EGF Transregulates Opioid Receptors through EGFR-mediated GRK2 Phosphorylation and Activation

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G protein-coupled receptor (GPCR) kinases (GRKs) are key regulators of GPCR function. Here we demonstrate that activation of epidermal growth factor receptor (EGFR), a member of receptor tyrosine kinase family, stimulates GRK2 activity and transregulates the function of G protein-coupled opioid receptors. Our data showed that EGF treatment promoted DOR internalization induced by DOR agonist and this required the intactness of GRK2-phosphorylation sites in DOR. EGF stimulation induced the association of GRK2 with the activated EGFR and the translocation of GRK2 to the plasma membrane. After EGF treatment, GRK2 was phosphorylated at tyrosyl residues. Mutational analysis indicated that EGFR-mediated phosphorylation occurred at GRK2 N-terminal tyrosyl residues previously shown as c-Src phosphorylation sites. However, c-Src activity was not required for EGFR-mediated phosphorylation of GRK2. In vitro assays indicated that GRK2 was a direct interactor and a substrate of EGFR. EGF treatment remarkably elevated DOR phosphorylation in cells expressing the wild-type GRK2 in an EGFR tyrosine kinase activity-dependent manner, whereas EGF-stimulated DOR phosphorylation was greatly decreased in cells expressing mutant GRK2 lacking EGFR tyrosine kinase sites. We further showed that EGF also stimulated internalization of μ -opioid receptor, and this effect was inhibited by GRK2 siRNA. These data indicate that EGF transregulates opioid receptors through EGFR-mediated tyrosyl phosphorylation and activation of GRK2 and propose GRK2 as a mediator of cross-talk from RTK to GPCR signaling pathway.

INTRODUCTION

G protein-coupled receptors (GPCR) form a large superfamily of heptahelical proteins that transduce a huge number of extracellular signals including hormones, neurotransmitters, and chemokines to the interior of cell and play fundamental roles in regulation of cellular functions. Activation of GPCR triggers a process termed desensitization, which is initiated by agonist-stimulated receptor phosphorylation by GPCR kinases (GRKs). Phosphorylation of GPCR promotes binding of β -arrestins to the receptor, which uncouples the receptor from G proteins and induces GPCR internalization and down-regulation (Kohout and Lefkowitz, 2003; Gao *et al.*, 2005). GRKs thus act as a critical modulator of GPCR signal transduction.

GRK2 is a ubiquitous member of the GRK family and its activity and subcellular location appear to be tightly controlled by stimulation of GPCR as well as its subsequent interaction with activated receptors, G $\beta\gamma$ subunits, lipids, anchoring proteins, caveolin, and calmodulin (Penela *et al.*,

2003). The interaction of GRK2 with G $\beta\gamma$ subunits through its C-terminal pleckstrin homology (PH) domain helps targeting the kinase to the membrane and enhances its activity toward the receptor (Pitcher *et al.*, 1992). The interaction of GRK2 with phosphatidylinositol 4,5-bisphosphate via the N-terminal portion of its PH domain seems to be necessary for its full activation (Pitcher *et al.*, 1995). Activity of GRK2 may also be regulated by its own phosphorylation. ERK could phosphorylate GRK2 at Ser670 and thus negatively regulate GRK2 activity (Pitcher *et al.*, 1999). Phosphorylation of GRK2 by protein kinase C (PKC) and PKA modulates its membrane-targeting and kinase activity (Chuang *et al.*, 1995; Cong *et al.*, 2001). Another important mechanism of GRK2 activation involves phosphorylation of GRK2 by c-Src. Src-mediated GRK2 phosphorylation at tyrosine in its N-terminal domain has been shown to promote the kinase activity of GRK2 toward both soluble and membrane-bound substrates in vitro (Sarnago *et al.*, 1999). The recruitment of Src to β_2 -adrenergic receptor (β_2 AR) and Src-mediated tyrosyl phosphorylation of GRK2 are obligate for agonist-induced desensitization of β_2 AR (Fan *et al.*, 2001).

Distinct from GPCRs in structure and function, receptor tyrosine kinases (RTKs) constitute another family of transmembrane receptors. As an important member of RTK family, epidermal growth factor receptor (EGFR) plays a critical role in regulation of many cellular processes such as proliferation, differentiation, motility, and survival (Schlessinger, 2000). It has been demonstrated that GRK2 could form a complex with c-Src and PDE γ to regulate EGF-stimulated activation of p42/p44 MAPKs (Wan *et al.*, 2003). GRK2 is able to phosphorylate EGFR and platelet-derived growth factor receptor (Freedman *et al.*, 2002). These data implicate that GRK2 can also regulate RTK signaling.

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Abbreviations used: DPDPE, D-Pen2, D-Pen5 enkephalin; GRKs, G protein-coupled receptor kinases; DOR, δ -opioid receptor; M4/5/6, a DOR mutant lacks GRK2 phosphorylation sites; MOR, μ -opioid receptor; EGFR, epidermal growth factor receptor; GST-EGFR, GST-tagged EGFR kinase domain; HEK293, human embryonic kidney 293 cells.

Our recent study showed that EGF stimulation induced translocation of GRK2 to the plasma membrane and the formation of GRK2-EGFR complex (Gao *et al.*, 2005). The current study is to explore whether GRK2 could be regulated by EGFR to exert influence on the GPCR signaling. Our results show that the activation of EGFR stimulates its interaction with GRK2 and the EGFR-mediated tyrosyl phosphorylation of GRK2, which activates GRK2 and regulates the internalization of δ -opioid receptor (DOR) and μ -opioid receptor (MOR). Our study thus reveals that GRK2 mediates a novel form of cross-talk between RTK and GPCR signaling pathways.

MATERIALS AND METHODS

Materials

D-Pen², D-Pen⁵ enkephalin (DPDPE) and DAMGO were purchased from Sigma (St. Louis, MO). 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-*d*] pyrimidine (PP2) was purchased from Calbiochem (La Jolla, CA). Modified Eagle's medium (MEM) and Dulbecco's MEM were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Protein A-Sepharose was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Rabbit and mouse monoclonal antibodies against FLAG epitope and Rabbit antibody against hemagglutinin (HA) epitope were purchased from Sigma. phospho-DOR (Ser 363) antibody was purchased from Cell Signaling Technology (Beverly, MA). Rabbit antibody against EGFR and GRK2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse antibody against HA was purchased from Covance (Berkeley, CA). Horseradish peroxidase (HRP)-conjugated mouse mAb against phosphotyrosine (pY20H) was supplied by BD Biosciences Pharmingen (San Diego, CA). Cy3-conjugated goat anti-rabbit IgG, Cy5-conjugated goat anti-mouse IgG, and FITC-conjugated goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Sulfo-NHS-SS-biotin was purchased from Pierce (Rockford, IL). Other chemicals were purchased from Sigma.

