, but not EGFR, both of which were required for the robust activation of ERK1/2. The EGFR-dependent permissive activation of Ras was mediated by the canonical Shc/Grb2/Sos system because SHC phosphorylation was not inhibited by PTX, as has been noted by other investigators too (20). The mechanism mediating the Gαi-controlled increase in Ras nucleotide exchange rate remains unclear but might involve the Ras-GRF/CDC25Mm exchange factor that is directly regulated by the Gβγ subunits (32–34). In this context, increased tyrosine phosphorylation of SHC seems to be a sensitive indicator whether the EGFR-dependent branch of the parallel pathway is activated by LPA or not.

In the present study, we evaluated the role of EGFR transregulation in three distinct ERK1/2-dependent cellular responses (antiproliferation, migration, and proliferation) elicited by LPA using a receptor add-back model in B82L cells that lack endogenous EGFR. The role of EGFR transregulation was tested by introducing wild-type human EGFR (35), a kinase-deficient EGFR(721A) mutant (36), the EGFR(T79F) receptor mutant, in which the sites of tyrosine autophosphorylation have been mutated to phenylalanines (37), and EGFR(845F) with its Src phosphorylation site mutated (38). Our data indicate that in EGFR-null B82L cells, LPA GPCR are capable of eliciting the three hallmark biological responses independent of the transactivation of PDGFR, ErbB2 and IGF RTK. However, LPA-elicited proliferative and migratory responses, but not the antiproliferative response, are markedly modulated by EGFR transactivation through a mechanism that does not require EGFR tyrosine kinase activity or a Src phosphorylation site but requires intact autophosphorylation sites to enable tyrosine phosphorylation of SHC for full-blown activation of ERK1/2.

MATERIALS AND METHODS

Reagents—LPA (oleoylethanolamine) was purchased from Avanti Polar Lipids Inc., (Alabaster, AL) and was applied to cells complexed with equimolar fatty acid-free bovine serum albumin (Sigma-Aldrich Chemical Co.). Recombinant rat TNF-α was purchased from BD Pharmingen. PD98059 and PD158780 were purchased from Calbiochem. PTX, AG1296, and AG1487 were from Biomol Laboratories Inc., (Plymouth Meeting, PA). Recombinant EGF and recombinant human PDGF-BB (PDGF) were purchased from Oncogene Research Products Inc., (San Diego, CA). Rabbit anti-p44/42 Tyr202/Tyr204 (Ab-2) monoclonal antibody and mouse anti-phospho-44/42 monoclonal antibodies were purchased from Cell Signaling Inc., (Beverly, MA). Chicken anti-IGF-1 receptor-a subunit IgY, rabbit anti-chicken IgY bridging antibody, and rabbit anti-chicken IgY horseradish peroxidase conjugate were all purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-PDGFR-β receptor was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit secondary antibody was purchased from Sigma. The anti-Erb B2 antibody (Ab-3) was purchased from Calbiochem. Rabbit anti-SHC polyclonal antibody used for immunoprecipitation was purchased from Upstate Biotechnology; mouse anti-SHC monoclonal antibody used for Western blotting was purchased from BD Transduction Laboratories. Mouse anti-PY99 monomeric antibody and goat anti-EGFR polyclonal antibody were purchased from Santa Cruz Biotechnology Inc.

Cell Culture—Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a humidified 95% air/5% CO2 atmosphere. B22L expressing the wild-type EGFR (B22-EGFR) or EGFR(721A) (B22-KA) were provided by Dr. Paul Bertics (University of Wisconsin, Madison, WI) and grown in the same media plus 20 nM methotrexate (35). B22L cells were transfected with pREK5-G8R(79F) construct (B22-YF), kindly provided by Dr. Ivan Dikic, (Goethe University Medical School, Frankfurt, Germany), or pCDNA3-EGFR(845F) (B22-YF), a gift from Dr. Sally Parsons (University of Virginia, Charlottesville, VA), and selected by supplementing the medium with 200 μg/ml G418. The G418-resistant cells were used without further subcloning. The medium was changed every other day. Subconfluent cells were washed twice and replaced by Dulbecco's modified Eagle's medium without serum just before experiments unless indicated otherwise.

RT-PCR Analysis of Receptor Transcripts—Total RNA was isolated from cells with the TRizol reagent (Invitrogen) and treated with deoxyribonuclease I and reverse-transcribed using the ThermoScript RT-PCR system (Invitrogen). The cDNA was synthesized from 250 ng of RNA amplified with TaKaRa Ex Taq polymerase (PanVera, Madison, WI) using 30 cycles as follows: 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 1 min. PCR primers were designed to amplify EGFR (5'-CTGGAAAGACGCTGATTACA-3') and 5'-TCTATGCTTCAATAACTCATGTCG-3', 277 bp), PDGFR (5'-AGATGACCCTTGAGACTAAGAG-3' and 5'-TTTACAGGAACCTCCCTC-3', 370 bp), and IGF (5'-ACCTTTGGCGATGTCCCGAGG-3' and 5'-TCTATGCGTCAAGGTCGCT-3), 332 bp). PCR products were analyzed by 10% agarose gel electrophoresis and ethidium bromide staining.

