

PHD THESIS

**MOLECULAR INTERACTIONS OF ERBB RECEPTOR TYROSINE
KINASES – WITH AN OUTLOOK ON THEIR THERAPEUTIC
TARGETING**

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1. INTRODUCTION

1.1. The ErbB family

The ErbB family of class I growth factor receptor tyrosine kinases consists of four members, EGFR (ErbB1, HER1) ErbB2 (HER2, neu), ErbB3 (HER3) and ErbB4 (HER4). Initial observations have suggested that these kinases are in the dimeric form when activated, and can form both homo and heterodimers within the family. The relative expression of the family members on a given cell, their respective affinity for each other, the presence of various ligands that can trigger signaling through them, and the diversity of signaling pathways initiated by the differentially activated molecular assemblies all influence the behavior and fate of ErbB-expressing cells.

ErbB kinases are large glycoproteins with an extracellular domain, a single-chain membrane spanning domain, and an intracellular tyrosine kinase domain which is flanked by noncatalytic regulatory regions. The extracellular domain consists of four (sub)domains designated L1, CR1, L2 and CR2, or - in another nomenclature -domains I-IV. Domains I and III serve as ligand binding sites. By binding simultaneously to both domains, the ligand may alter their relative orientation leading to the conformational change of domain II holding the dimerization arm. In all ErbB proteins except ErbB2, an intramolecular tether formed by domains II and IV buries this dimerization arm, so that the tethered form cannot dimerize and appears to be autoinhibited. After ligand binding, the extracellular domain of the molecule becomes “extended” exposing both ligand binding areas and also the dimerization arm. ErbB2 has unique properties within the family since it has no known soluble ligand, but even without binding a ligand, its dimerization arm is extended. These suggest that ErbB2 possibly functions as a dimerization partner and co-receptor for the other members of the family, and that ErbB2 alone can cause transformation if overexpressed.

According to the commonly accepted theory, dimerization is necessary for ErbB receptor autophosphorylation, and leads to activation of the kinase domain. ErbB3 is the only exception as it has an impaired kinase unit and can transmit signals only when a dimerization partner is present. The mechanism of ErbB activation is not yet known in detail. The classical model of allosteric oligomerization proposes that intramolecular interactions evoked upon EGF binding result in aggregation of ErbB molecules leading to higher affinity for the ligand and to kinase domain activation. This is supported by the activation kinetics of ErbB1 in solution being a secondary function of concentration and the activating effect of bivalent antibodies and chemical

cross-linking. The other model underlines the role of ligand binding and a still uncharacterized consequential conformational change in the transmembrane domain leading to activation of already pre-dimerized receptors. This theory is supported by the presence of high affinity preformed dimers shown by FRET measurements, and spontaneous activation of ErbB1 when diluted in detergents. There are also contradictory findings related to the further spreading of the signal from activated ErbB receptors. Some have found lateral spreading in the membrane after localized stimulation, which would require activation induced dimerization, while others have shown that activation remains local and spreads only "vertically", into the cytoplasm, which can be better explained by the presence of pre-formed signaling units – dimers or oligomers.

After EGF binding, ErbB1 should also be able to form a dimer with the ligandless ErbB2. Members of this ligand-induced (primary) dimer possess high tyrosine kinase activity, transphosphorylate each other starting intracellular signaling cascades, then dissociate. The activated ErbB2 may form a secondary dimer with the kinase domain impaired ErbB3 or another ErbB2, and by phosphorylating it, activate other, distinct signaling pathways. When neuregulins bind to ErbB3 or ErbB4, these can form a primary dimer with ErbB2, while ErbB2 thus activated can form secondary dimers with ErbB1 or other members of the family. Although transactivation via these heterodimers is very plausible, there is limited evidence that proves its occurrence *in situ* in living cells. However, there is biochemical evidence from cells with various ErbB expression spectra that intracellular activation patterns of the three primary dimers are probably different, and this is also true for the secondary dimers, which greatly diversifies the signaling capacity of the ErbB family.

1.2. Role of ErbB kinases in carcinogenesis

ErbB proteins often play an important role in the first steps of malignant transformation. In addition to deregulation of their signaling pathways, mutations causing constitutive or ligand-independent activation and overexpression – mostly based on gene amplification – of ErbB proteins are prominent causes of human tumors. It is generally accepted that overexpression of ErbB1 drives cell survival and proliferation pathways similar to those activated by the regularly expressed receptor. Mutations, however, also appear to play a distinct role in carcinogenesis. The most frequent class III mutants (ErbB1vIII, delta2-7 EGFR) contain a deletion within the extracellular domain leading to constitutive kinase activation. Amplification of the ErbB2 gene was first described in human breast and ovarian cancer, but it often occurs in other human malignancies. ErbB2 is overexpressed in 60% of the inflammatory and in 25% of the non-

inflammatory ductal invasive breast cancers and appears to be a relatively early event in cancer pathogenesis. It can drive tumor proliferation, and play an important role in aggressive tumor behavior associated with poor clinical outcome, thus substantiating the importance of early stage anti-ErbB2 therapies. ErbB1/ErbB2 and ErbB2/ErbB3 complexes have been implicated in some breast and lung cancers and are associated with decreased survival. The physiological and pathological role of ErbB4 is not yet clear.

