Luciferase Fragment Complementation Imaging of Conformational Changes in the Epidermal Growth Factor Receptor*

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Crystal structures of the epidermal growth factor (EGF) receptor suggest that its activation is associated with extensive conformational changes in both the extracellular and intracellular domains. However, evidence of these structural dynamics in intact cells has been lacking. Here we use luciferase complementation imaging to follow EGF-induced conformational changes in its receptor in real time in live cells. When the luciferase fragments are fused to the C terminus of an EGF receptor lacking the cytoplasmic domain, EGF stimulates a rapid increase in luciferase activity, consistent with ligand-induced receptor dimerization. However, when the luciferase fragments are fused to the C terminus of the full-length receptor, EGF induces a rapid but transient decrease in luciferase activity. The decrease requires tyrosine kinase activity, whereas the subsequent recovery requires MAP kinase activity. Our data demonstrate the utility of the luciferase system for in vivo imaging changes in EGF receptor dimerization and conformation. They also identify two sequential ligand-induced conformational changes in the EGF receptor.

The epidermal growth factor (EGF) receptor is a classical receptor tyrosine kinase that mediates cell proliferation, migration, and differentiation in response to growth factor stimulation (1–4). The EGF receptor consists of an extracellular ligand binding domain, a single-pass α-helical transmembrane region, an intracellular domain that contains the tyrosine kinase activity (5), and a C-terminal tail of ~200 amino acids. The receptor is thought to exist in cell membranes as a monomer (6, 7). However, upon binding EGF, the receptor undergoes ligand-induced dimerization (6). This promotes activation of the receptor tyrosine kinase activity and leads to autophosphorylation of the receptor on its C-terminal tail. Binding of SH2 and PTB domain-containing proteins to these phosphoryrosines results in the activation of downstream signaling pathways such as MAP kinase and Akt (1–4).

X-ray crystallography studies have provided a wealth of information on the structure of the EGF receptor. These studies suggest that receptor activation is linked to significant conformational changes in both the intracellular and extracellular domains of the receptor. The extracellular domain of the receptor is comprised of four subdomains, I through IV. Subdomains I and III are homologous and known to be involved in ligand binding (8). Subdomains II and IV are homologous high-cysteine regions. The crystal structure of the extracellular domain of the unliganded EGF receptor shows it in a tethered conformation in which an arm from subdomain II interacts with an arm from subdomain IV to hold the receptor in the closed configuration (8). Structures of the ligand-bound EGF receptor extracellular domain suggest that upon ligand binding, the tether between subdomains II and IV is released allowing the receptor to adopt an open, extended conformation. In this conformation, the receptor forms a back-to-back dimer with another ligand-occupied EGF receptor monomer (9, 10).

Activation of the tyrosine kinase also appears to be associated with conformational changes. Zhang et al. (11) showed that following dimerization of the extracellular domain, the kinase domain of the EGF receptor forms an asymmetric dimer in which the N-lobe of one kinase contacts the C-lobe of a second kinase. In this configuration, the first monomer becomes activated and phosphorylates the C-terminal tail of the second monomer. Presumably, a further conformational change leads to a shift in the position of the two kinase domains allowing activation of the second kinase.

In addition to the changes that occur in the extracellular and kinase domains upon receptor activation, studies on the isolated soluble intracellular domain of the EGF receptor indicate that the C-terminal tail of the EGF receptor undergoes a conformational change following phosphorylation (12–14). The data suggest that in the absence of phosphorylation, the C-terminal tail lies close to the kinase domain. However, upon phosphorylation, the C-terminal tail appears to move away from the kinase domain, adopting a more extended conformation. These findings are consistent with the different positions of the short segment of the C-terminal tail observed in the crystal structure of Wood et al. (15) and Stamos et al. (16).

Although evidence from crystal structures and purified systems suggests that the EGF receptor undergoes several confor-
EXPERIMENTAL PROCEDURES

Reagents—Murine EGF was purchased from Biomedical Technologies, Inc. and was dissolved in sterile water. U0126 was purchased from EMD Chemicals and dissolved in dimethyl sulfoxide. Erlotinib was obtained from OSI Pharmaceuticals and dissolved in dimethyl sulfoxide. Rapamycin was dissolved in dimethyl sulfoxide and was kindly provided by Dr. D. Piwnica-Worms (Washington University, St. Louis, MO). Doxycycline was purchased from Clontech and was dissolved in sterile water. d-Luciferin (Biosynth) was dissolved in phosphate-buffered saline and coelenterazine (Sigma) was dissolved in ethanol. The MAP kinase antibody was from Upstate. The EGF receptor was detected with phospho-specific MAP kinase antibody was from Promega. The phosphotyrosine antibody (PY20) was from BD Biosciences. The phospho-Tyr1173- and phospho-Thr669-specific antibodies were from Upstate. The EGF receptor was detected using Erbitux (ImClone) or a mixture of antibodies from Cell Signaling and Santa Cruz.

