Imaging Phosphorylation Dynamics of the Epidermal Growth Factor Receptor*

Epidermal growth factor receptor (EGFR) signaling is initiated by ligand binding followed by homodimerization and rapid receptor autophosphorylation. Monitoring EGFR phosphorylation was achieved by measuring translocation and binding of an enhanced yellow fluorescent protein (EYFP)-labeled phosphotyrosine-binding domain (PTB) to enhanced cyan fluorescent protein (ECFP)-tagged EGFR using fluorescence lifetime imaging microscopy or sensitized emission measurements. To simplify dynamic phosphorylation pattern measurements in cells, FLAME, a ratiometric sensor containing both EGFR-ECFP and PTB-EYFP in one molecule, was designed and examined in COS7 cells. Epidermal growth factor (EGF) treatment demonstrated rapid and reversible changes in the EYFP/ECFP fluorescence emission ratios, due to binding of the PTB domain to its consensus binding sites upon phosphorylation at the cell periphery, whereas perinuclear regions failed to respond to EGF but were responsive to tyrosine kinase inhibition. Long-term EGF treatment resulted in accumulation of dephosphorylated receptor in the perinuclear region due to active dephosphorylation occurring at intracellular sites. This indicates that the sensor closely approaches the true dynamics of tyrosine kinase auto-phosphorylation and dephosphorylation. Phosphatase inhibition by pervanadate resulted in an irreversible response in all cellular compartments. These data show that EGFR is under tonic phosphatase suppression maintaining the receptor in an unphosphorylated (silent) state and is dephosphorylated at endomembranes after ligand-mediated endocytosis.

The epidermal growth factor receptor (EGFR) tyrosine kinase is a transmembrane receptor, which exerts its activity through extracellular binding of epidermal growth factor (EGF) and EGF-like growth factors. Ligand-stimulated EGFR homodimerizes and subsequently phosphorylates itself and other cytoplasmic substrates at tyrosine residues, thus activating an intracellular signaling cascade and finally resulting in a wide range of responses, such as cytoskeletal rearrangements, changes in gene expression, and increased cell proliferation (1). Previous analysis of the phosphorylation/activation status of the EGFR using a number of biochemical approaches such as immunoprecipitation and/or immunoblotting has revealed the basic mechanisms of ligand-induced autophosphorylation. Moreover, cell-based assays using fluorescence lifetime measurements have yielded quantitative spatial information about EGFR phosphorylation in fixed cells (2). Unfortunately, biochemical as well as fixed cell approaches generally fail to capture the dynamics of tyrosine kinase activation in a single cell.

Therefore, the activity of EGFR has been elegantly monitored in live cells expressing genetically encoded ECFP- and EYFP-labeled ratiometric fluorescence resonance energy transfer (FRET) sensors on downstream targets such as Ras (3) or cytoplasmic substrates (4). The latter study specifically monitored the enzymatic activity of the EGFR tyrosine kinase toward soluble substrates in live cells. These studies successfully solved the above-mentioned problems but were focused on the analyses of substrate phosphorylation or Ras activation, representing later events in EGFR signaling.

Obviously, autophosphorylation of EGFR, representing the first step of the signaling cascade, can be determined by measuring the translocation of signaling molecules that interact with phosphotyrosine residues on EGFR to the plasma membrane in response to EGF. We therefore coexpressed the phosphotyrosine-binding domain (PTB) domain of human Shc fused to EYFP and EGFR-ECFP in MCF7 cells. Stimulation with EGF caused translocation of the PTB domain to the plasma membrane. Determination of FRET to show direct binding of the PTB domain to EGFR was done by measuring the fluorescence lifetime of ECFP or the sensitized emission of EYFP.

These quantitative FRET measurements are comparatively complex and involve a number of image processing/calculation steps. Moreover, these approaches require the coexpression of two constructs, the relative levels of which are difficult to control but have an influence on the dynamic range of the read out. To generate a sensor for EGFR tyrosine phosphorylation that can be imaged by a simple ratiometric approach, we fused the PTB-EYFP construct to EGFR-ECFP and thus created a monomolecular sensor, FLAME (fluorescent, linked autophosphorylation monitor for EGFR), representing a noninvasive approach to dynamically determine the phosphorylation state of the EGFR by intramolecular binding of the PTB domain to specific phosphotyrosine residues.