1.3. Trastuzumab – the first humanized monoclonal antibody against ErbB2

Although ErbB2 overexpression is a bad prognostic factor in breast cancer, ErbB2 is also a good target for personalized molecular therapies, as it is present in large amounts in the membrane of the tumor cells compared to healthy adult tissues. The mechanisms of action of trastuzumab (rhu4D5, Herceptin®), the first humanized anti-ErbB2 antibody probably include ErbB2 activation, internalization and down-regulation, which have previously been postulated as the primary mechanism of direct growth inhibition, as well as antibody dependent cellular cytotoxicity (ADCC). Although ErbB2 overexpression identifies patients who are likely to respond to therapy with trastuzumab, the occurrence of trastuzumab resistance is frequent. In contrast to the tight prognostic correlation and specific therapeutic opportunities, trastuzumab seems to be effective only in 20-50% of ErbB2 overexpressing breast tumor patients, and many of the initial responders have a relapse after a few months. The background of resistance is probably versatile and may differ from case to case. In the simplest approach, any molecular interaction at the cell surface that could hinder trastuzumab binding to ErbB2 is a candidate cause of trastuzumab resistance. Being the first of its class amenable to passaging *in vitro*, the JIMT-1 cell line isolated from a clinically trastuzumab resistant breast cancer patient vastly improved our possibilities of understanding resistance mechanisms.

2. AIMS

The interactions of ErbB kinases with each other and with various membrane proteins define their signaling activity and accessibility as therapeutic targets. Our aim was to contribute to the understanding of mechanisms behind ErbB1 and ErbB2 activation and transactivation, and compare, in this respect, trastuzumab resistant and sensitive breast tumor cell lines. In this context, the following questions were posed:

- Can fluorescence correlation spectroscopy (FCS) be used to detect the diffusion properties of ErbB1 in the membrane of living cells and reveal new information about the mechanism of ErbB1 activation?
- How do FCS and fluorescence recovery after photobleaching (FRAP) data on the mobility of ErbB1 correlate in the same cellular system?
- Does local activation of ErbB1 and ErbB2 by derivatized paramagnetic microspheres give rise to localized or laterally spreading signals?
- Can EGF and trastuzumab, binding to their targets, evoke transactivation of ErbB2 and ErbB1, respectively?
- On trastuzumab resistant JIMT-1 cells, is it the lack of function, or the diminished accessibility of ErbB2 to trastuzumab that causes resistance?
- In case of diminished accessibility, can the MUC4 sialomucin complex be responsible for the masking of ErbB2 on JIMT-1 cells?

3. MATERIALS AND METHODS

3.1. Cells

CHO cells, CHO transfected with ErbB1-eGFP (sub-clones F1-4 and F1-10 expressing ErbB1 with a C-terminally fused eGFP), A431 epidermoid carcinoma, its sub-clone A4-ErbB2-mYFP stably transfected with the ErbB2-mYFP fusion gene (expressing in addition to ErbB1 also ErbB2 with a C-terminal monomeric YFP fusion) SKBR-3 breast tumor cells and JIMT-1 trastuzumab resistant breast tumor cells (kind gift of Jorma Isola,) were cultured in DMEM with 10% FCS. The line F1-4 expressed 10^6 ErbB1-eGFP per cell, while F1-10 2×10^4 per cell; both were kept under selection pressure using 1 mg/ml G418. Culture medium of JIMT-1 was supplemented with F12 (1:1 with DMEM; Sigma) and 60 NE/L insulin. Cultures were

propagated every 3-4 days. For microscopic and fluorescence correlation spectroscopy measurements cells were seeded onto 12 mm diameter cover slips, or cover slip based chamber slides (Nunc), and used at sub-confluence (50-80% density). Before stimulation experiments, cultures were starved in serum-free DMEM for 12 or 24 h.

3.2. Western blot

Sub-confluent cultures were stimulated with 8 or 50 nM EGF for 5 minutes, then lysed and run on 10% polyacrylamide gels under reducing conditions. Proteins were blotted to PVDF Immobilon P membrane, blocked with 1% BSA-PBST and phosphotyrosine was labeled with horse radish peroxidase conjugated anti-phosphotyrosine antibody in 1% BSA-PBST. Signal was developed using ECL and detected on X-Ray films.

3.3. Ca²⁺ response measurements

Cells were loaded with 2 µg/ml Fura-2-AM for 30 minutes at 37 °C, washed twice with HBS and imaged with an Attofluor Digital Ratio Imaging System. Spectral ranges were 340 ± 12.5 nm and 380 ± 12.5 nm for excitation and larger than 520 nm for emission. To display activation-induced temporal changes in Ca²⁺ concentration ratio images (I_{334}/I_{380}) corrected for field flatness were calculated, and the average ratio of regions-of-interest covering the inside of each cell were graphed versus time.