DNA Constructs—To generate the EGFR-NLuc construct, Notch full-length NLuc (kindly provided by Dr. R. Kopan, Washington University, St. Louis, MO) was digested with BsiWI and XbaI. A flexible Gly-Ser-rich linker was generated (amino acid sequence WPRSYASRGGGSSGGG) (17) containing SacII, BsiWI, and XbaI sites. The linker was ligated into the Notch full-length NLuc construct using the BsiWI and XbaI sites. An EGFR-GFP construct (18) was digested with SacII and BsiWI and cloned into the EGFR-CLuc (pcDNA6/V5-His B) or EGFR-NLuc (pBI-Tet) constructs, resulting in the following linker, YASRGGSSGGG (17). The K721A-EGFR-CLuc construct was made using QuikChange site-directed mutagenesis (Stratagene) in the EGFR-CLuc pcDNA6/V5-His B construct. The K721A-EGFR-NLuc construct was made by digesting EGFR-NLuc (pcDNA3.1 TOPO) with BstEII and KpnI. The insert was ligated into the K721A pcDNA5.FRT (Invitrogen) construct digested with the same enzymes. The T669A-EGFR-CLuc construct was made using QuikChange site-directed mutagenesis (Stratagene) in the EGFR-CLuc pcDNA6/V5-His B construct. The T669A-EGFR-NLuc construct was made by digesting EGFR-NLuc (pcDNA3.1 TOPO) with BstEII and KpnI. The insert was ligated into the T669A pcDNA5.FRT (Invitrogen) construct digested with the same enzymes. All mutations were verified by sequencing. The FRB-NLuc and CLuc-FKBP constructs were kindly provided by Dr. D. Piwnica-Worms (17).

Cell Lines—CHO-K1 Tet-On cells (Clontech) were cotransfected with pTK-Hyg (Clontech) and EGFR-NLuc (pBI-Tet MCSI) using Lipofectamine 2000 (Invitrogen). Stable clones were isolated by selection in 400 μg/ml hygromycin (Invitrogen). A double-stable cell line was established by transfecting EGFR-CLuc (pcDNA6/V5-HisB) into EGFR-NLuc cells using Lipofectamine 2000 and selecting in 10 μg/ml blasticidin-S (Invitrogen). Double-stable lines were grown in DMEM containing 10% fetal bovine serum, 1000 μg/ml penicillin/streptomycin, 100 μg/ml G418, 50 μg/ml hygromycin, and 2 μg/ml blasticidin-S. ΔC-EGFR-NLuc (pBI-Tet MCSI) and c’973-EGFR-NLuc (pBI-Tet MCSI) were stably expressed as described above for EGFR-NLuc. Cells were maintained in DMEM containing 10% fetal bovine serum, 1000 μg/ml penicillin/streptomycin, 200 μg/ml G418, and 100 μg/ml hygromycin. ΔC-EGFR-CLuc (pcDNA6/V5-His B) and c’973-EGFRCLuc (pcDNA6/V5-His B) were transiently transfected into the appropriate parental cell line 24 h prior to luciferase complementation imaging using Lipofectamine 2000 (Invitrogen). The K721A-EGFR-NLuc/K721A-EGFR-CLuc and T669A-EGFR-NLuc/T669A-EGFR-CLuc constructs were transiently transfected into CHO-K1 Tet-On cells 24 h prior to luciferase complementation imaging using Lipofectamine 2000. To assess the transfection efficiency, cells were co-transfected with Renilla luciferase (pRLuc-N1, Packard Bioscience).

Kinase Activation and Western Blotting—EGFR-NLuc/CLuc cells were grown to confluence in 35-mm dishes. Cells were serum-starved in DMEM containing 1 mg/ml BSA for 3 h. Culture medium was removed and cells were washed twice in ice-cold phosphate-buffered saline and then scraped into RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 17 mM deoxycholate, and 2.7 mM EDTA) containing 20 mM p-nitrophenyl phosphate, 1 mM sodium orthovanadate, and protease inhibitors. Equal amounts of protein (BCA assay, Pierce) were loaded onto a 9% SDS-polyacrylamide gel and then
transferred to polyvinylidene difluoride (Millipore) or nitrocellulose (Osmonics, Inc.). Western blots were blocked for 1 h in TBST, 10% nonfat milk. The blots were incubated in primary antibody for 1 h, washed in TBST, 0.1% BSA, incubated in secondary antibody for 45 min and washed three times in TBST, 0.1% BSA. Western blots were detected using the ECL reagent from GE Healthcare.