Plasmid Construction

Plasmids encoding bovine GRK2, N-terminal 185-amino acid deletion mutant of GRK2 (Δ N-GRK2), and transducin α were prepared as described previously (Gao *et al.*, 2005). The HA-DOR and M4/5/6 constructs were prepared as described previously (Guo *et al.*, 2000). HA-MOR construct was provided by Dr. H. Loh (University of Minnesota School of Medicine). The Rab5-GFP construct was provided by Dr. D. Pei (Tsinghua University). The GRK2-GFP and GRK2-Flag cDNA constructs were generous gifts from Dr. Marc G. Caron (Duke University Medical Center). The mouse c-Src cDNA was kindly provided by Dr. Joan S. Brugge (Harvard Medical School) and human EGFR cDNA clone kindly provided by Dr. Neil J. Freedman (Duke University Medical Center) were subcloned into pcDNA3 (Invitrogen). The bovine GRK2 mutant GRK2-Y13F/Y86F/Y92F (GRK2-3Y/F) 127 HA-tagged GRK2-kinase domain (amino acid 186-513, HA-GRK2-CD), the GRK2 lacking PH-domain (Δ PH-GRK2), the dominant-negative c-Src mutant (K298R), the HA-MOR 363/370/375A, and the constitutively active c-Src mutant (Y527F) were constructed by PCR mutagenesis. Construction of RNAi plasmid for human GRK2 was performed as described (Sui *et al.*, 2002). The small interfering RNA (siRNA) sequence targeting GRK2 are 5'-GGCCATGAGGAAGAC-TACGCC-3' corresponding to the positions 1641-1661 relative to the start codon, respectively. The control nonspecific siRNA (5'-GGCCGCAAAGAC-CTTGTCCTTA-3') was prepared as described previously (Gao *et al.*, 2005).

Cell Culture and Plasmid Transfection

HEK293 cells and HEK293T cells were cultured in MEM and Dulbecco's MEM containing 10% FBS, respectively. Cells were seeded in 60- or 100-mm tissue culture dishes at $1-3 \times 10^6$ /dish 20 h before transfection. Transfection of cells with 2-5 μ g of each plasmid was performed with calcium phosphate/DNA coprecipitation method. Assays were performed 44-48 h after transfection, and the cells were maintained overnight in FBS-free medium before the assay. A431 cells were cultured in Dulbecco's MEM plus 10% FBS and were transfected using LipofectAMINE 2000 reagent (Invitrogen).

Immunofluorescence Imaging

A431 Cells grown on glass coverslips were transfected with HA-MOR or HA-MOR 363/370/375A plasmids. After 24 h, cells were incubated with mouse anti-HA antibody for 30 min and then treated with EGF (100 ng/ml) at 37°C for 30 min. After washed by phosphate-buffered saline (PBS), cells were fixed immediately and permeabilized with 0.3% (vol/vol) Triton X-100 in PBS. Cells were then incubated with rabbit anti-EGFR antibody overnight. Cells were washed by PBS, then stained with FITC-conjugated goat anti-

mouse and Cy3-conjugated goat anti-rabbit antibody at room temperature for 1 h, and washed with PBS. The coverslips were then mounted onto microscope slides with 50% glycerol in PBS. Fluorescence images were taken on a Zeiss 40 $\times$ oil/1.3 NA Fluar objective under a Zeiss 510 laser confocal microscope (Thornwood, NY). A431 cells transfected with GRK2-GFP alone or Rab5-GFP and GRK2-Flag were treated with EGF (100 ng/ml) at 37°C for the desired time and then were fixed and permeabilized. Cells were incubated with rabbit anti-EGFR antibody alone or with mouse anti-Flag antibody overnight and then stained with Cy3-conjugated goat anti-rabbit secondary antibody alone or with Cy5-conjugated goat anti-mouse antibody at room temperature for 1 h. The coverslips were then treated as described above. HEK293 cells grown on glass coverslips were transfected with HA-EGFR and GRK2-GFP plasmids. After 48 h, cells were incubated with rabbit anti-HA antibody for 1 h at 37°C and then treated with EGF (100 ng/ml) at 37°C for 5 min. Cells were fixed and permeabilized and then stained with Cy3-conjugated goat anti-rabbit secondary antibody at room temperature for 1 h. The coverslips were then treated as described above.

Immunoprecipitation and Western Blotting

Cells were washed with ice-cold PBS and lysed in 800 μ l NP-40 solubilization buffer (50 mM HEPES, pH 8.0, 250 mM NaCl, 0.5% NP-40, 10% glycerol, 2 mM EDTA, 1 mM Na₂VO₄, plus 10 μ g/ml aprotinin, 10 μ g/ml benzamide, and 0.2 mM PMSF) for 1.5 h as described (Gao *et al.*, 2005). The lysate was centrifuged and the supernatant was incubated with 1 μ g of anti-FLAG antibody and 15 μ l of 50% slurry of protein A-Sepharose beads at 4°C for 4 h. The beads were subsequently washed, and the proteins bound to the beads were eluted in PAGE in the presence of SDS-PAGE sample buffer and separated by SDS-PAGE. The samples were detected in the subsequent Western procedures with the corresponding antibody. To assess DOR phosphorylation, cells were lysed in cell lysis buffer (50 mM Tris, pH 7.4, 0.5% NP-40, 10% glycerol, 150 mM NaCl, 2 mM EDTA, 1 mM NaF, plus 10 μ g/ml aprotinin, 10 μ g/ml benzamide, and 0.2 mM PMSF). After centrifugation, receptors were immunoprecipitated with M2-conjugated Sepharose (Sigma) and detected with phospho-DOR (Ser 363) antibody.

For immunoblot analysis, protein bands were visualized by enhanced chemiluminescence. In some experiments, blots were incubated with IRDye 800CW-conjugated or 700CW-conjugated antibody (Rockland Biosciences, Gilbertsville, PA) and infrared fluorescence images were obtained with the Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE).

Fluorescence Flow Cytometry

Receptor internalization was quantitated using fluorescence flow cytometry assay as previously described (Zhang *et al.*, 2005). Cells were incubated in 1 μ M DPDPE or 10 μ M DAMGO at 37°C under the conditions indicated in figure legend. Then the cells were chilled on ice, and the surface receptors were labeled with mouse against HA antibody for 1 h at 4°C. After sufficient washing, the cells were incubated with FITC-conjugated goat anti-mouse IgG for another 1 h at 4°C. Then the cells were collected and fixed, and the surface receptor staining intensity was analyzed on a fluorescence flow cytometer (FACScan, Becton Dickinson). Basal cell fluorescence intensity was determined with cells stained with the secondary antibody alone.