3.4. Fluorescence Correlation Spectroscopy (FCS)

FCS measurements were performed with a custom-built instrument as well as with a Zeiss Confocor 2 using a 40×, NA 1.2 water immersion objective at defined positions of the plasma membrane over 60-200 seconds. A series of 3-10 consecutive measurements were acquired at each position. For assessing the effect of receptor stimulation, EGF was added at a final concentration of 50 nM. Data are presented for those experiments only, where no significant displacement of the membrane with respect to the confocal detection volume took place during the series of measurements. For excitation, the 488 nm line of an Ar ion laser at ~1 kW/cm² was used. Emission was detected through a 515-545 nm bandpass filter. To evaluate autocorrelation curves, we have used the fit function:

$$G(\tau) = a_0 + \frac{1}{\langle N \rangle} \cdot G_{tr} \cdot G_{bl} \cdot \sum_i^n \left[w_i \left(1 + \left(\frac{\tau}{\tau_{d,i}} \right)^{2/d_{w,i}} \right)^{-1} \left(1 + \frac{1}{S^2} \left(\frac{\tau}{\tau_{d,i}} \right)^{2/d_{w,i}} \right)^{-1/2} \right] \quad (1)$$

where fluctuations owed to the triplet state are described by

$$G_{tr}(\tau) = \frac{\left(1 - T + T e^{-\tau/\tau_{tr}}\right)}{1 - T} \quad (2)$$

and the protonation and light induced GFP blinking is taken into consideration as

$$G_{bl}(\tau) = \frac{\left(1 - \Theta_{bl} + \Theta_{bl} \cdot e^{-\tau/\tau_{bl}}\right)}{1 - \Theta_{bl}} \quad (3)$$

In Eq. (1) $\langle N \rangle$ is the average number of molecules in the detection volume, which is a great asset, since by fitting the autocorrelation function the concentration of labeled molecules can be obtained. The diffusion autocorrelation time, $\tau_{d,i}$ of the i^{th} diffusing species is also obtained from fitting; it represents the average time it takes for this species (of a weight fraction w_i) to traverse the detection volume. The detection volume is a rotational ellipsoid characterized by the structure parameter, $S = \omega_z/\omega_{xy}$ (ratio of axial and lateral radii). $d_{w,i}$ denotes the anomaly parameters of the diffusing components: a value of 2 is characteristic of free Brownian diffusion, while values larger than 2 hint at possible obstacles in the way of diffusing molecules.

The diffusional autocorrelation times, $\tau_{d,i}$ can be converted to a diffusion coefficient (D) using the equation:

$$D_i = \frac{\omega_{xy}^2}{4\tau_{d,i}} \quad (4)$$

3.5. Fluorescence recovery after photobleaching (FRAP)

FRAP measurements were performed using a custom-made instrument based on a Leica fluorescence microscope. The beam of a 488 nm argon ion laser was focused to a diffraction-limited spot using 40 mW for bleaching and a $10^4\times$ attenuation for monitoring. Emission was detected through the half-mirror and a 500 nm longpass filter. Data were fitted with a custom-written LabVIEW program employing a nonlinear Levenberg-Marquardt algorithm assuming a general model for the fluorescence intensity $f(t)$ at any time t :

$$f(t) = \frac{f_0 + f_\infty (t/t_{1/2})^\alpha}{1 + (t/t_{1/2})^\alpha} \quad (5)$$

where f_0 is the fluorescence intensity immediately after photobleaching, f_∞ is the maximum recovered intensity attainable after a long time, $t_{1/2}$ is the time of recovery to $(f_\infty + f_0)/2$, and α is

the anomaly parameter, which in the FRAP formalism has a value of 1 for free diffusion, and <1 for hindered diffusion. The diffusion constant is calculated as

$$D = \frac{\gamma\omega^2}{4t_{1/2}} \quad (6)$$

where ω is the radius of the laser beam and the correction factor γ was determined to be 1.147 ± 0.073 .

3.6. Antibodies

Antibody 528 against ErbB1 was prepared from the supernatant of the corresponding hybridoma line. Against ErbB2, we have used trastuzumab, 2C4, and Ab3/OP15, that binds to the intracellular domain. Activated form of ErbB1 was labeled with clone74, phosphorylated ErbB2 with Ab18, phosphotyrosine residues with PY99. MUC4 was detected using 1G8. Antibodies where indicated were conjugated with AlexaFluor488, AlexaFluor543, AlexaFluor633, Cy3, or Cy5 for direct labeling and used at final concentrations of 1-10 μ g/ml. Indirect immunofluorescence was performed by applying fluorophore conjugated goat anti mouse polyclonal antibodies.

3.7. Determination of receptor numbers and trastuzumab dissociation constants

Cells were harvested, washed once, resuspended at 5×10^5 cells in 100 μ l PBS and incubated for 10 minutes on ice with 2.5 to 50 μ g/ml Alexa-488 conjugated antibodies. Calibration was done with QIFIKIT. For determining Kd-s in Scatchard plots, background corrected histogram means, normalized to the maximal intensities were used; the bound fraction was estimated from the relative labeling intensities and known total receptor numbers.

3.8. Immunofluorescent labeling

Cells were washed, incubated with fluorescently labeled trastuzumab or 2C4 for 30 minutes on ice, fixed with 4% paraformaldehyde for 20 min and mounted in Mowiol. For intracellular labeling, cells were fixed with 100% methanol at -20°C, rehydrated, blocked and permeabilized for 20 min at room temperature using PBS-BSA with 0.1% TX-100. Primary antibodies against phospho-ErbB1, phospho-ErbB2, phosphotyrosine (PY) or MUC4 and secondary Cy3-GAMIG were used at 1 μ g/ml for 30 min at RT. Flow cytometry samples were labeled according to a similar protocol, the but the cells were harvested before labeling by incubation with trypsin-EDTA, washed in ice cold PBS, and pelleted at 600 \times g.