Measuring LPA Receptor Expression by Quantitative RT-PCR—Total RNA was isolated from 107 cells using TRizol reagent and digested with amplification grade DNase (Invitrogen). Reverse-transcription of 1 μg of total RNA from each sample was made using Thermoscript RT-PCR System (Invitrogen) with oligo-dT primer. Quantitative PCR was performed applying the real-time SYBR Green PCR technology with the use of an ABI sequence detection system 7700 (Applied Biosystems, Foster City, CA). The human LPA1, LPA2, and LPA3-specific primers were designed with Primer Express Software (Applied Biosystems), and their sequences were as follows: LPA1, 5'-CCACCTTGGACGATCCTGTTGTG-3' and 5'-AGGCGAGGCCTGACAGCTCTG-3'; LPA2, 5'-GGGCTCTGTGTTGCTCTG-3' and 5'-TTATTGGGTTAACGCTAC-3'; and for GAPDH, 5'-AAGGCTCTGCTGGCC-3' and 5'-GGGATGTTACATGCTCACAC-3'. Amplification reaction was performed with SYBR Green PCR Master Mix (Applied Biosystems) and thermal cycling conditions were: 95 °C for 15 s at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Abundance of mRNA was calculated based on cycle threshold values as described previously (39), and the expression of LPA receptor mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA expression. Every PCR assay was performed at least three times, and the results are expressed as mean ± S.E. Statistical comparison of mRNA expression for the target genes was evaluated with the use of analysis of variance.

Apoptosis Detection by DNA Fragmentation Enzyme-linked Immunosorbent Assay—To induce apoptosis, B82L cells were treated with 20 ng/ml TNF-α plus 25 μg/ml cycloheximide (Sigma-Aldrich) for 3 h. DNA fragmentation was measured by enzyme-linked immunosorbent assay following the procedure provided with the Cell Death Detection Enzo-chem kit (Enzo Life Sciences, New York, NY). DNA fragmentation was expressed as absorbance units per microgram of protein per minute.

Immunoprecipitation and Western Blotting—For detection of ERK1/2, cells were washed with phosphate-buffered saline once, then lysed in ice-cold M-PER (Pierce Chemical) protein extraction buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml each aprotinin, leupeptin, and pepstatin. For detection of EGFR, PDGFR, or IGFIR, cells were lysed in a modified radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 mM NaF, and 1 μg/ml each aprotinin, leupeptin and pepstatin. The protein concentration was quantified using the BCA kit (Pierce Chemical). ERK 1/2 and its phospho-
phorylated forms were detected by Western blotting. Twenty micrograms (50 μg for the detection of IGFR) of protein was mixed with an equal amount of 2× SDS-PAGE sample buffer, denatured by boiling for 5 min, followed by separation in SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% (w/v) bovine serum in 10 mM phosphate buffered saline, pH 7.4, and 0.05% Tween 20 for 30 min at room temperature and probed with the primary antibody for 1 h. After three washes with phosphate-buffered saline/Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibody diluted in phosphate-buffered saline/Tween 20 for 1 h, followed by four washes, and developed with the ECL reagent (PerkinElmer Life and Analytical Sciences, Boston, MA).

EGFR, IGFR, ErbB2, and PDGFR were also immunoprecipitated and then probed by Western blotting. In brief, 500 μg of protein/ml of cell lysate was precleared by adding 20 μl of 10% (w/v) protein A/G PLUS-Agarose. After gentle shaking for 30 min at 4 °C and centrifugation at 2500 × g for 5 min, the supernatant was incubated with 5 μg/ml chicken anti-IGFR IgY overnight with agitation followed by addition of 10 μg of rabbit anti-chicken IgG conjugated protein A/G PLUS-Agarose. The beads were collected by centrifugation, washed three times with ice-cold phosphate-buffered saline, and resuspended in 2× SDS-PAGE sample buffer and subjected to Western blotting as described above.

**Proliferation and Wound Healing Migration Assays—**LPA-stimulated DNA synthesis was measured by [3H]thymidine incorporation.

Fig. 1. Receptor profiling of B82L cells. Transcripts encoding LPA and RTK receptors were detected using RT-PCR and quantitative RT-PCR in B82L and its clones expressing EGFR mutants. Comparable expression of LPA receptors was found in all cell lines expressing EGFR constructs as indicated by mRNA abundance values normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each cell line (A). Transcripts encoding PDGFR and IGFR (but none encoding EGFR) were detected in B82L cells (B). Western blot (WB) analysis of cell lysates prepared from B82L-EGFR cells revealed the expression of an EGFR-immunoreactive band with an apparent molecular mass of 180 kDa that was not present in wild-type B82L cells (C). Only traces of IGFR were detectable by immunoprecipitation (IP) in cell lysates of B82L cells compared with F32 VIII C4 mouse hybridoma (C4) cells that abundantly expressed IGF-I (D, left). IGF (10 ng/ml) treatment of B82L cells for 5 min led to a barely detectable amount of phospho-tyrosine in the immunoprecipitated IGFR (D, right).

**RESULTS**

**Profiling of LPA and RTK Receptors in B82L Cells—**RT-PCR analysis revealed that B82L cells expressed transcripts for the three LPA receptors of the endothelial differentiation gene family (Fig. 1A). The expression of LPA receptor transcripts determined by quantitative RT-PCR was identical in wild-type B82L cells and in the B82L clones stably expressing wild-type or mutant EGFR (Fig. 1A). In agreement with previous reports (23, 35), B82L cells did not express EGFR transcript and protein (Fig. 1, B and C). In B82L cells stably transfected with wild-type EGFR, Western blot analysis readily detected the expression of wild-type receptor protein in the cell lysate (Fig. 1C). Abundant PDGFR and IGFR transcripts were detected in B82L cells (Fig. 1B). We also detected the expression of ErbB2 and PDGFR protein (see below). Although we found IGFR transcripts in B82L cells (Fig. 1B), we detected only trace amounts of IGFR protein using immunoprecipitation combined with Western blotting, although the antibody we used readily detected the mouse IGFR in F32 VIII C4 mouse hybridoma cells used as positive control (C4; Fig. 1D). Although 10 ng/ml IGF induced a marked increase in tyrosine phosphorylation of the IGFR in C4 cells, we failed to detect any increase in tyrosine-phosphorylated IGFR in B82L cells after a 5-min treatment (Fig. 1D). These results discount the IGFR as a major potential target of LPA transphosphorylation in B82L cells. Thus, we focused our studies on the PDGFR and ErbB2 receptors.

