Regulation of Epidermal Growth Factor Receptor Internalization by G Protein-Coupled Receptors†

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Received October 2, 2002; Revised Manuscript Received December 16, 2002

ABSTRACT: The epidermal growth factor (EGF) receptor (EGFR) plays a central role in regulating cell proliferation, differentiation, and migration. Cellular responses to EGF are dependent upon the amount of EGFR present on the cell surface. Stimulation with EGF induces sequestration of the receptor from the plasma membrane and its subsequent downregulation. Recently, internalization of the EGFR was also shown to be required for mitogenic signaling via the activation of MAP kinases. Therefore, mechanisms regulating internalization of the EGFR represent an important facet for the control of cellular response. Here, we demonstrate that EGFR is removed from the cell surface not only following stimulation with EGF, but also in response to stimulation of G protein-coupled lysophosphatidic acid (LPA) and β2 adrenergic (β2AR) receptors. Using a FLAG epitope-tagged EGFR to quantitate receptor internalization, we show that incubation with EGF, LPA, or isoproterenol (ISO) causes the time-dependent loss of cell surface EGFR. Internalization of EGFR by these ligands involves the tyrosine kinase activity of the receptor itself and c-Src, as well as the GTPase activity of dynamin. Unexpectedly, we find that internalization of the EGFR by EGF is dependent upon G$\beta\gamma$ and β-arrestin proteins; expression of minigenes encoding the carboxyl terminii of the G protein-coupled receptor kinase 2, or β-arrestin1, attenuates LPA-, ISO-, and EGF-mediated internalization of EGFR. Thus, G protein-coupled receptors can control the function of the EGFR by regulating its endocytosis.

Epidermal growth factor (EGF)[1] receptors (EGFRs) regulate cell growth and differentiation. Binding of EGF to extracellular domains of the receptor induces EGFR homodimerization, which leads to activation of the receptor's catalytic protein tyrosine kinase domain and to tyrosine autophosphorylation (*1*, *2*). Phosphorylated EGFR presents docking sites for Src homology 2 (SH2) domains of adapter molecules such as Gab1 and Grb2 (*3*). Recruitment of these proteins leads to assembly of a multiprotein signal transduction complex, one function of which is to regulate the small GTPase Ras. Activated, GTP-bound, Ras then initiates cell growth signaling cascades, in part, by activating the extracellular signal-regulated kinase 1 and 2 (ERK) MAP kinases through the sequential phosphorylation of Raf and MEK (*2*, *3*).

EGF binding also triggers efficient EGFR internalization that requires the coordinated interactions between several molecules at the cell surface. Autophosphorylated EGFR recruits proteins implicated in endocytosis such as EGFR pathway substrate 15 (Eps15), AP-2, and synaptojanin (*4*, *5*). Assembly of clathrin-coated pits following EGF stimulation requires the tyrosine kinase activity of c-Src (*6*) and fission of the pit from the plasma membrane is regulated by dynamin, a GTP hydrolyzing enzyme (*7*, *8*). Often, internalized EGFRs are routed to lysosomes where they are degraded. The resulting decrease in the number of receptors expressed at the cell surface is termed receptor downregulation and is believed to play an essential role in modulating the proliferative response to the growth hormone (*9*).

Recently, the process of EGFR internalization was also shown to be required for mitogenic signaling via the activation of ERKs (*10*, *11*); inhibition of vesicle trafficking attenuated EGF-mediated ERK phosphorylation. It has been postulated that movement of vesicles, containing EGFRs, from the cell surface into the cytosol serves as a shuttling mechanism, bringing activated enzymes (e.g., Raf and MEK) into close proximity to their cytosol-found substrates (*12*−*14*).

In addition to transducing signals initiated by EGF, recent evidence suggests that the EGFR is required for signal relay initiated by stimuli that do not directly interact with this receptor. Activation of ERK by adrenergic, thrombin, endothelin, and lysophosphatidic acid G protein-coupled receptors (GPCRs) and insulin-like growth factor 1 (IGF-1) tyrosine kinase receptor (*15*, *16*) induces the tyrosine phosphorylation of EGFR, and inhibition of EGFR tyrosine kinase activity markedly attenuates ERK activation. We recently demonstrated that activation of the α2A and β2 adrenergic receptors induces internalization of EGFR (*11*, *17*), albeit by undetermined mechanisms. This study was initiated to define how GPCRs promote internalization of the EGFR.

† This work was supported by a grant from the National Institutes of Health (R01 GM66231) to Y.D.

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[1] Abbreviation: GPCR, G protein-coupled receptor; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; ISO, isoproterenol; LPA, lysophosphatidic acid; AG1478, 4-(3-chloroanilino)-6,7-dimethyoxyquinazoline; PP2, 4-amino-5-(4-chlorophenyl)-7-(*tert*-butyl)pyrazolo-[3,4-D]pyrimidine; ERK, extracellular signal-regulated kinase 1 and 2.

10.1021/bi026942t CCC: $25.00
Published on Web 02/21/2003

To quantitate the amount of internalized EGFR, we generated a FLAG epitope-tagged receptor (FLAG-EGFR) and measured the rate of internalization following stimulation with EGF and the GPCR agonists lysophosphatidic acid (LPA) and isoproterenol (ISO). We also examined the roles of G$\beta\gamma$, β-arrestin, c-Src, and dynamin in the GPCR-mediated internalization of EGFR.

