Proteasome Inhibition Blocks Ligand-Induced Dynamic Processing and Internalization of Epidermal Growth Factor Receptor via Altered Receptor Ubiquitination and Phosphorylation

Aparna H. Kesarwala, Mustapha M. Samrakandi, and David Piwnica-Worms

Abstract

Epidermal growth factor (EGF) receptor (EGFR), a member of the EGF superfamily of receptor tyrosine kinases, is a critical regulator of cell growth and an important target for single agent and combination anticancer therapeutics. To further investigate the dynamics of ligand-induced EGFR processing and regulation noninvasively, we developed a chimeric EGFR-firefly luciferase (FLuc) fusion reporter to directly monitor processing of EGFR in real-time. In a stable HeLa cell line expressing the reporter at physiologically relevant levels, bioluminescence imaging continuously monitored reporter dynamics, correlating with the ligand-induced response of endogenous EGFR as determined by Western blot, subcellular localization of an EGFR-green fluorescent protein (GFP) fusion protein, and validated pharmacologic responses. The signaling competency of the reporter was confirmed by gene rescue experiments in EGFR-null cells. Bioluminescence analysis further showed that proteasome inhibition with bortezomib or MG132 attenuated overall ligand-induced degradation of EGFR. In cells expressing EGFR-GFP, pretreatment with proteasome inhibitors trapped essentially all of the receptor at the cell membrane both before and after ligand-induced activation with EGF. Furthermore, proteasome inhibition enhanced receptor ubiquitination in both the basal and ligand-activated states as well as delayed the processing of ligand-activated phosphorylation of the receptor, kinetically correlating with attenuated receptor degradation. These observations point to a potential mechanism for the synergistic therapeutic effects of combination EGFR- and proteasome-targeted therapies.

Introduction

Growth factor receptor tyrosine kinases (RTK) have been extensively studied for decades and continue to be a focus of investigation and drug targeting because of their central roles in development, proliferation, motility, survival, apoptosis, and differentiation. The epidermal growth factor (EGF) receptor (EGFR), also known as ErbB1, is one of four members of the ErbB family of RTKs, and is activated by a number of ligands, including EGF. Binding of EGF to EGFR activates the tyrosine kinase activity of the receptor, resulting in autophosphorylation of five tyrosine residues in the carboxy-terminal domain (1). The activated receptor initiates several downstream signaling cascades, notably the Ras–mitogen-activated protein kinase (MAPK) pathway; activation of Ras GTPase is the major signaling event initiated by EGFR (2).

Proteins in this pathway are also involved in receptor-mediated endocytosis, another major processing pathway triggered by ligand binding. After autophosphorylation, EGFR is ubiquitinated and targeted for internalization. Through use of differential antibody affinity, it had been suggested that EGFR was exclusively monoubiquitinated. However, mass spectrometric analysis showed that over half of all EGFR-associated ubiquitin is in the form of polyubiquitin chains rather than monoubiquitin; chains linked via lysine 63 represent the most prevalent form of polyubiquitination (3, 4). Although the significance and exact mechanisms of ubiquitination remain unresolved, the ubiquitin-dependent internalization of EGFR represents an example of the general contribution of ubiquitin to degradation of receptors.

Ubiquitination also is thought to act as a signal for post-endocytotic processing, leading EGFR through the endosomal pathway to either subsequent degradation in the lysosome or recycling back to the plasma membrane (5–8), where receptor functionality is maintained (9). This interplay between EGFR activation, degradation, and recycling form complex signaling layers interconnected by regulators with time and context restrictions (e.g., ligand type, cell density) that dictate EGFR-dependent responses (proliferation, survival, adhesion, differentiation, and migration; refs. 2, 10, 11). Perturbation of this interplay results in disease states, notably many types of cancer (12). For example, overexpression of EGFR is characteristic of 40% of gliomas and is especially correlated with poor prognosis in breast carcinoma, colon cancer, and non–small cell lung cancer (NSCLC; refs. 9–12). New drugs, such as the tyrosine kinase inhibitors (TKI), are at the forefront of the search for effective treatments for these cancers, especially NSCLC; both monoclonal antibodies and quinazoline-based small molecules targeting EGFR tyrosine kinase activity have been developed (13–16).

