Ligand-induced Dimer-Tetramer Transition during the Activation of the Cell Surface Epidermal Growth Factor Receptor-A Multidimensional Microscopy Analysis*

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The epidermal growth factor receptor (EGFR) is a member of the erbB tyrosine kinase family of receptors. For many years it has been believed that receptor activation occurs via a monomer-dimer transition that is associated with a conformational change to activate the kinase. However, little is known about the quaternary state of the receptor at normal levels of expression (<10⁶ receptors/cell). We employed multidimensional microscopy techniques to gain insight into the state of association of the human EGFR, in the absence and presence of ligand, on the surface of intact BaF/3 cells (50,000 receptors/cell). Image correlation microscopy of an EGFR-enhanced green fluorescent protein chimera was used to establish an average degree of aggregation on the submicron scale of 2.2 receptors/cluster in the absence of ligand increasing to 3.7 receptors/cluster in the presence of ligand. Energy transfer measurements between mixtures of fluorescein isothiocyanate-EGF and Alexa 555-EGF were performed using fluorescence lifetime imaging microscopy as a function of the donor:acceptor labeling ratio to gain insight into the spatial disposition of EGFR ligand binding sites on the nanometer scale. In the context of a two-state Förster resonance energy transfer (FRET)/non-FRET model, the data are consistent with a minimum transfer efficiency of 75% in the FRET population. The microscopy data are related to biophysical data on the EGFR in the A431 cell line and the three-dimensional structure of the ligated EGFR extracellular domain. In the context of a monomer-dimer-oligomer model, the biophysical data are consistent with a significant fraction of ligated EGFR tetramers comprising two dimers juxtaposed in a side-by-side (or slightly staggered) arrangement. Our data are consistent with a specific higher order association of the ligand-bound EGFR on the nanometer scale and indicate the existence of distinct signaling entities beyond the level of the EGFR dimer which could play an important role in receptor transactivation.

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The epidermal growth factor receptor (EGFR) is a member of the epidermal growth factor (EGF) receptor tyrosine kinase family (1, 2). Aberrant signaling from the EGFR network contributes to a number of processes important to cancer development and progression, including cell proliferation, apoptosis, angiogenesis, and metastatic spread. EGF overexpression and truncation (1) have both been observed in common cancers including brain, lung, breast, colon, and prostate, giving credence to the notion that a molecular understanding of EGFR activation will yield opportunities for developing new anticancer drugs (1).

Ever since Yarden and Schlessinger’s report (3) on ligand-induced aggregation of the EGFR, it has been considered that activation of the EGFR involves the formation of ligand-induced receptor oligomers resulting in kinase activation, transautophosphorylation, and a cascade of intracellular signaling (3–5). However, although experimental evidence for soluble EGFRs in solution is consistent with a ligand-induced allosteric dimerization model (6), there is a growing body of evidence that cell surface EGFR activation depends on conformational changes within preformed dimers, interactions between receptor dimers, heterodimerization, cross-talk with other receptor types, and ligand-independent lateral propagation of the activation processes (7–15). Single molecule studies have also identified a signal amplification mechanism involving the dynamic clustering of the EGFR (16). Recent crystallographic studies have yielded structures of several erbB ectodomains: EGFR (17–19), erbB2 (20), and erbB3 (21) in tethered and untethered conformations. Furthermore, a structure for the EGFR kinase domain has also been reported (22). Although the structures of the ectodomains reinforce a role for a dimer in the EGFR activation mechanism, they are not necessarily informative for the spatial organization of the full-length receptor in the context of the intact cell (23).

The importance of the aggregation states and spatial distribution of molecules to the input level of the erbB signal transduction network (“lateral signaling”) (1) becomes more apparent when it is recognized that the erbB receptors can form homo- and heterocomplexes and can be activated by different ligands (1). Furthermore, truncations of the extracellular do-

1 The abbreviations used are: EGFR epidermal growth factor receptor; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol; EGF, epidermal growth factor; eGFP, enhanced green fluorescent protein; FITC, fluorescein isothiocyanate; FLIM, fluorescence lifetime imaging microscopy; Fmoc, N-(9-fluorenyl)methoxycarbonyl; FRET, fluorescence resonance energy transfer; HPLC, high performance liquid chromatography; K35EGF, murine pGlu1K35EGF 1–48; RP, reversed phase; wt, wild type.
main also lead to partial activation of the EGFR kinase in the absence of ligands (24, 25).