Cell Surface Biotinylation

Surface biotinylation assay was performed as described (Zhang *et al.*, 2005). Cells were incubated at 4°C with 600 μ g/ml Sulfo-NHS-SS-biotin in PBS for 30 min. Unreacted biotin was removed by rinsing cells with Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). Cells were warmed to 37°C for 1 h followed by incubation in the presence or absence of 100 ng/ml EGF for 20 min. Then the cells were stimulated with 1 μ M DPDPE for 30 min. The biotin remaining on cell surface was cleaved by incubation in stripping buffer (50 mM glutathione, 100 mM NaCl, 60 mM NaOH, and 1% fetal bovine serum) at 4°C for 15 min. The remaining glutathione was quenched at 4°C for 30 min by 50 mM iodoacetamide resolved in PBS. Cells were extracted in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 25 mM KCl, 0.5% Triton X-100). After centrifugation, receptors were immunoprecipitated with M2-conjugated Sepharose and detected with HRP-conjugated streptavidin.

In Vitro Kinase Assays

Bovine GRK2-Flag was partially purified from HEK293T cells. Briefly, HEK293T cells overexpressing GRK2-Flag were lysed in lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 1% Triton, 10% glycerol, and 2 mM EDTA, plus 10 μ g/ml aprotinin, 10 μ g/ml benzamide, and 0.2 mM PMSF). After centrifugation, proteins were immunoprecipitated with M2-conjugated Sepharose (Sigma). After extensive wash in lysis buffer, beads were rinsed by TBS (50 mM Tris, pH 7.4, 50 mM NaCl) twice. Bound protein was eluted by a competition with 3 $\times$ Flag peptide (Sigma) as the product instructions suggested. 3 $\times$ Flag peptide and salt were removed by centrifugation in Amicon Ultra-4 (30-kDa cutoff size; Millipore, Bedford, MA). Protein was concentrated to a final concentration of 3 μ g/ml. Protein is >90% pure as determined by 10% SDS-PAGE. Recombinant N-terminal glutathione S-transferase (GST)-tagged human EGFR kinase domain (GST-EGFR) was purchased

from Millipore (Lake Placid, NY). In vitro kinase assay was performed as product instruction recommended. Briefly, reactions were performed (30°C, 30 min) in a total volume of 25 μ l of kinase buffer [8 mM MOPS, pH 7.0, 0.2 mM EDTA, 10 mM MnCl₂, 0.8 M (NH₄)₂SO₄] with or without 1 μ M GRK2-Flag, 0.1 mM ATP, or the desired concentration of GST-EGFR. Reactions were terminated by the addition of 25 μ l of 2 $\times$ SDS loading buffer and boiled. Half of samples were loaded onto 8% SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. The remaining samples were subjected to SDS-PAGE for subsequent immunoblotting for GST-EGFR and for GRK2.

Statistical Analysis

Data were analyzed using either Student's *t* test or two-way ANOVA for comparison of independent means with pooled estimates of common variances.

RESULTS

EGF Pretreatment Promotes Opioid-induced Internalization of DOR

It is well established that stimulation of GPCR induces GPCR internalization and desensitization. As shown in Figure 1A, stimulation of DOR with its selective agonist DPDPE induced a significant reduction of surface DORs stably expressed in HEK293 cells. Interestingly, flow cytometry data also showed that DPDPE-induced internalization of DOR

was significantly enhanced by EGF pretreatment in cells coexpressing EGFR and DOR. Although treating cells with EGF without DPDPE stimulation had no significant effect on the density of cell surface DOR (data not shown), pretreatment of EGF for 20 and 60 min significantly increased DPDPE-induced DOR internalization, whereas 0- or 5-min pretreatment had no significant effect (Figure 1B). The effect of EGF stimulation on internalization of DOR was also examined using receptor surface biotinylation approach. As shown in Figure 1, C and D, EGF pretreatment significantly increased the internalization of surface-biotinylated DOR, and this effect was attenuated by EGFR tyrosine kinase inhibitor AG1478.

GRKs play an important role in agonist-stimulated GPCR internalization. Activation of GPCR stimulates GRK catalytic activity and GRK-mediated receptor phosphorylation induces internalization of GPCR. The potential role of GRK-catalyzed DOR phosphorylation in the EGF-induced enhancement of opioid-dependent DOR internalization was explored next. As shown in Figure 1E, EGF pretreatment promoted DPDPE-induced internalization of the wild-type DOR but not M4/5/6, a DOR mutant that lacks GRK2

Figure 1. EGF pretreatment promotes DPDPE-induced DOR internalization. (A) HEK293 cells stably expressing HA-DOR were transiently transfected with EGFR plasmid and 48 h after transfection, and these cells were treated with PBS or 100 ng/ml EGF for 20 min before incubated at 37°C in 1 μ M DPDPE for the indicated time. EGF was not washed away before addition of DPDPE. Cell surface receptors were stained with antibody against HA- and FITC-conjugated goat anti-mouse IgG and analyzed by flow cytometry. Data are presented as percentage of total cell surface fluorescence measured in untreated cells. ***p* < 0.01 (by two-way ANOVA) compared with PBS pretreated control. (B) HEK293 cells transiently expressing HA-DOR and EGFR were pretreated with 100 ng/ml EGF for the indicated time and then stimulated with 1 μ M DPDPE for 40 min at 37°C. EGF was not washed away before addition of DPDPE. Cell surface receptors were analyzed by flow cytometry as described in A. The data are presented as a percentage of reduction of cell surface fluorescence, which represents the internalization of the indicated receptors. ***p* < 0.01 (by Student's *t* test). (C) HEK293 cells expressing Flag-DOR and EGFR were surface-biotinylated and incubated in the presence or absence of 100 ng/ml EGF for 20 min with or without a 20-min pretreatment of 100 nM AG1478 before incubation in 1 μ M DPDPE for 40 min at 37°C. EGF was not washed away before addition of DPDPE. After the biotin label remaining on the cell surface was stripped by glutathione, Flag-DOR was immunoprecipitated with M2-Sepharose and the Western blots was probed with HRP-conjugated streptavidin to detect (internalized) biotinylated Flag-DOR or with anti-FLAG antibody to detect total Flag-DOR. Direct Western analysis of the cell lysates was done using EGFR antibody. The data are representative of three independent experiments. (D) Band intensity for internalized biotinylated Flag-DOR in C was divided by cognate band densities for total Flag-DOR and normalized to those obtained from cells only stimulated with DPDPE. Data are presented as means $\pm$ SE of three independent experiments. **p* < 0.05 (by Student's *t* test). (E) HEK293 cells stably expressing HA-DOR or HA-M4/5/6 were transiently transfected with EGFR plasmid and 48 h after transfection, and these cells were treated with PBS or 100 ng/ml EGF for 20 min before incubated at 37°C in 1 μ M DPDPE for 40 min. EGF was not washed away before addition of DPDPE. The data are presented as a percentage of reduction of cell surface fluorescence, which represents the internalization of the indicated receptors. ***p* < 0.01 (by Student's *t* test) as compared with PBS pretreated control.