Given that the first generation of small molecule TKIs targeting EGFR seem to be effective in only a small subset of cancer patients (17–20), further exploration of the regulation of EGFR is warranted to better understand detailed mechanisms of receptor internalization and signal transduction. This may provide potential new targets to interfere with the increased signaling that is a hallmark of EGFR overexpression and tumorigenesis, and a better understanding of the kinetics of receptor activation would provide insight into potential ways to interfere with the altered equilibrium state characteristic of cancer. In this regard, a previously designed EGFR-based model has been used to follow glioma cell proliferation and burden in vivo (21). This model involved whole animal...
Dynamic Imaging of EGFR Processing

Figure 1. Characterization of an EGFR-FLuc fusion reporter. A, schematic illustrating pCMV-EGFR-FLuc fusion construct. B, bioluminescence images of HeLaEGFR-FLuc cells (n = 4) pretreated for 30 min with 150 μg/mL n-octyl-β-D-glucopyranoside and then activated with 100 ng/mL EGF starting at time t = 0 (left column) or vehicle (right column). C, concentration-response curves of HeLaEGFR-FLuc cells to ligand-induced activation with varying concentrations of EGF. Cells were treated with the following concentrations of EGF: 0.1 ng/mL (○), 1 ng/mL (△), 3 ng/mL (▲), 10 ng/mL (●), 30 ng/mL (◇), 100 ng/mL (⊗), or 300 ng/mL (○). SE was typically <7% and is not displayed for clarity. D, normalized concentration-response titration curves of percent maximum degradation 30 min postaddition of EGF. The four-variable ligand binding curve was y = 1.799 + 98.919/1 + 10^((LogEC50 - x)), where x = log[EGF]; EC50 = 10.4 ng/mL EGF; R^2 = 0.9996.

Materials and Methods

Plasmids. pCMV-EGFR-FLuc was a gift from Alexander Sorkin (University of Colorado). To generate pCMV-EGFR-FLuc, firefly luciferase sequences (FLuc) were PCR-amplified from the pGL-3 control plasmid (Promega) along with a SacII site and a sequence encoding for a 12 amino acid linker (PRSGGSSSSGGLA) upstream of FLuc and an XbaI site downstream of FLuc, and ligated into a TOP1 TA vector (Invitrogen). After excision of GFP from pCMV-EGFR-FLuc with SacII and Xbal, the FLuc PCR product was excised from the TOP1 TA vector with SacII and Xbal and ligated into the pCMV-EGFR-containing backbone. pCMV-FLuc was similarly constructed and used as a control plasmid, along with pcdNA3 (Promega). pMyc-abiquitin was a gift from Helen Piwnica-Worms (Washington University, St. Louis, MO).

Cell lines, transfections, and reagents. HeLa cells were cultured at 37°C under 5% CO2 in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% glutamine, and 0.1% penicillin/streptomycin solution. Cells were grown on glass coverslips for transient transfections (0.5 mg total DNA/100,000 cells) using Fugene 6 (Roche Applied Science) as recommended. Two stable HeLa lines expressing EGFR-FLuc (HeLaEGFR-FLuc) or FLuc alone (HeLaFLuc) were created by selection in 1 or 0.5 mg/mL G418, respectively. NR6 cells were a gift from Harvey Herschman (University of California, Los Angeles, CA) and were cultured at 37°C under 5% CO2 in low-glucose/high-bicarbonate DMEM supplemented with 10% heat-inactivated FBS, 1% glutamine, and 0.1% penicillin/streptomycin solution. Cycloheximide, tyrphostin AG1478, MG132, and wortmannin were purchased from Sigma. Bortezomib was a gift from Millennium Pharmaceuticals.

Fluorescence microscopy. 4',6-diamidino-2-phenylindole (DAPI) and rhodamine were added to a final concentration of 0.30 μg/mL in DMEM (Invitrogen) and used for imaging of nude mice into which glioma cells expressing either an EGFR-green fluorescent protein (GFP)-Renilla luciferase fusion (EGFR-GFP-RLuc) or mutant EGFRvIII-GFP-RLuc to monitor EGFR content and a constitutive firefly luciferase-red fluorescent protein fusion (FLuc-DsRed2) to monitor tumor cell mass were implanted into the brain. Although the authors showed increased EGFR expression and glioma growth over time both in vitro and in vivo, the bursting photonic activity profile of the Renilla luciferase reporter did not allow examination of the early kinetics of intracellular processing and degradation of EGFR. We herein present a firefly luciferase–based EGFR fusion reporter strategy for real-time imaging of ligand-induced processing and regulation of EGFR in live cells. The chimera reporter was used to examine the effect of proteasome inhibition on receptor processing.

Imaging of nude mice into which glioma cells expressing either an EGFR-green fluorescent protein (GFP)-Renilla luciferase fusion (EGFR-GFP-RLuc) or mutant EGFRvIII-GFP-RLuc to monitor EGFR content and a constitutive firefly luciferase-red fluorescent protein fusion (FLuc-DsRed2) to monitor tumor cell mass were implanted into the brain. Although the authors showed increased EGFR expression and glioma growth over time both in vitro and in vivo, the bursting photonic activity profile of the Renilla luciferase reporter did not allow examination of the early kinetics of intracellular processing and degradation of EGFR. We herein present a firefly luciferase–based EGFR fusion reporter strategy for real-time imaging of ligand-induced processing and regulation of EGFR in live cells. The chimera reporter was used to examine the effect of proteasome inhibition on receptor processing.

