Imaging Epidermal Growth Factor Receptor Phosphorylation in Human Colorectal Cancer Cells and Human Tissues*

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In tumor cells, high phosphorylation levels of receptor tyrosine kinases may occur in the absence of exogenous ligands due to autocrine signaling or enhanced tyrosine kinase activity. Here we show that the phosphorylation state of the endogenous epidermal growth factor receptor (EGFR) can be quantitatively imaged in tumor cells and tissues by detecting fluorescence resonance energy transfer between fluorophores conjugated to antibodies against the receptor and phosphotyrosine, respectively. Five different human colorectal cell lines were analyzed for activity and expression of EGFR. All cell lines exhibited basal EGFR phosphorylation under serum starvation conditions. Phosphorylation levels increased after stimulation with EGF or pervanadate, dependent on the level of basal EGFR phosphorylation in the respective cell lines. This basal activity correlated inversely with receptor expression. Using the acceptor photobleaching fluorescence resonance energy transfer imaging approach, a significantly higher phosphorylation state of EGFR was also found in resected human colorectal tumor samples as compared with adjacent healthy tissue. Imaging of EGFR phosphorylation may thus serve as a valuable tool to investigate the role of receptor tyrosine kinase activity in malignant cell growth.

Upon ligand binding EGFR dimerizes, leading to receptor phosphorylation on multiple tyrosyl residues catalyzed by its intrinsic tyrosine kinase activity. These phosphorylated tyrosyl residues recruit proteins with Src homology 2 and phosphotyrosine-binding domains, thereby assembling multiprotein complexes that propagate the signal inside the cell. The amount of phosphorylated receptors is determined by the balance between tyrosine kinase and specific protein-tyrosine phosphatase activities (1). Therefore, lowering the protein-tyrosine phosphatase activity or increasing the receptor kinase activity should lead to enhanced phosphorylation of the receptor in the absence of exogenous growth factors. Up-regulation and over-expression of EGFR has been correlated to many processes related to cancer, including uncontrolled cellular proliferation, autocrine stimulation of tumors producing their own growth factors (e.g. TGF-β, EGF), and prevention of apoptosis (2–4). This also appears to protect cancer cells from the toxic actions of chemotherapy and radiotherapy, rendering these treatment modalities less effective. Many epithelial tumor entities including gastric and cervical cancer as well as cancers of the head, neck, breast, and lung express high EGFR densities, which are associated with advanced disease and poor clinical prognosis (5). Extensive expression studies have also been performed in colorectal carcinoma in vivo and in vitro (6–12). A higher level of EGFR expression has been inversely correlated to survival in these patients and high expression levels have been found in advanced tumor stages (13, 14). However, expression level of the receptor is not necessarily an indicator of its signaling activity in tumor cells. Especially in the view of the novel EGFR targeting treatment modalities no study has so far provided a method for direct imaging of endogenous EGFR phosphorylation in tumor tissues to validate treatment efficiency. To close this gap, we introduce a fluorescence resonance energy transfer (FRET)-based method for quantifying EGFR phosphorylation within individual tumor cells. Spatially resolved FRET measured in the microscope (15) has provided an approach for tracing the reaction state of fluorescently tagged proteins inside single cells (16, 17). Using this novel approach we investigate the endogenous phosphorylation state of EGFR in tumor cells and correlate it to its expression.

**Experimental Procedures**

Reagents—Human recombinant EGF and the EGFR kinase inhibitor AG1478 was purchased from Calbiochem. Vanadate was obtained from Sigma (Taufkirchen, Germany). Cell culture materials were derived from Invitrogen. EGFR antibodies binding to the sequence DVAADAEYLIQP, which corresponds to amino acid residues 985–996 (referred to as F4) and generic phosphotyrosine antibodies (referred to as PY72) were obtained from the monoclonal cell facility of Cancer Research UK (London, UK). For immunoprecipitation a polyclonal anti-EGFR antibody from sheep was used (Upstate Biotechnology, Lake Placid, NY). Monoreactive dyes Cy3 and Cy5 were purchased from Amersham Biosciences (Buckinghamshire, UK). All other reagents were purchased from Sigma unless indicated otherwise.