Considering the importance of macromolecular oligomerization as a biological control mechanism and the unresolved questions of its mechanistic role in signal transduction, it is essential to perform experiments to assess the stoichiometry, extent of oligomerization, and symmetry of the EGFR and its ligands on the cell surface at the nanometer scale. We have used fluorescence microscopy to determine the submicron and nanometer scale association of the EGFR in intact BaF/3 cells. This EGFR/BaF/3 cell system has been used as a model system to examine the biology of the recombinant EGFR in the absence of other EGFR family members and autocrine ligands (26). We produced stable BaF/3 cell lines expressing the EGFR-eGFP chimera at a level of 50,000 receptors/cell and have used image correlation microscopy to determine the average association state of the EGFR-eGFP on the submicron scale (27) in the absence and presence of EGF. To determine the association of the ligand binding sites on the nanometer scale, a biologically active, EGFR derivative bearing a unique monovalent fluorophore conjugation site positioned to avoid interference with receptor binding and activation (see "Materials and Methods") was synthesized by Fmoc chemistry and labeled with suitable donor and acceptor probes. Förster resonance energy transfer (FRET) depends on the inverse sixth power of separation between interacting donor and acceptor fluorophores on the nanometer scale (28) and is therefore sensitive to protein oligomerization. We quantitated ligand FRET using a novel, microscope-based method, fluorescence lifetime imaging microscopy (FLIM) with an LED excitation source (29) and used a multicomponent analysis (30) that enabled us to determine FRET in the presence of fluorescence from non-FRET states. In the context of a monomer-dimer-oligomer model, the biophysical data are consistent with a significant fraction of ligated EGFR tetramers. The results support previous observations of preformed EGFR dimers (9–11, 32) and provide quantitative evidence for the existence of specific higher order nanoscale clustering of the EGFR in the presence of ligand, even in cells expressing normal levels of receptor. We propose that such an arrangement may facilitate trans-dimer activation and/or phosphorylation of the tyrosine kinase (33), as proposed on the basis of some biochemical (14) and single molecule studies (16).

**Materials and Methods**

*Generation of Stable Cell Lines—*Wild type EGFR contained within the mammalian expression vector eGFP-N3 (Clontech) was kindly donated by Prof. Phillipe Bastiaens (EMBL, Heidelberg). The EGFR-eGFP vector was transfected into the interleukin-3-dependent murine hemopoietic cell line BaF/3 using electroporation as described previously (26). Methods of cell selection and determination of receptor numbers are described in Ref. 26. EGFR-eGFP receptor numbers were determined by reactivity with monoclonal antibody 528/Alexa 536 anti-EGFR, Upstate Biotechnology, Inc.), followed by horseradish peroxidase-coupled secondary antibody (Bio-Rad) and ECL reagent (Amersham Biosciences). Immunoreactive bands were quantitated by scanning densitometer and wide line integration in ImageQuant.

*Refolding and Purification of K35EGF—*The EGFR derivative (K35EGF) is a truncated version of murine EGFR (residues 1–48) containing an Asn to pyroglutamic acid substitution to block the N terminus and a single lysine at position 35, which serves as a unique monovalent conjugation site for fluorophores, which is unlikely to interfere with the binding characteristics of the EGF (17). Marine phyto1K35EGF 1–48 (K35EGF) was prepared commercially by manual Fmoc synthesis (Auspep, Australia). The commercial peptide was dissolved in aqueous 10 mM dithiothreitol to a final concentration of 1 mg/ml and left at 25 °C for 2 h to ensure full reduction. The reduced peptide was then purified by RP-HPLC using a LiChrosart 100 RP-18 column (250 × 4-mm inner diameter) installed in an Agilent 1100 Series HPLC fitted with a diode array detector (Agilent Technologies, Australia). The reduced peptide was recovered using a linear 35-min gradient between 0.1% (v/v) trifluoroacetic acid and 65% aqueous CH3CN containing 0.09% (v/v) trifluoroacetic acid. The flow rate was 1 ml/min, and the column temperature was 25 °C. Detection was at 215 and 280 nm using a reference wavelength of 320 nm. Eluting peptides were recovered manually, conditions being made for the desired volume material. Mass spectrometry analysis of the major peak (retention time 28.5 min, data not shown) gave the anticipated mass of 5,286 Da. The recovered peptide was evaporated to dryness using a Savant SpeedVac concentrator (Thermo Electron Corporation). For refolding, the dried peptide was dissolved in 1% (w/v) ammonium bicarbonate, and aliquots (50 μg, based on integrated peak area) were left at room temperature for 18 h to refold under aerobic conditions. The refolded material was recovered by micropreparative RP-HPLC using a Brownlee RP-300 (30 × 2.1-mm inner diameter) column using a linear 60-min gradient between 0.15% (v/v) trifluoroacetic acid and 60% aqueous CH3CN containing 0.125% (v/v) trifluoroacetic acid. The flow rate was 100 μl/min. A major peak with a characteristic retention time of 30.1 min was recovered. Rechromatography of this material under identical conditions gave essentially homogeneous material. Mass spectrometry analysis indicated a mass of 5,280 Da, in agreement with the formation of the three disulfide bonds. The overall yield was typically around 15–20%.