Statistics and curve fitting. Data are presented as mean photon flux of treated wells as a percentage of photon flux of untreated wells plotted as a function of time after treatment ± SE of quadruplicate wells. Curve fitting was performed using the sigmoidal concentration-response nonlinear regression [y = Min + (Max – Min)/(1 + 10^(LogIC50 – x)), where x = log concentration] in GraphPad Prism 4 (GraphPad Software).

Western blots and immunoprecipitation. EGFR was detected in whole cell lysates derived from HeLa or HeLaEGFR-FLuc cells using rabbit anti-EGFR antibody at 1:1,000 (Cell Signaling Technology). Phospho-MAP/ERK kinase was detected in whole cell lysates derived from NB6 cells using rabbit anti-phospho-ERK antibody at 1:100 (Cell Signaling Technology). Western blots were probed with anti-rabbit secondary antibodies at 1:1,000 (Cell Signaling Technology). Phospho-MAP/ERK kinase was detected in whole cell lysates derived from NB6 cells using rabbit anti-phospho-ERK antibody at 1:100 (Cell Signaling Technology). Western blots were probed with anti-rabbit secondary antibodies at 1:1,000 (Cell Signaling Technology). Phospho-MAP/ERK kinase was detected in whole cell lysates derived from NB6 cells using rabbit anti-phospho-ERK antibody at 1:100 (Cell Signaling Technology). Western blots were probed with anti-rabbit secondary antibodies at 1:1,000 (Cell Signaling Technology). Phospho-MAP/ERK kinase was detected in whole cell lysates derived from NB6 cells using rabbit anti-phospho-ERK antibody at 1:100 (Cell Signaling Technology).
Blotting reagent (Amersham Biosciences), and imaged using the IVIS 100 imaging system (exposure time, 1 min; binning, 4; no filter; f/stop, 1; FOV, 15 cm). Blots were also stripped and reprobed with anti-actin antibody at 1:400 (Sigma).

For immunoprecipitation, cells were washed with ice-cold PBS and lysed on ice in lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 5% glycerol (w/v), 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L NaF, 30 mmol/L glycerol phosphate, 1 mmol/L Na3VO4, and 1 µL/106 cells protease inhibitor cocktail (Sigma)]. Lysates were shaken for 20 min at 4°C and then centrifuged at 10,000 × g for 15 min at 4°C. Supernatant equivalent to 1 mg of protein was adjusted to 1 mL with lysis buffer, mixed with 2.5 µg anti-EGFR antibody (Clone 528; Calbiochem) or 40 µL anti-FLAG antibody slurry (M2 Affinity Gel; Sigma), and incubated for 2 to 3 h at 4°C. Complexes were precipitated overnight at 4°C with 30 µL protein A agarose conjugate (Sigma). Complexes were washed three times with lysis buffer before being loaded for fractionation and immunoblotting. Immunoblotting was carried out using P4D1 rabbit anti-ubiquitin antibody at 1:1,000 (Santa Cruz) or mouse anti-Myc antibody at 1:1,000 (Sigma). Blots were probed with anti-rabbit or anti-mouse secondary antibodies at 1:1,000 (Amersham Biosciences) and developed and imaged as above.

Results

Characterization of an EGFR-FLuc reporter construct. To monitor ligand-induced processing and degradation of EGFR, we engineered a new fusion reporter construct, pCMV-EGFR-FLuc, in which firefly luciferase was fused to the COOH terminus of EGFR driven by a cytomegalovirus (CMV) promoter. A 12 amino acid glycine-serine–rich linker between the receptor and the luciferase allowed flexibility of the protein domains with the intent to minimize steric constraints on receptor processing (Fig. 1A; refs. 22, 23). This construct was then stably expressed in HeLa human cervical carcinoma cells (HeLaEGFR-FLuc).

To accurately image changes in receptor levels in real-time, time course imaging of HeLaEGFR-FLuc cells was performed with a cooled CCD imaging system (IVIS) at 37°C before and upon addition of EGF (100 ng/mL). To account for nonspecific changes in photon output from the luciferin-luciferase reaction over time as d-luciferin was consumed, the photon flux from the ligand-treated cells was divided by the photon flux from cells treated with vehicle. This ratio was then normalized so that the initial value was 100%, and data presented as a percent of the photon flux of untreated cells. The response of the EGFR-FLuc reporter to treatment with EGF (100 ng/mL) shows a highly reproducible initial decrease in photon flux, which correlated with the expected time course of receptor internalization and degradation over the first 30 to 45 min (Fig. 1B), followed by a signal rebound. We hypothesized that the rebound phase was a period of net reporter synthesis driven by the constitutive CMV promoter and posttranslational stabilization, exceeding the diminishing dynamics of the degradation events after 30 to 45 min, a phenomenon we have observed with other kinetically regulated bioluminescent fusion reporters (24, 25).