Cell Culture—Colon cancer cell lines were provided by the American Type Culture Collection (Manassas, VA) and were maintained in minimal essential medium (HT-29, WiDr), Dulbecco’s modified Eagle’s medium (A431), RPMI (Colo 320, SW-480), or McCoy (Caco-2) medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% (v/v) glutamine (ICN, Irvine, CA) in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Stock cultures were stored in liquid nitrogen and used for experiments within 5 passages. Cell viability (at least 95%) was assessed by trypan blue dye exclusion before cells were processed further.
Cells were grown to 70% confluence in 6-well dishes containing glass coverslips, starved in 1% fetal calf serum for 24 h, and then exposed to EGF (100 ng/ml) or pervanadate (final concentration of 1 mM after oxidation of vanadate by addition of 30% H₂O₂ to the 200 mM stock solution). Controls obtained the carrier PBS. After incubation cells were fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton X-100 before staining.

**Cancer Samples—**Human tumor tissues and adjacent normal tissues were collected from specimens that were resected at the Surgical Clinic Mannheim. Tissues were classified as normal or cancerous by hematoxylin and eosin staining of frozen sections. Adjacent sections of the tissue blocks were stained with the antibodies as described below. Samples were collected with informed patient consent, and the study was approved by the local ethics committee of the Mannheim University Hospital.

**Antibody Labeling—**F4 antibody binding a conserved intracellular domain of EGFR was labeled with Cy3, and antiphosphotyrosine antibody PY72 was labeled with Cy5. For labeling, 10-μl aliquots of 1 mM sodium Bicine buffer, pH 9.0, were added to 90 μl of antibody solution in PBS and a 20-fold molar excess of the monofunctional N-hydroxysuccinimidyl ester of the chromophores was added from a 10 mM stock solution in N,N-dimethylformamide. Conjugation reactions were allowed to proceed for 40 min in the dark at room temperature. Labeled antibody was separated from free unreacted dye by gel exclusion chromatography using Econo-Pac 10DG columns (Bio-Rad) and concentrated to ~50 μg/ml using Centrinor centrifugal filter devices (Amicon, Bedford, MA). The dye/protein labeling ratio was determined to be calculated by normalizing the difference of the donor post- and pre-bleach fluorescence to the increase in the Cy3 fluorescence.

**Fluorescence Lifetime Imaging Microscopy (FLIM)—**FLIM sequences were obtained at a modulation frequency of 80 MHz with an Olympus IX 70 microscope (Olympus, Hauppauge, NY) using a 100X/1.32 NA oil immersion objective. Simultaneous images with the pinholes set at 2 Airy units were obtained at a modulation frequency of 80 MHz with an Olympus IX 70 microscope (Olympus, Hauppauge, NY) using a 100X/1.32 NA oil immersion objective. The donor (Cy3) was excited by the 514 nm argon laser line. Fluorescence was detected with a dichroic beamsplitter (Q 570 LP; Chroma Technology Corp., Rockingham, VT) and a narrow-band emission filter (HQ 610/75 Chroma Technology Corp.). The Cy5 images were recorded using a 100-watt mercury arc lamp with a High Q Cy5 filter set (exciter: HQ 620/60, dichroic: Q 660 LP, emitter: HQ 700/75 LP; Chroma Technology Corp.). We analyzed FLIM data by calculating phase and modulation lifetimes as described previously (18, 19).

**RNA Isolation—**5 × 10⁶ cells were lysed with 1 ml of TRIzol (Invitrogen). After adding 0.2 ml of chloroform and mixing for 3 min, the RNA was extracted with phenol/chloroform extraction was performed, followed by an isopropanol alcohol precipitation. Total RNA samples containing pellets were air-dried and dissolved in water, and purity was determined by gel electrophoresis. Samples were treated with DNase before storage at −80 °C.

