LIGAND-INDUCED EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) OLIGOMERIZATION IS KINASE-DEPENDENT AND ENHANCES INTERNALIZATION*

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The current activation model of the epidermal growth factor (EGF) receptor (EGFR) predicts that binding of EGF results in dimerization and oligomerization of EGFR leading to the allosteric activation of the intracellular tyrosine kinase. Little is known about the regulatory mechanism of receptor oligomerization. In this study, we have employed Förster resonance energy transfer between identical fluorophores (homo-FRET) to monitor the dimerization and oligomerization state of the EGFR before and after receptor activation. Our data show that in the absence of ligand, approximately 40% of the EGFR molecules is present as inactive dimers or predimers. The monomer/predimer ratio was not affected by a deletion of the intracellular domain. Ligand binding induced the formation of receptor oligomers, which were found both in the plasma membrane and in intracellular structures. Ligand-induced oligomerization required tyrosine kinase activity and nine different tyrosine kinase substrate residues. This indicates, that the binding of signaling molecules to activated EGFRs results in EGFR oligomerization. Induction of EGFR predimer or preoligomers using EGFR fused to the FK506 Binding Protein (FKBP) did not affect signaling but was found to enhance EGF-induced receptor internalization. Our data show, that EGF receptor oligomerization is the result of EGF signaling and enhances EGF internalization.

The epidermal growth factor receptor (EGFR or ErbB1) has an essential role in the regulation of growth and differentiation of a large range of cell types. EGFR belongs to the family of ErbB of which all four members have been implicated in the development of different cancers (1). The first step in the signal transduction cascade is the binding of its ligand such as EGF or TGF-α to the ectodomain, which provokes receptor dimerization and oligomerization. Deletion of the dimerization domain, which is present in domain II of the EGFR ectodomain, blocks receptor activation completely, demonstrating that receptor dimerization is critical for the allosteric activation of the tyrosine kinase (2,3). Activation of the receptor tyrosine kinase (RTK) results in crossphosphorylation of the receptors and the phosphotyrosines in the intracellular domain serve subsequently as docking sites for adaptor proteins such as Grb2 and Shc and enzymes like phospholipase Cγ (PLCγ), which contain phosphotyrosine-specific SH2 or PTB domains. Eventually, the active ligand/receptor complex becomes internalized via both clathrin-dependent and clathrin-independent pathways, followed by the intracellular transport to lysosomes where the receptor ligand complexes are degraded (4).

Although EGF-binding and dimerization seem to be strictly connected, both microscopical and biochemical studies have demonstrated that in resting cells the receptor is already found on the cell surface as non-active dimers, the so-called predimers. This phenomenon was initially discovered using electron microscopy and immunogold labeling of the EGFR: in the resting cell ~35% of the total receptor population was present as receptor predimers (5). These observations were confirmed by chemical cross linking and co-immunoprecipitation studies with differentially tagged EGFRs (6-8). More recently, also advanced light microscopical methods have been used to address this question. EGFR predimerization has now been demonstrated using fluorescence correlation spectroscopy (FCS), steady-state fluorescence anisotropy,
Förster resonance energy transfer (FRET) and single molecule imaging (9-15). Recent structural data showed that the dimerization of the C-terminal part of the kinase prevents kinase activation and represents a mechanism how the EGFR tyrosine kinase is inhibited in resting cells (16). Factors controlling EGFR predimer formation, ligand-induced oligomerization and the function of both phenomena are poorly understood.

In this paper we have used homo-FRET imaging to investigate the regulation of EGFR predimerization and oligomerization. We have recently developed a homo-FRET imaging method that allows quantification of the degree of protein clustering on a subcellular level (17). It is based on Förster resonance energy transfer between identical fluorophores (homo-FRET), meaning that the nanometer proximity between identical reporter fluorophores such as GFP is detected with high sensitivity. Application of this method showed that in the resting cell approximately 40% of the total EGFR population is already present as predimers (17). A large increase in receptor oligomerization is seen after ligand binding. EGFR predimers were formed independent of kinase activity whereas EGF-induced receptor clustering was found to require both kinase activity and the substrate tyrosine residues. Inducing predimerization/oligomerization using FKBP dimerization domains demonstrates that receptor predimer formation is enhancing EGFR internalization.