Expanding the range of ligand concentrations used to activate subconfluent HeLaEGFR-FLuc cells showed a concentration-dependent decrease in photon flux during the first 30 min (Fig. 1C). Normalizing to the percentage of maximum degradation at 30 min, titration curve fitting showed an EC50 of 10.4 ng/mL (R2 = 0.999; Fig. 1D) for the EGFR-FLuc reporter degradation. This compared favorably to the known EC50 of 16 ng/mL for EGFR-induced EGFR dimerization documented in the literature (26). Consequently, a saturating concentration of EGF (100 ng/mL) was used in all subsequent experiments.

To confirm the signaling competency of the EGFR-FLuc fusion reporter, gene rescue experiments were carried out in NR6 fibroblast cells, an EGFR-null cell line (27). In subconfluent NR6 cells transiently transfected with EGFR-FLuc, exposure to EGF confirmed bioluminescence profiles of degradation and rebound kinetics of reporter photon flux similar to those observed in HeLaEGFR-FLuc cells (data not shown). The NR6 line was then used to examine the capacity of the EGFR-FLuc reporter to activate downstream targets of EGFR signaling, such as members of the MAPK pathway. Using an anti–phospho-MEK antibody, Western blots of whole cell lysates from transfected NR6 cells directly showed activation of the MAPK pathway by EGFR-FLuc, but not control vector (Fig. 2A), confirming the EGF-induced signaling competency of the expressed chimera reporter.

Further analysis of pharmacologic effects on EGFR-FLuc processing was also performed. Treatment of HeLaEGFR-FLuc cells with tyrphostin AG1478 (10 µmol/L), a specific inhibitor of EGFR kinase activity (28), indicated that ligand-induced receptor phosphorylation and data presented as a percent of the photon flux of untreated cells. This ratio was then normalized so that the initial value was 100%, and data presented as a percent of the photon flux of untreated cells. The response of the EGFR-FLuc reporter to treatment with EGF (100 ng/mL) shows a highly reproducible initial decrease in photon flux, which correlated with the expected time course of receptor internalization and degradation over the first 30 to 45 min (Fig. 1B), followed by a signal rebound. We hypothesized that the rebound phase was a period of net reporter synthesis driven by the constitutive CMV promoter and posttranslational stabilization, exceeding the diminishing dynamics of the degradation events after 30 to 45 min, a phenomenon we have observed with other kinetically regulated bioluminescent fusion reporters (24, 25).

Expanding the range of ligand concentrations used to activate subconfluent HeLaEGFR-FLuc cells showed a concentration-dependent decrease in photon flux during the first 30 min (Fig. 1C). Normalizing to the percentage of maximum degradation at 30 min, titration curve fitting showed an EC50 of 10.4 ng/mL (R2 = 0.9996; Fig. 1D) for the EGFR-FLuc reporter degradation. This compared favorably to the known EC50 of 16 ng/mL for EGFR-induced EGFR dimerization documented in the literature (26). Consequently, a saturating concentration of EGF (100 ng/mL) was used in all subsequent experiments.

To confirm the signaling competency of the EGFR-FLuc fusion reporter, gene rescue experiments were carried out in NR6 fibroblast cells, an EGFR-null cell line (27). In subconfluent NR6 cells transiently transfected with EGFR-FLuc, exposure to EGF confirmed bioluminescence profiles of degradation and rebound kinetics of reporter photon flux similar to those observed in HeLaEGFR-FLuc cells (data not shown). The NR6 line was then used to examine the capacity of the EGFR-FLuc reporter to activate downstream targets of EGFR signaling, such as members of the MAPK pathway. Using an anti–phospho-MEK antibody, Western blots of whole cell lysates from transfected NR6 cells directly showed activation of the MAPK pathway by EGFR-FLuc, but not control vector (Fig. 2A), confirming the EGF-induced signaling competency of the expressed chimera reporter.

Further analysis of pharmacologic effects on EGFR-FLuc processing was also performed. Treatment of HeLaEGFR-FLuc cells with tyrphostin AG1478 (10 µmol/L), a specific inhibitor of EGFR kinase activity (28), indicated that ligand-induced receptor phosphorylation...
degradation was dependent on kinase activity (Fig. 2B). Again, blockade of the rebound phase by kinase inhibition was considered to be a nonspecific effect on CMV promoter–driven synthesis and posttranscriptional processing of the reporter system.