3.9. Preparation of chemically coupled trastuzumab and EGF microspheres

Carboxy-functionalized superparamagnetic 1 μ m microspheres (SERA-MAG) were activated with 0.1 M sulfo-N-hydroxysulphosuccinimide, 0.1 M 1-ethyl(-3-[3-dimethylaminopropyl])carbodiimide hydrochloride in 0.1 M MOPS, pH 6, for 1 h at RT. After two washes in MOPS, microspheres were equilibrated in 0.1 M Na-phosphate, pH 8 and coupled overnight at 4°C with 50 μ g EGF or 250 μ g trastuzumab for 30 μ l of the 5% microsphere slurry with constant agitation. After 2 washes, the remaining reactive groups were capped with 1 M ethanolamine for 2 h at room temperature followed by further washings in PBS.

3.10. Cell stimulation with derivatized paramagnetic microbeads

Uncoupled, EGF-linked, and trastuzumab-linked magnetic microspheres or free trastuzumab, dye-conjugated trastuzumab or EGF in solution were used in Tyrode's buffer at a final concentration of 10 to 30 microspheres per cell. To provide a specific time of interaction and initiation of stimulation, cover slips were positioned on a magnet at time 0. After a specified reaction time, between 2 and 45 min at 37°C, cells were rinsed with PBS and fixed with ice-cold methanol for 20 min at -20°C.

3.11. Confocal Laser Scanning Microscopy

For confocal microscopy, Zeiss LSM 510 systems were used. For AlexaFluor488, YFP, Cy3/AlexaFluor543 and Cy5/647 excitation was at 488, 514, 543 and 633 nm, emission was detected through 505-550, 530 \pm 15, 560-615 and 650 LP filters. 512 \times 512 pixel, 0.5-1 μ m thick optical slices were acquired with 6.4 μ s pixel times in multi-track mode with minimized channel cross-talk. Reflection images of the microsphere distribution were taken using the 633 nm line without filtering the reflected light.

3.12. Image Analysis

The cross-correlation coefficient

$$C = \frac{\sum_i \sum_j (x_{i,j} - \bar{x})(y_{i,j} - \bar{y})}{\sqrt{\sum_i \sum_j (x_{i,j} - \bar{x})^2 \sum_i \sum_j (y_{i,j} - \bar{y})^2}} \quad (7)$$

where $x_{i,j}$ and $y_{i,j}$ are fluorescence pixel values at coordinates i,j in images x and y , characterizes the coincidence of signals in two channels of an image. This was calculated in a program custom

written in the LabView environment. The theoretical maximum is $C=1$ for identical images, and a value close to 0 implies random spatial localization of the two labels relative to each other.

Other image processing tasks were performed using SCIL-Image (TNO, Delft, The Netherlands). For determining ErbB phosphorylation in stimulated and nonstimulated areas of cells, background-corrected fluorescence of immunolabeled phospho-ErbB was used as an input channel. The histogram mean of cells from images of unlabeled samples taken with identical instrument parameters were taken as background values. Binary masks on cells were created by thresholding reflection images. Threshold values were chosen manually to exclude all background pixels; occasional holes in cell masks were filled with dilation-erosion cycles using a 3×3 mask and a connectivity of 8 pixels. Additional binary masks for separating stimulated and nonstimulated areas were generated by thresholding reflection images of microspheres when microsphere-coupled ligands were used and by thresholding Cy5-trastuzumab images when trastuzumab in solution was used as stimulus. For calculating the proportion of pixels above the resting level of phosphorylation, overall maximum values from corresponding nonstimulated but equivalently labeled cells were used as threshold.

4. RESULTS

4.1. Fluorescence correlation spectroscopy gives new insight into the activation mechanism of ErbB1

For studying how EGF binding alters the mobility and aggregation state of ErbB1, we have used as model system the ErbB1-eGFP chimeric protein stably transfected into CHO cells (F1-10 and F1-4 lines). The presence of endogenous fluorescent label was intended to obviate unwanted artifacts of antibody labeling, however, it also required that the functionality of the construct is confirmed.

4.1.1. The ErbB1-eGFP fluorescent chimera created for FCS measurements is functionally active

Signal transduction through ErbB1 is initiated by activation of the tyrosine kinase and subsequent phosphorylation of tyrosine residues in the receptor C-terminal domain and downstream substrates. Lysates of EGF-treated and control cells were probed for tyrosine phosphorylated proteins by Western blot. The human epidermoid carcinoma cell line A431 served as a positive control. In A431 cells, as well as F1-10 cells, ligand-induced tyrosine phosphorylation of bands with ~170-190 kDa molecular weight, corresponding to ErbB1 was

dose-dependent. Other proteins at ~60, 65, 90 and 100 kDa were also phosphorylated in a dose-dependent manner.

Phospholipase C γ is one of the early down-stream effectors of EGF receptor signaling, eliciting an increase of intracellular calcium concentration. Resting intracellular Ca $^{2+}$ levels of 80-120 nM were characteristic of all three cell lines. Positive control A431 cells showed prominent Ca $^{2+}$ elevation upon 50 nM EGF, in comparison, F1-10 cells expressing ErbB1-eGFP produced lower but still measurable Ca $^{2+}$ peaks. At 8 nM EGF a calcium response was detectable in A431 cells only. CHO negative controls did not exhibit an increase in intracellular calcium at any EGF concentration. The cause of the weaker tyrosine phosphorylation and calcium response in the case of F1-10 is likely the order of magnitude lower level of receptor expression. This is not a disadvantage, however, if one wants to examine the process of receptor activation at more physiological expression levels.