Phosphatases clearly play an important role in controlling receptor tyrosine kinase activity at various levels. First, phosphatase activity confers tonic suppression on tyrosine kinases to prevent spontaneous autophosphorylation in the absence of ligand (5) and thereby controls the phosphorylation levels. This is supported by the well-known observation that application of

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§ The abbreviations used are: ECFP, epidermal growth factor receptor; EGF, epidermal growth factor; PTB, phosphotyrosine-binding domain; PBS, phosphate-buffered saline; FRET, fluorescence resonance energy transfer; YFP, yellow fluorescent protein; EYFP, enhanced yellow fluorescent protein; CFP, cyan fluorescent protein; ECFP, enhanced cyan fluorescent protein; PTP, protein-tyrosine phosphatase.

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tyrosine phosphatase inhibitors, such as pervanadate, to cells induces ligand-independent, high tyrosine phosphorylation levels. A second function of phosphatases is receptor silencing after growth factor stimulation (signal termination). Because recent data indicate that EGFR activation/phosphorylation and signal termination by phosphatases occur at different subcellular localizations (6, 7), we decided to use the newly generated sensor to analyze the spatiotemporal distribution of phosphorylated (activated) and dephosphorylated (inactivated) EGFR upon growth factor stimulation.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF7 and COS7 cells were routinely cultivated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 IU/ml penicillin-streptomycin, and 2 mM glutamine. For microscopy experiments, cells were transferred to low bicarbonate minimal essential medium without phenol red and riboflavin, supplemented with 25 mM HEPES (pH 7.4).

Preparation of Constructs—EGFR-ECFP was produced in the same manner as the previously described EGFR-EYFP. The citrine variant of EYFP was used for all constructs due to its low pKa and salt independence. PTB-EYFP was constructed in two steps. First, a flexible linker was inserted into pcDNA3.1-Citrine in front of citrine by annealing the following oligonucleotides:

\[
\text{CTGACCCACCAGAACCGGTG-3} \\
\text{CAGGAGGGGGCGGAGGTA-3}
\]

PTB domain binding site (Tyr1114) is highlighted in light red because this site does not represent a major autophosphorylation site. In the FLAME_F5 constructs, all major autophosphorylation sites were knocked out by replacing tyrosine residues with phenylalanine residues.

Cell Transfection and Preparation for Imaging— Constructs were transiently transfected into MCF7 or COS7 cells using FuGENE 6 reagent (Roche Applied Science) according to the manufacturer's instructions. Alternatively, cells were microinjected with a mixture of plasmids encoding FLAME_F5 and EGFR at 1 ng/μl using an Injectman/Femtojet system (Eppendorf). Cells were then serum-starved for 12 h before all experiments in Dulbecco's modified Eagle's medium lacking fetal calf serum and antibiotics. A 250 mM pervanadate stock solution was prepared directly before use by mixing sodium orthovanadate and 30% hydrogen peroxide (approximately 10 s; Sigma) at a molar ratio of 2:1 and kept cold before application to cells. Stock solutions of human recombinant EGF (Promega) were prepared at 100 μg/ml in phosphate-buffered saline (PBS). AG1478 (CaliBiochem) was dissolved at 10 mM in DMSO and further diluted to 100 μM in PBS directly before use. A 25 mM stock solution of monensin (ethanol) was directly diluted to 25 μM in imaging medium.

Microscopy—FLIM sequences were obtained at a modulation frequency of 80 MHz with an IX70 microscope (Olympus), using a 100×/1.4 NA oil objective. ECFP was excited using the 457.9-nm line of an argon ion laser. ECFP fluorescence was detected using a dichroic beam splitter (467; Delta) and a narrow-band emission filter (HQ 480/20; Chroma). The EYFP images were recorded using a 100-watt mercury arc lamp with a filter set (excite, HQ 515/20; dichroic, Q 530 LP; emission filter, 467; Delta). ECFP and EYFP images were recorded simultaneously using CARIBA 530 nm excitation and monochrome detection (excite, HQ 515/20; dichroic, Q 530 LP; emiter, HQ 560/40). Confocal laser scanning microscopy was performed on a TCS SP2 microscope (Leica) equipped with a 63×/1.4 NA oil immersion lens. For sensitized emission measurements, CFP and YFP were excited sequentially by using the 457.9- and 514-nm argon laser line, respectively. Fluorescence emission was detected at 460–495 nm (CFP channel) and 520–550 nm (YFP channel). Calibrated sensitized emission measurements were performed as described previously (9, 10). Thus, the total amount of light emitted by sensitized emission from EYFP was calculated. For quantitative evaluation, the total amount of light calculated as sensitized emission was normalized by division by EYFP fluorescence to generate an apparent FRET efficiency EAPP.