**Reverse Transcription-PCR for EGFR—**cDNA was derived by a reverse transcriptase reaction with avian myeloblastosis virus reverse transcriptase and oligo(d-T) primers (25 °C 10 min, 42 °C 1 h, 95 °C 5 min), cooling to 4 °C). An EGFR-specific primer pair designed by Tib Molbiol Syntheselabor (Berlin, Germany) was used for amplification in a T-gradient cycler (Biometra, Gottingen, Germany): 5’TCT CAGCAA CAT GTGATGG 3’ and 5’TCC GAC TTC CAC TT ACG GGC 3’. PCR conditions were as follows: 94 °C 2 min, 38 cycles 94 °C 30 s, 66 °C 45 s, 72 °C over 45 s with a final elongation time of 10 min. PCR products were analyzed on a 1% agarose gel.

**Cloning a Standard for EGFR—**A RNA standard was cloned from the 479-base pair PCR fragment. In brief, PCR fragments were purified according to the QiAquick PCR purification kit (Qiagen, Hilden, Germany). 50 ng of the purified fragment were incubated with 50 ng of the pDRIVE cloning vector for 10 min. Ligation mix was added. 50 μl of bacteria were co-transfected with 2 μl of plasmid solution in SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO₄, 20 mM MgSO₄, 20 mM glucose) and heat-shocked (42 °C, 30 s). The transformation mixture was plated on ampicillin containing LB agar dishes for overnight culture. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside negative clones were derived and amplified in 15 ml of

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medium. Plasmids were isolated with the QIAprep Miniprep kit (Qiagen). The length of the plasmids were determined by gel electrophoresis, and the sequence was determined (Seqlab, Gottingen, Germany). For in vitro transcription, plasmids were treated with AVRII (2 IU/μg plasmid, 37 °C, 60 min) before incubation with RNA polymerase T7. RNA was purified as described above.

Real-time PCR—Kinetic reverse transcription-PCR was performed with a LightCycler (Roche Diagnostics, Mannheim, Germany) by using SYBR Green I as a double-strand DNA-specific binding dye. Since the EGFR primer pair used for the cloning step described above showed dimmer formation in the melting curve analysis, a different EGFR-specific primer pair coding for a 304-base pair fragment of EGFR-cDNA contained within the region of EGFR used as a 479-base pair standard was used. Primers were designed by Tib Molbiol Syntheselabor: 5’-GAG GAG AAC TGC CAG AAA CTG A-3’ and 5’-CCG TGA TCT GTC ACC ACA TAA TTA C-3’. The following single step reverse transcription-PCR protocol was used for detection: amplification was carried out in a total volume of 20 μl in glass capillaries containing a 0.5 μM concentration of each primer, 3.25 μM dNTP, SYBR Green I (Roche Diagnostics) and 10 ng of RNA diluted in 2 μl. RT was performed at 50 °C over 20 min. Probes were denatured at 95 °C over 15 min. Amplification conditions were 95 °C 15 s, 52 °C 20 s, 72 °C over 15 s, 10 s with temperature transition rates of 20 °C/s with acquisition of the intensities at 80 °C. Additionally for control, PCR products were visualized after agarose gel electrophoresis.

ELISA—Protein levels were measured using an EGFR-ELISA in quadruplet (BIOSOURCE International). Assays were performed in duplicate for each measurement using 100 μl per well in accordance with the manufacturer’s instructions. Cells were trypsinized and resuspended in PBS, and protein was extracted by incubation in cell extraction buffer (BIOSOURCE International) for 30 min at 0 °C. Samples were centrifuged at 10,000 × g for 10 min, and protein concentration in the supernatant was determined using a BCA protein assay. Samples used for ELISA contained 5–10 mg/ml protein. Values were read on a Diagnostic Products Corp. (Los Angeles, CA) microplate spectrophotometer at 450 nm.

Statistics—The data were analyzed for statistical significance with the Student’s t test using the software package SPSS™ (Chicago, IL). p values of <0.05 were considered significant. In the box plots used for graphical data representation a box is drawn around the interquartile range. A line inside the box indicates the median value. Error bars are drawn at the 5% and the 95% confidence intervals.