**LPA Prevents Apoptosis in B82L Cells—**LPA is a potent survival factor in many cell types (27–30). To evaluate whether the EGFR receptor was required for the LPA-elicted antiapoptotic activity, we examined its effect on TNF-α-induced apoptosis in both B82L and B82L-EGFR cells by measuring DNA fragmentation. Treatment of both cell lines with 20 ng/ml TNF-α in the presence of 25 ng/ml cycloheximide for 3 h resulted in a severalfold increase in DNA fragmentation compared with controls (p < 0.001; Fig. 2A). A 15-min pretreatment of the cells with 1 μM LPA before TNF-α exposure attenuated DNA fragmentation by more than 70% in both the B82L and B82L-EGFR cells (p < 0.001, Fig. 2A). There was no statistically significant difference in DNA-induced protection between the wild-type B82L and the EGFR expressing cells or when LPA was co-applied with EGF or PDGF to either cell line (Fig. 2B). The EGFR was immunoprecipitated from B82L-EGFR cells and its EGFR- or LPA-induced tyrosine phosphorylation was evaluated (Fig. 2C). Although 5-min exposure to 10 ng/ml EGF induced tyrosine phosphorylation, 1 μM LPA caused no detectable level of transphosphorylation of the EGFR with this technique. These results indicate that heterologous expression of the EGFR receptor does not affect the LPA-elicted antiapoptotic effect and that the EGFR, although readily activated by its cognate ligand, does not show detectable transphosphorylation in response to LPA.

**EGF and PDGF Do Not Prevent Apoptosis in B82L and B82L-EGFR Cells—**To further characterize the antiapoptotic effects of EGF and PDGF in B82L and B82L-EGFR cells, we...
comparing the effect of these two RTK ligands with that of LPA on TNF-α-induced apoptosis. Whereas LPA (1 μM) exerted a significant reduction in TNF-α-induced DNA fragmentation, neither EGF (100 ng/ml) nor PDGF (10 ng/ml) caused significant reduction regardless of whether the cells expressed the EGFR (Fig. 2B). B82L cells express the ErbB2 receptor that can heterodimerize with and thus become transactivated by the EGFR (41, 42). The ErbB2 receptor can be directly activated by H₂O₂ (500 μM) treatment, leading to tyrosine phosphorylation of the receptor (Fig. 3A). Exposure to 1 μM LPA for 5 min failed to elicit a detectable phosphorylation of the ErbB2 receptor. These results corroborate the observation that EGFR or ErbB2 activation is not necessary and does not alter LPA-induced protection from TNF-α-induced apoptosis.

Although PDGF did not elicit a protective effect in B82L cells or augment the effect of LPA (Fig. 2B), LPA exposure has nevertheless been documented to elicit the transphosphorylation of the PDGFR in some cell lines, including bronchial epithelial cells, mesangial cells, and L cells (21–23). This raises the possibility that in the absence of EGFR, PDGFR transactivation might somehow be involved in mediating LPA signals, as described previously by Herrlich and colleagues (23) in a different subclone of B82L cells. We tested this hypothesis in our B82L cells that express functional PDGFR receptor (Figs. 1B and 3B). Western blot analysis of the immunoprecipitated PDGFR demonstrated that exposure of B82L cells to 1 μM LPA for 5 min did not increase PDGFR phosphorylation (Fig. 3B). At the same time, the PDGFR-specific inhibitor tyrphostin AG1296 (1 μM) efficiently blocked PDGF-elicted receptor autophosphorylation. Although 1 μM LPA significantly inhibited TNF-α-induced DNA fragmentation in B82L cells, pretreatment of the cells with 1 μM PDGF kinase inhibitor AG1296 neither showed antiapoptotic activity nor attenuated the LPA-induced antiapoptotic response (Fig. 3C). These results argue against the idea that PDGF is a substrate of LPA-induced transphosphorylation and that it mediates the protective effect of LPA in our B82L cells.

PTX- and PD98059-sensitive ERK1/2 Activation Is Required for the LPA-elicted Antiapoptotic Effect in B82L Cells—Acti-
EGFR Tyrosine Kinase Inhibitors Block LPA-induced Apoptotic Protection against Apoptosis in Wild-type B82L Cells—

The EGFR receptor tyrosine kinase inhibitors AG1478 and PD158780 have been widely used to study EGFR receptor-mediated signal transduction, and many investigators have applied them at high concentrations (>1 μM) to obtain pharmacological evidence for EGFR transactivation. Our results with B82L cells show that the antiapoptotic response to LPA does not require the presence of EGFR. Therefore, the B82L cells provide an ideal model to identify the nonspecific activities by EGFR kinase inhibitors AG1478 and PD158780. Application of 1 μM LPA to TNF-α-treated wild-type B82L cells reduced DNA fragmentation by 70% (Fig. 4B). Pretreatment with AG1478 or PD158780 brought about a dose-dependent inhibition of LPA-induced protection against DNA fragmentation (Fig. 4B). At concentrations below 1 μM, no significant inhibition was detected; concentrations in excess of 3 μM, however, significantly inhibited the LPA-elicited protection against apoptosis. Because the activation of ERK1/2 was required for the antiapoptotic response (Fig. 4A), we tested whether AG1478 and PD158780 would inhibit this signaling pathway. Consistent with the inhibition of DNA fragmentation, pretreatment of B82L cells with AG1478 at concentrations in excess of 500 nM dose-dependently inhibited the LPA-elicited phosphorylation of ERK1/2 (Fig. 4C). Similar concentration-dependent inhibition was found with PD158780 (data not shown). These results caution against the use of concentrations of these two EGFR tyrosine kinase inhibitors in excess of only 500 nM, because they exert nonspecific inhibitory effects on ERK1/2 signaling.