MATERIALS AND METHODS

Materials. Tyrphostins AG1478 (EGFR inhibitor) and AG1295 (PDGFR inhibitor), and PP2 (Src family inhibitor) were from Calbiochem (San Diego, CA). Recombinant EGF was from Boehringer Mannheim (Indianapolis, IN). Isoproterenol (ISO) and lysophosphatidic acid (LPA) were from Sigma (St Louis, MO), and alkaline phosphatase substrate kit was from BIO-RAD (Hercules, CA). Sources for the antibodies were as follows: anti-phospho-ERK, Cell Signaling (Beverly, MA); anti-ERK 2 and anti-EGFR, Upstate Biotechnology (Lake Placid, NY); anti-phosphotyrosine PY20H, Transduction Laboratories (Lexington, KY); M1 and M2 mouse anti-FLAG antibodies and goat anti-mouse-conjugated alkaline phosphatase, Sigma; anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies, Jackson ImmunoResearch (West Grove, PA). All other reagents were standard laboratory-grade.

Plasmids. Plasmids were obtained as follows: Transducin, American Type Culture Collection; pRC-CMV-EGFR, J. Schlessinger; β-arrestin1 and β-arrestin2, R. J. Lefkowitz; and β-arrestin1 318−419, J. L. Benovic. c-Src, Csk, c-Src K298M, c-Src Y530F, GRK2ct, and Dynamin I (wild type, K44A, and Y231F/Y597F) were described before (*18*). The FLAG-EGFR was generated by standard polymerase chain reaction using the pRC-CMV-EGFR cDNA as template.

Tissue Culture. Human embryonic kidney (HEK)-293 cells were maintained in Modified Eagle's Medium (MEM) containing 10% fetal bovine serum and 100 μg/mL penicillin-streptomycin. Cells were transiently transfected using calcium phosphate, as previously described (*12*). Experiments were performed 2 days after transfection and cells were serum-starved overnight in culture medium containing 10 mM HEPES, pH 7.5, 100 μg/mL penicillin-streptomycin, and 0.1% bovine serum albumin (BSA).

Immunofluorescence Microscopy. Cells transiently expressing FLAG-EGFR were grown on sterile glass coverslips and were deprived of serum for 16 h prior to stimulation for 30 min at 37 °C with ISO (10 μM), LPA (10 μM), or EGF (10 ng/mL). Cells were fixed in 3% paraformaldehyde, labeled with a 1:100 dilution of mouse monoclonal M2 antibody in phosphate buffered-saline (PBS) containing 0.2% BSA, 0.1% Triton X-100, and visualized by fluorescein isothiocyanate-conjugated goat anti-mouse antibody (1:250 dilution) in PBS containing 0.2% BSA and 0.1% Triton X-100. After washing with PBS, coverslips were mounted on standard glass slide using Moviol (Molecular Probes, OR). Confocal microscopy was performed on a Zeiss LSM-510 laser scanning microscope using a Zeiss 100X oil immersion lens. Fluorescent signals were collected using the Zeiss LSM software in the line switching mode using dual excitation (488 nM) and emission (515−540 nM) filter sets.

Immunoprecipitation and Immunoblotting. Serum-starved cells were exposed to agonist, washed once with ice cold PBS, and lysed in glycerol lysis buffer (5 mM HEPES, pH 7.5, 250 mM NaCl, 10% glycerol (v/v), 0.5% Nonidet P-40, 2 mM EDTA, 100 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). Cell lysates were clarified by centrifugation and FLAG-EGFR immunoprecipitated using 20 μL of anti-FLAG M2-conjugated affinity beads (Sigma), rotated for 2 h at 4 °C, and immune complexes washed three times with ice-cold lysis buffer. Proteins were denatured in Laemmli sample buffer, separated on 4−20% SDS−PAGE gels, and transferred onto nitrocellulose filters. Filters were probed for both tyrosine phosphorylation (PY20H) and FLAG-EGFR (M2 antibodies), visualized using the Renaissance Enhanced Luminol reagent (NEN; Boston, MA), and quantitated by scanning laser densitometry.

Sequestration Assay. Sequestration is defined as the number of receptors removed from the cell surface after agonist exposure, as determined by flow cytometry or enzyme-linked immunosorbent assay (ELISA). Internalization experiments using flow cytometry were performed exactly as described (*12*). ELISA assays were performed as described (*19*). Cells were seeded at 6×10^5 cells per 60-mm dish, transfected with 6 μg total DNA and split after 24 h into 6 wells of 24-well tissue culture dishes precoated with 0.1 mg/mL poly-L-lysine (Sigma). After another 24 h, the cells were washed once with PBS and incubated in MEM at 37 °C for several minutes. Agonists were added for the indicated times at 37 °C and reactions stopped by removing the culture medium followed by fixing cells in 3.7% formaldehyde/TBS (20 mM Tris, pH 7.6, 150 mM NaCl) for 5 min at room temperature. Cells were washed three times with TBS followed by blocking with TBS containing 1% BSA for 45 min at room temperature. Mouse monoclonal M1 anti-FLAG IgG was added at a dilution of 1:1000 in TBS containing 1% BSA and 1 mM $CaCl_2$ for 1 h at room temperature. Next, cells were washed three times with TBS, briefly reblocked (15 min), and incubated with goat anti-mouse-conjugated alkaline phosphatase (1:1000 in TBS/BSA) for 1 h at room temperature. Cells were washed three times with TBS, and a colorimetric alkaline phosphatase substrate was added. When the adequate color change was reached, 100 μL samples were taken for colorimetric readings at 405 nm using a scanning multiwell spectrophotometer. Cells transfected with empty vector pcDNA3 were studied concurrently to determine background signal and all experiments were done in triplicate.