RESULTS

Detection of the co-occurrence of a specific Cy3-tagged antibody and a generic Cy5-coupled anti-phosphotyrosine antibody on EGFR by FRET allows a highly specific quantification of EGFR phosphorylation in cells. Here, EGFR phosphorylation was imaged by this two-antibody FRET approach (20) using acceptor photobleaching (15). FRET in the prebleach images quenches the fluorescence intensity, which can be visualized by photobleaching the acceptor. It was first determined whether FRET can take place between the two chromophore-tagged antibodies bound to phosphorylated receptor. For this, unstimulated SW-480 cells were incubated with both antibodies after fixation and permeabilization. The data from a typical FRET experiment are shown in Fig. 1. The initial anti-EGFR-Cy3 image (Fig. 1a) represents the fluorescence of the donor in the presence of the acceptor Cy5. The Cy3 fluorescence distribution shows a partial co-localization with that of Cy5-antiphosphotyrosine antibody (Fig. 1b). After complete photobleaching of the acceptor, a second image was taken in both channels. Fig. 1d shows the bleached region in the acceptor Cy5 image, and Fig. 1c shows the resulting Cy3 fluorescence. The difference in intensity of the donor is shown in Fig. 1e. Increased donor fluorescence after photobleaching of the acceptor Cy5 indicates that the fluorescence of anti-EGFR-Cy3 was indeed quenched by FRET. We also used fluorescence lifetime imaging microscopy (16, 17) to measure the decrease in Cy3 lifetime as an independent confirmation that FRET took place between the chromophores in HT-29 cells. An increase in fluorescence lifetime of Cy3 after photobleaching the acceptor Cy5 shows that FRET took place between the chromophores. Cy3 fluorescence increased due to a loss of FRET after photobleaching (Fig. 2 showing the false color phase lifetime images and the wide field fluorescence images). Using this optical assay we now characterized and compared EGFR phosphorylation in different colorectal cell lines. Experiments were carried out in HT-29, WiDr, Colo 320, SW-480, and Caco-2 cells. To determine the degree of autonomous EGFR autophosphorylation in these tumor cells lines, all cells were serum-starved for 24 h before imaging experiments. The cell lines showed a significantly different pattern of autonomous phosphorylation (Fig. 3a). WiDr cells and HT-29 cells maintained high levels of phosphorylation with FRET efficiencies of over 50%. Caco-2 cells showed FRET efficiencies close to 30%. SW-480 and Colo 320 cells showed a low residual phosphorylation with FRET efficiencies of 10% and 20%, respectively.

