Cell Surface Epidermal Growth Factor Receptors Increase Src and c-Cbl Activity and Receptor Ubiquitylation*

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Background: The endocytic pathway spatially regulates EGFR signaling.

Results: Activated, cell surface, EGFRs stimulate Src and c-Cbl better than intracellular receptors and increase receptor ubiquitylation.

Conclusion: Sustained signaling from the plasma membrane enhances receptor ubiquitylation, a key component of EGFR down-regulation.

Significance: Cell surface EGFRs signal key regulatory mechanisms for receptor degradation.

There is an established role for the endocytic pathway in regulation of epidermal growth factor receptor (EGFR) signaling to downstream effectors. However, because ligand-mediated EGFR endocytosis utilizes multiple “moving parts,” dissecting the spatial versus temporal contributions has been challenging. Blocking all endocytic trafficking can have unintended effects on other receptors as well as give rise to compensatory mechanisms, both of which impact interpretation of EGFR signaling. To overcome these limitations, we used epidermal growth factor (EGF) conjugated to polystyrene beads (EGF beads). EGF beads simultaneously activate the EGFR while blocking its endocytosis and allow analysis of EGFR signaling from the plasma membrane. Human telomerase immortalized corneal epithelial (hTCEpi) cells were used to model normal epithelial cell biology. In hTCEpi cells, both cell surface and intracellular EGFRs exhibited dose-dependent increases in effector activity after 15 min of ligand stimulation, but only the serine phosphorylation of signal transducer and activator of transcription 3 (STAT3) was statistically significant when accounting for receptor phosphorylation. However, over time with physiological levels of receptor phosphorylation, cell surface receptors produced either enhanced or sustained mitogen-activated protein kinase kinase (MEK), Casitas B-lineage lymphoma (c-Cbl), and the pro-oncogene Src activity. These increases in effector communication by cell surface receptors resulted in an increase in EGFR ubiquitylation with sustained ligand incubation. Together, these data indicate that spatial regulation of EGFR signaling may be an important regulatory mechanism in receptor down-regulation.

The epidermal growth factor receptor (EGFR) is one of the most physiologically important receptor tyrosine kinases. It has biological roles in developmental biology and tissue homeostasis; EGFR knockout mice are embryonic lethal or die shortly after birth. Further, many cancers are characterized by constitutively active mutants and/or overexpression of the EGFR and forecast a poor patient prognosis. Thus, understanding EGFR function and activity has important implications for multiple research areas.

The basic mechanisms that govern activation of the EGFR have been well delineated. Ligand binds to the extracellular portion of the receptor to induce a conformational change that exposes cysteine-rich domains. This allows dimerization with another EGFR and the kinase domain of one receptor to phosphorylate tyrosine residues on its partner. The phospho-tyrosines serve as docking sites for downstream signaling proteins (effectors). The magnitude and duration of the effector activity integrate to produce specific cellular responses such as proliferation, differentiation, and migration.

Fine-tuning of EGFR signaling ensures that the proper biological response occurs after receptor stimulation. One of the key mechanisms is the endocytic pathway, as it both temporally and spatially regulates EGFR activity. When endocytosis is disrupted, EGF-mediated cell biology is altered. Signaling to some effectors is enhanced because ligand-stimulated receptor activity is not properly attenuated; other downstream effectors (i.e. MAPK and PI3K) require endocytosis for maximal activity, presumably allowing the receptors and effectors to co-localize for efficient communication.

Studying the endocytic pathway is inherently difficult. First, there are multiple mechanisms that regulate EGFR endocytosis, and simply inhibiting one does not completely block receptor internalization. Second, many inhibitors of endocytic trafficking disrupt the intracellular movement of multiple proteins, so determining EGFR-specific effects can be challenging. Finally, the introduction of dominant negative proteins or RNAi requires time to maximally inhibit trafficking. During this time, typically 48–72 h, the cell can undergo compensatory mechanisms and/or constitutive membrane trafficking can be disrupted. Such changes can result in a cell that is vastly different from the one observed under physiological conditions.

To overcome many of the limitations of previous studies, we used EGF covalently linked to 900-nm polystyrene beads (EGF beads). EGF beads specifically activate the EGFR, but the
Cell Surface EGFRs Preferentially Activate Src and c-Cbl

The ligand-receptor complex is too large to enter the 50–100-nm clathrin-coated pit, and the active receptor is retained on the cell surface (14–16). Not only does treatment with EGF beads prevent compensatory mechanisms from arising, other endocytic processes are unperturbed. Further, signaling can be assessed from the moment that ligand binds, allowing meaningful dose–response and kinetic analyses to be performed.