EGF Receptor Expression Augments LPA-stimulated DNA Synthesis—Our results in B82L cells indicated that EGFR transactivation was not required for LPA-induced protection against apoptosis, which raised questions about the necessity of EGFR in the mitogenic response to LPA. In B82L cells, treatment with LPA modestly stimulated DNA synthesis in a dose-dependent manner (Fig. 5A). To evaluate the effect of EGF on LPA-induced mitogenic responses, we compared the effects of LPA, EGF, and PDGF on DNA synthesis in B82L and B82L-EGFR cells (Fig. 5B). Treatment with 5 μM LPA induced a significantly bigger increase of DNA synthesis in B82L-EGFR cells compared with that in B82L cells (p < 0.001). PDGF-induced DNA synthesis was neither significantly different nor inhibited by PTX in the two cell lines (Fig. 5B). Treatment with EGF stimulated DNA synthesis only in B82-EGFR cells and had no effect in the wild-type B82L cells, which is in agreement with a previous report (25). These findings indicate that forced expression of the EGFR plays a modulatory role in LPA-induced DNA synthesis. PTX (100 ng/ml) pretreatment significantly reduced (60%) LPA-induced DNA synthesis in B82L-EGFR cells and abolished it in B82L cells (Fig. 5B). Inhibition of mitogen-activated protein kinase kinase activation by 20 μM PD98059 completely blocked the LPA stimulated mitogenic effect in both B82L and B82L-EGFR cells. The EGF-induced increase in DNA synthesis in B82L-EGFR cells was not inhibited by PTX but was abolished by the mitogen-activated protein kinase kinase inhibitor PD98059 (Fig. 5B). Our results indicate that stable expression of the EGFR in B82L cells enables EGF to stimulate DNA synthesis and that EGFR also augments the mitogenic actions of LPA.

Co-expression of the EGF Receptor Augments LPA-stimulated Cell Migration—LPA elicited only a modest increase in DNA synthesis that was substantially enhanced by the co-expression of the EGFR. Therefore, we sought a more robust EGFR-modulated LPA response and investigated LPA-induced cell migration in wild-type B82L and B82L-EGFR cells using a wound healing assay (Fig. 6A). In B82L cells, 5 μM LPA induced a

![Diagram](https://via.placeholder.com/150)
Selective Modulation of LPA Responses by EGF Receptor Mutants

deficient EGFRK721A mutant, EGFRY5F that lacks the five autophosphorylation sites, and EGFRY845F that lacks the Src phosphorylation site, and evaluated the cellular responses to LPA, EGF, and PDGF. We found no difference in LPA-induced attenuation of TNF-α-elicited DNA fragmentation among wild-type B82L, B82-EGFR (Fig. 2), or cells expressing receptor mutants including B82-KA, B82-Y5, and B82-YF (Fig. 6C). Application of EGF or PDGF to any of the five EGFR-transfected cell lines failed attenuate TNF-α-elicited DNA fragmentation (Figs. 2 and 6C).

Although PDGF-induced DNA synthesis was not statistically different in any of the five cell lines tested, regardless of which EGFR construct they expressed, in B82-KA and B82-YF cells, LPA elicited significantly higher [3H]thymidine incorporation than in wild-type B82L cells (Fig. 6B). EGF elicited significant DNA synthesis in B82-EGFR (Fig. 5), B82-YF, and B82-Y5F cells (Fig. 6B). The augmented responses in LPA-induced DNA synthesis were not statistically different between B82-EGFR, B82-KA, and B82-YF cells. In every cell line, PD98059 inhibited LPA- and PDGF-induced DNA synthesis, whereas PTX inhibited only the LPA-induced increase in [3H]thymidine incorporation. In B82-Y5F cells, LPA-induced [3H]thymidine incorporation was indistinguishable from that found in wild-type B82L cells, indicating that this EGFR mutant failed to augment LPA-induced DNA synthesis. Altogether, these results suggest that the tyrosine kinase activity of the transfected EGFR is not necessary for the augmentation of LPA-induced DNA synthesis. However, deletion of the five autophosphorylation sites but not the Src phosphorylation site abolished the augmentation of LPA-induced [3H]thymidine incorporation seen in B82-EGFR cells.

We also compared LPA-elicited cell migration in the different B82 cell lines. In B82-KA cells, LPA elicited a 4-fold increase in the rate of cell migration that was identical to that seen in B82-EGFR cells, although the basal rate of cell migration was also identical in both types of cells (Fig. 6A). EGF failed to induce a significant change in cell migration in either cell line with or without EGFR expression. In contrast, PDGF elicited a significant migratory response in all cell lines that was abolished by 1 μM AG1296. These experiments reinforced the principles found in the DNA synthesis experiments and indicate that the tyrosine kinase activity of the EGFR is not required for EGF to augment LPA-induced cell migration.

Several reports demonstrating LPA-induced transphosphorylation of the EGFR implicated Src or Src-family tyrosine kinases in transphosphorylation (6, 16, 17). Therefore, we hypothesized that an LPA activated kinase might lead to the transphosphorylation of the EGFR, which in its phosphorylated state could augment LPA signaling by acting as a scaffold enhancing the assembly of signaling complexes. We tested this hypothesis in B82-Y5F and B82-YF cells. However, LPA-induced an increase of ~5-fold in the migration of B82-YF cells, indicating that the Src phosphorylation site was not required for the augmented migration. In contrast, LPA induced only a 2-fold increase in B82-Y5F cells that was identical to that seen in wild-type B82L cells (Fig. 6A), whereas the basal and PDGF-induced rate of migration was not statistically different in any of the B82L cells transfected with different EGFR variants.