**EXPERIMENTAL PROCEDURES**

**Plasmid construction**- To create the FKBP-mGFP containing constructs, mGFP was first PCR amplified from pEGFP-N1 using the following primers:

5’-atatactagtatgtagacacccgtagctgc-3’ and 5’-atatggatccctactgtgctcctgcagcag-3’,

which introduced flanking restriction sites SpeI and BamHI (underlined). The eGFP PCR product was inserted into the corresponding sites of pC4-Fv1E (Ariad Pharmaceuticals, Cambridge, MA) to produce pC4-Fv1E-GFP. A monomeric variant of eGFP (mGFP) was constructed by site-directed mutagenesis using the primers:

5’-cagtccagctgacaaagaggcagaggtgc-3’ and 5’-gtgatcgcgtctgtgagctgttggtg-3’,

(mutated codon in bold) as described previously (18), resulting in the FKBP-mGFP plasmid.

pCDNA3-EGFR-9YF was made by site-directed mutagenesis (Stratagene mutagenesis kit) of the human EGFR cDNA, resulting in tyrosine to phenylalanine transitions at position Y845, Y974, Y992, Y1045, Y1068, Y1101, Y1148 and Y1173. The EGFR constructs were PCR amplified with the primers:

5’-atatataaatgtgcagcctccyggcaggcggg-3’ and 5’-atatattctagatgcctcaataactaacttttggtg-3’ ,

introducing flanking MunI and XbaI sites (underlined), and inserted into pC4-Fv1E-mGFP. To construct EGFR-mGFP and EGFR K721A-mGFP, the FKBP domain was removed by digestion with XbaI and self-ligation. For cells stably expressing the gene products, the EGFR constructs were subcloned into pCDNA3.1-zeo (Invitrogen, Carlsbad, CA). Final constructs were amplified in E.coli, purified using an endotoxin-free plasmid isolation kit and confirmed by sequencing.

**Cell culture**- A431 cells (ATCC #CRL 1555), NIH 3T3 2.2 and Her14 were grown in DMEM supplemented with 2 mM L-glutamine and 7.5% fetal calf serum at 37°C in 5% CO2 under humidified conditions. Transient transfection with all constructs was performed with Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). Cells stably expressing EGFR-FKBP-mGFP or its mutants were produced using selective growth conditions (500 μM Zeocine), and FACS-sorting. For microscopy, cells were grown on coverslips for 2 days to 50% confluency.

**EGFR internalization and phosphorylation**- The internalization rate constant (K_i) was determined as described previously ((19). Briefly, cells grown were grown on 24-wells CellBind plates to 80% confluency serum starved over night in 0.5% FCS/ DMEM. Cells were incubated for 1 hr with ice-cold binding medium (DMEM, 0.1 % BSA, 20 mM HEPES pH 7.4) supplemented with either 1 μM AP20187, or 0.1% ethanol (mock). EGF (Oxford Biotechnology) was labeled with 125I by the chloramine T method yielding a typical specific activity of >400,000 cpm/ng. EGB4 nanobody (15 μg) was labeled with 1 mCi 125I in Iodogen-coated glass tubes with a typical specific activity of >1500 cpm/ng. Subsequently, 1 ng/ml 125I-EGF or 125I-EGB4 was added in a total volume of 0.5 ml for 3, 6, 9 and 12 min at 37°C. To collect surface bound EGF, cells were incubated for 5 min with ice-cold acid wash buffer (for EGF: 150 mM NaCl, 25 mM NaOH/CH3COOH pH 3.8; for EGB4: 250 mM NaCl, 100 mM glycine pH2.5), and
internalized EGF was collected in 1M NaOH. The ratio of internalized and surface radioactivity was plotted against time yielding the internalization rate constant ($K_e$).

NIH 3T3 2.2 cells were transfected using Lipofectamine 2000 with the indicated constructs, incubated overnight in 0.5% FCS/DMEM, followed by an incubation with 1 μM AP20187 for 1 hr or with 8 nM EGF for 10 min, or a combination of both. After washing, cells were lysed in lysis buffer (1% Triton X-100, 100 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, pH 7.4, Complete™ mixture of protease inhibitors). The EGFR-FKBP-mGFP constructs were immunoprecipitated using anti-GFP (Roche), size-separated with SDS-PAGE and immunoblotting with indicated antibodies.

RESULTS

EGFR forms predimers in the plasma membrane. The dimerization/oligomerization state of the EGFR in the cell was analyzed by homo-FRET imaging, a method that we have previously developed (17). For these studies the EGFR was fused with a monomeric variant of eGFP (A206K), which has a reduced dimerization binding affinity ($K_D$~74 mM) (18). Homo-FRET imaging was done with a confocal time-resolved fluorescence anisotropy imaging microscope (20), which allows for a direct quantification of the number of fluorophores in a nanometer scale cluster (17). This method is based upon the fact that GFP monomers have high anisotropy, which decreases with increasing degree of clustering. The measured (steady state) anisotropy $r$ relates directly to cluster size $N$, which in addition depends on the efficiency of the energy transfer and the relative orientation of the fluorophores (22). This relation is simplified when the limiting anisotropy ($r_{inf}$) in the time-resolved anisotropy decay is measured instead of the steady-state anisotropy, since $r_{inf}$ is not affected by variations in the efficiency of energy transfer (20). The calibration value for the anisotropy of GFP monomers ($r_{mono}$, theoretically 0.4 but often lower due to high NA objectives) is determined using a solution of 10 μM GFP in 50/50 glycerol/buffer. At this concentration GFP can be considered as monomeric since this concentration is well below the affinity value for GFP dimerization ($K_D$ = 110 μM) (18). To determine the degree of protein clustering in the cell we used reference proteins that contain one copy of mGFP and one or two copies of the dimerization domain from the FK506-binding protein, FKBP. With these reference proteins either dimerization or oligomerization can be induced by the addition of the ligand for the FKBP domain: AP20187 (17). Control experiments revealed that our EGFR-FKBP-mGFP constructs were not activated by the addition of AP20187 (Fig. S1A).