We found that blocking endocytosis of the activated EGFR slowed the kinetics of receptor dephosphorylation. At early time points, there are modest differences in effector activation by cell surface and intracellular phosphorylated EGFRs (15 min). However, there is a statistically significant increase in STAT3 serine phosphorylation by cell surface versus intracellular receptors. A kinetic analysis (0–120 min) reveals a more pronounced role for the spatial placement of the activated EGFR; cell surface receptors produce sustained Src activity and a greater magnitude of c-Cbl activity. These enhancements in signaling culminate in greater EGFR ubiquitylation over time. These data are consistent with the spatial regulation of EGFR-effector communication playing an important role in the downregulation of receptors.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—hTCEpi cells were obtained from Geron Corp. (Menlo Park, CA) and grown at 37 °C in 5% CO_2_, as described previously (17). Cells were maintained in keratinocyte serum-free media, containing defined keratinocyte serum-free media growth supplement and 100 units/ml penicillin and streptomycin.

**Cell Lysate Preparation and Immunoblotting**—Cell lysates were prepared as described previously (18). Protein concentrations were determined by BCA assay (Pierce), and samples were diluted in 6× SDS sample buffer. In each experiment, 40 μg of protein/sample was separated by SDS-page, transferred to nitrocellulose, and detected with the indicated antibody. Antibodies and sources are as follows: EGFR (SC-03, Santa Cruz Biotechnology, Santa Cruz, CA), EGFR-phospho-tyrosine 1068, EGFR-phospho-tyrosine 1045, phospho-c-Cbl, c-Cbl, phospho-MEK, MEK, phospho-c-Src, c-Src, phospho-serine Stat3, phospho-tyrosine Stat3, and Stat3 (Cell Signaling Technology, Danvers, MA). Antibodies were then probed with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Pierce). Proteins were detected and visualized by Enhance Chemiluminescence (ECL) using the Luminary/FX imaging system (FOTODYNE Inc., Hartland, MI). Blots were quantified using ImageJ software (National Institutes of Health, Bethesda, MD), taking care to make sure the exposures were in the linear range (19).

**Immunoprecipitations**—EGFRs were immunoprecipitated as described previously (20). Following ligand stimulation, EGFR (Ab-1) and protein A/G-agarose beads (Santa Cruz Biotechnology) were used to immunoprecipitate the EGFR. Immunoprecipitates were resolved by 7.5% SDS-PAGE and immunoblotted with ubiquitin, EGFR (SC-03), and phospho-tyrosine (PY99) (Santa Cruz Biotechnology) as described above.

**Indirect Immunofluorescence**—Localization of the EGFR was described previously (12). Briefly, cells grown on coverslips were fixed in 4% paraformaldehyde, permeabilized with 0.1% saponin, 0.5% fetal bovine serum, 0.25 m CaCl_2_, 0.25 m MgCl_2_, PBS, pH 7.4. Cells were incubated in the following primary antibodies: EGFR-phospho-tyrosine 1068 XP (Cell Signaling) and clathrin heavy chain (GeneTex); Secondary antibodies used were Alexa Fluor 568 and Alexa Fluor 647 (Life Technologies), respectively. After rinsing several times, cells were mounted on microscope slides using ProLong Gold with DAPI (Life Technologies). Carboxylate-modified polystyrene fluorescent yellow-green latex beads (L4655, Sigma), conjugated to EGF, were visualized using the Alexa Fluor 488 channel. Images were captured using a Nikon A1R-A1 confocal microscope using Nikon NIS-Elements software (Nikon, Melville, NY). Images were exported to Photoshop, pseudo-colored for clarity, and constructed into a figure.

**EGF Bead Preparation**—Nine hundred-nm carboxylate-modified polystyrene beads (CLB-9, Sigma) were conjugated to human recombinant epidermal growth factor (Invitrogen) as described previously (15). One thousand-nm fluorescently labeled beads (L4655, Sigma), used for imaging experiments (see Fig. 1), were also conjugated to EGF and prepared the same as above. New batches of EGF beads are routinely made and used within 2 weeks of synthesis. The bioactivity of each batch is measured by its ability to induce EGFR phosphorylation. Differences in the amount of EGF beads used in assays reflect the bioactivity of the individual EGF bead preparation.

**Statistical Analysis**—Data analysis was performed using GraphPad Prism Software (GraphPad Software, Inc., La Jolla, CA).