125I-EGF Binding—Murine EGF was radioiodinated and ligand binding assays were performed as described previously (20).

Luciferase Complementation Imaging—Cells were plated 48 h prior to use at 5 × 10^4 cells per well in DMEM containing doxycycline in a black-walled 96-well plate. On the day of the assay, the wells were serum-starved for 3 h and then incubated for 20 min in 175 μl of DMEM without phenol red, containing 1 mg/ml BSA, 25 mM Hapes, and 0.6 mg/ml β-luciferin at 37 °C. To establish a baseline, cell radiance (photons/s/cm^2/steradian) was measured using a cooled CCD camera and imaging system at 37 °C (IVIS 50; Caliper) (30 s exposure; binning, 8; no filter; f-stop, 1; field of view, 12 cm). EGF was added in a volume of 25 μl in the same media (DMEM, 1 mg/ml BSA, 25 mM Hapes, 0.6 mg/ml β-luciferin). Radiance was measured as described above. For the ΔC-EGFR, c′973-EGFR, K721A-EGFR, and T669A-EGFR experiments involving transient transfection, the transfection efficiency was assessed by monitoring Renilla luciferase expression. Media was replaced with cells from those of EGF-treated cells. Standard errors were determined using the formula for the calculation of the unpoole standard error.

RESULTS

EGF Induces a Rapid Increase in Luciferase Complementation in ΔC-EGFR-NLuc/CLuc Cells—The firefly luciferase complementation system utilizes two fragments of luciferase termed NLuc and CLuc. These fragments are inactive by themselves but are capable of reconstituting an active luciferase if the fragments are brought into close proximity (17, 21). Initially, the luciferase fragments were independently fused to the C terminus of an EGF receptor lacking the entire cytoplasmic domain yielding the ΔC-EGFR-NLuc and ΔC-EGFR-CLuc cDNA constructs. CHO-K1 Tet-On cells were transfected with ΔC-EGFR-NLuc and a line that stably expressed ΔC-EGFR-NLuc in a doxycycline-inducible manner was selected. Twenty four hours prior to use, these cells were transiently transfected with ΔC-EGFR-CLuc. These cells are referred to as ΔC-EGFR-NLuc/CLuc CHO cells.

For imaging experiments, cells were first incubated with luciferin for 20 min at 37 °C to allow equilibration of the intracellular and extracellular pools of this substrate. This preincubation assures a stable baseline during the subsequent 20-min observation period. Luciferase activity was measured by monitoring the photon flux from ΔC-EGFR-NLuc/CLuc co-expressing CHO cells. Readings were taken approximately every 30 s, providing a continuous readout of luciferase activity through bioluminescence imaging of live cells.

Luciferase activity was detectable in cells co-expressing ΔC-EGFR-NLuc and ΔC-EGFR-CLuc even in the absence of added EGF (Fig. 1A, open circles). This suggests the presence of receptor-receptor interactions in the basal state. The addition of 10 nM EGF led to a rapid increase in light production that plateaued between 10 and 15 min (Fig. 1A, closed squares). Both the rate and extent of ligand-induced dimer formation were dependent on the concentration of EGF (Fig. 1B). This increase in luciferase complementation following EGF stimulation is consistent with the canonical model of EGF-induced dimerization of its receptor (6). Importantly, the rate of dimer formation detected by luciferase fragment complementation was similar to the rate of 125I-EGF binding observed in these cells (Fig. 1C), indicating that this imaging technique accurately reflects the kinetics of ligand-induced dimerization of the EGF receptor.

EGF Elicits a Rapid but Transient Decrease in Luciferase Activity in Full-length EGF-NLuc/CLuc Cells—To assess the contribution of the cytoplasmic domain of the EGF receptor to receptor-receptor interactions, the NLuc and CLuc fragments were independently fused to the C terminus of the full-length EGF receptor. To ensure that equal levels of the two chimeric receptors were expressed, a double-stable CHO-K1 Tet-On cell line was established that will be referred to as the EGF-NLuc/CLuc cell line. In this cell line, EGFR-CLuc was constitutively expressed, whereas EGF-NLuc was expressed from a doxycycline-inducible plasmid. This allowed adjustment of EGF-NLuc expression levels to match those of EGFR-CLuc.