Ratiometric imaging was done using an automated Axiosvert 135 microscope (Zeiss) equipped with a 63×/1.4 NA oil immersion lens or a 100×/1.4 NA oil immersion lens.
FIG. 2. Translocation and binding of soluble PTB domain to EGFR-ECFP in MCF7 cells. A, fluorescence lifetime imaging data showing the fluorescence images of PTB-EYFP and EGFR-ECFP and the fluorescence lifetime maps of ECFP calculated before EGF treatment (first row), after 18 min of EGF (100 ng/ml) treatment (second row), and after photodestruction of EYFP (third row). Lifetimes (in ns) were calculated independently as described from the phase shift (\(\text{Tau\_Phase}\)) and the demodulation (\(\text{Tau\_Mod}\)). B, sensitized emission data as obtained by confocal microscopy after stimulation with 100 ng/ml EGF followed by treatment with 100 nM AG1478. EGFR, EGFR; PTB-EYFP, PTB; calculated sensitized emission, \(S\). C, quantitation of sensitized emission data in B was performed by normalizing the sum over all pixels (B, third row) against the total EYFP intensity (B, second row) and normalizing the resulting values against time = 0 to obtain relative changes (C, solid line). EYFP emission intensities, dashed line.
40×/0.2 NA water immersion lens, two automatic filter wheels (Visitron systems), and a Cool Snap HQ cooled charge-coupled device camera (Photometrics). Samples were illuminated by a 100-watt mercury arc lamp through a D436/10 excitation filter, a 90% neutral density filter, and a CFP/YFP/Cy5 triple-band beam splitter and imaged sequentially through 470/30 (CFP) and 535/30 (YFP) emission filters with the charge-coupled device camera set to 4 binning. Background values were calculated within a region of interest outside the cell. Ratios were calculated after background subtraction by pixel-wise divisions of the images in the YFP and CFP channels.

RESULTS

PTB Domain Translocation by Binding to EGFR-ECFP—EGF-induced binding of the PTB domain to EGFR-ECFP (Fig. 2A) was analyzed by measuring FRET using fluorescence lifetime microscopy (11). MCF7 cells were cotransfected with EGFR-ECFP and PTB-EYFP (see Fig. 1) and starved as described. Treatment with 100 ng/ml EGF resulted in a drop in the fluorescence lifetime of ECFP, indicating binding of the PTB domain to the activated receptor. This drop in lifetime was fully reversed when EYFP was bleached by intense illumination at 515 nm, indicating that the observed lifetime drop was due to FRET.

Confocal microscopy (Fig. 2B) demonstrated the EGF-induced rapid translocation of the PTB domain to the plasma membrane. Moreover, sensitized emission measurements were carried out as described previously (9, 10) to observe FRET at confocal resolution. The addition of 100 ng/ml EGF resulted in complete translocation and maximal sensitized emission after 30 s. Quantitation of the sensitized emission demonstrated that a maximum of 150% of the starting signal was reached after EGF treatment (Fig. 2C), whereas the EYFP fluorescence emission (dashed line) remained constant. The subsequent addition of 100 nM AG1478, an EGFR-specific tyrosine kinase inhibitor (12), 2 min after EGF stimulation resulted in a gradual reversion of the PTB domain translocation, which was complete 2.5 min after AG1478 addition. These data indicate that EGF induces binding of the PTB domain to EGFR-ECFP, which can be slowly reversed by inhibition of EGFR kinase activity.