Ubiquitinated EGFR is recruited for sorting to multivesicular endosomes (MVE) by components of the endosomal sorting complex required for transport-1, specifically, hepatocyte growth factor-regulated tyrosine kinase substrate bound to phosphatidylinositol-3-phosphate (29, 30). The phosphatidylinositol-3-kine inhibitor wortmannin thereby prevents the formation of intraluminal vesicles in MVEs and precludes proper sorting of EGFR targeted for lysosomal degradation. Pretreatment of HeLaEGFR-FLuc cells with wortmannin (1 μM/L) resulted in a delay in peak degradation by ~30 min and an inhibition of the level of degradation by approximately two-thirds, directly indicating the dependence of ligand-induced EGFR-FLuc degradation on proper intracellular sorting of the reporter (Fig. 2C).

EGF (100 ng/mL) was then used to test the effect of cell contact–dependent modulation of EGFR internalization and signaling (31, 32) on the magnitude of EGFR-FLuc degradation dynamics. As expected, the maximum dynamic range of signaling (31, 32) on the magnitude of EGFR-FLuc degradation by approximately two-thirds, directly indicating the dependence of ligand-induced EGFR internalization and signaling (31, 32) on the magnitude of EGFR-FLuc degradation by approximately two-thirds, directly indicating the dependence of ligand-induced EGFR internalization and signaling (31, 32)

The bioluminescence signal was also analyzed in the presence of the translation inhibitor cycloheximide to confirm that the observed ligand-induced decrease in signal was a function of the EGFR component of the reporter. In HeLaEGFR-FLuc cells, pretreatment with cycloheximide for 1 h did not effect the first 45 min of degradation after EGF treatment but completely abrogated the rebound of bioluminescent signal over the subsequent 75 min (Fig. 3B). By contrast, in HeLa cells stably expressing control pCMV-FLuc (HeLaFLuc), pretreatment with cycloheximide for 1 h followed by addition of EGF showed no dynamic minimum. Thus, upon stimulation of HeLaEGFR-FLuc cells with EGF, the lack of response in the presence of cycloheximide indicated that there was essentially no effect of EGFR on FLuc degradation, whereas the lack of response in the presence of vehicle (DMSO) showed that there was no significant effect of EGFR on FLuc protein synthesis. Similar results were obtained after pretreating HeLaEGFR-FLuc cells with the transcription inhibitor actinomycin-D (Fig. 3C). Overall, these experiments were again consistent with a biphasic response of the EGFR-FLuc reporter consisting of an initial processing/degradation phase and a subsequent rebound phase, the latter being a result of CMV promoter–driven EGFR-FLuc synthesis. Although both cycloheximide and actinomycin-D blocked the rebound phase of signal increase, neither had an effect on the physiologically-relevant posttranslational processing and early degradation phase of the reporter.

Fluorescence microscopy was used to characterize an identical construct containing enhanced GFP instead of FLuc fused to EGFR. After transient transfection into HeLa cells, the fluorescent reporter protein showed correct localization to the plasma membrane under basal conditions (Fig. 4A). In response to treatment with EGF, a submembranous punctuate pattern developed along with loss of fluorescence intensity over the ensuing 20 to 60 min, consistent with ligand-induced endocytosis, relocalization to internal vesicles, and degradation of EGFR-GFP. As this GFP isoform requires several hours to mature fully (33), newly translated fluorescent fusion protein failed to be visualized over the time frame of these experiments. As a result, the kinetic pattern of photonic output of the GFP-based reporter system was...
analogue to the bioluminescent system in the presence of cycloheximide.

To show that EGFR-FLuc reporter levels were an accurate reflection of the response of endogenous EGFR to EGF over time, we compared the dynamic response of bioluminescence of HeLa\textsuperscript{EGFR-FLuc} cells in the presence of cycloheximide (Fig. 4B) to Western blot analysis of whole cell lysates of parental HeLa cells (Fig. 4C), each obtained after treatment with EGF (100 ng/mL). Semiquantitative densitometric analysis of Western blots (anti-EGFR) showed excellent correlation between endogenous EGFR and EGFR-FLuc chimera protein levels ($R^2 = 0.97$; Fig. 4D). Similarly, in HeLa cells transiently transfected with EGFR-FLuc-FLAG, excellent correlation was observed between EGFR-FLuc bioluminescence output and FLAG-tagged chimera protein levels ($R^2 = 0.89$; data not shown).