4.1.2 Diffusion rate of ErbB1 decreases upon EGF stimulation

FCS measurements imply the continuous illumination of fluorophores in a sub-micron sized spot. During the initial part of this illumination, we have experienced photobleaching of a fraction of ErbB1-eGFP fluorescence that hindered the correct fitting of ErbB1 diffusion times and thus needed to deliberately photobleach the low mobility and / or immobile receptor fractions until fluorescence was constant. This fraction was proportional to the initial fluorescence intensity of the cell. We must note that this strategy restricts FCS measurements to reasonably rapidly diffusing species, or at least hinders the exact determination of proportions among species with varying diffusion constants. In fact, we have determined that at higher monitoring laser powers, where more of the slowly diffusing species are photobleached, the overall diffusion determined in FCS appears to be faster, and if more components are differentiated, the slower components will be more affected.

Using the general mathematical model in eq. 1., we have found that the autocorrelation curves can be best fitted, on the grounds of minimum number of necessary components and lowest mean square error, by a four-component model. This model takes into account a fast and a slow diffusion component ($n=2$ in eq. 1), in addition to triplet state formation ($G_{triplet}$), and a blinking component (G_{blink}) that apparently covers both protonation dependent blinking terms as well as light induced blinking. Of the diffusion terms, the fast one is a 3 dimensional diffusion, originating mostly from the cytoplasm, whereas the slow one is a 2D motion in the plane of the

membrane. We allowed for the possibility of anomalous subdiffusion or obstructed diffusion for both diffusing species.

The faster diffusion was characterized by a diffusion constant of $1.97 \pm 0.83 \times 10^{-7} \text{ cm}^2/\text{s}$. The cytoplasmic nature of this fast component was confirmed by the position dependence of the relative contribution of this component to the autocorrelation function; in the cytoplasm of resting cells, the fast component contributed $59 \pm 8\%$ to the autocorrelation function, compared to only $33 \pm 5\%$ when the beam waist was placed on the plasma membrane. This component could be attributed to free cytoplasmic ErbB1-eGFP that had lost its signal sequence, proteolytic release of GFP into the cytoplasm, or GFP transcribed alone owing to the strong promoter, which is coherent with earlier data on the diffusion constant of free cytoplasmic GFP.

The slow diffusion term gave rise to a diffusion constant of $1.17 \pm 0.51 \times 10^{-9} \text{ cm}^2/\text{s}$. Control measurements have indicated co-diffusion of the intracellular GFP tag on ErbB1 and AlexaFluor647 labeled 528-Fab attached to the extracellular domain of ErbB1 on F1-10 cells, confirming that this component of the autocorrelation spectrum originates from movement of receptors in the plane of the cell membrane. The diffusion constant derived was consistent with that of a relatively freely diffusible transmembrane protein. In coherence with this, for the membrane diffusion component the anomaly parameter was 2, which hints that in the small area examined by FCS, overall there are no obstacles to diffusion. Upon EGF stimulation, the most obvious change occurred in the this slow membrane diffusion component, which on average slowed to about half of the original value immediately upon EGF stimulation. This observation is coherent with the predicted di- and oligomerization of the ErbB1 receptors during their activation, but does not exclude the possibility that ErbB1 exists also in the form of pre-associated dimers that upon EGF stimulation associate to larger aggregates, other proteins, or cytoskeletal elements, leading to their decreased lateral mobility.

4.1.3 ErbB1 could exist also as pre-formed di- or oligomers

In addition to slowed diffusion, EGF also caused a decrease of the number of molecules in the detection volume to 78% on average, which is also consistent with the more widely accepted theory that EGF receptors aggregate upon EGF stimulation. Receptor oligomerization was further evaluated by calculating the mean fluorescence per diffusing particle normalized to laser power, the ratio of which after and before stimulation was 0.94 ± 0.36 . These findings may reflect activation of the receptors according to the conformational activation model (not involving a change in the aggregation state of the receptor) rather than by induced receptor dimerization or

oligomerization. In fact, a very recent study applying a modified fluorescence intensity distribution analysis (FIDA) approach has also demonstrated that under physiological conditions, the EGF receptor exists in a complex equilibrium involving single molecules and clusters of two or more receptors. On the other hand, the two-fold decrease of D even exceeds the theoretically predictable value for dimerization ($\sim\sqrt[3]{2} = 1.26$). A higher order oligomerization of the receptors could explain the drastic decrease of D but is inconsistent with the meek 22% decrease of the number of independently diffusing species.

This discrepancy is best resolved by hypothesizing the immobilization or at least hindrance of the motion of the ErbB1-eGFP fusion proteins by interaction with static structures such as the underlying cytoskeleton. However, we have to keep in mind that the chromophore of a large fraction of the receptors is bleached and therefore not subject of the FCS studies. Thus, it is equally plausible that receptor aggregation / dimerization also occurs, but it is only reflected by the decrease in diffusion constant, as the detected ErbB1-eGFP molecules, or preformed dimers adhere to the more slowly moving higher order clusters that are already photobleached in the pre-bleach period and thus do not contribute to the specific fluorescence per particle value.