As shown in Fig. 2A, Scatchard analysis of 125I-EGF binding to the uninduced EGF-NLuc/CLuc cells indicated that EGF-RCLuc was expressed at a level of ~100,000 receptors per cell. When the cells were treated with 1 μg/ml doxycycline, binding experiments demonstrated the presence of ~200,000 EGF
receptors per cell. These data suggest that under these conditions, EGFR-NLuc and EGFR-CLuc are expressed at roughly equivalent levels of $\frac{1}{100,000}$ receptors/cell. As can be seen from Fig. 2A, both Scatchard plots were curvilinear, demonstrating that these receptor fusion proteins retain this characteristic feature of EGF binding to the wild type receptor (20).

The effect of the luciferase fragments on the kinase activity of the EGF receptor was determined independently in CHO cells that expressed only the EGFR-NLuc or the EGFR-CLuc receptors. The data in Fig. 2B show that EGF stimulated the autophosphorylation of both EGFR-NLuc and EGFR-CLuc, indicating that both receptors retain kinase function. EGFR-NLuc showed two distinct bands, both of which were phosphorylated and both of which reacted with anti-luciferase antibodies (not shown). This suggests that the lower molecular weight form is not the result of proteolytic removal of the luciferase fragment. It is possible that differences in glycosylation may be responsible for the different forms. Both NLuc- and CLuc-EGF receptors mediated the activation of MAP kinase (Fig. 2B).

Thus, addition of the luciferase fragments did not substantially alter the biochemical behavior of the EGF receptor. When EGFR-NLuc/CLuc cells were incubated with luciferin, light production was observed in the absence of EGF (Fig. 3A). However, in contrast to the results obtained with the truncated AC-EGF receptor, addition of EGF to EGFR-NLuc/CLuc cells resulted in a rapid, but transient, decrease in luciferase activity (Fig. 3, B–G). The decrease was observed at the earliest time point following EGF addition and reached a nadir 2 to 4 min after EGF stimulation. Subsequently, light production recovered to essentially the same level as that observed prior to EGF addition. The magnitude of the decrease in luciferase activity was dependent on the dose of EGF, exhibiting an EC$_{50}$ of $\frac{1}{100,000}$ EGF. This value is similar to that observed for the stimulation of EGF receptor autophosphorylation ($\frac{1}{100,000}$).

Because EGF induces a variety of changes in ion transport and metabolic pathways, it was possible that the decrease in light production observed after the addition of EGF was due to changes in pH or some other metabolite within the cells. To examine this possibility, HeLa cells that express $\frac{1}{100,000}$ endogenous EGF receptors/cell were transfected with FRB-NLuc and CLuc-FKBP (17) 24 h prior to imaging. Cells were pretreated with rapamycin (80 nM for 5 h). Before collecting data, cells were incubated for 20 min with 0.6 mg/ml o-luciferin. Photon flux was immediately measured over time after the addition of vehicle, 10 nM EGF, or rapamycin. Error bars represent the S.E. of four independent measurements for each condition.

**FIGURE 2.** Characterization of the full-length EGFR-NLuc and EGFR-CLuc. A, Scatchard analysis of $^{125}$I-EGF binding to CHO cells stably expressing EGFR-CLuc (no doxycycline, open circles) or EGFR-NLuc/CLuc (closed circles) in which EGFR-NLuc expression had been induced by the addition of 1 $\mu$g/ml doxycycline for 48 h. B, CHO cells stably expressing either EGFR-NLuc or EGFR-CLuc were stimulated with 10 nM EGF at 37 °C for the indicated lengths of time. Western blot analysis was performed with the indicated antibodies.