Thus, introduction of the EGFRY5F mutant into B82L cells failed to cause an enhancement in LPA-induced rate of cell migration. Together, these data suggest that the presence of the EGFR receptor can positively modulate cell migration and DNA synthesis elicited by LPA; however, intrinsic tyrosine kinase activity and the Src phosphorylation site are not necessary but the tyrosine autophosphorylation sites are necessary for the augmentation.

2-fold increase in migration rate. This activation by LPA was abrogated by 100 ng/ml PTX (Fig. 6A) or 20 μM PD98059 (data not shown) pretreatment. In B82L-EGFR cells, 5 μM LPA induced a 5-fold increase in migration rate that was significantly higher than that in B82L cells. In B82L-EGFR cells, the LPA-induced migration was significantly attenuated with PTX pretreatment (Fig. 6A) or 20 μM PD98059 (not shown). EGF alone failed to induce a migratory response in either wild-type B82L or B82L-EGFR cells, which is in agreement with a previous report (35). Furthermore, pretreatment of B82L-EGFR cells with 1 μM AG1478 did not inhibit LPA induced migration and had no effect on the basal rate of cell migration. In contrast to EGF, PDGF elicited a significant increase in migration rate, although smaller than that elicited by 5 μM LPA. The PDGF-elicited migratory response in wild-type B82L cells and B82L-EGFR cells was abolished by AG1296 treatment, whereas this tyrophostin had no significant inhibitory effect on the rate of LPA-induced cell migration (Fig. 6A).

Effect of Mutant EGFR on LPA-induced Cellular Responses in B82L Cells—We were intrigued by the observation that although EGF stimulated DNA synthesis in B82-EGFR cells, it failed to increase cell migration, yet the LPA-induced cell migration was augmented. The B82L cells and B82L-EGFR cells used in our study have been shown not to secrete EGF or TGF-α (35). One possible explanation of this cooperation between LPA GPCR and EGFR could involve activation of the intrinsic kinase activity and/or the transphosphorylation of the EGFR. To evaluate this hypothesis, we obtained or generated stable transfectants of B82L cells expressing either kinase-deficient EGFRK721A or EGFRY5F that lacks the five autophosphorylation sites, and EGFRY845F that lacks the Src phosphorylation site, and evaluated the cellular responses to LPA, EGF, and PDGF. We found no difference in LPA-induced attenuation of TNF-α-elicited DNA fragmentation among wild-type B82L, B82-EGFR (Fig. 2), or cells expressing receptor mutants including B82-KA, B82-Y5, and B82-YF (Fig. 6C). Application of EGF or PDGF to any of the five EGFR-transfected cell lines failed attenuate TNF-α-elicited DNA fragmentation (Figs. 2 and 6C).

Although PDGF-induced DNA synthesis was not statistically different in any of the five cell lines tested, regardless of which EGFR construct they expressed, in B82-KA and B82-YF cells, LPA elicited significantly higher [3H]thymidine incorporation than in wild-type B82L cells (Fig. 6B). EGF elicited significant DNA synthesis in B82-EGFR (Fig. 5), B82-YF, and B82-Y5F cells (Fig. 6B). The augmented responses in LPA-induced DNA synthesis were not statistically different between B82-EGFR, B82-KA, and B82-YF cells. In every cell line, PD98059 inhibited LPA- and PDGF-induced DNA synthesis, whereas PTX inhibited only the LPA-induced increase in [3H]thymidine incorporation. In B82-Y5F cells, LPA-induced [3H]thymidine incorporation was indistinguishable from that found in wild-type B82L cells, indicating that this EGFR mutant failed to augment LPA-induced DNA synthesis. Altogether, these results suggest that the tyrosine kinase activity of the transfectected EGFR is not necessary for the augmentation of LPA-induced DNA synthesis. However, deletion of the five autophosphorylation sites but not the Src phosphorylation site abolished the augmentation of LPA-induced [3H]thymidine incorporation seen in B82-EGFR cells.

We also compared LPA-elicited cell migration in the different B82 cell lines. In B82-KA cells, LPA elicited a 4-fold increase in the rate of cell migration that was identical to that seen in B82-EGFR cells, although the basal rate of cell migration was also identical in both types of cells (Fig. 6A). EGF failed to induce a significant change in cell migration in either cell line with or without EGFR expression. In contrast, PDGF elicited a significant migratory response in all cell lines that was abolished by 1 μM AG1296. These experiments reinforced the principles found in the DNA synthesis experiments and indicate that the tyrosine kinase activity of the EGFR is not required for EGF to augment LPA-induced cell migration.

Several reports demonstrating LPA-induced transphosphorylation of the EGFR implicated Src or Src-family tyrosine kinases in transphosphorylation (6, 16, 17). Therefore, we hypothesized that an LPA activated kinase might lead to the transphosphorylation of the EGFR, which in its phosphorylated state could augment LPA signaling by acting as a scaffold enhancing the assembly of signaling complexes. We tested this hypothesis in B82-Y5F and B82-YF cells. However, LPA-induced an increase of ~5-fold in the migration of B82-YF cells, indicating that the Src phosphorylation site was not required for the augmented migration. In contrast, LPA induced only a 2-fold increase in B82-Y5F cells that was identical to that seen in wild-type B82L cells (Fig. 6A), whereas the basal and PDGF-induced rate of migration was not statistically different in any of the B82L cells transfected with different EGFR variants.