*Preparation and Purification of K35EGF and K35EGF Fluorescent Conjugates—*FITC-K35EGF and Alexa 555-K35EGF were prepared from the parent reactive fluorophores (fluorescein isothiocyanate (FITC) and Alexa Fluor 555 carboxylic acid, such as fluorescent probes (Molecular Probes) and purified by HPLC using methods similar to those described previously (37–39). The oxidized K35EGF recovered from the micropreparative RP-HPLC columns (~10 μg) was evaporated to dryness using the Savant SpeedVac concentrator and redissolved in 0.1 M sodium bicarbonate buffer (25 μl, pH 10.0). FITC (Molecular Probes) was then added (75 μl, 3.3 mg/ml in dimethyl sulfoxide) and the mixture was allowed to stand for 25 °C in the dark. The sample was then buffer exchanged into phosphate-buffered saline using a Naf 5 column (Amersham Biosciences) and the FITC-K35EGF recovered by micropreparative RP-HPLC as described above for the purification of the K35EGF. A major product eluting at 39.0 min was recovered. Mass spectrometry analysis of this peak indicated that the anticipated mass was 5,671 Da. The yield was then assessed: Alexa 555-K35EGF was prepared from the 555 carboxylic acid, succinimidyl ester according to the manufacturer’s instructions (Molecular Probes) and purified by micropreparative RP-HPLC as described above. The Alexa 555-K35EGF was resolved from the K35EGF and had a characteristic retention time of 31.7 min. The potency of the fluorescently labeled K35EGF conjugates was compared with full-length FITC-EGF in binding to a high affinity truncated form of the soluble ectodomain EGFR501 (40). Using a fluorescence anisotropy binding assay (method as described in Ref. 37) the FITC-K35EGF-EGFR501 and Alexa Fluor 555-K35EGF-EGFR501 interactions had affinities in the nanomolar range (Kd of 60 ± 30 nM), compared with a Kd of 50 nM for the full-length FITC-EGF.

*Biological Activity of EGF Analogues—*Mitogenic assays were performed using the LIM1215 colon cancer cell line (42). The cells were initially grown to confluence, trypsin treated, and resuspended in RPMI 1640 with ATG supplement (bovine serum albumin, iron-saturated transferrin, and l-glutamine). The cells were then seeded into 96-well microtiter plates at 5 × 104 cells/well using the Biomek 2000 and incubated for 20 h at 37 °C in 5% CO2 before the addition of the EGF or EGF analogues. The plates were then incubated for another 22 h at 37 °C in a humidified chamber with 5% CO2. Cell proliferation was determined by incubation for a further 2 h, 10 mM NaOH (30 μl/well) was then added and the plates left for 30 min at 25 °C before harvesting onto Unifilter 96GF/C plates (PerkinElmer Life Sciences). The filter plates were dried, and 20 μl of Microsprint 20 (Perkin Elmer Life Sciences) scintillation mixture was added per well and the plates counted using a TopCount NXT beta counter (Perkin Elmer Life Sciences). The data were fitted to a sigmoidal function using Origin (OriginLab). mEGF, or the K35-EGF,
Alexa 555-K35EGF, and FITC-K35EGF analogs were recovered from micropreparative RP-HPLC immediately prior to assay for quantitation and mitogenic assay (43). All compounds were found to have a potency similar to the mEGF control (EC_{50} = 0.021, 0.015, 0.015, and 0.013 nm, respectively).

Cell Staining and Fixation Protocol for K35EGF Fluorescent Conjugates—BaF3 cells stably transfected with wtEGFR were resuspended in serum-free/phenol red-free RPMI (Invitrogen), supplemented with 1 mg/ml bovine serum albumin, and serum starved at 37 °C for 3 h. Cells were washed and then incubated on ice with fluorescein- and Alexa 555-K35EGF (phosphate-buffered saline, 1% bovine serum albumin, in serum-free/phenol red-free RPMI, supplemented with 1 donor and acceptor EGFs. In a simple two-state model, this will give the presence of FRET (9, 48).