Mouse NIH 3T3 fibroblasts, which are devoid of endogenous EGFR (clone 2.2), were stably transfected with a vector encoding mGFP or EGFR-mGFP (for an overview of all EGFR constructs used in this study see Fig. 1A). FACS sorting was performed to have comparable expression levels of the fluorescent constructs, which related to ~50,000 proteins/cell. Control experiments demonstrated that activation of the

Homo-FRET- Fluorescence anisotropy microscopy was essentially performed as described before (20). A 473 nm pulsed diode laser (Becker and Hickel BDL-473; Berlin, Germany) operating at 50 MHz was directly coupled into the modified confocal scan head (C1, Nikon Instruments Europe). A linear polarizer (Meadowlark, Frederick, CO, USA) was positioned in the laser beam to define the excitation polarization direction. The microscope was equipped with a 60x, NA=1.20 water immersion objective (Plan Apo, Nikon). For GFP a value of $r_0 = 0.38$ was found instead of the theoretical value of 0.4. The emission light was split into a parallel and perpendicular channel with a broadband polarizing beam splitter cube (PBS, OptoSigma, Santa Ana, CA, USA). The two emission channels were coupled to LIMO detection systems (LIMO, Nikon Instruments BV, Badhoevedorp, The Netherlands) (21), equipped with an internal photon counting photomultiplier tube. All images were recorded in a 160x160 pixel mode, covering an area of 50x50 μm. The procedures of data analysis, synchronization and correction for sensitivity differences between the two channels were based on using reference dyes. Anisotropy analysis showed that $r_{inf}/r_{mono}$ values between 1 and 0.87 were found in the monomeric situation, whereas the dimers generate values between 0.87 and 0.77, and values between 0.77 and 0, with a mean value of 0.72, correspond to oligomers. The cluster size images were calculated using anisotropy images. Binning with a factor of 2 was required to obtain sufficient signal to discriminate $N_{AV}=1$, $N_{AV}=2$ and $N_{AV} \geq 3$. 
EGFR-mGFP construct is indistinguishable from the wild type EGFR, which is in agreement with other publications (15). To determine the anisotropy \( r_{\text{inf}} \) at least five cells per condition were analyzed, and the average value of \( r_{\text{inf}} \) per cell was used for statistical analysis. The results are presented as the degree of polarization (\( r_{\text{inf}}/r_{\text{mono}} \)). Comparing cells expressing mGFP or EGFR-mGFP, show that EGFR-mGFP has a significant higher loss of anisotropy than cytoplasmic mGFP (Fig. 1B). To prove that the anisotropy loss is due to homo-FRET in receptor predimers, we recorded the average time resolved anisotropy decay (Fig. S2). A typical homo-FRET profile is observed: an immediate drop of \( r \) (< 1 ns) that levels off to \( r_{\text{inf}} \) demonstrating that homo-FRET occurs with high efficiency. This is an important parameter that confirms that the reference constructs can be used. In previous work, we showed that the amount of depolarization due to homo-FRET can be directly related to the degree of clustering (17). For EGFR-mGFP in resting cells, the relative anisotropy value \( r_{\text{inf}}/r_{\text{mono}} \) is 0.89. This corresponds to a fraction of clusters of 0.4, which means that 40% of all EGFR molecules is part of a predimer or precluster.

As a control experiment, we co-expressed EGFR-mGFP in cells with a high level of endogenous EGFRs (3x10^5 and 2x10^6 receptors/cell for Her14 and A431 cells respectively). This is based upon the idea that heterodimerization of EGFR-mGFP with endogenous EGFR does not result in homo-FRET, resulting in anisotropy values similar to the mGFP control values (10). Anisotropy analysis demonstrated that this is indeed the case: the anisotropy of EGFR-mGFP in these cells was increased in Her14 as well as in A431 cells to the situation observed for mGFP (Fig. 1B). In addition, we analyzed whether this predimer formation was dependent upon the concentration of EGFRs by plotting the intensity values of individual pixels against their anisotropy value. No differences in anisotropy values were apparent in pixels with higher intensities indicating that EGFR predimer formation is concentration independent (Fig. S3). In conclusion, our data show that approximately 40% of the total EGFR-mGFP population is predimerized in the plasma membrane of non-stimulated NIH 3T3 2.2 cells.