Intramolecular Recognition of Phosphorylation in FLAME—A ratiometric sensor, FLAME, was designed as outlined in Fig. 1 to monitor EGFR phosphorylation by intramolecular binding of the linked PTB domain to its consensus binding sites present...
in the tail of EGFR upon phosphorylation. FLAME has several advantages for measuring phosphorylation of EGFR compared with translocation experiments as indicated above. First, the physical link between the PTB domain and EGFR renders the interaction of PTB-EYFP with EGFR-ECFP independent of long-range diffusion, such that PTB domain binding kinetics should more closely approach that of EGFR phosphorylation. Second, the relative amounts of ECFP and EYFP are constant throughout the cell when FLAME is expressed, enabling the recording of EYFP/ECFP emission ratios as a direct measurement of changes in FRET. Finally, the stoichiometry of binding between EGFR and the PTB domain is constant in FLAME, whereas binding of several PTB domains to one EGFR could occur during coexpression of PTB-EYFP and EGFR-ECFP.

Biochemical characterization of FLAME was performed by immunoblotting as shown in Fig. 3. Transfected COS7 cells were lysed, and protein (10 μg/lane) was loaded and immunostained with a GFP antibody recognizing all spectral variants or the Py72 anti-phosphotyrosine antibody. Immunoblotting confirmed that both FLAME and FLAME_F5 were expressed at the correct size and became tyrosine-phosphorylated in response to EGF (100 ng/ml, 2 min). It should be noted that FLAME_F5 showed a very low basal level of tyrosine phosphorylation but still became phosphorylated upon EGF stimulation. This is due to the fact that EGFR contains more than five tyrosine residues that become phosphorylated upon growth factor stimulation (13). Two of these five major autophosphorylation sites correspond to the consensus-binding site of the PTB domain (Tyr1086 and Tyr1148), thus allowing specific interaction. A third potential binding site at Tyr 1114 is still present in FLAME_F5 (see Fig. 1) but represents a minor phosphorylation site and was shown not to contribute to the observed ratio changes (see below).

Fig. 4A demonstrates that FLAME expressed in COS7 cells reversibly measures EGFR autophosphorylation. Transfected COS7 cells were observed by ratiometric imaging using 436/10 nm excitation. Recombinant human EGF (100 ng/ml) was added at the indicated time points, and YFP/CFP emission ratios were recorded. After 2 min of EGF stimulation, the EGFR-specific tyrosine kinase inhibitor AG1478 (100 nM) was added, which resulted in rapid and complete reversion of the ratio changes even beyond the initial values. These data indicate that PTPs are able to access and dephosphorylate phosphotyrosine residues in the EGFR-PTB domain com-
plex, resulting in rapid reversibility. Furthermore, Fig. 4A demonstrates that in the absence of ligand, EGFR has basal phosphorylation levels that can be reduced by inhibition of its tyrosine kinase activity. Quantitation of four regions, as indicated in the images, demonstrated that EGF-induced ratio changes were more pronounced at the periphery (i.e. close to the membrane) of the cell than in the perinuclear regions, yielding maximal ratio changes of 5% and 8%, whereas the perinuclear regions hardly responded to EGF treatment and yielded ratio changes of 2%. The S.D. within the regions of interest was 2% for all four regions (Fig. 4C).

In contrast, replacing the five major tyrosine autophosphorylation sites (see Fig. 1) with phenylalanine (13) residues resulted in an inactive sensor termed FLAME_F5, as shown in Fig. 4B., indicating that the observed ratiometric changes in FLAME are due to binding of the PTB domain to phosphorylated tyrosine residues on the receptor, phospho-Tyr1086 and/or phospho-Tyr1148 (14). Furthermore, these findings indicate that the potential PTB binding site at Tyr 1114 (a minor phosphorylation site of EGFR) does not contribute to the observed FRET response.

Phosphatase inhibition by treatment with 1 mM pervanadate (Fig. 5) resulted in a rapid drop of the YFP/CFP ratio, which was stable even after the subsequent addition of AG1478 for another 60 s. Irreversibility arises from the absence PTP activity, which could dephosphorylate the receptor in the cell. Furthermore, quantitation of four regions of interest (two on the periphery and two in the perinuclear region) yielded irreversible ratio changes in a similar range for both peripheral and perinuclear regions, indicating that pervanadate treatment, in contrast to growth factor treatment, induces full phosphorylation of EGFR on all membrane compartments in the cell.