4.1.4. Complementarity of Fluorescence Recovery After Photobleaching and FCS in resolving the mobility of ErbB1

Our fluorescence recovery after photobleaching (FRAP) experiments in resting cells yielded diffusion coefficients of $0.7\text{-}0.9 \times 10^{-10} \text{ cm}^2/\text{s}$. Earlier reports on the lateral diffusion coefficients of EGF receptors determined by FRAP are available for several cell types and in the range of $1.8 \times 10^{-10} - 4.5 \times 10^{-10} \text{ cm}^2/\text{s}$. The differences are probably attributable to the use of different cell types and experimental conditions, as well as the possibly biased nature of FRAP measurements as in the earlier reports the ligand EGF was used to fluorescently label the receptor. However, we also note that our data obtained on similar resting with FRAP and FCS also differ by an order of magnitude.

To understand this discrepancy, the different nature of the experiments must be considered: in FCS, the local environment of a relatively small area of the membrane is probed, which may be smaller than the diameter of a membrane domain. Therefore mainly diffusion inside a membrane microdomain determines the diffusion correlation time, and this diffusion was found to be unhindered. In FRAP, the long-range diffusion rate of unbleached molecules arriving from more distant areas and having to cross domain boundaries is also examined. Thus, the rate of long-range receptor motion is not only affected by the intradomain diffusion rate, but also by

the rate of crossing domain boundaries. The obtained anomaly parameter lesser than unity ($\alpha=0.6$ or 0.8 depending on the model used) supports the possibility of obstructed diffusion. It also has to be kept in mind that in the FCS measurements receptors with low mobility were pre-bleached to avoid artifacts in the autocorrelation function, so the motion of this fraction of the molecules was not subject of the FCS studies.

Thus FCS can be said to be an adequate probe of local diffusion rates and molecular mechanisms. However, the large fraction of receptors with low mobility demonstrates a practical limitation of FCS in cellular studies set forth by the sensitivity of the method to the displacement of the membrane with respect to the detection volume. These factors prevent measurements of diffusion times as slow, or slower than a few seconds. For investigating such slow diffusion processes FRAP may be a more adequate approach.

4.2. Derivatized microbeads as a novel tool for assessing ErbB activation

Microsphere-based techniques allow localized stimulation of cells with precise control of timing and location, providing a versatile tool for kinetic assays of signal spreading and downstream propagation. In our studies, we used EGF and trastuzumab coupled paramagnetic microspheres to explore the activation properties of ErbB1 and ErbB2 *in situ*.

4.2.1. EGF and trastuzumab conjugated microspheres efficiently evoke ErbB autophosphorylation

To test the functionality of trastuzumab- and EGF coupled microspheres, we first used a cell line derived from the A431 epidermoid carcinoma by stably transfecting it with the ErbB2-mYFP chimera gene. The transfected line A4-ErbB2-mYFP exhibits a somewhat downregulated expression of ErbB1 (1.2×10^6) and 9.5×10^5 ErbB2 per cell. Applying EGF microspheres, ErbB1 could be focally activated as detected both via total tyrosine phosphorylation and specific phosphorylation of ErbB1. The correlation between the phosphorylation signal and location of microspheres was high ($C = 0.68$ and $C = 0.57$ for phospho-ErbB1 and phosphotyrosine). Trastuzumab microspheres on the same cells also evoked local total tyrosine phosphorylation ($C=0.47$) and specific ErbB2 phosphorylation ($C=0.68$). Correlation between microspheres and specific ErbB phosphorylation was always higher than that between microspheres and generic phosphotyrosine labeling, implying that signal spreading from the activated receptors may be attributed to other phosphorylated molecular species.

4.2.2. Focal stimulation by EGF and trastuzumab microspheres yields a well-localized activation process without lateral signal spreading

We observed highly localized activation of the receptors in the immediate proximity of the microspheres, confirmed by the high cross-correlation coefficients. There was no spreading of phospho-ErbB1 activation over the plasma membrane in any cell line expressing ErbB1 and stimulated with EGF-linked magnetic microspheres regardless of whether the microspheres were applied with or without a magnet. Transactivation of ErbB2 induced by EGF-linked magnetic microspheres was also well localized spatially. Similarly, trastuzumab microspheres recruited ErbB2-mYFP to their neighborhood and initiated phosphorylation of the receptor, which was then followed by apparent efforts to internalize the microspheres. Clusters of ErbB2-mYFP were also observed outside the microsphere-covered areas but there was no sign of phosphorylation in these clusters. Stated quantitatively, in microsphere-covered areas the ratio of background corrected YFP/phosphotyrosine signal was 1.2 ± 0.2 , whereas in areas outside the microspheres that were above background for YFP, the corresponding ratio was 6.8 ± 1.4 .

Verveer et al. experienced that after focal stimulation, ErbB1 activation has spread rapidly to the unstimulated areas of the cell membrane suggesting further associations and secondary dimer formation after ligand binding. In contrast, others have detected local patches of stimulation and localized polymerization of actin. We have confirmed these latter observations using several cell lines, and extended them also to trastuzumab microspheres. The absence of lateral spreading confirms the notion derived from FCS measurements that preformed ErbB1 di- or oligomers could be instrumental in EGF-based signaling.