Figure 2. EGF stimulates the interaction of GRK2 with EGFR and translocation of GRK2 to the plasma membrane. (A) HEK293 cells transiently expressing GRK2 alone or coexpressing GRK2 and Flag-EGFR were incubated in the presence or absence of 100 ng/ml EGF for 5 min at 37°C. The cell lysates were immunoprecipitated with anti-FLAG antibody and then the EGFR immunocomplexes and the input cell lysates were analyzed by Western blotting. The top and the bottom parts of the same membrane were probed with antibodies against GRK2 and FLAG, respectively. (B) HEK293 cells transiently expressing GRK2 and Flag-EGFR were stimulated with 100 ng/ml EGF for the indicated time. The cell lysates were treated and analyzed as described in A. (C) HEK 293 cells coexpressing HA-EGFR and GRK2-GFP were incubated with rabbit anti-HA antibody for 1 h at 37°C and then treated with 100 ng/ml EGF at 37°C for 5 min, fixed, permeabilized, and stained with Cy3-conjugated goat anti-rabbit secondary antibody for laser confocal microscopy. (D) A431 cells were transfected with GRK2-

GFP. After 24 h, cells were treated with EGF (100 ng/ml) at 37°C for 5 min, fixed, permeabilized, and labeled with a primary EGFR Rabbit antibody, followed by a secondary layer of Cy3-conjugated goat antibody anti-rabbit IgG for laser confocal microscopy. The white arrows indicate GRK2-GFP translocated to the membrane.

phosphorylation sites (Guo *et al.*, 2000), suggesting that GRK-catalyzed DOR phosphorylation is essential for the enhancement of DOR internalization by EGF.

EGF Stimulates GRK2 Translocation to the Plasma Membrane and Association with EGFR through Its Catalytic Domain

Immunoprecipitation experiment showed that stimulation of 100 ng/ml EGF induced an increase in GRK associated with EGFR in HEK293 cells coexpressing GRK2 and Flag-EGFR. The maximum association of GRK2 and EGFR was observed around 5–10 min of EGF stimulation (Figure 2, A and B). Confocal microscopy data obtained in HEK 293 cells transfected with GRK2-GFP and HA-tagged EGFR revealed that in response to EGF treatment, GRK2 translocated to the membrane and became colocalized with EGFR (Figure 2C). These data indicate that activation of EGFR stimulates GRK and induces the formation of GRK-EGFR complex on the membrane. We also examined GRK2 distribution in A431 cells, which express endogenous EGFR. After a 5-min exposure to EGF, a portion of the GRK2 appeared in plasma membrane and colocalized with the distribution of the endogenous EGFR (Figure 2D). These data indicate that GRK2 can be recruited to the membrane and form a complex with EGFR in an EGFR agonist-dependent manner.

To explore the molecular mechanism of EGF-induced interaction of GRK2 with EGFR, we mapped the EGFR-binding region of GRK2. Previous study has shown that the N-terminal domain (consisting of 185 residues) of GRK2 is involved in the interaction with agonist-occupied GPCR and Gαq (Pitcher *et al.*, 1998), and the PH domain and its α-helix extension (residues 561–670) can bind acidic lipids and Gβγ (Pitcher *et al.*, 1992, 1995; Carman *et al.*, 2000), indicating that these domains are essential for the membrane targeting of GRK2 and phosphorylation of GPCR. Thus we first examined the interaction of ΔN-GRK2 (the N-terminal deletion mutant) and ΔPH-GRK2 (the PH domain and its α-helix extension deletion mutant; Figure 3A). As shown in Figure 3B, both ΔN-GRK2 and ΔPH-GRK2 could be detected in the Flag-EGFR

immunoprecipitates. ΔN-GRK2 and ΔPH-GRK2 appeared to be associated with EGFR more efficiently than with the wild-type GRK2. This result suggests that neither the N-terminal domain nor the PH domain of GRK2 is essential for the association of GRK2 with EGFR. To explore if the catalytic domain of GRK2 is responsible for the binding of EGFR with GRK2, an HA-tagged peptide encoding GRK2 catalytic domain (residues 185–514, HA-GRK2-CD) was constructed (Figure 3A). A large amount of HA-GRK2-CD was detected in the Flag-EGFR immunoprecipitates but not in the control one (Figure 3C), confirming that GRK2 associates with EGFR through its catalytic domain, and indicating the catalytic domain is sufficient for GRK interaction with EGFR. We examined the EGF-stimulated interaction of EGFR with GRK2 truncation mutants, and the results showed that EGF stimulation increased the association of EGFR with these GRK2 truncation mutants (Figure 3, D and E), suggesting that the increased association of EGFR with GRK2 in response to EGF treatment may be largely attributed to the elevated affinity of GRK2 catalytic domain to the activated EGFR.

EGF Stimulates EGFR Tyrosine Kinase Activity-dependent Tyrosyl Phosphorylation of GRK2 N-Terminus

Tyrosyl-phosphorylation of GRK2 by c-Src has been shown to enhance the catalytic activity of GRK2 (Sarnago *et al.*, 1999). To determine whether EGFR could phosphorylate GRK2, HEK293 cells coexpressing EGFR and GRK2-Flag were incubated with EGF, and phosphorylation of GRK at tyrosine was assessed with tyrosyl-phospho-specific antibody after immunoprecipitation. As shown in Figure 4A, after EGF treatment, GRK2 was strongly tyrosyl-phosphorylated and GRK phosphorylation reached a plateau (~20-fold) during 15–30 min of stimulation. AG 1478, a specific inhibitor of EGFR tyrosine kinase, completely blocked EGF-induced tyrosyl phosphorylation of GRK2 (Figure 4B). Significant tyrosyl phosphorylation of GRK2 was not detected in HEK293 cells after EGF treatment, likely due to low abundance of endogenous GRK2 and endogenous EGFR in these cells. In SK-BR3 cells, which express abundant endoge-

nous EGFR, significant tyrosyl phosphorylation of endogenous GRK2 was detected after EGF treatment (Figure 4C). These results suggest that EGFR-mediated tyrosyl phosphorylation of GRK2 requires tyrosine kinase activity of EGFR, and it could occur in cells under physiological conditions.