Inhibition of EGFR dephosphorylating protein-tyrosine phosphatases by pervanadate is expected to give maximal EGFR phosphorylation even in the absence of exogenous ligands due to the basal kinase activity of EGFR (21–24). Therefore, maximal FRET efficiency after treatment with pervanadate corresponds to 100% EGFR phosphorylation, which can be used to calibrate the assay. Cells with low levels of autonomous autophosphorylation responded well to pervanadate treatment with the highest increase in FRET efficiency in relation to the basal activity in Caco-2 and SW-480 cells. Colo 320 and HT-29 cells showed a lesser increase, and no significant increase could...
Since aggregating of labeled antibody might also contribute an unspecified signal to the total FRET efficiency, we tested whether blocking of the epitope would attenuate the FRET signal. SW-480 cells were grown on coverslips, starved, and treated with pervanadate before fixation and permeabilization. Cells were incubated with a 10-fold excess of unlabeled F4 or PY 72 antibody at 30 and 90 µg/ml, respectively, before staining was performed with Cy3-F4 and Cy5-PY72. No FRET could be found in these cells. Specific PY-72-Cy5 staining of pervanadate-stimulated SW-480 cells was also lacking by preincubation of the labeled antibodies with a 10-molar excess of phosphotyrosine-albumin for 60 min at room temperature. The Cy3-F4 staining remained unchanged. Again, no FRET could be detected in these samples. In all cell lines, FRET efficiencies dropped to zero when cells were incubated for 180 min with the specific EGFR kinase inhibitor AG 1478 ad 5 µM prior to stimulation with pervanadate. Immunoblots against phosphotyrosine using the PY72 antibody of immunoprecipitated EGFR showed concordance with the optical assays in that a high level of basal phosphorylation was found for WiDr cells, which could not be stimulated further by pervanadate, and a low basal phosphorylation was found for SW-480 cells with weak bands only appearing at long exposure times. In SW-480 cells, EGFR phosphorylation increased in a time-dependent manner after stimulation with EGF (Fig. 3b). Pervanadate treatment gave the highest phosphorylation levels on blot in agreement with the FRET experiments. EGFR phosphorylation in response to the ligand EGF was also followed over time in all cell lines. Here again, cells with a lower level of autonomous autophosphorylation that had responded to pervanadate (Colo 320, HT-29, and SW-480 cells) showed a significant increase in FRET efficiency after EGF stimulation. Caco-2 cells showed a delayed response to EGF stimulation with a marginal increase in FRET efficiency after 10 min. WiDr cells, which had shown the highest autonomous EGFR phosphorylation level after serum starvation, did not further respond to EGF. In SW-480 cells, the phosphorylation response after EGF was lower than after pervanadate treatment as shown by both the FRET experiments (Fig. 3a) and immunoblotting (Fig. 3b). EGFR immunofluorescence images showed that these cells had a particular high level of intracellular EGFR localized to endomembranes that can be phosphorylated by protein-tyrosine phosphatase inhibition with pervanadate but not by extracellular EGF. Taken together, these results show that cell lines that exhibit a high degree of autonomous phosphorylation cannot be stimulated further.

The same optical FRET assay was used to determine EGFR phosphorylation levels in human tissue samples (Fig. 4, a and b). Since in fixed tissues the FRET efficiency cannot be calibrated by pervanadate treatment, FRET signals in tissue sections from human colon tumor samples were compared with tissue samples taken from the mucosa of tumor-free resection margins (Fig. 4c). Significantly higher FRET efficiency was found in sections of tumor tissues as compared with healthy tissue (Fig. 4d). This indicates an increased endogenous EGFR phosphorylation also in human colorectal cancer tissue.

High EGFR expression in tumor cells had been discussed by several earlier studies (6–12). However, high EGFR expression is not necessarily an indication of high EGFR activity. Instead, we found an inverse correlation between residual EGFR phosphorylation and EGFR expression. The highest amount of mRNA copies could be found in SW-480 and Colo 320 cells, which had the lowest autonomous EGFR activity in the FRET experiments. Caco-2 and HT-29 cells, which had shown little
response toward stimulation due to a high level of autophosphorylation, showed lower levels of EGFR mRNA, and the EGF-unresponsive WiDr cells had the lowest mRNA content (Fig. 5a). EGFR mRNA content correlated well with EGFR protein expression as quantified by ELISA (Fig. 5b).

DISCUSSION

The principle novelty of the present work is the development and application of a single cell based functional assay that allows quantification of endogenous EGFR phosphorylation in human cancer cell lines and tissues as compared with earlier studies that mainly focused on expression levels (6–12). Structurally, EGFR is a transmembrane molecule composed of an extracellular ligand-binding domain, a transmembrane membrane-anchoring domain, and an intracellular domain with intrinsic tyrosine kinase activity. Ligand binding to the extracellular domain of the receptor initiates a process of receptor homo- or heterodimerization to other EGFR family members that activates the intracellular tyrosine kinase domain of the receptor and results in autophosphorylation (17). The phosphorylated receptor thus serves as a docking site for binding and activation of several downstream signaling mediators that ultimately results in cell proliferation (25, 26) and survival (27). Therefore, quantification of EGFR phosphorylation can be used as a direct indicator of intracellular EGFR activity, which is the essential parameter for growth control exerted by the receptor.