Ligand-induced receptor oligomerization.

We next investigated the effect of EGF on the oligomerization state of EGFR using homo-FRET imaging. Cells expressing EGFR-mGFP were treated for 10 min at 37°C with 8 nM EGF and fixed with 4% formaldehyde. In the resting cell, EGFR-mGFP is primarily located in the plasma membrane with more intense staining in membrane ruffles (Fig. 2A, top panels). In EGF-stimulated cells, EGFR-mGFP becomes increasingly present in intracellular vesicles reflecting the EGF-induced internalization of the active EGFR (Fig. 2A, top panels). Effects on anisotropy \( r_{\text{inf}} \) are indicated in false colors: a clear effect of EGF on the anisotropy is observed reflecting the EGF-induced oligomerization (Fig. 2A, bottom panels). Note that also the internalized receptors in the early endosomes are oligomerized. Using the reference \( r_{\text{inf}} \) values of monomers, dimers and oligomers, we converted the anisotropy values into a cluster size image (Fig. 2C). In the resting state, the EGFR population is found as a mixture of monomers and dimers. After treatment of the cell with EGF, the majority of EGF receptors was found in nanoscale clusters of 3 or more receptors per cluster (Fig. 2C).

From a direct comparison of the average depolarization \( r_{\text{inf}}/r_{\text{mono}} \) to reference values (Fig. 2B) we can conclude that the EGFR-mGFP receptor forms large clusters upon stimulation with EGF. As described in Experimental procedures the \( r_{\text{inf}} \) is calculated on basis of Gaussian fitting of the anisotropy values of all pixels in the image. To demonstrate the homogeneity in the response the anisotropy values of two representative cells from both conditions are presented and show a true shift in the mean value of \( r_{\text{inf}} \) (Fig. S4). In conclusion, comparison of the calculated average anisotropy values from non-stimulated and EGF-stimulated EGFR-mGFP expressing cells demonstrate a significant decrease in anisotropy and consequently an increase in EGFR clustering in EGF-stimulated cells.

Receptor oligomerization is kinase-dependent. To analyze the role of EGFR tyrosine kinase activity in the oligomerization of EGFR, a kinase dead (K721A) EGFR construct was fused with mGFP. Cells expressing wild type EGFR-mGFP or EGFR-K721A-mGFP were incubated for different time periods with 8 nM EGF, fixed and analyzed with homo-FRET imaging. For EGFR-K721A-mGFP, a distribution pattern was found that was at time point 0 similar to that of the wild type receptor (Fig. 3A+B). After EGF-stimulation
intracellular vesicles appeared after 10-20 min indicative for receptor internalization (Fig. 3A+B), which is in agreement with our previous studies (23). In the absence of ligand, the K721A receptor and wild type receptor display similar anisotropy values (Fig. 3C). This indicates that the kinase dead EGFR forms predimers in a similar extent as wild type EGFR, thus, predimer formation is kinase independent. For the wild type receptors, the anisotropy values decreased within 5 min to values corresponding with receptor oligomers (Fig. 3A-C). Ligand-induced EGFR oligomerization was maintained for at least 20 min after stimulation.

A gradual increase in anisotropy is seen from 10 to 45 min after activation, which might be caused by receptor dissociation or lysosomal degradation of receptor oligomers. The effect of EGF on the oligomerization of the kinase dead mutant was analyzed for the same period of time. Remarkably, this mutant did not display any change in anisotropy for the entire observation period of 45 min, indicating that neither the amount of receptor predimers is affected nor that kinase dead EGFRs oligomerize. Based on these results we conclude that the EGFR predimer formation is kinase independent while the ligand-induced EGFRs oligomerize. EGFR clustering stimulates receptor internalization. An important question concerns the possible function for both receptor predimer formation and receptor oligomerization. Modeling of EGFR activation via monomers and predimers predicts that activation via the predimers would result in a faster phosphorylation of tyrosine residues in the intracellular domain of the EGFR.