**RESULTS**

Although there have been numerous studies of endocytic pathway-regulated EGFR signaling, many have used cancer cells (10, 14, 18, 21). To better understand endocytic regulation under physiologic conditions, we chose to use corneal epithelial cells as EGFR activity is both necessary and sufficient for corneal epithelial homeostasis (22–24). Human corneal epithelial cells were immortalized by the stable transfection of hTCEpi cells (17). By all accounts, these cells faithfully recapitulate corneal epithelial biology and eliminate the inherent genetic variability that accompanies cancer cells.

Prior to experimentation, each newly synthesized batch of EGF beads was tested for its ability to induce EGFR phosphorylation. As part of the preparation, the EGF beads were washed extensively to remove free EGF; the final wash of each preparation was tested to ensure that there was no remaining, unconjugated EGF (Fig. 1A). Confocal microscopy was used to determine the distribution of phosphorylated EGFRs following treatment with EGF beads and EGF (Fig. 1B). After 60 min of treatment with EGF beads, the phosphorylated EGFRs are retained at the plasma membrane similar to what is seen with EGF treatment on ice (Fig. 1B). In contrast, cells treated with EGF at 37 °C have phosphorylated EGFRs localized almost entirely in the cytoplasm, although a small percentage appears in the nucleus. The presence of ligand-stimulated EGFRs appearing in the nucleus has been reported previously (25); however, in those studies the nuclear fraction of EGFRs is much larger (26). The EGF bead-mediated retention of the phosphor-
ylated EGFR at the plasma membrane is similar to what has previously been reported (14).

Past studies have indicated that EGF beads are less efficacious mediators of EGFR phosphorylation than EGF (14). This is likely due to the steric hindrance of the 900-nm beads that physically limit the amount of ligand accessible to the receptor. In addition, because the beads are not soluble, they do not move with Brownian motion in the media. This is evident in the dynamic range of EGFR phosphorylation (EGFR phospho-Tyr-1068) following treatment of hTCEpi cells with varying concentrations of soluble EGF or EGF beads (Fig. 2). Although both ligands were able to induce EGFR phosphorylation in a dose-dependent manner, the EGF bead dose-response curve is linear, whereas EGF alone has a characteristic sigmoidal shape.

For these experiments, we concluded it was not meaningful to monitor the differences in EGF beads and soluble EGF signaling as a function of ligand concentration. It was more appropriate to study the difference in receptor-effector coupling when normalized to the magnitude of EGFR phosphorylation. More specifically, for subsequent kinetic studies (see Figs. 4–7), we used EGF bead concentrations that yielded a level of EGFR phospho-Tyr-1068 that is comparable with 3 ng/ml EGF. This ligand concentration was chosen because it promotes clathrin-mediated endocytosis over non-clathrin-mediated endocytosis (27).

Using the same concentrations of ligands as in Fig. 2, effector activity was monitored as a function of effector phosphorylation (Fig. 3). Only a subset of EGFR effectors was examined. We specifically selected those effectors that play a role in cell growth, proliferation, and migration and contribute to corneal epithelial homeostasis (28–31). These include the transcription factor STAT3 (Fig. 3, B and C), the MAP kinase kinase, MEK (Fig. 3D), and the ubiquitin ligase, c-Cbl, which targets the EGFR for degradation (Fig. 3E). In all cases, effector phosphorylation (activity) increased with receptor phosphorylation; however, it was not always directly proportional.
To better assess whether there was a spatial component to effector activation, equivalent levels of EGFR phosphorylation were compared (i.e. 80 μl/ml EGF beads and 3 ng/ml EGF, Fig. 3, B–E, shaded in gray). At this level of receptor phosphorylation, only serine phosphorylation of STAT3 was activated to a greater extent by cell surface EGFRs (Fig. 3F), albeit with a limited dose dependence. However, at 15 min after the addition of ligand, the subcellular location of the receptor does not significantly impact activity of the other effectors.

Following treatment with EGF and EGF beads, we examined the kinetics of receptor phosphorylation (Fig. 4) and effector activity (Fig. 5). Both ligands were able to induce comparable levels of EGFR phosphorylation that attenuated over time with similar kinetics (Fig. 4, A and B). At these low ligand concentrations, there was no appreciable receptor degradation, which permits analysis of receptor phosphorylation without the confounding variable of changing receptor levels (quantified in Fig. 4C). The dephosphorylation of the receptor in the absence of endocytic trafficking may indicate that in hTCEpi cells, phosphatases play a significant role as negative regulators of EGFR signaling.