Immunoblotting experiments were performed to confirm the reversibility of FLAME phosphorylation response to EGF. FLAME-expressing COS7 cells were subjected to either single treatments with AG1478, EGF, or pervanadate (2 min) or two consecutive treatments using the same compounds (2-min treatment with the first compound, followed by an additional 2-min treatment with the second compound) and lysed, and protein (10 μg/lane) was loaded and immunostained with the Py72 anti-phosphotyrosine antibody, stripped, and reprobed with GFP antibody.

Fig. 6 demonstrates that EGF induced a 2.5-fold increase in phosphoryrosine levels, whereas AG1478 reduced the band intensity to ~50%. Pervanadate resulted in a very strong induction of phosphorylation (>5-fold). However, consecutive treatment of the cells with EGF and AG1478 did not result in increased phosphorylation compared with control, whereas consecutive application of pervanadate and AG1478 resulted in a strong induction of tyrosine phosphorylation relative to control. These data again suggest that EGF induces reversible phosphorylation, whereas pervanadate, by inhibiting phosphatases, leads to a very strong and irreversible phosphorylation.
tion of the receptor, and these results are in good accordance with those obtained by ratiometric imaging (Figs. 3 and 4).

Moreover, we assessed whether FLAME responds to changes in tyrosine phosphorylation following an intra- or intermolecular mechanism. The latter could occur by binding of the linked PTB domain to phosphorylated consensus binding sites present on proteins in the vicinity of FLAME, e.g. as a result of homodimerization (representing a well-described mechanism in EGFR signaling). This is important to establish because an intermolecular binding mechanism would depend on local FLAME concentration differences within different compartments in addition to phosphorylation, thus complicating the interpretation of results, whereas an intramolecular mechanism would be concentration-independent. To achieve this aim, we coexpressed FLAME_F5 with EGFR by microinjecting equal amounts of both plasmids and performed ratiometric imaging (Fig. 7). Quantitative image analysis was performed by subtracting the relative ratio (R/R0) after EGF treatment (last frame of EGF exposure) from the relative ratio after AG1478 addition (first frame after addition) obtained in EGF-treated COS7 cells within peripheral regions to evaluate the maximal dynamic range in the ratio changes. Even in the presence of coexpressed EGFR, FLAME_F5 expression did not yield any ratiometric response, in contrast to expression of FLAME. These data indicate that FLAME responds to phosphorylation via an intramolecular binding mechanism and therefore does not require dimerization for its activity.

Ligand-induced receptor phosphorylation was followed for a longer period to observe dephosphorylation of the receptor by endogenous PTPs. To obtain more rapid internalization of the receptors upon EGF treatment, these experiments were performed in the presence of 25 μM monensin, which blocks EGFR recycling (15). Fig. 8 shows that FLAME was largely internalized after 24 min of EGF treatment. Dephosphorylation was found to take place in the central area of the cell, where dephosphorylated FLAME accumulated in a broad central region. The peripheral region showed a rapid EGF-induced ratio drop, which became even more pronounced upon longer incubation. Addition of AG1478 after 24 min of EGF treatment resulted in a global increase of the YFP/CFP emission ratio. Addition of pervanadate after 24 min of EGF treatment, on the other hand, resulted in a dramatic ratio drop for both the peripheral and central regions (Fig. 8B). In summary, these data indicate that the observed ratio changes were indeed due to changes in the phosphorylation status of the receptor caused by changed local balances between EGFR kinase and PTP activities and not to protein degradation or other mechanisms involved in receptor down-regulation.

**DISCUSSION**

Here we used EGFR-ECFP (2) and the PTB domain from human Shc tagged with citrine (8) encoded either as separate proteins or as a fusion construct separated by a flexible linker to monitor the EGFR phosphorylation state in cells. Because human EGFR harbors three potential binding sites for the Shc PTB domain (14), activation of the receptor by EGF or phosphatase inhibition results in binding of the PTB domain to the phosphorylated receptor.