**Effect of proteasome inhibitors on EGFR processing.** Partial blockade of EGFR-FLuc degradation was also achieved pharmacologically by pretreatment of HeLa\textsuperscript{EGFR-FLuc} cells with two proteasome inhibitors. At concentrations of bortezomib or MG132 at which proteasomal activity was nearly completely inhibited (34), some inhibitors. At concentrations of bortezomib or MG132 at which proteasomal activity was nearly completely inhibited (34), some inhibitors. At concentrations of bortezomib or MG132. At various time points after addition of EGF, Western blot analysis of whole cell lysates of parental HeLa cells, and pretreated with MG132 or vehicle and then activated with EGF. A Western blot of whole cell lysates from various postactivation time points using an anti–phospho-EGFR antibody indicated that EGFR was still phosphorylated in the presence of bortezomib (Fig. 6A). However, densitometry analysis of both the endogenous EGFR as well as the EGFR-FLuc reporter indicated that bortezomib significantly delayed degradation of phosphorylated species of both proteins approximately equally (Fig. 6B).

To test the hypothesis that proteasome inhibition affected the ubiquitination of EGFR, HeLa\textsuperscript{EGFR-FLuc} cells as well as HeLa cells transiently transfected with both EGFR-GFP and Myc-tagged ubiquitin, were pretreated with MG132 or vehicle and then activated with EGF. Western blot analysis of cell lysates for ubiquitinated EGFR-FLuc (Fig. 6C, left) or EGFR-GFP (Fig. 6C, right) indicated that EGFR was rapidly ubiquitinated post-EGF activation, and furthermore, MG132 pretreatment enhanced the ubiquitination state post-EGF activation (Fig. 6C, compare lanes 2 and 4 in both panels). The smearcd band visible only after activation with EGF is consistent with EGFR protein that was both phosphorylated and ubiquitinated at multiple sites. Notably, treatment with MG132 alone increased the basal level of ubiquitination even in the absence of EGF (Fig. 6C, lane 3 in both panels). Together, the confocal microscopy and Western blot data indicated that treatment with proteasome inhibitors alone changed not only the localization and compartmentalization of EGFR, but also its basal ubiquitination state, which alone resulted in essentially complete internalization of the protein by 30 min (Fig. 5D, second column). In contrast, pretreatment with bortezomib or MG132 alone for 1 hour resulted in trapping of essentially all of the protein at the plasma membrane. Remarkably, there was a distinct absence of cytosolic receptor and punctate endosomal compartments after 1 hour of treatment with proteasome inhibitor (Fig. 5D, fourth column). Stimulation of proteasome-inhibited cells with EGF resulted in modest internalization of EGFR, but a majority of the protein remained at the cell membrane, even after 60 to 100 min (Fig. 5D, third column).

To test the hypothesis that proteasome inhibition affects the phosphorylation status of EGFR, HeLa\textsuperscript{EGFR-FLuc} cells were pretreated with either bortezomib or vehicle and then activated with EGF. A Western blot of whole cell lysates from various postactivation time points using an anti–phospho-EGFR antibody indicated that EGFR was still phosphorylated in the presence of bortezomib (Fig. 6A). However, densitometry analysis of both the endogenous EGFR as well as the EGFR-FLuc reporter indicated that bortezomib significantly delayed degradation of phosphorylated species of both proteins approximately equally (Fig. 6B).

To determine if proteasome inhibition affected the ubiquitination of EGFR, HeLa\textsuperscript{EGFR-FLuc} cells as well as HeLa cells transiently transfected with both EGFR-GFP and Myc-tagged ubiquitin, were pretreated with MG132 or vehicle and then activated with EGF. Western blot analysis of cell lysates for ubiquitinated EGFR-FLuc (Fig. 6C, left) or EGFR-GFP (Fig. 6C, right) indicated that EGFR was rapidly ubiquitinated post-EGF activation, and furthermore, MG132 pretreatment enhanced the ubiquitination state post-EGF activation (Fig. 6C, compare lanes 2 and 4 in both panels). The smearcd band visible only after activation with EGF is consistent with EGFR protein that was both phosphorylated and ubiquitinated at multiple sites. Notably, treatment with MG132 alone increased the basal level of ubiquitination even in the absence of EGF (Fig. 6C, lane 3 in both panels). Together, the confocal microscopy and Western blot data indicated that treatment with proteasome inhibitors alone changed not only the localization and compartmentalization of EGFR, but also its basal ubiquitination state, which alone resulted in essentially complete internalization of the protein by 30 min (Fig. 5D, second column). In contrast, pretreatment with bortezomib or MG132 alone for 1 hour resulted in trapping of essentially all of the protein at the plasma membrane. Remarkably, there was a distinct absence of cytosolic receptor and punctate endosomal compartments after 1 hour of treatment with proteasome inhibitor (Fig. 5D, fourth column). Stimulation of proteasome-inhibited cells with EGF resulted in modest internalization of EGFR, but a majority of the protein remained at the cell membrane, even after 60 to 100 min (Fig. 5D, third column).