Thus, introduction of the EGFRY5F mutant into B82L cells failed to cause an enhancement in LPA-induced rate of cell migration. Together, these data suggest that the presence of the EGFR receptor can positively modulate cell migration and DNA synthesis elicited by LPA; however, intrinsic tyrosine kinase activity and the Src phosphorylation site are not necessary but the tyrosine autophosphorylation sites are necessary for the augmentation.

Fig. 5. LPA induces a dose-dependent increase in DNA synthesis in B82L cells that is enhanced in B82-EGFR cells. A, serum-starved wild-type B82L cells were exposed to increasing concentrations of LPA, and DNA synthesis was evaluated 24 h later using [3H]thymidine incorporation (A). LPA concentrations in excess of 1 μM caused a statistically significant increase in [3H]thymidine incorporation over vehicle controls (*, p < 0.05). LPA elicited higher DNA synthesis in B82-EGFR cells than in B82L cells and B82-EGFR cells (B). Sister cultures received either pretreatment with 100 ng/ml PTX (overnight) or 20 μM PD98059 (45 min). LPA (5 μM) elicited ~2-fold higher [3H]thymidine incorporation in B82-EGFR cells compared with wild-type B82L cells. LPA-induced DNA synthesis in both cell lines was inhibited by PTX and PD98059. Data represent the mean ± S.E. of three independent experiments. *, p < 0.001 compared with vehicle controls; †, p < 0.01 compared with LPA treatment.
FIG. 6. A, LPA responses are differentially affected by mutant EGFR receptors. To test cell migration, confluent cells were serum-starved overnight, wounded, and exposed to 5 μM LPA, 25 ng/ml EGF, or 10 ng/ml PDGF for 8 h. Parallel cultures were pretreated with 100 ng/ml PTX pretreatment (overnight), 1 μM AG1478, 1 μM PD158780, or 1 μM AG1296 for 45 min before treatment with the different receptor ligands. Cell migration was photographed and measured using NIH Image software. LPA induced a 2-fold increase in cell migration in wild-type B82L cells. In contrast, in B82-EGFR and B82-KA cells, LPA-induced migration was 5-fold over control and sensitive to PTX but not significantly attenuated by either 1 μM each of AG1478 or AG1296. PDGF alone induced a slight increase in cell migration that was abolished by 1 μM AG1296. However, in B82-Y5F cells, LPA induced only a modest increase in migration that was comparable with that seen in wild-type cells. Data shown are the means ± S.E. of three independent results. *, p < 0.001 compared with vehicle controls; †, p < 0.01 compared with LPA or PDGF treatment.

B, LPA elicits protection similar to TNF-α-induced DNA fragmentation in B82-KA, B82-Y5F, and B82-YF cells. Cells stably expressing the different EGFR mutants were starved in serum-free Dulbecco’s modified Eagle’s medium overnight. LPA was applied at a concentration of 1 μM 15 min before TNF-α, and DNA fragmentation was measured 3 h after the induction of apoptosis. Note that LPA caused an ~80% reduction in DNA fragmentation in all three cell types, which is similar to that found in wild-type B82L and B82-EGFR cells (see Fig. 2, A and B). Neither EGF (100 ng/ml) nor PDGF (10 ng/ml) attenuated DNA fragmentation and synergized with LPA. Data shown represent the mean ± S.D. of three independent experiments, and asterisks represents p < 0.001 compared with TNF-α. Abs, antibodies.

C, LPA-elicited [3H]thymidine incorporation was augmented in B82-KA cells and B82-YF cells, whereas it remained at the level of the wild-type cells in B82Y5F cells (see Fig. 5 B). Sister cultures received 100 ng/ml EGF, 10 ng/ml PDGF, or pretreatment with 100 ng/ml PTX (overnight) or PD98059 (20 μM, 45 min). EGF increased [3H]thymidine incorporation in B82-EGFR, B82-Y5F, and B82-YF cells. LPA-induced DNA synthesis was inhibited by PTX and PD98059. The mitogenic effect of EGF and PDGF was not affected by PTX but was abolished by PD98059. Data represent the mean ± S.D. of three independent experiments. *, p < 0.001 compared with vehicle controls; †, p < 0.01 compared with LPA, EGF, or PDGF treatment.
pretreated with vehicle or PTX (100 ng/ml) overnight and exposed to 1 μg/ml LPA for 5 min. Cell lysates containing 500 μg of total protein for each sample were immunoprecipitated (IP) with monoclonal EGFR antibody, followed by Western blotting (WB) with anti-SH3 or anti-phospho-SH3 antibodies. IB, immunoblot. Total SHC was also probed with monoclonal anti-SH3 antibody, and the ratio of phospho-SHC to total SHC (data not shown) was calculated. LPA-elicited SHC phosphorylation was present in B82-EGFR, B82-KA, and B82-YF cells but was absent in wild-type and B82-Y5F cells. SHC phosphorylation was not sensitive to PTX. SHC was recruited into a stable complex with the EGFR in B82-EGFR and B82-KA cells but not in the other cell lines. LPA elicits different levels of ERK1/2 phosphorylation in B82L cells (Fig. 4, C and D). These results suggest that enhancement of LPA-induced responses, SHC phosphorylation, and full-blown ERK1/2 activation require some or all of the five autoregulatory tyrosines of the EGFR.