We first analyzed PTB domain binding by observing translocation of PTB-EYFP to the plasma membrane concomitant with sensitized emission measurements or fluorescence lifetime measurements. Using sensitized emission measurements, we demonstrated that EGF treatment resulted in rapid translocation of the PTB domain to the plasma membrane, which was shown to be due to binding to phosphorylated EGFR-ECFP, as indicated by a gain of FRET due to close proximity of ECFP and EYFP. Reversibility was demonstrated by subsequent treatment of the cells with the EGFR inhibitor AG1478; however, full reversion of the translocation/binding took more than 2 min. In several independent experiments (data not shown), maximal sensitized emission also occurred only after a few minutes of EGF treatment. These observations suggest that the translocation experiments partially fail to monitor the true dynamics of EGFR autophosphorylation, possibly due to diffusion limited steps (diffusion out of the nucleus is rather slow) (Fig. 1B) and/or partial block of receptor dephosphorylation by PTPs after binding of up to three PTB domains to the phosphorylated receptor. On the other hand, quantitation of the sensitized emission signals demonstrates, depending on the relative expression levels, a high dynamic range (in the range of 50% change compared with the initial signal) of the measurement.

Our data indicate that the construction of the ratiometric sensor FLAME incorporating the PTB domain into the EGFR is monitoring receptor phosphorylation (activation) with a faster response time than the two-component system presented here.
or sensors that monitor phosphorylation of cytoplasmic substrates (4). In the latter study, several minutes of EGF treatment were required before maximal phosphorylation was observed, suggesting that the activity of EGFR was monitored at substrate saturation. Because dimerization between members of the EGFR family is a hallmark of EGF signaling, we analyzed whether the mechanism by which FLAME responds to tyrosine phosphorylation involves cis-binding and/or trans-binding (in a homodimer) of the linked PTB domain to its phosphorylated consensus binding sites. Cells coexpressing FLAME lacking the five major autophosphorylation sites (FLAME_F5) with EGFR failed to respond to EGF and AG1478, indicating that FLAME mainly monitors tyrosine phosphorylation by intramolecular binding of the linked PTB domain. Phosphatase inhibition experiments in the absence of ligand (as shown in Fig. 5), which resulted in very efficient responses, also support an intramolecular binding mechanism because structural data suggest that EGFR dimerization requires ligand-induced rearrangements of the extracellular domain (16, 17). We therefore conclude that the observed ratio-

Fig. 8. Ratiometric imaging of COS7 cells during EGF-induced FLAME internalization. A, COS7 cells expressing FLAME were stimulated with 100 ng/ml EGF for 24 min, followed by treatment with AG1478. Quantitation was performed in two regions of interest (center and periphery). B, COS7 cells expressing FLAME were stimulated with 100 ng/ml EGF for 24 min, followed by treatment with pervanadate. Quantitation was performed in two regions of interest (center and periphery).
metric changes of FLAME are due to intramolecular binding of the linked PTB domain to its consensus binding sites upon phosphorylation.

Our data furthermore demonstrate that the interplay between the EGFR tyrosine kinase and EGFR-specific phosphatases is highly dynamic because both EGF-induced phosphorylation and dephosphorylation upon inhibition of the tyrosine kinase activity occur at the same time scale of ~30 s, when applying ratiometric imaging, but could also be demonstrated, albeit at lower time resolution (approximately 2 min), using immunoblotting.

Imaging these effects with the sensor described here is possible only due to a specific property of the PTB domain phosphotyrosine interaction. Zhou et al. (14) pointed out previously that both the association and dissociation rates of the PTB domain from its binding sites are very high, resulting in a dynamic equilibrium, with a sufficiently low stability of the complex to enable PTPs to interact with and dephosphorylate specific phosphotyrosine residues, despite their high affinity for the PTB domain ($K_D$: 140 nM to 5.3 μM).

Using this sensor, we reproducibly measured a drop in the YFP/CFP emission ratio, indicating a loss of FRET upon PTB domain binding to the phosphorylated receptor, in contrast to the two-component approach, in which an increase in FRET upon binding of the PTB domain was observed. The response in the two-component system involves binding by translocation of the FRET acceptor (i.e. PTB-EYFP) to the donor (EGFR-ECFP), which obviously results in a gain of FRET upon phosphorylation. However, FLAME, which contains both ECFP and EYFP within one molecule, exhibits FRET in both an active and inactive state due to the monomolecular design. Intramolecular rearrangements between ECFP and EYFP induced by PTB domain binding to phosphotyrosine residues can therefore result in either increased or decreased FRET efficiency.