**FIGURE 3.** Reconstitution of luciferase activity in response to EGF in EGFR-NLuc/CLuc cells. EGFR-NLuc/CLuc cells were plated into DMEM containing 1 $\mu$g/ml doxycycline 48 h prior to imaging. Before collecting data, cells were incubated for 20 min with 0.6 mg/ml o-luciferin. A, photon flux (photons/s; p/s) in the absence of ligand. B–G, change in photon flux following addition of varying concentrations of EGF. H, HeLa cells were plated 48 h prior to bioluminescence imaging in DMEM. Cells were transiently transfected with FRB-NLuc and CLuc-FKBP (17) 24 h prior to imaging. Cells were pretreated with rapamycin (80 nM for 5 h). Before collecting data, cells were incubated for 20 min with 0.6 mg/ml o-luciferin. Photon flux was immediately measured over time after the addition of vehicle, 10 nM EGF, or rapamycin. Error bars represent the S.E. of four independent measurements for each condition.
assay, cells were serum starved for 20 min with 0.6 mg/ml doxycycline 48 h prior to assay. On the day of assay, cells were serum starved for 3 h before stimulation with 10 nM EGF for the indicated lengths of time. Cells were lysed and equal proteins were loaded on a 9% SDS-PAGE gel. Proteins were transferred and Western blot analysis was done with a phosphotyrosine antibody (pY20, BD Biosciences). Densiometric analysis was done using ImageJ (National Institutes of Health). For luciferase imaging, EGFR-NLuc/CLuc cells were plated in DMEM containing 1 μg/ml doxycycline 48 h prior to imaging. Before collecting data, cells were incubated for 20 min with 0.6 mg/ml luciferin. Photon flux (photons/s; p/s) was measured after addition of 10 nM EGF. Error bars represent the S.E. of four independent measurements for each condition.

The Decrease in Luciferase Activity Is Dependent on EGF Receptor Kinase Activity—Fig. 4 compares the time course of EGF-stimulated receptor autophosphorylation with that of the EGF-induced decrease in luciferase activity in EGFR-NLuc/CLuc cells. As can be seen from the figure, maximal autophosphorylation occurred within 30 s after the addition of EGF. By contrast, the decrease in luciferase activity required 2 to 4 min to reach its nadir. Thus, receptor activation precedes the agonist-induced decrease in luciferase activity. This suggests that the decrease in luciferase activity might be a consequence of receptor tyrosine kinase activation.

To assess the involvement of receptor kinase activity in the decreased luciferase complementation, EGFR-NLuc/CLuc cells were incubated with 5 μM erlotinib to inhibit receptor tyrosine kinase activity. Treatment with erlotinib induced a ~3-fold increase in basal luciferase activity indicating that it enhanced the interaction between EGF receptors under resting conditions (Fig. 5A). This increase was specific for the EGF receptor, because treatment of HeLa cells expressing FRB-NLuc and CLuc-FKBP with erlotinib did not alter basal light production (not shown). As expected, stimulation with EGF led to a transient decrease in luciferase activity in control cells (Fig. 5B). However, this effect was largely eliminated in cells pretreated with erlotinib. Similar results were obtained with AG1478 (not shown).

Because the effect of erlotinib on basal luciferase complementation complicated the interpretation of its effect on the EGF-stimulated decrease in luciferase activity, the requirement for EGF receptor tyrosine kinase activity was further examined using the K721A-EGF receptor. The K721A mutation abolishes the tyrosine kinase activity of the EGF receptor (22). The NLuc and CLuc fragments were fused to the C terminus of the K721A-EGF receptor and were transiently transfected into CHO cells. As was seen for the wild type EGF receptor, erlotinib enhanced basal luciferase activity in this kinase-dead EGF receptor mutant (Fig. 5C). However, in contrast to the situation with wild type receptor, addition of EGF to the K721A-EGF receptor led to an increase in luciferase activity (Fig. 5D), similar to that seen in the ΔC-EGF receptor (Fig. 1). Erlotinib enhanced the EGF-stimulated increase in luciferase activity in the K721A-EGF receptor system suggesting that the effect of this tyrosine kinase inhibitor is independent of the effects elicited by EGF. These data confirm the involvement of receptor tyrosine kinase activity in the ligand-induced decrease in luciferase complementation.

The observed decrease in luciferase activity is consistent with the hypothesis that EGF induces a conformational change in the receptor that leads to separation of the luciferase fragments. To determine whether the EGF-stimulated decrease in luciferase complementation was due to movement of the C-terminal tails of the receptors or involved changes in the relative positions of the kinase domains, the luciferase fragments were fused to an EGF receptor truncated at residue 973 just beyond the kinase domain. The constructs were expressed in CHO cells. Luciferase activity was detectable in these cells in the absence of EGF (Fig. 6A). As was seen with the wild type receptor, treatment of the c’973-EGFR-NLuc/CLuc cells with EGF led to a decrease in luciferase activity (Fig. 6B). Although there was some recovery of luciferase activity over time, it was slower and less extensive than that seen for the wild type receptor. These data suggest that, at a minimum, the EGF-induced
A decrease in luciferase activity involves movement of the kinase domains relative to each other.