EGFR oligomerization requires receptor tyrosine phosphorylation. Since kinase activity was found to be essential for EGFR oligomerization, we wanted to see whether preventing tyrosine phosphorylation of the EGFR had similar effects on receptor oligomerization. To investigate this, we used an EGFR mutant in which nine C-terminal tyrosine residues at the following positions Y845, Y974,Y 992, Y1045, Y1068, Y1086, Y1101, Y1148, Y1173 were mutated into phenylalanines (EGFR-9YF). Control experiments show that phosphorylation of the wild type EGFR-FKBP-mGFP protein is induced by EGF, in contrast to the K721A and 9YF mutants (Fig. 4A). 3T3 2.2 cells, stably expressing these constructs were stimulated with 8 nM EGF for 10 min, or left untreated, and fixed. In the absence of ligand, the anisotropy of the wild type receptor, K721A and 9YF mutants is similar, reflecting a similar degree of predimer formation (Fig. 4B). As expected, the wild type receptor shows a decrease in anisotropy after EGF stimulation, reflecting an increase in homo-FRET and consequently in receptor oligomerization. EGF-treatment of the cells expressing the K721A or 9YF mutants did not result in a change in anisotropy as compared to untreated cells. This observation demonstrates that the kinase dependent EGFR oligomerization requires the phosphorylation of tyrosine residues in the intracellular domain of the EGFR.

EGFR clustering stimulates receptor internalization. An important question concerns the possible function for both receptor predimer formation and receptor oligomerization. Modeling of EGFR activation via monomers and predimers predicts that activation via the predimers would result in a 100x faster activation or phosphorylation of the receptor (13). Consequently, the predimerization or even pre-oligomerization would result in a faster signaling and higher sensitivity of the cell for EGF. To test a role for EGFR predimer/oligomer formation in signalling, we expressed EGFR fused with one or two copies of FKBP (Fig. 1A). Cells were either pretreated with 1 μM AP20187 for 1 hr to induce predimer/oligomer formation, or left untreated, followed by an incubation with 8 nM EGF for 9 min at 37°C. Anisotropy analysis already showed that incubation of the cells expressing this construct leads to reduction in anisotropy reflecting the increase in EGFR predimers (Fig. 2B). However, combined treatment with AP20187 and EGF did not, at least under the conditions used, enhance receptor activation when compared to EGF alone (Fig. 5A, and Fig. S5). Also down stream targets of EGFR signaling such as Akt and MAP kinase were not affected, indicating that no function for receptor predimerization in the EGFR activation process can be discerned (Fig. 5A, Fig. S5). Moreover, similar results were obtained with an EGFR construct with 2xFKBP, which becomes pre-oligomerized (Fig. 5A, Fig. S5). Further refinement of this analysis with additional time intervals (3-12 min) and lower ligand concentrations (0.25 - 8 nM EGF) yielded similar results.

This approach was subsequently used to investigate a possible role for EGFR clustering in receptor internalization. The internalization rate constant (K_e) was determined using a single domain Llama antibody, or nanobody, that does not activate the receptor nor competes for EGF binding (24). Cells stably expressing the
indicated EGFR-FKBP-mGFP or EGFR-2xFKBP-mGFP constructs were either untreated or pretreated with AP20187 to induce EGFR predimerization or pre-oligomerization respectively, and then incubated with radiolabeled anti-EGFR nanobody (125I-EGb4), either in the absence of EGF or in the presence of a high concentration EGF (8 nM EGF). The data show that in the control situation the monovalent EGb4 nanobody is internalized with at a low rate, reflecting fluid phase endocytosis (Fig. 5B). Addition of EGF stimulates the internalization rate constant significantly demonstrating the dominant role for kinase activity in the internalization process. However, internalization rate constants were dramatically increased after predimerization of the EGFR prior to EGF treatment. Remarkably, pre-oligomerization of the receptor did not further increase this K_e value, indicating that predimerization is already sufficient to stimulate internalization.

To further substantiate the role for EGFR predimer formation in the internalization process, we made use of the fact that kinase dead EGFR does not oligomerize upon EGF-binding and is internalized upon binding of EGF albeit at a low rate. Internalization rate constants were determined for wild type and kinase dead EGFRs and compared with rate constants from the same receptors that were predimerized by a preincubation with 1 μM AP20187 for 1 hr, performed at 4°C to prevent receptor internalization. The cells were incubated with a high dose of EGF (8 nM EGF) for confocal microscopy and a low dose (1 ng/ml) 125I-EGF to determine the internalization rate constant (K_e). While the non-treated EGFR-FKBP-mGFP is predominantly present in the plasma membrane, the AP-treated cells show internalized EGFR as judged from the presence of intracellular endocytic vesicles (Fig. 5C). Also at a low dose, the addition of EGF to cells with predimerized EGFR induces a prominent internalization of the EGFR. At this low EGF dose the K_e of wt-EGFR internalization is higher than at a high EGF dose (Fig. 5C), which is in agreement with previous work (25). As expected, the kinase dead EGFR-FKBP-mGFP construct is endocytosed at low rate (K_e, kd = 0.10 min⁻¹), which is much slower than the internalization of the wild type EGFR (K_e, wt = 0.22 min⁻¹). Increasing the amount of EGFR predimers using AP20187 resulted in a ~30% increase in the K_e of the wild type receptor (K_e = 0.29 min⁻¹). The K_e of the K721A construct was even doubled from 0.10 min⁻¹ in the absence to 0.20 min⁻¹ in the presence of AP20187. In summary, these results show that predimerization of the EGFR increases the internalization rate of the EGFR, independent of ligand concentration or kinase activity.