4.2.3. EGF-bound ErbB1 transactivates ErbB2 but trastuzumab-bound ErbB2 does not transactivate ErbB1

Measuring the activation of ErbB1 and ErbB2 upon stimulation allowed the assessment of the degree of transactivation of ErbB1 to ErbB2 and of ErbB2 to ErbB1. In the latter case, trastuzumab-coupled microspheres provided a unique new tool because ErbB2 has no physiological ligand, but trastuzumab is able to induce specific ErbB2 phosphorylation. We have observed quite strong ErbB2 phosphorylation upon EGF-microsphere stimulation, for which the cross-correlation between microsphere locus and phospho-ErbB2 was $C = 0.43$. In the reverse direction, however, very little activation of ErbB1 was seen upon trastuzumab-microsphere stimulation: phospho-ErbB1 specific immunofluorescent signal was not observed above the level in starved, resting cells. Because on these cells ErbB1 and ErbB2 were expressed at roughly the

same levels, the asymmetric results indicate a much higher affinity of EGF bound ErbB1 for ErbB2 than that of the trastuzumab bound ErbB2 for ErbB1. A possible explanation could be that bivalent trastuzumab binding to ErbB2 favors ErbB2 dimer formation and sterically hinders its association with ErbB1, although probably not by obstructing the dimerization loop, but rather by preventing the formation of a multimer of at least one ErbB1 and an active ErbB2 dimer, based on earlier findings of our group on the molecular structure of ErbB2 dimers.

4.2.4. Transactivation of ErbB2 via ligand bound ErbB1 depends on receptor density

We have, as a next step, exploited the opportunity that both ErbB2 activation with trastuzumab linked microspheres and ErbB1 activation with EGF-microspheres was feasible only on A4ErbB2-mYFP but also on SKBR-3 and JIMT-1 cells and examined whether transactivation of ErbB1 and ErbB2 occurred on these cell lines. A lack of specific ErbB1 phosphorylation in SKBR-3 and JIMT-1 cell lines indicated that trastuzumab microspheres did not activate ErbB1, a result consistent with that obtained on A4ErbB2-mYFP cells. EGF-linked microspheres were effective in directly activating ErbB1 on SKBR-3 and JIMT-1, albeit at very low levels. The signal intensity of phospho-ErbB1 immunofluorescence was roughly proportional to the level of ErbB1 expression on the two cell lines, whereas the fraction of pixels under EGF microspheres that showed ErbB1 phosphorylation above resting levels was $59 \pm 22\%$ and $43 \pm 19\%$ for SKBR-3 and JIMT-1 respectively, comparable to the results with trastuzumab microspheres. On SKBR-3 and JIMT-1 cells, phospho-ErbB2 was generated at EGF-linked microsphere loci and reached 40% and 25%, respectively, of the hypothetical maximum intensity that was generated by trastuzumab microspheres. In addition, the proportion of pixels under EGF-microspheres that exhibited ErbB2 phosphorylation above resting levels was significantly higher than outside the microspheres. The differences in the activation of ErbB2 through ErbB1 in the cell lines examined appeared to be proportional to the differences in ErbB1 expression levels. We conclude that ErbB2 transactivation in cells with similar ErbB2 levels is dependent on the expression level of ErbB1, and EGF-bound ErbB1 readily forms heterodimers with ErbB2 – an *in situ* proof of the formation of ErbB1-ErbB2 secondary dimer.

4.3. Steric hindrance: a possible trastuzumab resistance mechanism

The SKBR-3 cell line is a well-known trastuzumab sensitive model of ErbB2 overexpressing mammary carcinoma. This cell line expresses 1.9×10^5 ErbB1 and 1.1×10^6 ErbB2 molecules per cell as measured by flow cytometry. While *in vitro* proliferation of SKBR-3 and several other

breast tumor lines is inhibited by trastuzumab, good *in vitro* models of trastuzumab resistance were unavailable until the recent establishment of the JIMT-1 line.

4.3.1. ErbB2 expression and affinity for trastuzumab in trastuzumab sensitive and resistant cell lines

Although JIMT-1 cells express about half of the amount of ErbB2 as SKBR-3 cells do (0.62 and 1.1 million receptor per cell, respectively), we found that trastuzumab binds much weaker to them: quantifying fluorescently labeled trastuzumab bound to ErbB2 on JIMT-1 yields $\sim 8 \times 10^4$ targets per cell. We have extended this measurement to trastuzumab Fab and two other ErbB2 targeting antibodies. OP-15 binds to the intracellular domain of the ErbB2, thus, its labeling intensity truly represents ErbB2 expression; accordingly, proportion of ErbB2 on JIMT-1 to SKBR-3 was ~ 0.5 . The antibody 2C4, which binds to the dimerization arm of ErbB2, gave similar results. The same ratio for trastuzumab was ~ 0.1 , however, it could be increased to ~ 0.4 by treating the cells with Triton X-100, and trastuzumab Fab fragments could also bind 2.5 times better to JIMT-1 cells than the whole trastuzumab antibody.

Dissociation constant of trastuzumab was determined on JIMT-1 and SKBR-3 cells in Scatchard experiments to be 2.5×10^{-8} M, one order of magnitude greater than on SKBR-3. This latter is still over an order of magnitude greater than the 0.1 nM determined for expressed ErbB2 extracellular domain in solution, which draws the attention to the highly important role of local molecular environment actually experienced by therapeutic antibodies at the surface of living cells. In line with this, it is reasonable to propose that in JIMT-1 cells the reason of the resistance could be decreased trastuzumab binding. However, the cause of resistance could also be the absence of ErbB2 functionality in these cells.