To test the hypothesis that proteasome inhibition affects the phosphorylation status of EGFR, HeLa\textsuperscript{EGFR-FLuc} cells were pretreated with either bortezomib or vehicle and then activated with EGF. A Western blot of whole cell lysates from various postactivation time points using an anti–phospho-EGFR antibody indicated that EGFR was still phosphorylated in the presence of bortezomib (Fig. 6A). However, densitometry analysis of both the endogenous EGFR as well as the EGFR-FLuc reporter indicated that bortezomib significantly delayed degradation of phosphorylated species of both proteins approximately equally (Fig. 6B).

To determine if proteasome inhibition affected the ubiquitination of EGFR, HeLa\textsuperscript{EGFR-FLuc} cells as well as HeLa cells transiently transfected with both EGFR-GFP and Myc-tagged ubiquitin, were pretreated with MG132 or vehicle and then activated with EGF. Western blot analysis of cell lysates for ubiquitinated EGFR-FLuc (Fig. 6C, left) or EGFR-GFP (Fig. 6C, right) indicated that EGFR was rapidly ubiquitinated post-EGF activation, and furthermore, MG132 pretreatment enhanced the ubiquitination state post-EGF activation (Fig. 6C, compare lanes 2 and 4 in both panels). The smearcd band visible only after activation with EGF is consistent with EGFR protein that was both phosphorylated and ubiquitinated at multiple sites. Notably, treatment with MG132 alone increased the basal level of ubiquitination even in the absence of EGF (Fig. 6C, lane 3 in both panels). Together, the confocal microscopy and Western blot data indicated that treatment with proteasome inhibitors alone changed not only the localization and compartmentalization of EGFR, but also its basal ubiquitination state, which
subsequently affected ligand response. These data provided further mechanistic insight into the observation that proteasome inhibition attenuated ligand-induced degradation of EGFR.

Discussion

The degradation pathway of EGFR, long used as a model of RTK processing, is highly regulated in both subcellular localization and kinetics by numerous extracellular and intracellular processes. Even as EGFR continues to be a highly desirable therapeutic target, many aspects of receptor regulation remain poorly defined. Understanding the dynamics of this receptor will be crucial not only in evaluating new drugs in cellular and animal models but also in identifying potential new targets. Thus, we have developed a reporter (EGFR-FLuc) that allows monitoring of EGFR processing and degradation in intact cells in real-time, thereby providing a transcriptionally uncoupled readout of the degradation of the receptor.

Overall, EGFR-FLuc bioluminescence indicated that the reporter protein provided an accurate readout of the total population of receptor in living cells. Temporal profiles of the effects of various pharmacologic agents were made possible by real-time imaging of photon output from the reporter population allowed for essentially immediate phenotypic readout reflecting key posttranslational modifications in receptor processing, such as ubiquitination (Figs. 5 and 6). Importantly, because bioluminescence imaging allowed for readout of the total receptor population, whole cell lysates were used in Western blots to correlate with the bioluminescence data. The large smear representing the multi-phosphorylated and polyubiquitinated EGFR visible on the Western blots provided further evidence for the complexity of the status of the receptor post ligand engagement. Many typical biochemical protocols used to study EGFR involve immunoprecipitation followed by immunoblotting, thereby selecting for subpopulations of receptor; results from such protocols may be further confounded by differential antibody affinity for these subpopulations.

The new observation from this study was that pretreatment of HeLaEGFR-FLuc cells with the pharmacologically relevant concentrations of proteasome inhibitors bortezomib or MG132 significantly decreased ligand-induced degradation of EGFR by ~50%, but enhanced both basal and ligand-induced ubiquitination of the receptor (Figs. 5 and 6). The data were consistent with a major role for the proteasome in the processing and degradation of EGFR. It remains to be determined if the contribution of the proteasome was direct, where a certain subpopulation of receptor was targeted to the proteasome for degradation, or if the contribution of the proteasome was indirect. An indirect mechanism would suggest that a proteasomally regulated protein.