DISCUSSION

To monitor the dimerization and oligomerization of EGFR before and during receptor activation, we applied a novel non-invasive homo-FRET based technique that allows the imaging of receptor dimers and oligomers in its cellular context. A time-resolved anisotropy analysis was chosen since data obtained with this method are more accurate in the determination of protein clustering than steady-state measurements (20). Analysis of EGFR clustering was done using reference EGFR constructs that allow for controlled dimerization or oligomerization by induced binding (17). For both the reference and the EGFR-mGFP constructs, a high homo-FRET efficiency was observed, which means that the GFP fluorophores are in close proximity and their relative orientation is very similar. This is the major prerequisite for using the reference anisotropy values for monomer, dimers and oligomers. The reporter protein that we have used in this study is the monomeric version of the enhanced GFP, GFP-A206K, or mGFP. This mutant was found to have a reduced homo-association constant as compared to wild type GFP, and was therefore considered to be strictly monomeric (18). The reliability of this determination is high, despite the fact that in an ideal case membrane bound GFP would be preferred. In the plasma membrane, however, artifacts can be introduced when membrane anchoring induces clustering (18). The homo-FRET data demonstrate that in resting cells approximately 40% of the total population of EGFR is present as predimer. Evidence for the presence of EGFR predimers is based upon different arguments. Firstly, the anisotropy of EGFR-mGFP is predominantly present in the plasma membrane, the AP-treated cells show internalized EGFR as judged from the presence of intracellular endocytic vesicles (Fig. 5C). Also at a low dose, the addition of EGF to cells with predimerized EGFR induces a prominent internalization of the EGFR. At this low EGF dose the K_e of wt-EGFR internalization is higher than at a high EGF dose (Fig. 5C), which is in agreement with previous work (25). As expected, the kinase dead EGFR-FKBP-mGFP construct is endocytosed at low rate (K_e, kd = 0.10 min⁻¹), which is much slower than the internalization of the wild type EGFR (K_e, wt = 0.22 min⁻¹). Increasing the amount of EGFR predimers using AP20187 resulted in a ~30% increase in the K_e of the wild type receptor (K_e = 0.29 min⁻¹). The K_e of the K721A construct was even doubled from 0.10 min⁻¹ in the absence to 0.20 min⁻¹ in the presence of AP20187. In summary, these results show that predimerization of the EGFR increases the internalization rate of the EGFR, independent of ligand concentration or kinase activity.
associates with equal preference to EGFR and EGFR-mGFP, the formation of hetero- or homodimers is proportional to the relative expression levels of both receptor types. As the amount of non-fluorescent receptor increased, a decrease in apparent cluster size was found, which approximated the level of cytosolic monomeric mGFP. Similar data were provided by Lidke et al, who used CHO cells instead of 3T3 fibroblasts (10). Thirdly, the clustering is concentration independent, indicating that predimer formation is not the result of overexpression of the receptor.

Our homo-FRET results are in perfect agreement with both light and electron microscopical data obtained from A431 cells (5,15). Recently, the AP-2 binding region in the C-terminal part of the tyrosine kinase domain has been shown to interact with each other in the predimer, resulting in an autoinhibited receptor dimer (16). Mutations in this ‘electrostatic hook’ were found to activate the tyrosine kinase. Thus, predimerization of the EGFR is a mechanism to inhibit the EGFR in the resting cell (16). An interesting question is the mechanism that controls EGFR predimer formation. First of all, predimerization is not the result of basal receptor activity in the absence of EGF. This can be concluded from the observation that for the kinase-dead EGFR, no significant differences in anisotropy values were obtained when compared to the non-stimulated wild type EGFR (Fig. 3). Recently, cholesterol levels were found to regulate the number of dimers (11). We have previously demonstrated that the EGFR colocalizes with the lipid raft marker GM1 (24). Based on these data we hypothesize that the partitioning of EGFR in lipid raft domains might play a role in the stabilization of the EGFR predimer.

EGFR activation modeling studies have suggested that signaling via the predimer might occur two orders of magnitude faster than via the monomeric receptor simply because the time to find a binding partner is not required (13). To study the functional importance of EGFR predimerization we have increased the amount of receptor preimers using the FKBP domain. Importantly, our EGFR-FKBP-mGFP construct was not activated by the FKBP mediated predimerization, which is in contrast to previous studies (26). This may be caused by the fusion with mGFP or by differences in orientation of the dimerization domain. Thus, our FKBP-mediated predimerization system generates functional, non-active EGFR predimers. The amount of EGFR predimers was enhanced by treatment of the cells with the FKBP ligand, which did not affect EGF-mediated signaling. Also further refinement of the assay, by checking at shorter time intervals or different EGF concentrations did not reveal any difference. However, Chung et al. have showed recently that at very short time intervals (14.6 s) differences in EGFR phosphorylation were noticeable (27). Although dimer formation itself is essential for receptor activation, an increase in the amount of receptor predimers did not affect signaling at longer time intervals.