Transferrin Uptake. Internalization of transferrin was assessed by confocal microscopy to measure the uptake of Texas red-transferrin in HEK-293 cells, as previously described (*20*). In short, cells transiently expressing GFP-β-arrestin 318−419 peptide were incubated with Texas red-transferrin for 15−30 min at 37 °C and processed as described above. For quantitation of transferrin uptake, cells expressing comparable amount of GFP-β-arrestin 318−419, or not, were analyzed. The level of transferrin and GFP-β-arrestin 318−419 fluorescence in the cytoplasm was determined using the LSM 510 confocal microscope software, exactly as described (*21*).

RESULTS

As shown in Figure 1A, FLAG epitope containing the DYKDDDDK amino acids was inserted after the signal

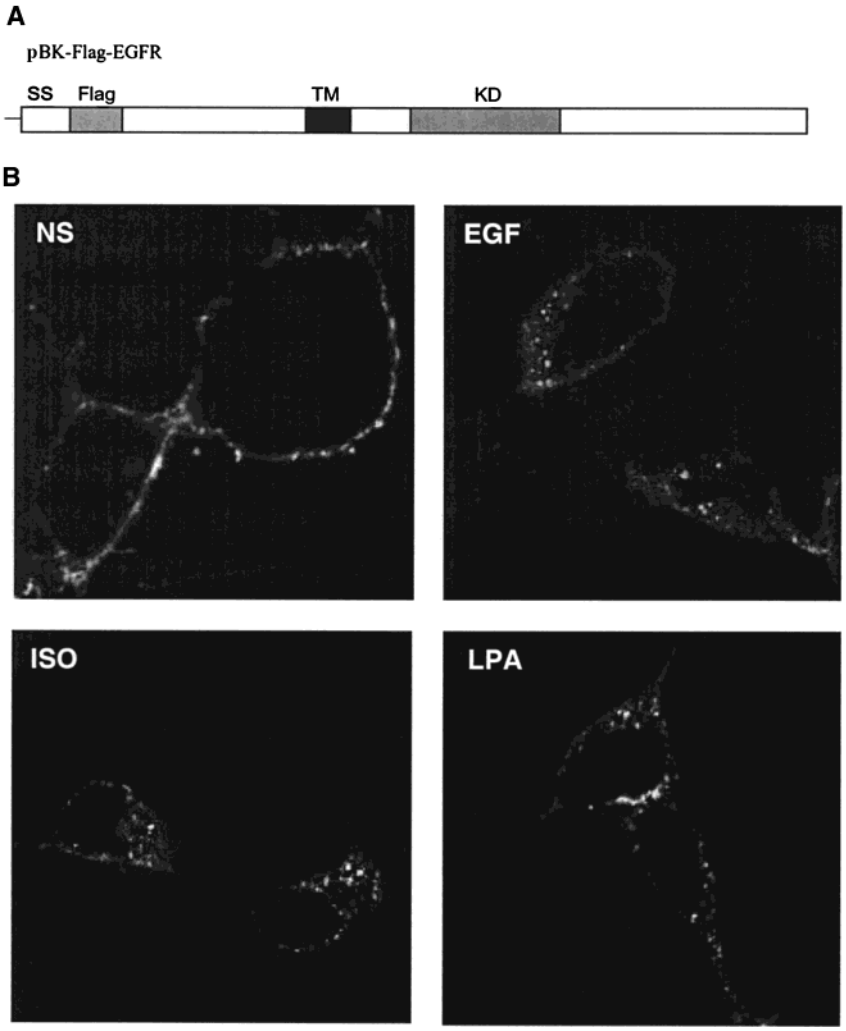

FIGURE 1: Effect of ISO and LPA on the subcellular localization of FLAG-EGFR. (A) Schematic representation of the FLAG-EGFR. The FLAG-EGFR was generated by polymerase chain reaction incorporating the FLAG epitope downstream of the signal sequence. SS, signal sequence; TM, transmembrane domain; KD, kinase domain. (B) Activation of β2 adrenergic and LPA receptors induces translocation of FLAG-EGFR into the cytosolic compartment. Confocal microscopic images depicting the cellular distribution of FLAG-EGFR before (NS) and after 30 min exposure to EGF, ISO, and LPA in HEK-293 cells. In the absence of agonist (NS), EGFR staining was predominantly confined to the plasma membrane. After exposure to the different agonists, the EGFR redistributed to the intracellular compartment. Panel B shows the representative pictures of three separate experiments.

sequence of human EGFR using polymerase chain reaction. Amplified DNA fragments were verified by sequencing, and expression of the FLAG-EGFR is depicted in Figure 1B using confocal microscopy. In the absence of stimulant, FLAG-EGFR was present on the plasma membrane. Following exposure to EGF for 30 min, the majority of EGFR was redistributed to the intracellular compartment (Figure 1B-EGF). In addition to EGF, the GPCR ligands ISO and LPA also induced the redistribution of FLAG-EGFR into the cytosol (Figure 1B-ISO/LPA). We confirmed these results using confocal microscopy with chimeric EGFR-GFP expressed in HEK-293 cells; the presence of EGFR-GFP on the cell surface decreased following ISO and LPA stimulation (data not shown). To expand on these qualitative data, we used flow cytometry to quantitatively determine characteristics of agonist-induced internalization of EGFR. Figure 2A shows that internalization of the FLAG-EGFR by LPA or ISO was time-dependent with maximal internalization achieved 20−30 min after stimulation. Internalization by EGF was more delayed and reached equilibrium after 45 min of ligand application. Average internalization in response to EGF was calculated to be 25−30% and, by LPA or ISO, 12−16% (Figure 2A). These data clearly show that EGFR is sequestered from the plasma membrane in response to direct stimulation with EGF, as well as following activation of β2 adrenergic and LPA receptors.