The consequence of energy transfer is that the excited state lifetime of the donor fluorophore is given by

\[ \tau_d = \frac{1}{2} \frac{1}{\tau_1 + \frac{1}{\tau_2}} \]  
(30).

This analysis is equivalent to two-state global analysis models that have been used elsewhere in cellular FRET-FLIM investigations to extract populations and transfer efficiencies (13, 30). Although a distribution of lifetime states might be expected from a complex biological system, the recovery of even an average lifetime that represents the FRET population is needed to provide quantitative estimate of energy transfer efficiencies.

Two transfer efficiencies that reflect the energy transfer (in the limit of no non-FRET states) and the average energy transfer (which includes fluorescence from non-FRET states) may be defined.

The energy transfer efficiency (E_{\text{FRET}} in the FRET population is defined as

\[ E_{\text{FRET}} = 1 - \left( \frac{\tau_r}{\tau_2} \right) \]  
(4).

This effectively is the energy transfer efficiency that would be measured in the absence of resolving the FRET and non-FRET contributions, and slope \( \theta \) of the straight-line fit to the AB plot, in Equation 2, using the relationship in Equation 3 (30).

\[ \tau_2 = \frac{1}{1 + \frac{4u(u + v)}{\theta^2}} \]  
(3).

where \( \tau_1 \) is the lifetime of the donor in the FRET population (derived from the two-component analysis), \( \tau_r \) is the lifetime of the donor in the absence of FRET and by definition \( \tau_r < \tau_2 \).

The intensity-weighted average energy transfer efficiency includes the presence of fluorescence from non-FRET states in the calculation. This effectively is the energy transfer efficiency that would be measured in the absence of resolving the FRET and non-FRET contributions,

\[ E = aE_{\text{FRET}} + (1 - a)E_{\text{non-FRET}} \]  
(5).

\[ E_{\text{FRET}} = \frac{1}{2} \frac{1}{\tau_1} + \frac{1}{\tau_2} \]  
(6).

where \( E_{\text{FRET}} \) is the FRET efficiency in the non-FRET population (= zero), \( a \) is the average \( (A/\langle A \rangle) - (B/\langle B \rangle) = B(B) - B(\tau_1) \) is the fractional fluorescence caused by the FRET states, and \( 1 - a \) is the fractional fluorescence from non-FRET states. This definition is used to compare with other measures of energy transfer such as obtained from an intensity-weighted average lifetime (from time-resolved fluorescence measurements) (9) or average donor photo-bleaching time constant (11).

The advantage of the AB plot method, as used here, is that it is insensitive to errors in donor acceptor molar ratio or labeling as long as a sufficient spread of AB values is produced. The AB plot method makes no assumptions about oligomerization state. The significance of this approach is that we are able to FRET efficiencies between labeled ligand binding sites in the presence of fluorescence from non-FRET states.

RESULTS

EGFR Clustering on the Submicron Scale—The classical time-resolved phosphorescence anisotropy decay experiments of Jovin and colleagues (31) established that the EGF-bound EGFR was aggregated in clusters containing 10–50 receptors on the surface of A431 cells. These cells express 2–3 million receptors. Subsequently, Petersen and colleagues (27) used image correlation microscopy to determine the aggregation states of immuno-tagged EGFR on the surface of A431 cells, showing that EGFR is also microclustered (degree of association = 10–50 receptors/cluster, cluster density = 10–19 clusters/μm²) on the submicron scale (27).