The potential tyrosyl phosphorylation residues in GRK2 were examined. After EGF treatment, significant phosphorylation of ΔN-GRK2 was not observed (data not shown), suggesting that EGFR-mediated GRK2 phosphorylation occurs in the N-terminal tyrosyl residues of GRK2. It has been shown that GRK2 can be phosphorylated by c-Src at N-terminal Tyr13, Tyr 86, and Tyr 92 residues (Penela *et al.*, 2001). Further experiment showed that EGF-induced tyrosyl phosphorylation of GRK2-3Y/F-Flag, a mutant with Tyr13, Tyr 86, and Tyr 92 substituted

to Phe residues, was reduced more than 80% compared with that of its wild-type counterpart (Figure 4, D and E). The residual phosphorylation signal of GRK2-3Y/F-FLAG suggests the presence of additional, minor tyrosyl phosphorylation sites in GRK2. Data obtained from mutant GRK2 carrying single substitution mutation at Tyr13, Tyr 86, or Tyr 92 (not shown) indicate that Tyr13, Tyr 86, and Tyr 92 residues located at the N-terminal domain of GRK2 are EGFR-mediated primary phosphorylation sites.

EGFR-mediated GRK2 Tyrosyl Phosphorylation Occurs on the Plasma Membrane

Some of EGFR signaling events such as full tyrosine phosphorylation of EGFR and the activation of MAPKs occur

Figure 4. EGFR activation stimulates phosphorylation of GRK2 at tyrosyl residues. (A, B, and D) HEK293 cells coexpressing EGFR and GRK2-Flag (A and B) or EGFR and GRK2-Flag or GRK2-Y3/F-Flag (D) were incubated in the presence or absence of 100 ng/ml EGF for the time indicated (A) or 15 min (B and D), with or without a 20-min pretreatment of 100 nM AG1478. (C) SK-BR3 cells were pretreated with 50 μM pervanadate for 15 min and incubated in the presence or absence of 100 ng/ml EGF for 5 min. Cell lysates were immunoprecipitated with antibody against FLAG or GRK2. The tyrosine phosphorylation of GRK2-Flag was analyzed with PY 20, and the same blot was striped and reprobed with anti-FLAG or GRK antibody. Direct Western analysis of the cell lysates was done using GRK2 and EGFR antibodies. The data are representative of three similar experiments. (E) Phosphotyrosine band intensity in D was divided by the corresponding GRK2-3Y/F-Flag, or GRK2-Flag band intensity and normalized to those obtained from EGF-stimulated cells expressing GRK2-Flag and EGFR. Data are presented as means ± SE of three independent experiments. **p < 0.01 (by Student's *t* test).

Figure 5. EGFR-mediated GRK2 tyrosyl phosphorylation occurs on the plasma membrane. (A) HEK293 cells transiently expressing EGFR, GRK2-Flag, and β -gal or HA-tagged dynamin I K44A were treated with or without 0.4 M sucrose for 30 min before incubation in the absence or presence of 100 ng/ml EGF for 15 min at 37°C. GRK2-Flag was immunoprecipitated with antibody against FLAG and the tyrosine phosphorylation of GRK2-Flag was analyzed with PY20. The blots were striped and reprobed with FLAG antibody for total GRK and direct Western analysis of the cell lysates was done using antibodies as indicated. (B) Phosphotyrosine band intensity in A was divided by the corresponding GRK2-Flag band intensity and normalized to those obtained from cells expressing β -gal, stimulated with EGF, but not treated with sucrose. Data are presented as means $\pm$ SE of three independent experiments. * p < 0.05 (by Student's t test). (C) A431 cells were transfected with GRK2-Flag and Rab5-GFP. After 24 h, cells were treated with EGF (100 ng/ml) at 37°C for 30 min, fixed, permeabilized, and labeled with a Rabbit anti-EGFR antibody and mouse anti-FLAG antibody, followed by a secondary layer of Cy3-conjugated goat antibody anti-rabbit IgG and Cy5-conjugated goat antibody anti-mouse IgG for laser confocal microscopy.

Bar, 10 μ m. The boxed areas are magnified in the corresponding bottom panels, Bar, 5 μ m. The white arrows indicate EGFR positive endosomes.

during the endocytosis process of the receptor (Vieira *et al.*, 1996). EGFR is endocytosed through both the clathrin-dependent and lipid raft-dependent route (Sigismund *et al.*, 2005). We used hypertonic sucrose, which inhibits clathrin-dependent endocytosis, and dominant negative dynamin mutant K44A, which inhibits both clathrin-dependent and lipid raft-dependent endocytosis, to explore whether EGFR-mediated GRK2 phosphorylation also occurs on the endocytic vesicles. As shown in Figure 5, A and B, treatments of 0.4 M sucrose has no effect on EGF-induced GRK2 phosphorylation, and dynamin K44A significantly elevated the phosphorylation of GRK2. Confocal microscopy data obtained in A431 cells coexpressing Rab5-GFP and GRK2 revealed that 30-min EGF stimulation resulted in redistribution of EGFR in the Rab5 positive early endosomes. However, no obvious accumulation of GRK2 at these positions could be observed (Figure 5C). These data suggest that endocytosis of EGFR is not required for EGFR-mediated GRK2 tyrosyl phosphorylation, and EGF induced tyrosyl phosphorylation of GRK2 may occur on the plasma membrane, not on the endocytic vesicles. These data are in agreement with that GRK2 colocalized with EGFR on the membrane, what we observed in Figure 2, C and D. The elevated phosphorylation of GRK2 in cells expressing dominant negative dynamin mutant K44A is likely a result from accumulation of the activated EGFR on the plasma membrane. It also supports our hypothesis that EGF-induced tyrosyl phosphorylation of GRK2 occurs on the plasma membrane.