EGF-induced internalization of EGFR is regulated by tyrosine autophosphorylation of the receptor (*1*, *9*). Therefore, we tested whether GPCR stimulation is capable of inducing tyrosine phosphorylation of the EGFR. Cells transiently expressing FLAG-EGFR were stimulated with EGF, ISO, or LPA and EGFR immunoprecipitated using M2 anti-FLAG antibody and immunoblotted with anti-phosphotyrosine PY20H antibody. FLAG-EGFR became tyrosine phosphorylated in response to direct activation with EGF, as well as in response to activation of the β2 adrenergic or LPA receptors (Figure 2B,C), in agreement with published data (*17*, *22*). On average, EGF induced (10.53 $\pm$ 3.12)-fold increases ($n = 3$, $P < 0.01$), whereas ISO and LPA induced (3.11 $\pm$ 0.37)- ($n = 3$, $P < 0.01$) and (3.78 $\pm$ 1.2)-fold increases ($n = 3$, $P < 0.01$), respectively, in the tyrosine phosphorylation content of the FLAG-EGFR (Figure 2B).

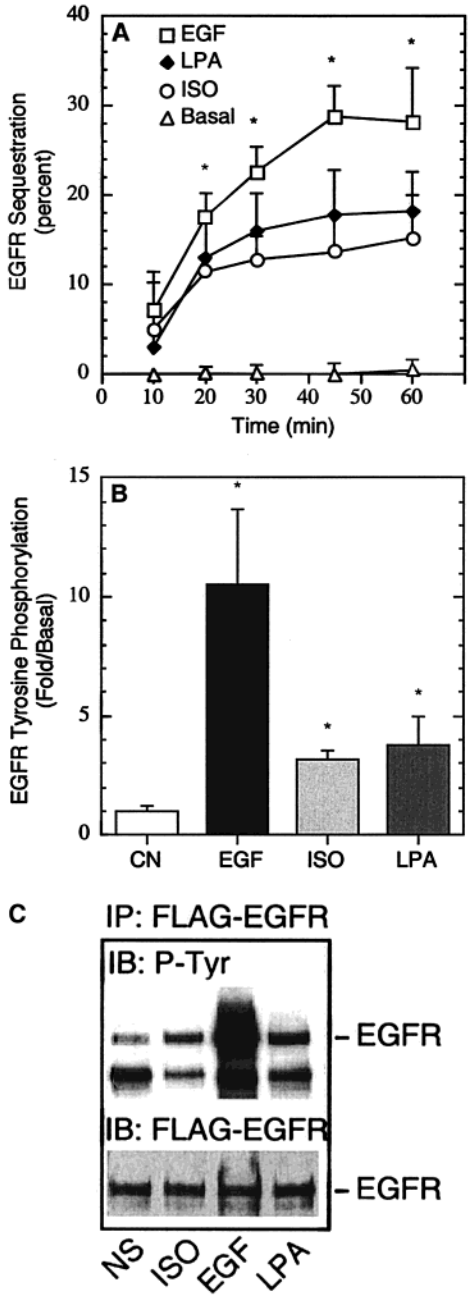

FIGURE 2: ISO and LPA regulate the expression of EGFR on the plasma membrane. (A) ISO and LPA induce internalization of the EGFR. Internalization of FLAG-EGFR was quantitated as the percent loss of cell-surface fluorescence in agonist-treated cells using flow cytometry. Serum-starved HEK-293 cells transiently expressing the FLAG-EGFR were stimulated for the indicated times with EGF, ISO, or LPA. The data are expressed as mean ± S.E. of seven (basal, EGF) and five (ISO, LPA) independent experiments. * $P < 0.05$ compared to unstimulated samples. (B) ISO and LPA induce tyrosine phosphorylation of the EGFR. HEK-293 cells transiently expressing FLAG-EGFR were serum-starved for 24 h and exposed to EGF, ISO, or LPA at 37 °C. FLAG-EGFR immunoprecipitates were resolved by SDS−PAGE and tyrosine phosphorylation of EGFR detected with PY20H antibody. The filters were stripped of immunoglobulin and expression of FLAG-EGFR determined by blotting with M2 antibodies. Band intensity was determined by densitometry and the graph represents agonist-induced fold increases in tyrosine phosphorylation of EGFR above basal level. Data are expressed as mean ± S.E. of three independent experiments. * $P < 0.01$ compared to control values (CN). (C) A representative western blot showing the tyrosine phosphorylation of EGFR in HEK-293 cells.