4.3.2. Trastuzumab microspheres are effective even if trastuzumab in solution has no effect

To differentiate between dysfunctional, and functionally intact, but sterically hindered ErbB2, we have made use of the derivatized paramagnetic microspheres characterized earlier. Stimulation of JIMT-1 cells with trastuzumab-coupled microspheres led to pronounced phosphorylation of ErbB2, similarly to the case of SKBR-3. The cross-correlation between microspheres and phospho-ErbB2 specific immunofluorescence signal was $C=0.73$ and $C=0.57$ for SKBR-3 and JIMT-1, respectively. As expected, the application of trastuzumab in solution led to activation of ErbB2 in SKBR-3 but hardly any in JIMT-1, which was confirmed by quantitative comparison of stimulated (bead loci, or labeled trastuzumab signal) and non-stimulated areas for phospho-

ErbB2 signal and for the number of pixels above the maximal resting ErbB2 phosphorylation. Thus, ErbB2 appears to be fully functional also on JIMT-1 cells, and apparently by applying a higher density of ligand and, possibly, as a result of the steric effects arising from the microspheres, it can be activated.

4.3.3. Membrane-associated mucin, MUC4, can be one cause of decreased trastuzumab binding and efficiency

Fluorescence microscopy showed that MUC4 was expressed at a substantially higher level in JIMT-1 than in the trastuzumab-sensitive SKBR-3 cells. JIMT-1 cells labeled with 1G8 against MUC4 and with trastuzumab showed an inverse correlation between the local density of MUC4 and trastuzumab binding implying that high MUC4 expression may interfere with the binding of trastuzumab. The large O-glycosylated ascites sialoglycoprotein 1 (ASGP1) domain of MUC4 is connected non-covalently to the smaller ASGP2 domain, thus, its removal could improve trastuzumab binding, which is consistent with the increased trastuzumab labeling ratio of JIMT-1/SKBR-3 upon Triton X-100 pre-treatment. The role of MUC4 in steric hindrance was further substantiated in experiments where downregulation of MUC4 by siRNA improved trastuzumab binding. As MUC4 expression is routinely not yet tested, its precise role in big populations cannot be estimated. However, based on our findings it appears to be important to investigate this aspect of trastuzumab resistance further as it might be a good prognostic factor, and a possible indication that tyrosine kinase inhibitors might be better applied than ErbB2-targeted antibodies.

5. CONCLUSIONS

- ErbB1-eGFP chimeric molecules are functionally intact, and can serve as a good model system for mobility measurements when stably expressed in CHO cells. Fluorescence correlation spectroscopy (FCS) could resolve a slow, $1.17 \pm 0.51 \times 10^{-9}$ cm²/s intramembrane diffusion component of the fused receptor in addition to fast cytoplasmic diffusion and photochemical processes in the eGFP tag. FCS measurements were selective for diffusible molecular species as not or very slowly diffusing eGFP tags needed to be photobleached for average fluorescence to stabilize.
- Stimulation with EGF resulted in a reduction by 50% of ErbB1 membrane diffusion and by 25% of the concentration of diffusing particles, coherent with the idea of ligand

induced aggregation. However, this would also cause an increase of the relative fluorescence per particle, but this parameter did not change. Consequently, it is likely that pre-formed ErbB1 dimers are present on the cell surface, that upon EGF binding get conformationally activated and associate with less mobile ErbB1 clusters that have already been photobleached, or interact with the cytoskeleton yielding slower average diffusion.

- FCS disclosed ErbB1 diffusion rates an order of magnitude faster than FRAP measurements for the same ErbB1-eGFP chimera. Also, diffusion resolved by FCS exhibited no anomaly (spatial hindrance) within the small sub-micron detection spot, while FRAP showed that influx of fluorescent species from distal areas into the bleached spot is a result of anomalous diffusion. Thus FCS and FRAP are complementary both in terms of assessing local and larger range diffusion, as well as being selective to faster and slower fluctuations, respectively.
- In various cell lines expressing ErbB1 and ErbB2 at different quantities, EGF and trastuzumab coated paramagnetic microspheres proved to be functional and stimulated local phosphorylation of the targeted ErbB1 or ErbB2 receptors. The signaling events remained laterally localized, and activated the internalization machinery.
- EGF coated microspheres transactivated also ErbB2, while trastuzumab coated ones did not transactivate ErbB1, indicating that trastuzumab binding favors ErbB2 homodimers. The extent of ErbB2 transactivation by EGF beads was directly proportional to increasing surface density of ErbB1, implying that in the presence of activated ErbB1, formation of ErbB1-ErbB2 heterodimers is favorable.
- Trastuzumab binds less on the surface of JIMT-1 cells, its dissociation constant is a magnitude higher than on SKBR-3. ErbB2 phosphorylation is low on JIMT-1 and is not enhanced substantially by trastuzumab. However, ErbB2 on these cells is functional, since high ligand density on trastuzumab-coated microspheres could evoke local ErbB2 activation. The steric effect likely exerted by the beads also hints at the possibility of hindered trastuzumab binding on JIMT-1.
- The mucosialoprotein MUC4 was found to be overexpressed on JIMT-1 cells and its expression was negatively correlated with trastuzumab binding. Given the large size and abundance of MUC4, it could well play a role in sterically hindering trastuzumab binding, and as such, might be a useful predictor of the success of ErbB2-directed antibody therapies.

6. PUBLICATIONS

6.1. Related In extenso publications

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