**DISCUSSION**

The concept of RTK transregulation by GPCR is based on increased tyrosine phosphorylation of RTK consequent to ligand activation of the GPCR. Although the mechanisms causing RTK phosphorylation induced by the authentic polypeptide growth factor or GPCR ligands and its consequences on ERK1/2 activation have been the subject of intense investigations, the functional consequences of this receptor cross-talk have not been investigated. Our long-standing interest in elucidating the signal transduction mechanisms mediating the different cellular responses to LPA, a GPCR ligand that elicits RTK phosphorylation in many types of cells, has prompted us to investigate the effect of EGFR in the modulation of LPA-induced antiapoptotic protection, mitogenesis, and migration using a receptor add-back approach in B82L cells that lack endogenous EGFR.

B82L cells express all three types of proven LPA receptors in addition to ErbB2, PDGFR, and IGFR (Fig. 1). However, in our B82L cells (35), LPA failed to elicit a detectable increase in tyrosine phosphorylation of these endogenous RTKs or heterologously expressed human EGFR, although each receptor was autophosphorylated in response to its authentic ligand. Notwithstanding, LPA-induced cell migration and DNA synthesis but not antiapoptotic protection was augmented in cells after heterologous expression of EGFR, regardless of whether it possessed an active or dead tyrosine kinase or whether Tyr411, the Src phosphorylation site, was intact. It is important to note that transregulation of RTK by GPCR shows cell type specificity (13, 20); not only the EGFR but also other RTKs can be substrates of GPCR-induced transphosphorylation, including the PDGFR (21–23, 43). ERK1/2 activity was required for LPA-induced antiapoptotic protection, mitogenesis, and migration (Figs. 4A, 5B, and 6D and data not shown). In wild-type B82L cells, LPA elicited ERK1/2 activation (Fig. 4), which, although less robust than in B82-EGFR cells (Fig. 7), was sufficient to protect cells from TNFα-induced apoptosis (Fig. 2, A and B). It seems that enhanced LPA-elicited cell migration and DNA synthesis correlates with LPA-induced SHC phosphorylation and a more robust activation of ERK1/2 (Fig. 7). However, to substantiate this correlation, a quantitative comparison of the magnitude and duration of LPA-elicited ERK activity, along with the quantitative mechanistic analysis of the canonical SHC/Grb2/Sos/Ras signaling axis, will have to be done in future experiments.

Heterologous expression of the EGFR, and any of its three mutants we tested, had no impact on LPA-induced protection against apoptosis (Figs. 2 and 6). The B82L cell line and its EGFR- and EGFR-KA-expressing subclones have been thoroughly characterized previously (35, 44–48). These cells, unlike the other EGFR-deficient L-cell clone used by Herrlich et al. (23), show no LPA-induced transphosphorylation of the PDGFR (Fig. 2C). Furthermore, we found no evidence for the

**FIG. 7.** LPA stimulates SHC phosphorylation and recruitment to EGFR (A and B). Serum-starved wild-type B82L cells and mutants were pretreated with vehicle or PTX (100 ng/ml) overnight and exposed to 1 μg/ml LPA for 5 min. Cell lysates containing 500 μg of total protein for each sample were immunoprecipitated (IP) with monoclonal anti-EGFR antibody, followed by Western blotting (WB) with anti-SHC antibody, and the ratio of phospho-SHC to total SHC (data not shown) was calculated. LPA-elicited SHC phosphorylation was present in B82-EGFR, B82-KA, and B82-YF cells but was absent in wild-type and B82-Y5F cells. SHC phosphorylation was not sensitive to PTX. SHC was recruited into a stable complex with the EGFR in B82-EGFR and B82-KA cells but not in the other cell lines. LPA elicits different levels of ERK1/2 phosphorylation in B82L cells (35), LPA failed to elicit a detectable increase in tyrosine phosphorylation of these endogenous RTKs or heterologously expressed human EGFR, although each receptor was autophosphorylated in response to its authentic ligand. Notwithstanding, LPA-induced cell migration and DNA synthesis but not antiapoptotic protection was augmented in cells after heterologous expression of EGFR, regardless of whether it possessed an active or dead tyrosine kinase or whether Tyr411, the Src phosphorylation site, was intact. It is important to note that transregulation of RTK by GPCR shows cell type specificity (13, 20); not only the EGFR but also other RTKs can be substrates of GPCR-induced transphosphorylation, including the PDGFR (21–23, 43). ERK1/2 activity was required for LPA-induced antiapoptotic protection, mitogenesis, and migration (Figs. 4A, 5B, and 6D and data not shown). In wild-type B82L cells, LPA elicited ERK1/2 activation (Fig. 4), which, although less robust than in B82-EGFR cells (Fig. 7), was sufficient to protect cells from TNFα-induced apoptosis (Fig. 2, A and B). It seems that enhanced LPA-elicited cell migration and DNA synthesis correlates with LPA-induced SHC phosphorylation and a more robust activation of ERK1/2 (Fig. 7). However, to substantiate this correlation, a quantitative comparison of the magnitude and duration of LPA-elicited ERK activity, along with the quantitative mechanistic analysis of the canonical SHC/Grb2/Sos/Ras signaling axis, will have to be done in future experiments.