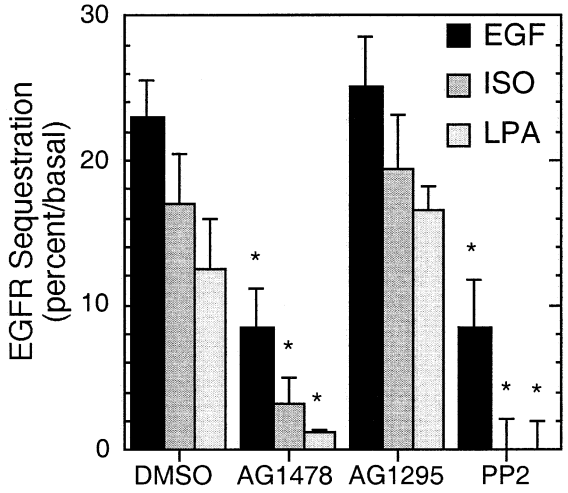

FIGURE 3: Effect of tyrosine kinase inhibitors on internalization of EGFR. HEK-293 cells transiently expressing FLAG-EGFR were exposed to EGF, ISO, or LPA for 30 min at 37 °C after treatment with tyrphostins AG1478 (250 nM), AG1295 (250 nM), or PP2 (5 μM) for 30 min. Plasma membrane content of FLAG-EGFR was analyzed by ELISA as described. Data shown represent means ± S.E. from five different experiments. * $P < 0.01$ compared with agonist-stimulated values without inhibitors (DMSO).

Stimulation with EGF, ISO, and LPA also increased ERK phosphorylation by (6.74 ± 0.86)-fold, (2.97 ± 0.25)-fold, and (4.87 ± 0.62)-fold ($n = 5$; $P < 0.01$), respectively, demonstrating that the increase in ERK phosphorylation by the GPCRs correlates with their abilities to induce tyrosine phosphorylation of the EGFR. Together, these data show that EGFR internalizes and becomes tyrosine phosphorylated not only in response to EGF stimulation, but also following GPCR activation.

Accumulating evidence suggests that c-Src tyrosine kinase activity is required for internalization of cell surface receptors, including RTKs (*6*, *23*) and GPCRs (*18*, *24*). In an attempt to characterize the role of c-Src in the GPCR-mediated endocytosis of EGFR, we utilized the chemical PP2, a selective Src family tyrosine kinase inhibitor. Tyrphostins AG1478 and AG1295, selective EGFR and PDGFR inhibitors, respectively, were used as controls. Pretreatment of cells with AG1478 or PP2 attenuated the EGF-mediated internalization of EGFR by 60% and completely blocked the ISO- and LPA-stimulated internalization of the EGFR. However, pretreatment with AG1295 did not affect EGF-, ISO-, or LPA-mediated internalization of EGFR (Figure 3). The reason for the partial inhibition by AG1478 following stimulation with EGF is not clear and could be a limitation of the experimental approach. However, we employed two independent techniques (flow cytometry and ELISA), and both gave similar results. Pretreatment of the cells with AG1478 or PP2, but not with AG1295, also attenuated ISO- and LPA-induced EGFR and ERK phosphorylation (data not shown), in agreement with published results (*17*, *22*). Together, these data imply that internalization of the EGFR, and subsequent activation of ERK, are regulated by intrinsic tyrosine kinase activity of the receptor itself and c-Src.

To further implicate Src kinases as regulators of GPCR-mediated internalization of EGFR, cells were cotransfected with FLAG-EGFR and either of two biologic inhibitors of c-Src: C-terminal c-Src kinase (Csk) or a catalytically inactive kinase, c-Src K298M. Control cells were trans-

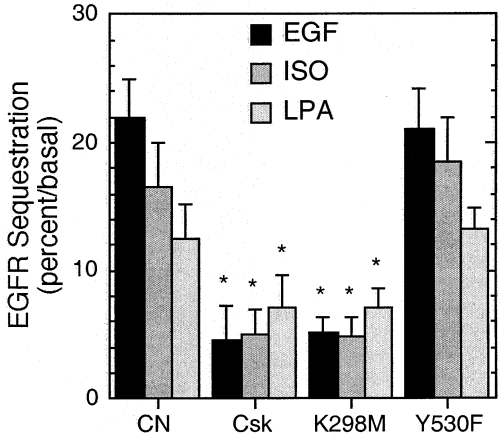

FIGURE 4: Role of c-Src tyrosine kinase in agonist-induced EGFR internalization. FLAG-EGFR was cotransfected with plasmids expressing either C-terminal c-Src kinase (Csk), catalytically inactive c-Src K298M, or constitutively active c-Src Y530F. Transiently transfected HEK-293 cells were exposed to either EGF, ISO, or LPA for 30 min at 37 °C. The plasma membrane content of FLAG-EGFR was analyzed by ELISA. Data represent means ± S.E. from seven independent experiments performed in triplicate. * $P < 0.05$ compared with agonist-stimulated values obtained from untransfected cells (CN) .

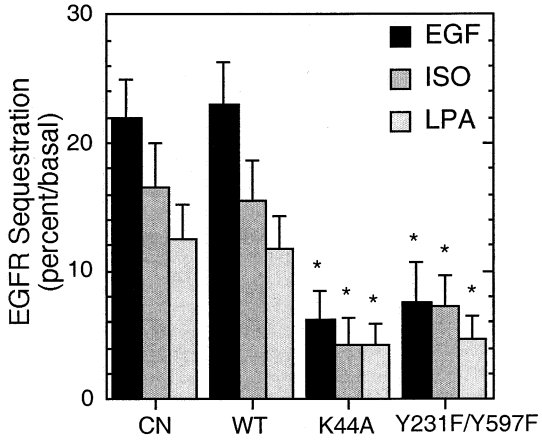

FIGURE 5: Effect of dynamin function on agonist-induced EGFR internalization. FLAG-EGFR was cotransfected with plasmids expressing either wild type, K44A, or Y231F/Y597F dynamin I. Transiently transfected HEK-293 cells were exposed to either EGF, ISO, or LPA for 30 min at 37 °C, and the plasma membrane content of FLAG-EGFR was analyzed by ELISA and flow cytometry. Data represent means ± S.E. from seven independent experiments performed in triplicate. * $P < 0.05$ compared with agonist-stimulated values obtained from untransfected cells (CN).