We used image correlation microscopy to determine the cluster density and degree of aggregation of the EGFR-eGFP chimeric receptor in the absence and presence of ligand, in cells expressing the EGFR at normal levels (50,000 receptors). Previous investigations have shown that the key biological properties of the EGFR, including its phosphorylation (13), recruitment of signaling molecules, and internalization (35, 36), are not perturbed by attachment of EGFR to enhanced GFP via a flexible linker. In accordance with these reports, we observed that the EGFR-dependant tyro-
In the absence of EGF, the cluster density of receptors on the cell surface cannot be explained by an isolated ligand. The presence of significant FRET between receptor-bound EGF ligands on the cell surface cannot be explained by an isolated ligand model tumorgenic cell lines. To test for fluorophore-fluorophore interactions caused by oligomerization of the EGFR on the nanometer scale, we measured FRET between the FITC-K35EGF donor and the Alexa 555-K35EGF acceptor using in BaF3 cells expressing relatively low levels of wild type receptor. Increasing the proportion of Alexa 555-K35EGF resulted in a faster fluorescence decay of the FITC-K35EGF donor because of FRET between proximal EGF ligands. This is clearly evidenced by the decrease in the phase lifetime as the ratio of Alexa 555-K35EGF increased (Table II). A fit of the fluorescence decay data to a two-state model (Equation 2) yielded two lifetimes for the FITC-K35EGF (in the presence of Alexa 555-K35EGF) of 3.95 ± 0.2 ns (3.9–4.0 ns) and 0.1 ± 0.2 ns (~0.6 to 1.1 ns) (lifetime limits obtained by nonlinear regression analysis). The extrapolated longer lifetime of the FITC-K35EGF on cells reflects the lifetime of the chromophore in the absence of FRET and is similar to that observed for FITC-K35EGF in solution (phase lifetime = 3.9 ± 0.2 ns, modulation lifetime = 4.1 ± 0.2 ns). The limiting short lifetime reflects the rapid energy transfer process between closely interacting EGF ligands within a population of oligomers on the BaF3 cell surface. From the upper boundary of 1 ns we calculate that the energy transfer efficiency in the FRET population is at least 73% (range $E_{\text{FRET}}$ ~ 97 ± 15%, Equation 4). Table II also lists the computed average energy transfer efficiencies (Equation 5). As expected, the average energy transfer efficiency increases from 0 to about 20% as the proportion of acceptor is added. At the highest acceptor to donor ratio we estimate that greater than 90% of molecules are clustered on the nanometer scale in the presence of ligand.

**DISCUSSION**

Using biophysical techniques, we have obtained evidence for submicron and nanoscale clustering of the EGFR in cells expressing a normal level of receptor. It is important to stress that these results were obtained in the absence of other EGFR family members, in the absence of secreted or cell surface ligands, and under conditions in which the EGFR is maintained at the cell surface. In these respects our study differs from analogous biophysical studies on EGFR, which have mainly used model tumorgenic cell lines.

We shall attempt to provide an interpretation of our data in terms of a structural model of the ligated receptor complex. The occurrence of significant FRET between receptor-bound EGF ligands on the cell surface cannot be explained by an isolated ligated EGFR dimer because the distance between the ligands is greater than 10 nm in the x-ray crystal structure (17, 18), and this is outside the detectable distance range of FRET (15). Moreover, the analysis of the image correlation data is compatible with the existence of submicron domains containing multiple copies (N > 2) of the EGFR in the presence of EGF, which could serve as sites for higher order association on the nanometer scale. To gain an estimate of the association state(s) we use the FRET-FLIM-derived result that 90% of the molecules are oligimerized and the image correlation result that the mean degree of association in the present of ligand is 3.7. In the context of a bimodal monomer-oligomer model (49), the combined FRET and image correlation data are compatible with an oligomer number of 4. An alternative model, the dimer-oligomer model, discloses an oligomer number of 3.9. These cal-
Calculations show that even in the presence of minor contributions from monomers and/or dimers, there is a significant fraction of tetrameric ligated EGFR on the surface of BaF/3 cells. We wish to stress that these conclusions are model-dependent (simple aggregation model; smooth spherical surface model for cell membrane) and that more work is needed to specify fully the distribution of aggregate sizes.