Furthermore, long-term stimulation experiments indicated that active dephosphorylation occurs in a central region after ligand-induced internalization of the receptor via the endocytic pathway, probably by specific phosphatases localizing to internal membranes (6). At the beginning of the experiment, the receptor is largely localized to the plasma membrane, where it displays basal phosphorylation levels (see Fig. 4, A and C). EGF treatment subsequently induces plasma membrane-localized receptor phosphorylation followed by internalization. Dephosphorylated receptor finally accumulates (see Fig. 5A) in a central area of the cell, which was observed by high ratio values exceeding the starting levels.

On the other hand, EGFR kinase inhibition experiments (as shown in Fig. 4) demonstrate the existence of a second, functionally different phosphatase activity. This suppressing PTP activity was found to dephosphorylate ligand-stimulated EGFR only in the absence of its tyrosine kinase activity (demonstrated by specific inhibition), which is distinguishable from signal terminating PTP activity that acts on endocytosed receptors.

These experiments thus provide evidence for two functionally different phosphatase activities controlling EGFR phosphorylation (and thus activity) levels present in cells, and the regulation of these two functionally different phosphatase activities is spatially distinct. At the cell membrane, the phosphorylation state of the EGFR, which is linked to its tyrosine kinase activity (18–20), is under tonic phosphorylation control (5) by a subset of phosphatases localizing to the plasma membrane or in the cytoplasm. EGF binding is able to overcome this constant suppression due to an increase of the EGFR kinase activity and/or inhibition of PTP activity (5, 21). Phosphatase inhibition studies support the above-outlined principle of a constant competition between the EGFR tyrosine kinase and its specific phosphatases because removal of phosphatase activity by pervanadate resulted in a rapid ratio change, demonstrating that the basal kinase activity of EGFR is sufficient for complete and irreversible (after AG1478 addition) autophosphorylation, if phosphatase suppression is removed (see Figs. 4 and 6). Although a number of phosphatases such as RPTPα or SHP-1 have been described to reduce EGFR phosphorylation (22, 23), it is presently unclear which PTPs are responsible for tonic suppression of EGFR activation, and several PTPs could most likely act together on phosphorylated EGFR.

Secondly, ligand-induced EGFR signaling needs to be shut down after signaling. Therefore, phosphatases capable of dephosphorylating ligand-activated EGFR are localized to endomembranes, which allows them to exert their activity only after receptor internalization, as observed experimentally. This is in good accordance with a previous study pointing out that dephosphorylation is a step preceding receptor degradation (7). PTP1B, which localizes to the endoplasmic reticulum, is a candidate phosphatase for EGFR signal termination because it has previously been shown to interact with internalized EGFR after EGF stimulation (6).

Previously described ratiometric FRET sensors have generally proven very useful for monitoring dynamic changes of signaling processes. These studies, in contrast to the sensor presented here, focused mainly on small molecules with a well-described structure facilitating construction of fusion proteins and rational design of probes (4, 24–28).

Kinase activity of protein kinase A (27) and of several tyrosine kinases including the EGFR has been monitored using ratiometric probes that serve as specific substrates (4, 28). Our study compares well with these observations and uses a ratiometric FRET sensor to monitor EGFR activity but has focused on analysis of the (auto)phosphorylation changes of the receptor instead of monitoring substrate phosphorylation by EGFR kinase activity (4) representing a later step of the signal transduction cascade. Furthermore, due to the different design and the exclusive intramolecular response mechanism, FLAME is capable of monitoring receptor phosphorylation independent of diffusion processes directly on the receptor. This enabled us to dynamically monitor local differences in receptor phosphorylation levels induced by EGF, which can typically not be achieved with freely diffusing (i.e. unlinked) constructs that interact only transiently with the receptor. We thus observed active dephosphorylation concomitant with endocytosis in a central cellular compartment, whereas the not-yet-internalized receptors on the periphery were found to remain phosphorylated.

Finally, the strategy presented here measuring EGFR auto-phosphorylation using a linked sensor approach could thus possibly be extended to different transmembrane receptor tyrosine kinases to study the spatio-temporal regulation of their activation state.

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