fected with constitutively active c-Src Y530F. In these experiments, agonist-dependent internalization of EGFR was determined by ELISA (*19*). Both Csk and c-Src K298M attenuated the GPCR-induced internalization of the FLAG-EGFR by 60−80%, whereas expression of constitutively active c-Src Y530F showed no additive effect (Figure 4). These data support the notion that c-Src family tyrosine kinases are involved in the GPCR-mediated internalization of EGFR.

In the case of ISO-induced internalization of β2AR, we recently found that dynamin serves as a substrate for c-Src to facilitate receptor internalization (*18*, *25*). Therefore, FLAG-EGFR was cotransfected with a plasmid expressing tyrosine phosphorylation-deficient dynamin I Y231F/Y597F protein. Wild-type and GTPase-deficient K44A dynamin I were used as controls. As shown in Figure 5, both dynamin

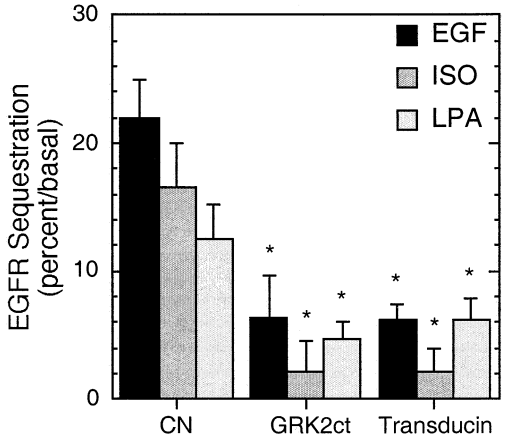

FIGURE 6: GRK2ct and transducin inhibit internalization of EGFR. HEK-293 cells were transfected with FLAG-EGFR cDNA alone, or together with plasmids expressing GRK2ct peptide or transducin. Transiently transfected cells were exposed to EGF, ISO, or LPA for 30 min at 37 °C and plasma membrane content of FLAG-EGFR was analyzed by ELISA. Data represent means ± S.E. from five independent experiments performed in triplicate. * $P < 0.01$ compared with agonist-stimulated values obtained from untransfected cells (CN).

I K44A and Y231F/Y597F mutants inhibited EGF- and GPCR-mediated internalization of EGFR by 50−76%. Overexpression of wild-type dynamin I showed no effect on the agonist-promoted internalization of EGFR. Thus, the GPCR- and EGF-mediated internalization of EGFR requires dynamin being tyrosine phosphorylated.

Free G$\beta\gamma$ subunits regulate clathrin-mediated endocytosis (*26*), and EGFR is thought to internalize via clathrin-dependent mechanisms (*6*, *9*). Therefore, we examined the role of G$\beta\gamma$ subunits in the GPCR-regulated EGFR internalization. Expression of GRK2ct or transducin, which serve as G$\beta\gamma$ subunit-sequestering polypeptides, inhibited the ISO- and LPA-induced internalization of FLAG-EGFR by 67−87% (Figure 6). Unexpectedly, expression of the GRK2ct peptide and transducin also inhibited the EGF-mediated internalization of the EGFR.

Agonist-dependent internalization of some GPCRs, such as the β2AR (*27*, *28*) and RTKs, such as the IGF-1R (*29*) is dependent upon β-arrestin proteins, which target receptors to clathrin-coated pits and recruit active c-Src to the plasma membrane (*30*). We tested the role of β-arrestins in the internalization of EGFR by expressing a peptide encompassing the carboxyl terminus of β-arrestin1 (amino acids 318−419) that sequesters clathrin (*31*). As shown in Figure 7A, expression of the β-arrestin1 318−419 peptide inhibited the ISO- and LPA-, as well as the EGF-mediated internalization of the EGFR by 60−70%. Overexpression of β-arrestin proteins only modestly affected the agonist-promoted tyrosine phosphorylation content of EGFR (Figure 7B).

Our data show that internalization of EGFR is inhibited in the presence of β-arrestin1 318−419 peptide (Figure 7A). However, it is not clear whether the β-arrestin1 318−419 peptide acts in a specific manner to interfere with active receptor endocytosis, or nonspecifically by sequestering proteins involved in vesicle recycling. To address this question, we examined the effect of β-arrestin1 318−419 peptide on the constitutive recycling of transferrin receptor in HEK-293 cells. The data show that expression of the β-arrestin1 318−419 peptide does not interfere with recycling of the