c-Src Activity Is Not Required for EGFR-mediated Tyrosyl Phosphorylation of GRK2

It has been documented that c-Src is capable of phosphorylating GRK2 at Tyr13, Tyr 86, and Tyr 92 and that c-Src has been implicated in EGFR-mediated signal transduction (Belsches *et al.*, 1997). Therefore, c-Src is likely the candidate

kinase that catalyzes EGFR-mediated GRK2 phosphorylation. However, overexpressing K298R, a dominant negative mutant of c-Src (Fan *et al.*, 2001), failed to inhibit EGF-induced GRK2 tyrosyl phosphorylation (Figure 6A). Furthermore, dose-effect experiments showed that EGF-stimulated GRK2 tyrosyl phosphorylation was not sensitive to pretreatment of PP2, a Src-selective tyrosine kinase inhibitor, in contrast to what observed with GRK2 phosphorylated by constitutively active c-Src (Y527F; Figure 6, B–D). High concentration PP2 (5 μ M) not only completely blocked tyrosyl phosphorylation of GRK2 by c-Src Y527F but also greatly inhibited EGF-stimulated tyrosyl phosphorylation of EGFR and GRK2 (Figure 6, B–D). This result is consistent with a previous report that PP2 at high concentrations inhibits EGFR tyrosine kinase activity (Sorkina *et al.*, 2002). The loss of EGFR-mediated tyrosyl phosphorylation of GRK2 after 5 μ M PP2 pretreatment could be attributed to the inhibition of EGFR kinase activity by PP2. The above data thus suggests that c-Src activity is not required for EGFR-mediated phosphorylation of GRK2.

GRK2 Is a Substrate of EGFR

In vitro pulldown and kinase assays were done to explore if GRK2 is a direct interactor and a substrate of EGFR. GST-tagged human EGFR kinase domain (GST-EGFR) and GRK2-Flag were purified as described in *Materials and Methods* (Figure 7A). Recombinant GST-EGFR could pull down purified GRK2-Flag, in the reciprocal way purified GRK2-Flag alone could pull down recombinant GST-EGFR (Figure 7B). These data demonstrate that EGFR can directly interact with GRK2. We next examined if GRK2 is a substrate of EGFR. We incubated purified GRK2-Flag and ATP with recombinant GST-EGFR and detected phosphorylation by immunoblotting with anti-phosphotyrosine antibody. In the presence of ATP, GST-EGFR was autophosphorylated and

Figure 6. The role of Src in EGFR-mediated tyrosyl phosphorylation of GRK2. (A) HEK293 cells transiently expressing GRK2-Flag, EGFR, and β -gal or c-Src K298R were incubated in the presence or absence of 100 ng/ml EGF for 15 min. (B) HEK293 cells transiently expressing GRK2-Flag and EGFR were pretreated with or without the indicated concentration of PP2 for 15 min before incubation in the presence or absence of 100 ng/ml EGF for 15 min. (C) HEK293 cells transiently expressing GRK2-Flag and c-SrcY527F were incubated in the indicated concentration of PP2 for 30 min. GRK2-Flag was immunoprecipitated with antibody against FLAG, and the tyrosine phosphorylation of GRK2-Flag was analyzed with PY 20. The blots were stripped and reprobed with FLAG antibody for total GRK and direct Western analysis of the cell lysates was done using antibodies as indicated. (D) Phosphotyrosine band intensity in B and C was divided by the corresponding GRK2-Flag band intensity and normalized to those obtained from cells incubated in the absence of PP2. Data are presented as means $\pm$ SE of three independent experiments. ** $p < 0.01$ (by Student's t test).

shifted to a high molecular weight (Figure 7C, lane 1 and lane 2, the arrow indicated); these data confirm that the GST-EGFR we used is active. As expected, GRK2 was phosphorylated by recombinant GST-EGFR (Figure 7C), and 10 nM GST-EGFR could result in a robust tyrosyl phosphorylation of GRK2 (Figure 7C, lane 4). These data indicate that GRK2 is a substrate for EGFR *in vitro*.

To confirm that GRK2 serves as a substrate of EGFR tyrosine kinase *in vivo*, we used HA-GRK2-CD to compete

with the wild-type GRK2 for binding of EGFR. As shown in Figure 7D, overexpression of HA-GRK2-CD greatly inhibited basal and EGF-induced tyrosyl phosphorylation of GRK2. Furthermore, NT-GRK2-Flag, a truncated GRK2 containing only the N-terminal 1–185 amino acid residues, could only be phosphorylated by c-Src Y527F but not EGFR (Figure 7E). These results indicate that the binding of GRK2 to EGFR is necessary for the phosphorylation of GRK2 mediated by EGFR *in vivo* and hint that the mechanisms of

Figure 7. GRK2 is a substrate of EGFR. (A) Purity was assessed by SDS-PAGE and Coomassie Blue staining using 1.5 μ g of GRK2-Flag. (B) Purified GRK2-Flag and recombinant GST-EGFR alone or were combined *in vitro* for GST and M2 pull-down assay as indicated. Immunoblots depict GST-EGFR coimmunoprecipitated with GRK2-Flag. (C) Phosphorylation of GRK2 by EGFR *in vitro*. *In vitro* kinase assay was performed as describe in *Material and Methods*. (D–F) HEK293 cells transiently expressing GRK2-Flag, EGFR and 127 β -gal or HA-GRK2-CD (D), NT-GRK2-Flag and the wild-type c-Src (WT-Src), c-Src Y527F, or EGFR (E), or EGFR and GRK2-Flag or Δ PH-GRK2-Flag (F) were incubated in the presence or absence of 100 ng/ml EGF for 15 min. GRK2-Flag was immunoprecipitated with antibody against FLAG and the tyrosine phosphorylation of GRK2-Flag was analyzed with PY20. The blots were stripped and reprobed with FLAG antibody for total GRK and direct Western analysis of the cell lysates was done using antibodies as indicated.

tyrosyl phosphorylation of GRK2 mediated by Src and EGFR are different.

As shown in Figure 7F, Δ PH-GRK2-Flag, which lacks the PH domain, was significantly phosphorylated after EGF treatment. This implies that neither $G\beta\gamma$ nor PH domain of GRK2 is required for EGFR-mediated GRK2 tyrosyl phosphorylation. Consistent with the above result, transducin α , $G\beta\gamma$ scavenger, showed no significant effect on the level of EGFR-mediated tyrosyl phosphorylation of GRK2 (data not shown).