**MAP Kinase Activity Is Required for the Recovery of Luciferase Complementation**—The recovery of luciferase activity after treatment of EGFR-NLuc/CLuc cells with EGF occurred over a time course that was similar to that of the activation of MAP kinase by EGF (Fig. 7, top left). MAP kinase is known to catalyze the phosphorylation of the EGF receptor on Thr<sup>669</sup> (23, 24) and the phosphorylation of this residue on the EGF receptor paralleled the activation of MAP kinase in EGFR-NLuc/CLuc cells (Fig. 7, top left).

To determine whether the activation of MAP kinase played a role in the recovery of luciferase complementation, EGFR-NLuc/CLuc cells were pretreated with the MEK inhibitor, U0126, to block the activation of MAP kinase. As shown in Fig. 7, top right, U0126 completely prevented both the activation of MAP kinase and the phosphorylation of Thr<sup>669</sup> when cells were pretreated with U0126 prior to assay of luciferase activity, U0126 had little effect on basal light production (Fig. 7, bottom left). However, this inhibitor completely blocked the recovery of luciferase activity after the EGF-stimulated decrease (Fig. 7, bottom right). The initial decrease was of a greater magnitude than that seen in control cells and there was no recovery toward baseline levels over the 20-min observation period. These data indicate that MAP kinase activity is involved in the recovery phase of luciferase complementation.

The requirement for MAP kinase activation in the recovery of luciferase activity was further examined using the T669A-EGFR receptor. The T669A mutation removes the MAP kinase phosphorylation site from the EGF receptor (23–25). The NLuc and CLuc fragments were fused to the C terminus of the EGF receptor. The T669A-EGF receptor also displayed basal luciferase activity, which was relatively stable over the 20-min time course of the experiment. Addition of EGF led to a rapid decrease followed by a slow recovery of luciferase activity (Fig. 8B). The T669A-EGF receptor also displayed basal luciferase activity (Fig. 8C). But in this mutant, treatment with EGF led to a decrease in luciferase complementation with no significant recovery back toward baseline levels of activity (Fig. 8D). Furthermore, the decrease observed in the T669A-EGFR-NLuc/CLuc cells was greater in magnitude than that observed in the wild-type EGFR-NLuc/CLuc cells (Fig. 8, B and D). This pattern was similar to that seen in cells expressing wild type EGFR-NLuc/CLuc cells but treated with U0126 (Fig.

**FIGURE 6.** Effect of truncation of the C-terminal tail of the EGF receptor on reconstituted luciferase activity. CHO cells stably expressing c’973-EGFR-NLuc were plated in DMEM containing 2 μg/ml doxycycline 48 h prior to luciferase imaging. Cells were transiently transfected with c’973-EGFR-CLuc 24 h prior to imaging. Cells were treated for 20 min with 0.6 mg/ml α-luciferin prior to imaging. A, basal photon flux (photons/s; p/s); B, luciferase activity in cells treated with 10 nM EGF. Error bars represent the S.E. of four independent measurements for each condition.

**FIGURE 7.** Contribution of MAP kinase activation to the recovery of reconstituted luciferase activity. EGFR-NLuc/CLuc cells were plated 48 h prior to assay in DMEM containing 1 μg/ml doxycycline. Cells were serum starved for 3 h prior to assay. EGFR-NLuc/CLuc cells were pre-treated with vehicle (control), top left or 10 μM U0126 (top right) for 20 min prior to addition of 10 nM EGF at time = 0. Western blot analysis was performed with the indicated antibodies. For the luciferase imaging, EGFR-NLuc/CLuc cells were plated 48 h before imaging in DMEM containing 1 μg/ml doxycycline. EGFR-NLuc/CLuc cells were pretreated with vehicle (control) or 10 μM U0126 for 20 min (both in the presence of 0.6 mg/ml α-luciferin). The photon flux in the absence of ligand (bottom left) or after addition of 10 nM EGF (bottom right) was monitored over time. Error bars represent the S.E. of four independent measurements for each condition.

**FIGURE 8.** Effect of mutation of Thr<sup>669</sup> of the EGF receptor on reconstituted luciferase activity. Panels A and B, CHO-K1 Tet-On cells were transiently transfected with EGFR-NLuc and EGFR-CLuc 24 h prior to imaging. Cells were treated for 20 min with 0.6 mg/ml α-luciferin prior to imaging. A, basal photon flux (photons/s; p/s); B, luciferase activity in cells treated with 10 nM EGF. Error bars represent the S.E. of four independent measurements for each condition.
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7B). These data suggest that the recovery in luciferase complementation is a result of the phosphorylation of the EGF receptor on Thr\(^{669}\) by MAP kinase.