Figure 5. Effect of proteasome inhibition on ligand-induced EGFR processing. A to B, effect of proteasome inhibition on HeLaEGFR-FLuc cells. Cells were pretreated for 1 h with (A) 5 μmol/L bortezomib (open) or vehicle (solid) or (B) 20 μmol/L MG132 (open) or vehicle (solid). C, concentration-response of HeLaEGFR-FLuc cells to varying concentrations of bortezomib. Cells were pretreated for 1 h with 100 ng/mL cycloheximide and the following concentrations of bortezomib: 0 μmol/L (○), 0.001 μmol/L (●), 0.01 μmol/L (▲), 0.1 μmol/L (▲), 1 μmol/L (○), or 10 μmol/L (■). The four-variable ligand binding curve was y = 73.62 + 16.66/1 + 10^{-(x-1.203)/4}, where x = log(bortezomib); EC_{50} = 0.063 μmol/L bortezomib; R^2 = 0.962. SE was typically <7% and is not displayed for clarity. D, effect of proteasome inhibition on HeLa cells transiently transfected with EGFR-GFP. Cells in the third and fourth columns were pretreated for 1 h with 10 μmol/L bortezomib (BZ; top) or 20 μmol/L MG132 (MG; bottom). Fluorescence images of DAPI and GFP were obtained at the indicated times after treatment with EGF (100 ng/mL; second and third columns) or vehicle (first and fourth columns). Magnification, ×40.
was a key modulator of one or more of the early steps of the EGFR degradation pathway. Our data further indicated that correct subcellular localization was a requirement for proper processing of the receptor and it was strikingly altered in the presence of proteasome inhibition (Fig. 5D). The decreased degradation in the presence of bortezomib or MG132 could therefore be attributed to trapping of EGFR at the cell membrane, thereby preventing it from proceeding into the endosomal pathway for appropriate processing/degradation.

Bortezomib (Velcade), an approved therapy for multiple myeloma in both the United States and Europe, is now under investigation for efficacy in the treatment of solid tumors, including non–small cell lung, breast, pancreatic, and colon cancers (35), all cancers that potentially overexpress EGFR (9–12). Although the mechanism of the antitumor activity of bortezomib remains poorly understood, possibly significant molecular sequelae include inhibition of nuclear factor-κB activation, induction of cell cycle arrest, and effects on Bax and Bcl-2 leading to induction of apoptosis (36–39). Enhanced apoptosis was more recently observed when bortezomib was combined with both small molecule TKIs and monoclonal antibodies targeted against EGFR (35).

We propose several possible mechanisms for the promising effects of bortezomib in combination with EGFR inhibitors in the treatment of solid tumors, based on our observation that proteasome inhibition decreases degradation and significantly alters subcellular localization of EGFR, specifically by trapping receptor at the plasma membrane in a ubiquitination-dependent manner. EGFRs at the plasma membrane therefore remain accessible to inhibition by monoclonal antibodies targeting extracellular motifs of the receptor surface. Furthermore, small molecule TKIs may also have increased ability to access the ATP-binding site of the tyrosine kinase domain of EGFR localized to the plasma membrane, as accessibility for drugs may be compromised when the receptor is internalized and localized within the MVE.

Alternatively, it is possible that phospho- and ubiquitination-dependent allosteric conformations of the receptor or kinase domains specifically induced by proteasome inhibitors favor binding or increase the affinity of TKIs for EGFR. Fundamentally, the basal state of the cell is significantly altered after proteasome inhibition, given the changes in ubiquitination and subcellular localization that are visible after proteasome inhibition, even in the absence of ligand. This proteasome-inhibited cell can therefore also be expected to respond differently to receptor-targeted therapy,
which may enhance the efficacy of drug combinations targeting EGFR. These models also provide implications for targeted therapies against a multitude of cell surface receptors, which may be similarly affected by proteasome inhibition.

Considerable effort is under way to develop effective modulators of EGFR for treatment of various cancers; no fewer than eight drugs targeting the protein have been approved or are in clinical trials, with modest benefits thus far. We envision that EGFR-Fluc could be used in cellulo for screening of compounds that would modulate the deleterious effects of enhanced stimulation of downstream signaling pathways, as well as in vivo for further testing of lead compounds, including combination therapy with proteasome inhibitors such as bortezomib. The effects of any particular agent or gene on EGFR could be monitored in real-time on a variety of genetic backgrounds. In combination with readouts of downstream pathway activity, this reporter has the promise to provide insight into the well-studied yet still enigmatic processing of EGFR that is central to both normal physiology and many forms of malignancy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments

Received 7/31/2008; revised 10/20/2008; accepted 10/23/2008.
Grant support: NIH grant P50 CA94056.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank colleagues of the Molecular Imaging Center for helpful discussions.

References


www.aacrjournals.org 983 Cancer Res 2009; 69: (3). February 1, 2009

Dynamic Imaging of EGFR Processing

Received OnlineFirst January 27, 2009; DOI: 10.1158/0008-5472.CAN-08-2938

Downloaded from cancerres.aacrjournals.org on April 15, 2017. © 2009 American Association for Cancer Research.
Proteasome Inhibition Blocks Ligand-Induced Dynamic Processing and Internalization of Epidermal Growth Factor Receptor via Altered Receptor Ubiquitination and Phosphorylation

Aparna H. Kesarwala, Mustapha M. Samrakandi and David Piwnica-Worms


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-08-2938

Cited articles
This article cites 39 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/69/3/976.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/69/3/976.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.