EGFR-mediated Tyrosyl Phosphorylation of GRK2 Enhances the Catalytic Activity of GRK2 in Intact Cells

To correlate EGFR-mediated tyrosyl phosphorylation of GRK2 with GRK2 activity directly, the change in DOR phosphorylation level after EGF treatment was assessed using an antibody specifically recognize phospho-Ser 363, a major GRK2 phosphorylation site of DOR (Guo *et al.*, 2000). As shown in Figure 8A, DPDPE stimulation induced phosphorylation of DOR in cells expressing endogenous GRK2, and overexpression of GRK2 enhanced basal and DPDPE-induced phosphorylation of DOR. However, M4/5/6, the DOR mutant lacking GRK2 phosphorylation sites, failed to undergo DPDPE-induced phosphorylation, even in cells overexpressing GRK2 (Figure 8B). The effect of EGF stimulation on DOR phosphorylation was tested next in cells coexpressing GRK2 or GRK2-3Y/F with DOR and EGFR. Previous study showed that neither the subcellular localization pattern nor the kinase activity of GRK2-3Y/F toward GPCR or soluble substrates was significantly altered when

compared with the wild-type GRK2 (Penela *et al.*, 2001). Our results showed that in the absence of EGF, the phosphorylation levels of DOR in cells expressing the wild-type GRK2 and GRK2-3Y/F were equivalent. After EGF treatment, the phosphorylation of DOR was significantly elevated in cells expressing the wild-type GRK2 (Figure 8, C and F), and this effect could be inhibited by AG1478 (Figure 8E). However, EGF-induced phosphorylation of DOR was greatly decreased in cells expressing GRK2-3Y/F compared with those expressing the wild-type GRK2 (Figure 8, C and F). Notably EGF stimulation also caused an increase of phosphorylation of DOR in cells expressing GRK2-3Y/F, likely resulted from EGF-induced membrane translocation of GRK2. As expected, EGF treatment failed to induce phosphorylation of M4/5/6 (Figure 8D). Taken together, these data strongly suggests that EGF stimulation enhances activity of GRK2, and that EGFR-mediated tyrosyl phosphorylation of GRK2 contributes to the increased catalytic activity of GRK2 toward GPCR substrates.

EGF Stimulates GRK-mediated Internalization of μ -Opioid Receptor

We next examined the effect of EGF on the internalization of μ -opioid receptor (MOR), another G protein-coupled opioid receptor. EGF treatment at 100 ng/ml induced internalization of MOR in HEK293 cells coexpressing HA-MOR and EGFR. Serine 363, threonine 370, and serine 375 residues within the carboxyl tail of MOR have been shown to be responsible for the basal- and agonist-induced phosphorylation of MOR (El Kouhen *et al.*, 2001; Schulz *et al.*, 2004). We

Figure 8. Activation of EGFR induces GRK-mediated DOR phosphorylation. (A) HEK293 cells transiently coexpressing Flag-DOR and β -glycosidase or GRK2 were incubated in the presence or absence of 1 μ M DPDPE for 10 min. (B) HEK293 cells transiently coexpressing Flag-DOR or Flag-M4/5/6 with β -glycosidase or GRK2 were incubated in the presence or absence of 1 μ M DPDPE for 10 min. (C) HEK293 cells transiently coexpressing Flag-DOR, EGFR, and GRK2 or GRK2-3Y/F were incubated in the presence or absence of 100 ng/ml EGF for the indicated time. (D) HEK293 cells transiently coexpressing EGFR, GRK2 and Flag-DOR or Flag-M4/5/6 were incubated in the presence or absence of 100 ng/ml EGF for 15 min. (E) HEK293 cells transiently coexpressing Flag-DOR, EGFR, and GRK2 were pre-treated for 20 min with PBS or 100 nM AG1478 and incubated in the presence or absence of 100 ng/ml EGF for 15 min. Flag-DOR was immunoprecipitated with M2 antibody-coupled Sepharose and the DOR phosphorylation was analyzed with phospho-DOR (Ser363) antibody. The blots were stripped and re-probed with FLAG antibody for total DOR and direct Western analysis of the cell lysates was done using antibodies as indicated. (F) EGF-induced phosphorylation of DOR at 15 min in C from three independent experiments is summarized. The band intensity was divided by the corresponding value of DOR-Flag and normalized to those obtained from cells expressing the wild-type GRK2 and not treated with EGF. Data are presented as means $\pm$ SE of three independent experiments. *p < 0.05 (by Student's t test).

Figure 9. EGF stimulates GRK-mediated internalization of μ -opioid receptor. (A) HEK293 cells were transiently cotransfected with EGFR and HA-MOR or HA-MOR363/370/375A. After 48 h, cells were stimulated with 100 ng/ml EGF or 10 μ M DAMGO at 37°C for 40 min. Cell surface receptors were stained with mouse antibody against HA- and FITC-conjugated goat anti-mouse IgG and analyzed by flow cytometry. Data are presented as percentage of total cell surface fluorescence measured in PBS-treated cells. $**p < 0.01$ (by Student's *t* test). (B) A431 cells were transfected with HA-MOR or HA-MOR363/370/375A. After 24 h, cells were incubated with mouse anti-HA antibody for 30 min and then treated with DAMGO (10 μ M) or EGF (100 ng/ml) at 37°C for 30 min, fixed, permeabilized, and labeled with a rabbit anti-EGFR antibody, followed by a secondary layer of Cy3-conjugated goat antibody anti-rabbit IgG and FITC-conjugated goat antibody anti-mouse IgG for laser confocal microscopy. Bar, 10 μ m. The boxed areas are magnified in the bottom of the pictures. The white arrows indicate internalized MOR. (C) HEK293 cells were transiently transfected with EGFR, HA-MOR and control siRNA or GRK2 siRNA, after 72 h, cells were stimulated with 100 ng/ml EGF at 37°C for 40 min. Cell surface receptors were analyzed by flow cytometry as described in A. The data are presented as a percentage of reduction of cell surface fluorescence, which represents the internalization of MOR $**p < 0.01$ (by Student's *t* test).

next examined the effect of EGF on the internalization of MOR phosphorylation-deficient mutant, HA-MOR363/370/375A. EGF stimulation failed to promote internalization of HA-MOR363/370/375A (Figure 9A). We next used confocal immunofluorescence microscope to examine the localization of MOR after EGF treatment. In unstimulated A431 cells transfected with HA-MOR and HA-MOR 363/370/375A, HA-MOR was localized primarily on the cell membrane. EGF treatment led to redistribution of cell surface HA-MOR to the intracellular structures. However, redistribution of HA-MOR 363/370/375A was not observed after EGF stimulation (Figure 9B). These results suggest that EGF-induced internalization of MOR critically depends on the phosphorylation of MOR. As shown in panels C and D of Figure 9, GRK2 siRNA significantly reduced EGF-induced MOR internalization. Taken together, these results further demonstrate that EGF stimulation results in internalization of MOR and this is mediated by GRK2-induced MOR phosphorylation.