The role of the endocytic pathway in regulating EGFR-effector communication becomes apparent with this kinetic analysis. The differences in cell surface and intracellular EGFR signaling were most obvious between 30 and 120 min (Fig. 5). Fig. 5, B–G, are graphic representations of the amount of phosphorylated effector generated from the densitometric analysis of multiple immunoblots. In Fig. 5, B, D, and F, the data are plotted as the density of the phosphorylated effector relative to the total effector (pEffector/tEffector) versus time and indicate the total effector activity. In Figs. 5, C, E, and G, the amount of phosphorylated effector has been normalized to the amount of total effector (pEffector/tEffector) and then corrected for the amount of EGFR phosphorylation (pEGFR/tEGFR). By correcting for the amount of phosphorylated EGFR, this more accurately corrects for the spatial contribution of signaling.

Total MEK phosphorylation was slightly higher from cell surface EGFRs, but when corrected for EGFR phosphorylation, the kinetics of MEK phosphorylation were nearly identical from both locations (Fig. 5, B and C). In contrast, both Src and c-Cbl had greater activity when stimulated by cell surface EGFRs (Fig. 5, D–G). Treatment with soluble EGF causes Src activity to peak at relatively early time points (15–30 min), whereas EGF bead treatment resulted in sustained Src activity. With regard to c-Cbl, treatment with EGF beads led to a greater magnitude of c-Cbl activity (30 min).

The increases in Src and c-Cbl activity led us to examine the downstream effects of these proteins, namely ligand-dependent EGFR ubiquitylation. Knowing that EGF levels must reach a certain threshold to induce EGFR ubiquitylation (32), multiple concentrations of each ligand were examined (Fig. 6A). Both EGF beads and EGF treatment were able to increase EGFR ubiquitylation. However, the increased Src and c-Cbl activity did not enhance EGFR ubiquitylation at 15 min. When data comparison of the indicated effector activities following stimulation with 80 μl/ml EGF beads or 3 ng/ml EGF. Data were analyzed using a Student’s t test; * indicates p value of < 0.05.
from multiple experiments are combined, there is a linear relationship between receptor phosphorylation and ubiquitylation (Fig. 6B). Thus, at early time points, EGFR ubiquitylation is independent of the subcellular location of the receptor.

We next examined the kinetics of spatially regulated, ligand-dependent EGFR ubiquitylation (Fig. 7). Consistent with the increases in Src and c-Cbl activity at 60 min of ligand treatment, there also was an increase in the amount of ubiquitylated EGFR. Thus, cell surface EGFRs specifically initiate one of the key post-translational modifications associated with receptor degradation.

**DISCUSSION**

How the endocytic pathway spatially regulates receptor signaling is a pressing question in the fields of signal transduction and membrane trafficking. To date, there has been no clear consensus as to exactly how this regulation occurs. The absence of a unified model indirectly highlights the challenges of this area of investigation. This study addresses this question while avoiding some of the limitations of previous studies. Using EGF beads, we can specifically activate the EGFR at the plasma membrane without impacting other endocytic processes. This approach allows us to directly compare the signaling events mediated by cell surface EGFRs with those that progress through the endocytic pathway. Although EGF beads do not have the same maximum efficacy as soluble EGF, they do activate the receptor in a dose-dependent manner (Fig. 2). Importantly, EGF beads induce physiological levels of receptor phosphorylation that preferentially promote clathrin-mediated endocytosis (27).

Both ligands have similar kinetic profiles in receptor phosphorylation/dephosphorylation in hTCEpi cells, with minor differences in the magnitude of total phosphorylation (Fig. 4). This allows the study of spatially restricted receptor signaling while minimizing the differences in the duration of receptor activity. In addition, this indicates that there is a role for receptor dephosphorylation, as well as lysosomal degradation, in signal attenuation.

The dose-response analysis at 15 min of ligand incubation reveals minimal differences in effector activity between the two subcellular locales. In general, effector activity is a function of both ligand concentration and EGFR phosphorylation. However, when the ratio of STAT3 serine phosphorylation versus EGFR phosphorylation is compared for the two ligands (Fig. 3, B and F), it is clear that STAT3 serine phosphorylation is enhanced by cell surface EGFRs. The absence of dynamic changes in STAT3 serine phosphorylation may reflect saturation of the signaling pathway due to either the duration of ligand treatment or a limitation of the more phosphorylated EGF-EGFR to sustain receptor activity due to movement away from the plasma membrane.