The FKBP system was subsequently used to analyze a possible function for the EGFR clustering in the internalization process of the EGFR. To directly monitor receptor internalization, we used a VHH or nanobody recognizing the ectodomain of the EGFR that does not block EGF-binding nor activate the receptor (24). While the induction of receptor dimerization prior to EGF binding clearly stimulated EGFR internalization, receptor oligomerization did not stimulate this process any further. These results show that predimerization of the EGFR enhances the ligand-induced internalization of the EGFR. Receptor oligomerization did not stimulate this process any further, indicating that predimer formation is already sufficient to prime EGFR for internalization. This might be mediated by the two clathrin-binding motifs that are present in the intracellular domain of the EGFR: a double leucine motif at position 1010/1011 and a tyrosine-based motif at position 974 (26,28). We suggest that receptor oligomerization contributes to the internalization process by enhancing the binding of EGF receptors to the AP2 complexes of the internalization machinery. This would result in a binding of EGFR to already preformed clathrin-coated pits, which is in line with recent observations (29).

In conclusion, while the formation of receptor dimers is essential for signaling, the number of EGFR predimers is not, but is involved in the onset of EGFR internalization, both for kinase dead and wild type EGFRs.

Ever since the report of Yarden and Schlessinger, ligand-induced aggregation of the EGFR was considered as the activation step of the tyrosine kinase (30). In contrast, our data show that receptor oligomerization after EGFR activation is the result of receptor tyrosine kinase activity rather than provoking it. The
results obtained with the 9YF mutant suggest that the kinase-induced oligomerization is exerted via the phosphotyrosines located in the intracellular domain of the EGFR. Based upon our data the following sequence of events during the process of EGFR activation is proposed. The receptor is present both as monomers and predimers in the plasma membrane. Factors involved in the regulation of this system may include the lipid composition of the membrane. Predimers are auto-inhibited and keep receptors in an inactive state (16). Ligand binding induces a conformational change of the ectodomain leading to the reorientation of the intracellular kinase domains, resulting in the activation of the asymmetric kinase dimer. As signaling proceeds, activated receptors will bind to phosphotyrosine binding proteins such as actin, Cbl and Grb2 and resulting in the oligomerization of the EGFR. This process is occurring more efficiently with increasing amounts of predimers. Receptor oligomerization enhances EGF-induced endocytosis via either the clathrin-dependent or -independent pathways, finally resulting in the downregulation of EGFR.

REFERENCES


FOOTNOTES

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‡ This manuscript is dedicated to prof.dr. A.J. Verkleij in recognition of his great contributions and devotion to science.

The abbreviations used are: EGFR: epidermal growth factor receptor; FACS: fluorescence-activated cell sorting; FCS: fluorescence correlation spectroscopy; FRET: Förster resonance energy transfer; FKBP: FK506 Binding Protein; GFP: green fluorescent protein; mGFP: monomeric green fluorescent protein; PLCγ: phospholipase Cγ; RTK: receptor tyrosine kinase; PTB: phosphotyrosine binding; VHH: VH domain of a heavy-chain-only antibody.

FIGURE LEGENDS

Figure 1.
EGFR forms predimers in the cell membrane.
(A) Overview of constructs used in this study. Full-length EGFR was fused to monomeric GFP (mGFP), in some constructs preceded by one or two FKBP dimerization domains. (B) mGFP or EGFR-mGFP was expressed in different cell lines: 3T3 2.2 fibroblasts devoid of endogenous EGFR, Her14 cells: 3T3 2.2 fibroblasts expressing ~3x10^5 human EGFR/cell, and A431 cells, expressing ~2x10^6 EGFR/cell. Values of r_{inf} were measured and expressed as fraction of r_{mono} (SEM, * p<0.005).
Figure 2. 
EGFR-mGFP is oligomerized after EGF stimulation. 
(A) Cellular distribution of GFP intensities and anisotropy values. 3T3 2.2 cells expressing indicated EGFR-mGFP constructs were stimulated with 8 nM EGF for 10 min, 2 hrs with 1 μM AP20187, or left untreated. Limiting anisotropy values ($r_{\text{inf}}$) were measured as described in Methods and expressed in false colors. (B) Average anisotropy values, expressed as fraction of $r_{\text{mono}}$ of indicated constructs (SEM, * p<0.005). (C) Representation of cluster size values of EGFR-mGFP before and after EGF stimulation in false colors. Anisotropy values of cells shown in (A) were classified as monomers: $N_{AV}$=1 (blue); dimers: $N_{AV}$=2 (green) and oligomers: $N_{AV}$≥3 (red) as described in Methods.