Imaging approaches to determine EGFR phosphorylation have been described using green fluorescent protein fusions to the receptor (24, 28, 29), which require ectopic expression of the fusion constructs. These approaches will perturb the overall EGFR phosphorylation level (24) and cannot be applied to tissue samples. We therefore developed an optical assay to quantitatively image endogenous EGFR phosphorylation based on chromophore-labeled antibodies against EGFR and phosphotyrosine. While the specific anti-EGFR antibody will bind to EGFR independent of its phosphorylation state, the generic anti-phosphotyrosine antibody will only bind on phosphorylated tyrosines. Therefore, the co-occurrence of both antibodies on EGFR as detected by FRET between the conjugated dyes reports on phosphorylated EGFR molecules. FRET was quantified by measuring the increase in donor intensity after acceptor photobleaching (15) and verified independently by FLIM (16, 17). The derived FRET efficiency therefore is proportional to the molar fraction of phosphorylated EGFR in a given cell type (16). In contrast to an absolute determination of phosphorylation levels with single antibodies against phosphorylated EGFR, different samples can be compared without external calibration. FRET imaging could therefore be carried out in this way in fixed tissue culture cells as well as in resected tumor tissues from human patients.

In the examined colorectal tumor cell lines (HT-29, WiDr, Colo 320, SW-480, and Caco-2) we found different levels of basal EGFR phosphorylation in the absence of exogenous growth factors. Cell lines with low levels of autonomous EGFR phosphorylation responded by the highest increase in receptor phosphorylation after EGF stimulation and vice versa. To determine the full dynamic range and thus the maximal responsiveness of endogenous EGFR toward phosphorylation, we employed pervanadate to inhibit protein-tyrosine phosphatases. Again, low basal EGFR phosphorylation gave the largest responsiveness in the cell lines, while cells with the highest levels of basal EGFR autophosphorylation showed no response. When we determined EGFR expression in the cell lines we surpris-
ingly found an inverse correlation between EGFR expression and activity. High EGFR expression was found in SW-480 and Colo 320 cells, which had displayed low levels of basal activity. Moderate levels could be determined for HT-29 and Caco-2 cells. The lowest EGFR mRNA levels were found for WiDr cells, the cell line with the highest EGFR autophosphorylation and a general unresponsiveness toward stimulation. Protein expression determined by ELISA correlated with the expression levels derived for mRNA. This essentially argues against post-translational regulation of EGFR expression in the cell lines investigated, which had been suggested elsewhere (30). The observed inverse correlation of EGFR activity with protein expression suggests a negative feedback loop between EGFR expression and activity in colorectal cancer cell lines.

A substantial number of studies have indicated that deregulation of EGFR function, either by overexpression (10–12), mutations that result in constitutive activation of the receptor (30) paracrine, or autocrine secretion of activating ligands (31, 32), are key processes in malignant transformation. For the cell lines that were the subject of the present study, a high production of EGF and intermediately high production of EGF has been demonstrated for HT-29, WiDr, Colo 320, and SW-480 cells as well as for Caco-2 cells, respectively (33). Therefore, the high levels of basal activity are most likely due to an autocrine stimulation in certain cell lines.

EGFR has been discussed as a promoting factor for cell growth and proliferation (34), tumor cell survival (35), angiogenesis (36), and the development of metastases (37). Studies using tumor tissues from cancer patients have shown a deregulation of EGFR expression in the majority of human epithelial neoplasms with frequencies ranging from 15% to as high as 90% (38). Specifically for colorectal carcinoma, EGFR-dependent autocrine growth stimulation has been shown to be mediated by coexpression of EGFR ligands and up-regulation of EGFR (5, 38). Here, we have shown that high expression levels for EGFR do not necessarily indicate a high level of intracellular EGFR activity. If novel EGFR-directed chemotherapies (39–41) are to be employed in clinical practice for the treatment of colorectal cancer, our method may prove to be useful to select expected responders to therapy by determining EGFR activity in biopsy samples or surgical specimens.

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