Although the spatial regulation of EGF-mediated STAT3 serine phosphorylation is striking, the underlying biology limits enthusiasm as an area of study. Phosphorylation of STAT3 serine 727 has previously been reported to potentiate the transcriptional activity of the tyrosine-phosphorylated protein (33). However, this process requires tyrosine phosphorylation of STAT3, which is only observed at high EGF concentrations in hTCEpi cells: levels that cannot be achieved with EGF bead stimulation, therefore preventing a meaningful analysis.

Although the dose-response curves indicate no difference for the other effectors, significant differences emerged when signaling was observed over longer periods of time. Cell surface EGFRs induced a greater duration and magnitude of Src and c-Cbl activity (Fig. 5). The soluble EGF-mediated activity for
Src decreases when the EGF-EGFR complexes move away from the plasma membrane, whereas Src activity by cell surface receptors is maintained, if not slightly increased. For c-Cbl, activity increases when the EGFRs are retained at the plasma membrane.

Both Src and c-Cbl have established roles in EGFR ubiquitylation (34, 35). This led us to hypothesize that increased Src and c-Cbl activity in the absence of receptor endocytosis invokes a secondary mechanism to attenuate signaling. At short time points, EGFR ubiquitylation is independent of receptor location and is most accurately predicted by the extent of receptor phosphorylation (Fig. 6). However, when longer time points were examined, the consequence of enhanced Src and c-Cbl activity became apparent as EGFR ubiquitylation was increased. Importantly, cell surface EGFR ubiquitylation increases between 30 and 60 min after ligand treatment. This indicates that the differences in ubiquitylation for cell surface and intracellular receptors are not due to degradation of the...
intracellular receptors, but due to enhanced ubiquitylation of those at the plasma membrane.

The notion that c-Cbl is activated by EGFRs at the plasma membrane is not unprecedented. de Melker et al. (36) reported that when the EGFR was retained at the plasma membrane using dominant negative mutants of dynamin, c-Cbl associated with the EGFR, albeit in the absence of ligand. It has been suggested that c-Cbl-mediated ubiquitylation of the EGFR is a critical step of receptor internalization, although there has not always been a consensus as to whether this was required for clathrin-mediated or non-clathrin-mediated endocytosis (27, 37). However, the fact that the differences in Src and c-Cbl activity do not culminate in enhanced EGFR ubiquitylation at early time points suggests that the role of ubiquitylation is not in promoting endocytosis. This is consistent with other reports that the loss of EGFR ubiquitylation does not affect the kinetics of receptor internalization (38).

We propose that sustained cell surface EGFR signaling increases activation of Src and c-Cbl at the plasma membrane as part of a negative feedback mechanism to ensure that there is proper receptor down-regulation. In contrast to many previous studies, this kinetic analysis of effector activity and receptor ubiquitylation uses relatively low levels of stimulatory ligand to ensure that the effects of clathrin-mediated endocytosis are being studied. It is likely that spatial differences in receptor ubiquitylation would be more dramatic when using saturating concentrations of ligand. Increased receptor ubiquitylation would be beneficial to cells if there were sustained growth factor release, increased EGFR expression, or perturbations delaying normal endocytosis. Any of these scenarios could potentially increase the amount of active cell surface receptors and stimulate necessary down-regulation mechanisms.

These data do not eliminate the possibility that EGFR ubiquitylation also occurs at the distal stages of the endocytic pathway (20). One possibility is that receptor ubiquitylation serves as a rheostat to control excessive EGFR activity and trigger its degradation. This may arise from intracellular locations as the result of high level of stimulatory ligand (27, 32), or as is described here, at the plasma membrane if signaling is sustained. The EGFR has multiple mechanisms to attenuate signaling (11).

Establishing biological links to spatially regulated EGFR signaling, beyond changes in effector activity, has been a major challenge. To date, there have only been a handful of studies that clearly demonstrate that spatial restriction of the activated EGFR, or any signaling receptor, affects cell biology. For the EGFR, the reported outcomes focus on cell viability. Data have been conflicting, both at the level of effector activity (10, 39) and at the level of biological consequence (18, 21, 40). Although this current study moves beyond effector activity, it does not indicate a clear biological change. Despite the EGF beads being an excellent tool for studying EGFR activation at the plasma membrane, the size of the ligand can impact the breadth of cell responses that can be studied. An alternative strategy is to directly conjugate the EGF to the matrix on which the cells grow as has been done by Stefonek and Masters (41).
This study provides clear evidence that in non-transformed cells, EGFR-effector communication is spatially regulated. Importantly, we demonstrate that these biochemical changes impact receptor biology by modifying the receptor so that it is targeted for lysosomal degradation. Our data highlight the complexities of growth factor receptor signaling by identifying a novel mechanism by which the cell can attenuate EGFR signaling.

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