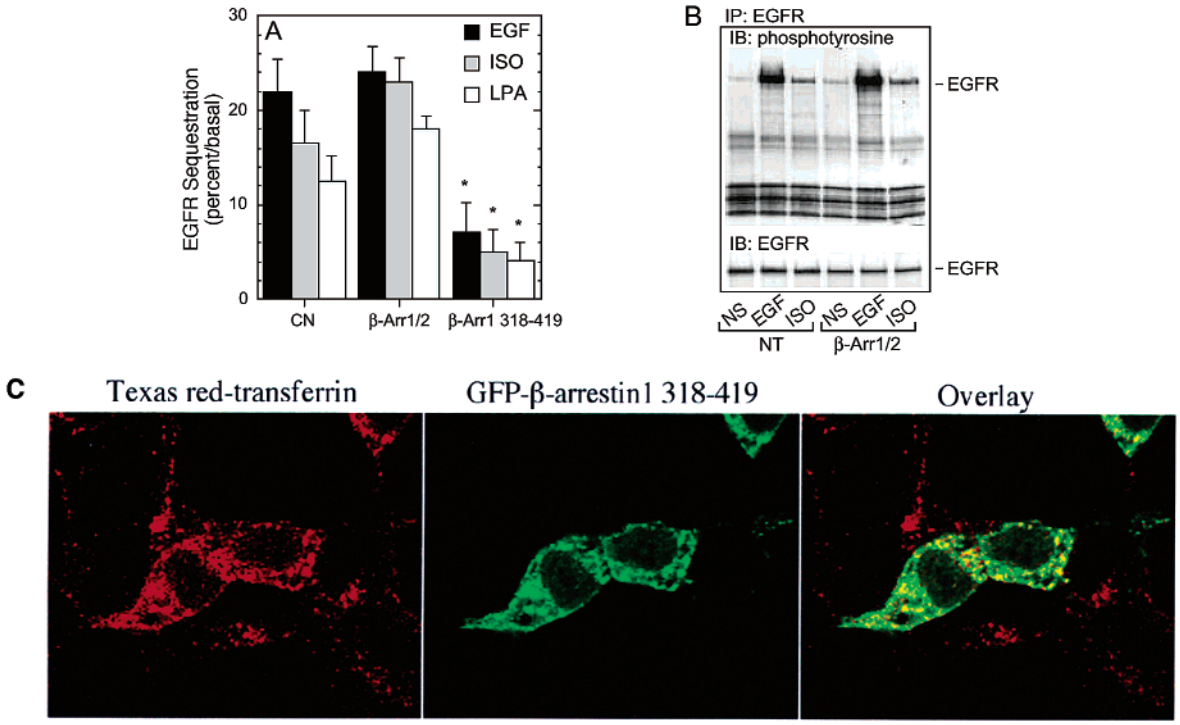

FIGURE 7: Regulation of EGFR internalization by β-arrestin. (A) β-arrestin-dependent internalization of EGFR. FLAG-EGFR was cotransfected with plasmids expressing wild type β-arrestin 1 and 2 or β-arrestin1 318−419 peptide. Transiently transfected HEK-293 cells were exposed to EGF, ISO, or LPA for 30 min at 37 °C and plasma membrane content of FLAG-EGFR analyzed by ELISA. Data represent means ± S.E. from three independent experiments performed in triplicate. * $P < 0.01$ compared with agonist-stimulated value in receptor alone-transfected cells (CN). (B) Effect of β-arrestins on tyrosine phosphorylation of EGFR. HEK-293 cells were transfected with plasmids expressing β-arrestin 1 and 2 and treated with EGF or ISO for 5 min at 37 °C and tyrosine phosphorylation content of EGFR determined as described. Data shown are representative of three experiments. NS, not stimulated; NT, receptor alone-transfected cells (CN). (C) Effect of β-arrestin1 318−419 on internalization of transferrin receptor. HEK-293 cells were transiently transfected with GFP-β-arrestin1 318−419 and incubated with Texas red-conjugated transferrin for 30 min at 37 °C. Expression of the GFP-β-arrestin1 318−419 peptide and transferrin uptake was determined using confocal microscopy. Results are representative of four independent experiments.

transferrin receptor (Figure 7C). Quantitation of data was done by qualitative assessment of the level of Texas red in 50 cells expressing the β-arrestin1 minigene construct and comparing it with the fluorescence signal of neighboring cells that were not transfected with the β-arrestin1 minigene, exactly as described (*20*). We found no significant difference in transferrin uptake between cells expressing the β-arrestin1 peptide or not. Together, these data indicate that the EGFRs, like many GPCRs, specifically utilize β-arrestin proteins for their internalization.

DISCUSSION

The process of EGFR internalization plays two important functions in the life cycle of the receptor. It induces receptor downregulation by decreasing the expression of EGFR present on the plasma membrane, and contributes to the EGF-mediated mitogenic signaling (*2*, *9*). Our results show that, in addition to EGF, EGFR is removed from the plasma membrane following activation of GPCRs. Since cells are simultaneously exposed to many ligands, these data suggest that GPCRs can act as regulators of EGFR cell surface expression and signaling under relevant physiologic conditions.

EGFRs are internalized in response to EGF stimulation, as well as following activation of certain GPCRs (*11*, *17*, *25*, *32*). Our quantitative analyses of EGFR internalization show that ISO and LPA caused at least half of the maximal effect achieved by EGF, suggesting their high efficiency to promote internalization of the EGFR. Interestingly, we find that maximal internalization of EGFR by EGF was more delayed (45 min), compared to the ISO- and LPA-induced response (20 min). The reason for this is not clear, although it may indicate existence of multiple endocytic routes for EGFR internalization (*33*).

Decreased numbers of detectable cell surface receptors following stimulation with agonist could result from either receptor sequestration or degradation. Stimulation with 5-hydroxytryptamine (5-HT) induced EGFR internalization and that was attenuated by inhibitors of endocytosis (*32*). However, exposure of cells to cycloheximide, a protein synthesis inhibitor, did not affect the 5-HT-induced EGFR internalization (*32*), demonstrating rapid protein synthesis does not play a significant role in the EGF- and GPCR-mediated EGFR internalization.