**DISCUSSION**

The use of firefly luciferase for enzyme complementation has allowed us to continuously monitor reversible conformational changes in the EGF receptor. When the luciferase fragments were fused to EGF receptors that contained only the extracellular and transmembrane domains of the receptor, the system permitted the direct imaging of EGF receptor dimerization in real time in living cells. The observed rate of receptor dimerization paralleled the rate of ligand binding suggesting that the luciferase complementation system responds rapidly to changes in proximity of the fragments, allowing an accurate temporal read-out of receptor dimerization in vivo. Previous studies that employed a β-galactosidase complementation system reported dimerization rates that were 5–10-fold slower than those observed here (26, 27). The time lag between ligand binding and receptor dimerization, as measured by the β-galactosidase assay, is likely due to the fact that the β-galactosidase assay must be performed ex vivo on lysed cells and is susceptible to artifacts resulting from the slow, continuous accumulation of product over time. Thus, the β-galactosidase system is suboptimal for monitoring rapid and dynamic changes in protein-protein interactions.

When the luciferase fragments were fused to the full-length EGF receptor, substantially different results were obtained than with the truncated EGF receptor. A significant basal luciferase activity was observed, suggesting that the unstimulated EGF receptor exists in a conformation in which the C-terminal tails of the two monomers are in close proximity to each other. This is consistent with previous reports that some fraction of cell-surface EGF receptors exist as pre-formed dimers (28–31). Addition of the tyrosine kinase inhibitor, erlotinib, significantly increased the basal luciferase activity. This is in agreement with the observation that the level of inactive, pre-formed EGF receptor dimers is increased by treatment of cells with 4-anilinoquinazoline tyrosine kinase inhibitors (32–35). Our finding that erlotinib also enhanced basal luciferase complementation in the kinase-dead K721A-EGF receptor system suggests that the effects of erlotinib are due solely to the binding of the inhibitor and do not require an active tyrosine kinase.

Addition of EGF to the EGFR-NLuc/CLuc cells resulted in a biphasic response to the ligand. Initially, EGF stimulated a rapid decrease in luciferase activity. This was followed by a slower recovery back to baseline levels of luciferase complementation. We interpret these findings as indicating the presence of two sequential, ligand-induced conformational changes in the EGF receptor.

For several reasons, we do not feel that the observed changes are related to internalization and/or degradation of the EGF receptor. First, the initial decrease in luciferase activity occurs much more rapidly than the transport to and degradation of the receptor in endosomes. Thus, it seems unlikely that the loss of luciferase activity is due to the dissociation of dimers or degradation of the receptors in this acidic compartment. Furthermore, the decrease in luciferase activity is reversible indicating that it cannot be due to an irreversible process such as proteolysis. The recovery phase could arise from clustering of the receptors in coated pits for internalization. However, both U0126 treatment and the T669A mutation lead to enhanced receptor internalization (36) and would thus be expected to promote the recovery phase. However, they actually abolished recovery of luciferase activity. It therefore seems probable that the observed changes in luciferase activity are the result of conformational changes in the EGF receptor.

Treatment with EGF initially led to a rapid decrease in luciferase activity consistent with the hypothesis that a conformational change had occurred that resulted in the separation of the luciferase fragments. The decrease in luciferase activity was dependent on the concentration of EGF and reached its maximum 2 to 4 min after the addition of EGF. This is well after receptor autophosphorylation has peaked, suggesting that this conformational change occurs as a result of receptor autophosphorylation rather than concomitant with kinase activation.

Consistent with this hypothesis, treatment of the cells with erlotinib blocked the decrease in light production. More importantly, mutation of the receptor to the kinase-dead K721A variant abolished the decrease in luciferase activity and actually led to a system in which EGF stimulated an increase in light production. This demonstrates that: (i) the conformational dynamics reported by the luciferase fragment complementation assay are the result of receptor tyrosine kinase activity, and (ii) EGF does induce dimer formation in the context of the full-length EGF receptor but its effects are masked by conformational changes within the intracellular domain. The observation that erlotinib blocked the decrease in luciferase activity but did not reveal the increase in receptor dimer formation in the wild type receptor may be due to the presence of residual kinase activity in the inhibitor-treated cells. A low level of kinase activity would promote a decrease in complementation thereby offsetting the increase in luciferase activity induced by EGF receptor dimerization.