To estimate the geometries permitted by the FRET data we...
performed a grid search over a range of interdimer distances and calculated the expected FRET-FLIM results. We used standard kinetic models employed with frequency-domain data (50) taking into account the statistical distribution of lifetime states at each labeling ratio (51). Several tetramer geometries are consistent with the experimental data, but these are bounded to geometries where two of the interfluorophore separations are close to 30–50 Å (0.6–0.7 R_F, R_F = 50–70 Å) and where the two dimers are parallel or slightly staggered with respect to each other. Fig. 5A represents the experimental data (Table II, represented by A_i = M_i sin(β_i), B_i = M_i cos(β_i) versus EGF acceptor:donor molar ratio) together with two simulated plots. The solid lines represent a simulation to a side-by-side dimer-of-dimers (for configuration, see Fig. 5B) with an interfluorophore separation of 0.6 R_F, and the dashed lines represent a simulation of a partially staggered dimer-of-dimers (for configuration, see Fig. 2B) with an interfluorophore separation of 0.7 R_F. A head-to-tail dimer-of-dimers model, composed of a co-linear arrangement of binding sites, is not consistent with the data. Given that the width of the ectodomain surface is ~40 Å and even allowing for some extension of the lysine-tethered fluorophores (~8 Å + fluorophore), the two dimers must be in close contact. We note that the simulation includes the presence of other non-FRET fluorescence populations (monomers and dimers) but does not rule out the existence of minor contributions from other oligomeric forms such as trimers. A significant population of larger oligomers (pentamers, hexamers, etc.) in the BaF/3 cells is excluded from the image correlation results. FRET between ligands bound to the cell surface EGFR has been reported previously (9, 11, 15). Whitson et al. (15), using a ligand-based homo-FRET method, found evidence for a significant degree of energy transfer between EGF derivatives bound to cells expressing an EGFR number, similar to that used in the present study (15). The data were interpreted in terms of a higher order oligomer of the EGFR (15). They showed that significant homo-FRET occurred in cells but not in membrane vesicles prepared from a high expression cell line. Although no figure for the magnitude of energy transfer or interfluorophore separation was provided, the low anisotropy measured at times greater than 1 ns suggests that the energy transfer must be occurring on a subnanosecond time scale, consistent with the results of the present study. Gadella and Jovin (11) reported that energy transfer between fluorescein and rhodamine-labeled EGF on the surface of A431 cells (expressing 2–3 million EGFRs) and measured average transfer efficiencies of 5% at 4 °C for the total population. Similar transfer efficiencies were reported by Martin-Fernandez et al. (9) at 4 °C using Alexa 488-EGF and eosin-EGF conjugates (efficiency 5%). In these studies the investigators used a single acceptor-to-donor labeled preparation (acceptor:donor labeling ratio of 2:1), average lifetimes or photobleaching times were used to compute apparent energy transfer efficiencies, and extrapolations were made

### Table II

| Acceptor:donor ratio | B_i = M_i cos(β_i) ± S.D. (n = 30) | A_i = M_i sin(β_i) ± S.D. (n = 30) | Lifetime (ps) | (E) |
|----------------------|----------------------------------|----------------------------------|--------------|-----|
| 0                    | 0.51 ± 0.03                      | 0.51 ± 0.03                      | 3.94         | NA b |
| 1                    | 0.48 ± 0.03                      | 0.51 ± 0.03                      | 4.26         | -0.02 |
| 2                    | 0.55 ± 0.05                      | 0.49 ± 0.05                      | 3.52         | 0.06 |
| 3                    | 0.54 ± 0.04                      | 0.47 ± 0.04                      | 3.46         | 0.07 |
| 5                    | 0.58 ± 0.03                      | 0.40 ± 0.03                      | 2.74         | 0.18 |

a Molar ratio of Alexa Fluor 555-K35EGF to FITC-K35EGF.

b NA, not applicable.
another model a distinct heterotetrameric species comprising two erbB2-erbB3 heterodimers is formed (14). In light of the present report, the observation of a significant population of homotetramers of the EGFR suggests that the latter model is a distinct possibility.

To conclude, we have provided evidence for a specific and nonrandom high order self-association of the EGFR on both the submicron and nanometer scales in cells expressing a normal level of receptor. The EGFR monomer-dimer activation model should be refined to include the higher order oligomers (Fig. 6). Although there are clearly inactive, unligated dimers on the cell surface, it is still to be established whether ligand-induced kinase activation occurs at the level of the dimer, or whether higher order oligomerization is required to produce a signaling complex. Earlier studies have emphasized the importance of heterotetramers in the formation of active complexes (e.g., EGFRerbB2 (14)). The juxtapositioning of two dimers into a side-by-side (or slightly staggered) configuration may have consequences for kinase activation, phosphorylation, and affinity modulation by intracellular (adaptors) and/or extracellular components (ligands). To understand quantitatively how EGFR oligomers modulate downstream signaling cascades will require development of hybrid imaging approaches. For example, by combining the image correlation analyses with FRET-based assays of phosphorylation state (13) the nexus between oligomerization/clustering and phosphorylation can be examined. Clearly more work is needed to specify fully the oligomeric distributions involved with respect to population, activity, and spatio-temporal disposition within the cell. Photon counting histogram or higher order moment analyses of fluorescence fluctuation data have the potential to provide important insights into this question (41, 53).

Techniques such as those presented here will enable a more thorough characterization of oligomerization states in other cell/receptor systems and lead to an increased understanding of the general role of oligomerization in signal transduction.

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High Order EGFR Oligomerization

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