Clathrin-mediated endocytosis of ligand-occupied receptors was recently shown to require the enzymatic activity of tyrosine kinases. c-Src phosphorylates clathrin causing assembly of clathrin lattices, which are required for EGF-induced internalization of the EGFR (*6*). Our results demonstrate that c-Src kinase activity is required for ISO- and LPA-mediated internalization of EGFR. Although the relevant substrate(s) for c-Src in the ISO- and LPA-mediated internalization of EGFR remain to be determined, our preliminary studies show that ISO and EGF induces c-Src-dependent tyrosine phosphorylation of clathrin (J. Kue and Y. Daaka, unpublished, 2001).

Another potential substrate for c-Src is the GTPase dynamin, which is required for fission of clathrin-coated vesicles from the plasma membrane. The c-Src is involved in phosphorylation of dynamin on Tyr231 and Tyr597 residues following stimulation with ISO (*18*), and that is required for GPCR (*18*) and EGFR (*11*, *24*, *25*) internalization. In this report we demonstrate that expression of dynamin Y231F/Y597F, which is impaired in its phosphorylation by c-Src, also inhibits EGF-, ISO-, and LPA-mediated internalization of the EGFR. These results suggest that the c-Src-dependent tyrosine phosphorylation of dynamin, similar to its phosphorylation of clathrin (*6*), is involved in EGFR internalization.

Our data demonstrate that free G$\beta\gamma$ subunits are required for the ISO- and LPA-mediated internalization of EGFR. Exact role of the G$\beta\gamma$ subunits in this reaction is not clear. One possibility is that they participate in transactivation of the EGFR (*17*) potentially by inducing the formation of a GPCR-β-arrestin-c-Src trimeric complex. Preliminary data show that sequestration of free G$\beta\gamma$ subunits, by expression of the GRK2ct, attenuates ISO-mediated phosphorylation of the β2AR (Y. Daaka, unpublished results, 2002), which is required for the formation of β2AR-β-arrestin-c-Src complex (*30*, *34*). Another possibility is that G$\beta\gamma$ subunits regulate activation of phosphatidylinositol kinase (*35*), which is required for receptor endocytosis (*36*).

Sequestration of G$\beta\gamma$ subunits also attenuates the EGF-mediated internalization of EGFR. Free G$\beta\gamma$ subunits have been shown to activate specific subtypes of phospholipase C (*37*) and to modulate the activity of various isoforms of adenylyl cyclase (*38*). These enzymes, however, are unlikely to affect the internalization of EGFR since recent data suggest that the EGF-induced activation of protein kinase A (PKA) and PKC has no effect on EGFR internalization (*39*, *40*). It is possible that overexpression of the GRK2ct, which contains a pleckstrin homology domain, leads to sequestration of factors (e.g., PIP_2) necessary for assembly of clathrin-coated pits. In addition to the G$\beta\gamma$ subunits, it is possible that Gα subunits may affect the internalization of EGFR. Recently, a regulator of Gαs protein signaling, RGS−PX1, was identified and shown to possess a Phox domain that regulates vesicle trafficking activity (*41*). Overexpression of the RGS−PX1 in HEK-293 cells delayed EGF-induced degradation of EGFR implying that activated Gαs subunits regulate EGFR internalization.

Cytosolic β-arrestin proteins mediate internalization of many GPCRs by direct binding to clathrin (*27*) and AP2 (*28*). Our data support the notion that the β-arrestin proteins are specifically required for active EGFR internalization, but not for the constitutive recycling of transferrin receptor. Therefore, these data suggest an expanded role for the β-arrestins in endocytosis. In further support to this conclusion is the recent finding that IGF-1-induced internalization of IGF-1R is regulated by β-arrestin (*29*).

The precise steps linking activated GPCRs to EGFR remain unclear, although matrix metalloproteinase-mediated shedding of mature EGFR ligands, such as heparin-bound (HB)-EGF, has been demonstrated (*42*, *43*). However, using specific antibodies to neutralize HB-EGF activity (*44*), we could demonstrate that HB-EGF does not account for all the ISO- and LPA-mediated transactivation of EGFR in HEK-293 cells (P. Kue and Y. Daaka, unpublished results, 2001). This implies existence of additional mechanisms involved in the ISO- and LPA-promoted transactivation and internalization of EGFR. Indeed, stimulation with ISO induces formation of β2AR-EGFR hetero-receptor complexes (*17*), which are required for signal transduction to ERK. Hence, it is reasonable to suggest that the ISO-induced internalized β2AR "carries" with it EGFR to intracellular compartments.

In summary, the results demonstrate a remarkable similarity in the mechanisms regulating the EGF- and GPCR-mediated internalization of EGFR. These internalization regulators include G$\beta\gamma$ subunits and β-arrestin proteins, previously thought to be required solely for agonist-dependent internalization of GPCRs, as well as tyrosine kinases and dynamin. Activation of GPCRs may regulate EGFR-dependent signaling pathways via the regulation of EGFR expression on the plasma membrane. The reduced expression of EGFR on the plasma membrane can, in principle, restrict the cell's responsiveness to extracellular EGF.

ACKNOWLEDGMENT

We thank Drs. J. Schlessinger, R.J. Lefkowitz, and J.L. Benovic for providing plasmids and Drs. J.L. Benovic and A. Marchese for help in setting up the ELISA assay. We also thank Ms. Jessica House for excellent secretarial assistance.

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BI026942T