It is noteworthy that EGF stimulated dimerization of the kinase-dead EGF receptor in the presence of erlotinib that itself enhanced receptor-receptor interactions. This suggests that the effects of EGF and erlotinib on the formation of receptor dimers occur through independent mechanisms. Presumably, EGF induces dimerization of the extracellular domains, whereas erlotinib promotes kinase-kinase interactions within the intracellular domain (33–35, 40). Whether these mechanisms can work additively within the same receptor dimer (or tetramer) is unclear. However, the fact that EGF stimulated luciferase complementation in receptors that were fully saturated with erlotinib suggests that this is likely the case.

The EGF receptor is extensively autophosphorylated on its C-terminal tail (38, 39, 40, 41) and previous studies on the isolated cytoplasmic domain of the receptor suggested that the C-terminal tail adopts a more extended conformation following phosphorylation (12–14). It was therefore possible that the decrease in luciferase activity was due to a phosphorylation-induced change in the position of the C-terminal tails within the activated EGF receptor dimer with no movement of the kinase domains. Fusion of the luciferase fragments to a truncated EGF receptor lacking the C-terminal tail resulted in a
system in which the initial decrease in luciferase activity was present but the subsequent recovery of luciferase complementation was noticeably dampened. This suggests that ligand binding induces a change in the relative positions of the kinase domains that separates the luciferase fragments. Although changes in the C-terminal tails may also contribute to this effect in the full-length receptor, the C-terminal tails appear to be relatively more important in the recovery phase of luciferase complementation.

Luciferase fragment complementation identified a second ligand-induced conformational change in the EGF receptor that followed the tyrosine phosphorylation-dependent separation of the C-terminal tails. This second phase was marked by the recovery of luciferase activity back to baseline levels. The recovery phase occurred over a time course that was similar to the activation of MAP kinase, a downstream signaling pathway stimulated by EGF. Inhibition of MAP kinase activation abolished the recovery of luciferase activity suggesting that a MAP kinase-catalyzed phosphorylation event is responsible for the increase in luciferase activity.

MAP kinase is known to phosphorylate the EGF receptor on Thr$^{669}$ (23–25). When the luciferase fragments were fused to the T669A-EGF receptor, EGF stimulated a decrease in luciferase activity but the recovery phase was completely ablated. These data are consistent with the interpretation that phosphorylation of the EGF receptor on Thr$^{669}$ by MAP kinase induces a conformational change in the receptor that allows the re-establishment of complementation between the luciferase fragments.

Recent studies have demonstrated that phosphorylation of the EGF receptor on Thr$^{669}$ leads to desensitization of the receptor (36). Our data suggest that this functional change in the EGF receptor is likely to be the result of a conformational change in the receptor. We hypothesize that phosphorylation of the EGF receptor on Thr$^{669}$ by MAP kinase induces a re-orientation of the cytoplasmic domains that results in the adoption of a post-activated conformation of the receptor in which the C-terminal tails are once again in close proximity. The relationship between the resting and the post-activated conformations of the receptor is not clear. However, because the activated receptor would be phosphorylated, ubiquitinated, and bound to a variety of interacting molecules, it seems likely that the final conformation of the activated receptor would differ substantially from that of an unstimulated receptor.

Li et al. (37) recently reported the use of the luciferase fragment complementation to study EGF receptor interactions. These workers reported that EGF did not elicit any change in the photon flux in cells expressing NLuc and CLuc fused to the full-length EGF receptor. However, their protocol involved treatment of their cells with EGF for 15 min prior to imaging. Thus, they only observed the system after it had recovered back to baseline levels of luciferase complementation and failed to see the early dynamics that follow ligand binding.

Our data demonstrate the utility of luciferase fragment complementation imaging for monitoring reversible conformational changes in the EGF receptor in real time in living cells. Utilizing this approach, we developed an assay for assessing dimerization of the EGF receptor in intact cells using either C-terminal truncated or kinase-dead receptors. This assay is superior to the chemical cross-linking studies normally used as it is more sensitive and yields accurate information on the temporal progress of the dimerization reaction. The ability to generate temporal information allowed us to identify two sequential ligand-induced changes in the conformation of the full-length EGF receptor. These observations reveal structural dynamics in the activated EGF receptor and provide insight into how MAP kinase may induce desensitization of the EGF receptor.

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REFERENCES
Imaging Conformational Changes in the EGF Receptor