Figure 3 
EGFR kinase activity is essential for EGF-induced oligomerization. 
(A) Cellular distribution of GFP intensities and anisotropy values of 3T3 2.2 cells expressing EGFR-mGFP, followed in time during stimulation with 8 nM EGF. The limiting anisotropy value is determined as described in Methods; (B). Cellular distribution of GFP intensities and anisotropy values of 3T3 2.2 cells expressing EGFR K721A-mGFP, followed in time during stimulation with 8 nM EGF. The limiting anisotropy value is determined as described in Methods; (C) Average anisotropy data of EGFR-mGFP and EGFR-K721A-mGFP, followed in time during stimulation with 8 nM EGF. The limiting anisotropy $r_{\text{inf}}$ is expressed as fraction of $r_{\text{mono}}$.

Figure 4 
EGFR tyrosine phosphorylation is essential for EGF-induced oligomerization. 
(A) 3T3 2.2 cells expressing wild type, K721A, ΔICD or 9YF EGFR-mGFP were treated with 8 nM EGF for 10 min or left untreated. The limiting anisotropy value $r_{\text{inf}}$ is determined as described in Methods and expressed as fraction of $r_{\text{mono}}$ (SEM, * p<0.005). (B) 3T3 2.2 cells expressing EGFR-mGFP wild type (WT), EGFR-mGFP kinase dead (K721A) and EGFR-mGFP-9YF were whether or not stimulated with 8 nM EGF for 10 min. Lysates were separated by SDS-PAGE, blotted onto PVDF membrane and analyzed using antibodies against the activated EGFR (pY1068) or against GFP.

Figure 5 
EGFR predimerization stimulates receptor internalization but not signaling. 
(A) 3T3 2.2 cells expressing EGFR-FKBP-mGFP were left untreated, incubated with 1 μM AP20187 for 2 hrs at 4°C and/or with 8 nM EGF for 9 min. at 37°C, or a combination of both. Cell lysates were analyzed by immunoblotting using antibodies against the activated EGFR receptor (pY1068), against GFP to detect EGFR-FKBP-mGFP and against the indicated signaling proteins (Akt and MAPK); representative blots are shown, quantification of signaling is shown in Figure S5; (B) 3T3 2.2 cells expressing the indicated FKBP-constructs of wild type EGFR were incubated for 2 hrs with 1 μM AP20187 at 4°C. Then, 1 ng/ml radio labeled nanobody against EGFR (125I- EGb4) was, whether or not in the presence of 8 nM EGF, added at 37 °C for different time periods (3, 6, 9, 12 min), and finally the internalization rate constant ($K_e$) was determined; (C) 3T3 cells expressing EGFR-FKBP-mGFP were incubated with 1 μM AP20187 for 2 hours or 8 nM EGF for 10 minutes, or a combination of both. Cells were analyzed by confocal immunofluorescence microscopy; (D) 3T3 2.2 cells expressing the indicated FKBP-constructs of wild type and kinase dead EGFR (K721A) were incubated for 2 hrs with 1 μM AP20187 at 4°C. Then, 1 ng/ml radio-labeled EGF (125I- EGF) was added for different time periods (3, 6, 9, 12 minutes) and finally the internalization rate constant ($K_e$) was determined.
Figure 1

A

EGFR-mGFP  EGFR-FKBP-mGFP  EGFR-2xFKBP-mGFP  EGFR-∆ICD-FKBP-mGFP

B

\[ \frac{r_{\text{inf}}}{r_{\text{mono}}} \]

3T3  3T3  Her14  A431
Figure 2

A

Intensity

EGFR-mGFP

EGFR-mGFP + EGF

EGFR-FKBP-mGFP

EGFR-FKBP-mGFP + AP20187

EGFR-2xFKBP-mGFP

EGFR-2xFKBP-mGFP + AP20187

B

\[ \frac{r_{\text{inf}}}{r_{\text{mono}}} \]

EGFR-mGFP

EGFR-mGFP + EGF

EGFR-FKBP-mGFP

EGFR-FKBP-mGFP + AP20187

EGFR-2xFKBP-mGFP

EGFR-2xFKBP-mGFP + AP20187

\[ N_w = 1 \quad N_w = 2 \quad N_w \geq 3 \]
Figure 3

A

<table>
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<th>0 min</th>
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Intensity

B

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Intensity

C

![Graph showing the ratio of $r_{inf}/r_{mono}$ for EGFR-mGFP and EGFR-K721A-mGFP over time.](graph.png)
Figure 4

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* p < 0.05
Figure 5

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C

D

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