DISCUSSION

In this study, we show that EGF induces translocation of GRK2 to the membrane and the activated EGFR recruits GRK2 by binding its catalytic domain and tyrosyl phosphorylates the N-terminal tyrosine residues of GRK2 on the membrane. The tyrosyl phosphorylation of GRK2 enhances GRK2 activity and then transregulates internalization of δ -opioid receptor. Our preliminary data showed that EGF also regulated internalization of other GPCRs including MOR. These data suggest that the transregulation of GPCR by EGFR is not restricted to δ -opioid receptor. Our results propose that GRK as a mediator of cross-talk from RTK to GPCR signaling pathway.

Changes in subcellular distribution and functionality of GRKs greatly affect GPCR functions, as reported under sev-

eral pathological conditions such as hypertension (Gros *et al.*, 1997), congestive heart failure (Ungerer *et al.*, 1993) and rheumatoid arthritis (Lombardi *et al.*, 2001). In addition to the well-documented roles of GRKs in GPCR signaling, GRKs have recently been recognized as a potential regulator of signal transduction mediated by certain receptor tyrosine kinases. GRK2 is capable to form a signaling complex with c-Src and PDE γ to regulate EGF-stimulated p42/p44 MAPK activation. GRK2 can interact with PDGFR and EGFR in vivo (Freedman *et al.*, 2002; Gao *et al.*, 2005) and phosphorylate PDGFR and EGFR (Freedman *et al.*, 2002). GRK2-mediated seryl phosphorylation of PDGFR inhibits tyrosyl phosphorylation of PDGFR, PDGFR-evoked phosphoinositide hydrolysis, and Akt activation (Hildreth *et al.*, 2004). Our results propose a novel mechanism for regulation of the GPCR signaling by receptor tyrosyl kinases via growth factor-mediated membrane translocation and phosphorylation of GRK2. We also demonstrated that tyrosyl phosphorylation of GRK2 induced by the activation of receptor tyrosine kinase stimulates the serine/threonine kinase activity of GRK2 and thus results in altered responsiveness of GPCR signaling.

C-Src has been implicated in EGFR signal transduction. We show that the activated EGFR phosphorylates GRK2 at the same tyrosyl sites as c-Src does. However, our data also demonstrate that Src contributes little, if any, in EGFR-mediated GRK2 tyrosyl-phosphorylation (Figure 6). Our in vitro pulldown and kinase assays further confirm that GRK2 is a direct binding partner and substrate of EGFR (Figure 7). These results are in consistent with those of Wu *et al.* (2005), who reported recently that PDGFR β can tyrosyl phosphorylate GRK2 directly. However, EGFR and PDGFR may employ distinct mechanisms to phosphorylate GRK2. Wu *et al.* postulate that PDGFR β localizes in caveolae after its activation and phosphorylates GRK2, which is localized to the caveolae by interaction with caveolin. It is more likely that

the activated and autophosphorylated EGFR forms a docking site for GRK2 by binding its catalytic domain and then phosphorylates the N-terminal tyrosine residues of GRK2. Support for this model comes from two lines of evidences: first, overexpression of the peptide encoding the catalytic domain of GRK2, which is responsible for the interaction with EGFR, greatly inhibited EGFR-mediated GRK2 tyrosyl phosphorylation. Second, the caveolin-binding domain containing peptide encoding N-terminal 1–185 amino acid residues of GRK2 (Carman *et al.*, 1999) could be phosphorylated by a constitutive active form of c-Src, but not the activated EGFR. These data demonstrate that GRK2 serves as a substrate for both receptor tyrosine kinase and nonreceptor tyrosine kinase. It further supports our hypothesis that GRK2 acts as a mediator of cross-talk from RTK to the GPCR signaling pathway.

We also postulate that EGFR-mediated GRK2 phosphorylation may occur on the membrane, several lines of evidence support this model: First, GRK2 was colocalized with EGFR on the membrane but not in the endocytic vesicles (Figures 2, C and D, and 5C). Second, inhibition of endocytosis of EGFR by dynamin K44A enhanced EGF-induced phosphorylation of GRK2 (Figure 5, A and B).

Tyrosyl-phosphorylation of GRK2 has been shown to play an important role in regulation of GRK2 activity. Tyrosyl-phosphorylation of GRK2 by c-Src promotes the kinase activity of GRK2 toward both soluble and membrane-bound substrates *in vitro* (Sarnago *et al.*, 1999). Fan *et al.* (2001) demonstrated that tyrosyl-phosphorylation and activation of GRK2 participate in agonist-induced desensitization of β_2 AR agonist triggers tyrosyl phosphorylation of β_2 AR and creates a canonical SH2-binding domain on the receptor that recruits and activates Src. Subsequently, Src phosphorylates and activates GRK2 (Fan *et al.*, 2001). PDGFR-mediated GRK2 tyrosyl phosphorylation activates GRK2 and enhances GRK2-mediated seryl phosphorylation of the PDGFR, an event that reduces PDGFR signaling (Wu *et al.*, 2005). In this study, we showed that EGF treatment regulated internalization of both δ - and μ -opioid receptors through EGFR-mediated GRK2 phosphorylation and activation. These data suggest that tyrosyl-phosphorylation of GRK2 may act as a common and important mechanism of GRK2 activation when cells mobilize GRK2 toward non-GPCR targeting extracellular stimulus to generate integrate cellular response that coordinates with GPCR signaling.

In the current study, we have used opioid receptors to explore the transregulation of GPCR by EGFR. Activation of opioid receptors results in a multitude of effects including analgesia, respiratory depression, and euphoria, feeding the release of hormones, inhibition of gastrointestinal transit, and effects on anxiety. Opioid receptors are widely expressed in many tissues such as brain, spinal cord, intestine, adrenal, kidney, lung, spleen, testis, ovary, and uterus, where EGFR is also expressed. It has long been known that EGFR is overexpressed in many tumors. Deletion mutations and point mutations resulting in constitutive activation of EGFR have been discovered in some malignancies, such as gliomas and non-small-cell lung cancers (Yun *et al.*, 2007). All three subtypes of opioid receptors δ , μ , and κ are expressed in non-small-cell lung cancers and other tumor cells (Campa *et al.*, 1996). Expression of opioid receptors correlated inversely with tumor growth *in vivo*, and administration of opioid agonists resulted in increased survival rates of tumor-bearing mice (Gomez-Flores *et al.*, 2005). Currently, opioid-based anticancer drugs are in early clinical trials (Smith *et al.*, 2004). Studies support that transregulation of opioid receptors by EGFR may play a role in tumorigenesis.

However, we must note that our results were obtained from model cellular systems, and the potential physiological significance of these findings remains to be demonstrated further.

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