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inhibition of the LPA responses by the PDGFR-specific tyrophostin AG1296, although it inhibited the PDGF response and the autophosphorylation of PDGFR (Figs. 2 and 6). We attribute this to a difference between the individual cell lines. It is interesting that Li et al. (35) have shown that EGF did not increase the migration in B82L-EGFR cells but enhanced their migration in response to fibronectin or laminin even in the absence of added EGF, which is consistent with a downstream role of EGFR in the signal transduction pathways mediating integrin-activated fibroblast migration. In our experiments, EGF also failed to activate migration in B82L-EGFR cells, yet LPA-induced migration was augmented in an AG1478- and AG1296-independent but PTX- and PD98059-dependent manner. Moreover, these pharmacological data suggest that heterotrimeric G proteins of the Gi/o subfamily and ERK1/2 activation are essential for this LPA effect. Thus, it seems that RTK can silently modulate GPCR responses that themselves do not elicit in a given cell type. Our findings in B82L cells are not without precedent, because in GD25 mouse fibroblast cells, despite the failure of EGF and PDGF alone to induce migration, LPA has been shown to enhance migration (26). The synergizing effect between LPA and EGF in GD25 fibroblasts was sensitive to PTX and C3 exotransferase, indicating the role of heterotrimeric G proteins and Rho signaling. Thus, our results are similar to those of Sakai and colleagues (26), who found no evidence for transregulation or sensitization of EGFR and PDGFR by LPA and found that the increase in ERK1/2 activation to a combination of these ligands was additive. Thus, modulation of LPA-induced cell migration without detectable RTK transphosphorylation is not unique to B82L cells.

Our experiments using the EGFR kinase inhibitors AG1478 and PD158780 raise caution, in that we found that LPA-induced antiapoptotic protection (Fig. 4) and other ERK1/2-dependent responses were inhibited in EGF-null B2L cells. Both inhibitors have been used extensively to probe the role of EGFR tyrosine kinase activity in LPA-induced ERK1/2 activation. Most investigators use these RTK inhibitors at 100–200 nM concentrations, and we found that when used at a slightly higher concentration, in excess of 500 nM, they cause a significant inhibition of ERK1/2. Whether these compounds inhibit ERK1/2 directly or via another kinase involved in ERK1/2 activation remains to be determined.

Our findings that the Src phosphorylation site was not required for the augmentation of LPA-induced migration and proliferation are consistent with reports that have shown that LPA activated ERK1/2 in cells derived from Src−/− or Src−/−Y5F−/−Y5F−/− animals (14) and that Src activity was not affected by LPA or by PD158780 in HeLa and NIH3T3 cells (25). These observations discount the role of the Src phosphorylation site in the enhancement of LPA responses. The other unexpected observation in our studies was that EGFR kinase activity was not required for the augmentation of the mitogenic and migratory response to LPA; however, the intact autophosphorylation sites were necessary. One possible explanation for the enhancement of the LPA response by the kinase-dead receptor might be that downstream signals from the LPA GPCR could activate soluble kinase(s), which could bypass the need of autophosphorylation and generate specific phosphotyrosine residues necessary for docking of various signaling molecules, including SHC (Fig. 7), to the EGFR (37, 49, 50). This also implies that tyrosine phosphorylation of EGFR is a required event for the augmentation of some (mitogenesis and migration) but not all (antiapoptosis) cellular responses to LPA. Indeed, kinase-deficient EGFR mutants have been found to promote cell proliferation (Fig. 6C) and SHC phosphorylation (Fig. 7) through heterodimerization-mediated transactivation of the ErbB2 receptor in B82L cells (37, 41, 49, 50). In our studies, however, we found no detectable transactivation of ErbB2 by LPA in B82L cells, although this does not exclude the possibility that such a transregulation could not occur in cells expressing EGFR or its mutants. Thus, future experiments will have to address the possibility of a triple transregulatory axis that involves LPA GPCR-EGFR and ErbB2.

The lack of augmentation in cells expressing the Y5F receptor mutant bring new attention to the importance of some, or all, EGFR residues Tyr992, Tyr1068, Tyr1086, Tyr1148 and Tyr1173 (51). The primary binding site for SHC has been mapped to Tyr1173 and a secondary site has been found at Tyr992 (52). SHC docking to the EGFR sets up a hierarchical relationship with Grb2 binding, and bound SHC augments Grb2 binding and phosphorylation (52). EGFR-Y5F cells showed a lack of LPA-activated SHC phosphorylation and a modest level of ERK1/2 phosphorylation similar to that seen in wild-type B82L cells and much less than that in those EGFR-expressing clones tested in the present study. This sets up an apparent contradiction: immunoprecipitation and Western blotting failed to detect LPA-induced tyrosine phosphorylation of the EGFR, yet intact autophosphorylation sites seem to be required for enhanced LPA responses. The reasons for this remain unclear. However, given the complicated nature of binding sites for these adaptor proteins on the EGFR, secondary sites with short-lived phosphotyrosines might be one hypothesis to explain our inability to detect receptor tyrosine phosphorylation. It is interesting that EGF stimulation of an EGFR lacking the autophosphorylation sites can still induce SHC phosphorylation and SHC-Grb2 association (49), and transactivation via heterodimerization with the ErbB2 receptor has been implicated in mediating the activation of SHC-Grb2 (50). Thus, autophosphorylation site mutant EGFRs have been shown to signal through auxiliary mechanisms (37). However, our data suggest that these auxiliary mechanisms are not effectively activated by LPA in the case of the Y5F EGFR mutant.

The lack of need for EGFR tyrosine kinase activity in eliciting several EGF responses is well documented in the literature (41, 50). Similarly, it is well documented that the phosphotyrosines on the EGFR required for the modulation of some LPA responses can be generated via redundant pathways that include not only the intrinsic tyrosine kinase of the EGFR but also other soluble tyrosine kinases. However, neither Src, Yes, nor Fyn was found to mediate EGFR phosphorylation, but Pyk-2 was, although it was not necessary for ERK1/2 activation by LPA (14). The substantial, although semiquantitative, differences in ERK1/2 phosphorylation among the different clones suggest that migration and DNA synthesis may require a more robust activation of the mitogen-activated protein kinase in which transregulation of the EGFR, with its permissive input to Ras activation described by Rubio et al. (31), plays a necessary role. The identification of the specific sites and the LPA GPCR-regulated molecules phosphorylating and/or docking to these EGFR tyrosines will be subject of future investigation.

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