THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Quantitative description of the constitutive and ligand-induced associations of ErbB receptors

by Ágnes Tímea Nagyné Szabó

Supervisors: János Szöllősi, D.Sc.

Péter Nagy, Ph.D.

UNIVERSITY OF DEBRECEN

Doctoral School of Molecular Medicine

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1. Introduction

Besides cardiovascular diseases malignant tumors lead mortality statistics in the developed countries. Breast cancer is the second most common after lung cancer, however the most prevalent among women. The ErbB receptor family plays an important role in malignant transformation and progression. In breast cancer ErbB2 is particularly important both from diagnostic and therapeutic point of view, since it is overexpressed in 20-30% of breast cancers.

There are 4 members of the ErbB receptor family which play an important role in regulating cell growth and development at low expression level. These proteins belong to receptor tyrosine kinases. Their intracellular tyrosine kinase domain is activated upon ligand binding, oncogenic mutation or at extremely high receptor expression level, and numberless signal transduction pathways will be activated. Homo- and heteroassociations of these receptors are pivotal in their activation. They are likely to exist in the absence of receptor stimulation but undergo significant rearrangement upon ligand binding (e.g. EGF, heregulin).

2. Background

2.1. The epidermal growth factor receptor family

The epidermal growth factor receptor family (EGFR) of receptor tyrosine kinases comprises four members: ErbB1 (EGFR, HER1), ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4). These transmembrane receptors play an important role in early embryogenesis and development of the heart, the central and peripheral nervous system and mammary gland function. Their mutation or overexpression can lead to cancer formation.

2.2. Ligands of ErbB receptors

ErbB protein ligands are synthesized as type I transmembrane proteins that can be proteolytically cleaved to release soluble growth factors. Both transmembrane and soluble ligands contain an EGF-like sequence which recognizes and binds to ErbB receptors. The ligands can be grouped based on their affinity to receptors.

Epidermal growth factor (EGF) belongs to the group of EGF-like ligands and binds to ErbB1 with high affinity.

Heregulin (HRG) is a neuregulin and has several isoforms. HRG-α and HRG-β bind to ErbB3 and ErbB4 with different affinities. The more affine HRG-β was used in my experiments.

2.3. Signal transductions activated by ErbB receptors

Various ligands, growth factors can bind to the extracellular domain of ErbB proteins leading conformational changes and the induction of receptor homo- and heteroassociations.

Dimerization is the steric condition of transphosphorylation since the tyrosine kinase domain located in the intracellular part phosphorylates tyrosine residues of the neighboring receptor. In the resulting signaling complex secondary messengers

are often phosphorylated then dissociated from the complex to activate additional proteins in the cytoplasm which initiate second messenger systems. Depending on which ligand activated the receptor and exactly which tyrosine was phosphorylated different signaling pathways can be activated influencing cell proliferation, migration, differentiation and apoptosis.

2.4. Interactions between ErbB receptors

According to the classical dogma ErbB receptors form homo- and heteroassociations upon ligand binding, but recently larger clusters were also detected. Domain I and III in the extracellular part of the receptor is responsible for ligand binding, while the cystein rich domain II and IV play an important role in dimerization.

ErbB2 is a ligandless member of the ErbB receptor family that functions as a coreceptor for ErbB1, ErbB3 and ErbB4 and can only participate in signal transduction processes in a heterocomplex. The kinase-dead ErbB3 is also unable to transmit signals on its own. ErbB2-containing heterodimers formed upon ErbB2 overexpression bind ligand with higher affinity than homodimers and heterodimers of ErbB2 are degraded effectively so the tyrosine kinase activity of these heterodimers is continuous and more potent.

2.5. Monoclonal antibodies against ErbB2

Trastuzumab (Herceptin®) was the first monoclonal antibody against the extracellular part of ErbB2, which was developed to treat solid tumors based on genomic research. Trastuzumab is the humanized version of the original mouse-derived antibody designated 4D5. The anti-tumor effect of trastuzumab was clearly demonstrated in phase II and III clinical trials, but its exact mechanism of action is still unknown.

Pertuzumab is a humanized monoclonal antibody against the extracellular part of ErbB2 binding to domain II. Thus, it sterically inhibits heterodimerization of ErbB2 even if ErbB2 is not overexpressed.

2.6. Fluorescence resonance energy transfer (FRET)

In FRET energy is transferred in a nonradiative fashion from an excited donor molecule to a nearby acceptor. During its relaxation the FRET excited acceptor can emit photons which can be detected by a flow cytometer, a fluorescence microscope or a spectrofluorimeter. FRET efficiency depends on the distance between the donor and the acceptor therefore it can be used to detect protein-protein interactions.

When the process takes place between a donor and a spectroscopically different acceptor, it is termed hetero-FRET. The fact that the energy transfer process in hetero-FRET is unidirectional limits its potential to distinguish between clusters with more than two subunits. Hetero-FRET can be determined from the decrease of fluorescence intensity and lifetime of the donor and from the increase of donor fluorescence anisotropy.

In homo-FRET, the interaction takes place between a donor and a spectroscopically identical acceptor which can serve as the donor in the next homo-FRET step. Because energy is distributed among the interacting molecules, homo-FRET can be used for the quantitative analysis of large protein clusters. The only manifestation of homo-FRET is decreased fluorescence anisotropy.

2.7. Theoretical background of fluorescence anisotropy

Fluorescence anisotropy is the measure of emission polarization which can be used to detect molecular rotation. Limiting anisotropy is the maximal anisotropy of a stationary fluorophore. Anisotropy depends on the rotational correlation time and the fluorescence lifetime of the fluorophore.

If there is a spectroscopically identical molecule (acceptor) close to an excited

fluorophore (donor) homo-FRET takes place between them and results in decreased anisotropy of the donor population, because the acceptor molecule is oriented in a non-polarized manner thus the photon emitted by the acceptor is depolarized. Cluster size can be derived from the rate of anisotropy decay as a function of fluorophore concentration.

We have developed a method for studying the dependence of fluorescence anisotropy on the concentration of labeled antibodies using flow cytometry and show how large scale homoclusters of ErbB proteins can be quantitatively characterized by this approach.

2.8. FRET-sensitized acceptor photobleaching (FSAB)

Mekler et al. described a method called PES (photochemical enhancement of sensitivity) which can precisely detect low FRET efficiencies. FRET efficiency was determined from the increased rate of a photochemical reaction of the acceptor sensitized by FRET from the donor.

In our method, called FSAB, the proteins to be studied were labeled by a photostable donor and a photolabile acceptor, and by exciting the donor only the acceptors within FRET distance to the donor will get photobleached. The decreased intensity of acceptor was examined at the emission wavelength of the acceptor and the fraction of acceptor molecules in the vicinity of donors were determined.

2.9. Aims

The ErbB receptor family plays an important role in cancer research, since their activation caused by overexpression leads to malignant cell proliferation. Their dimerization or higher order oligomerization is a key step in their activation. Although molecular clusters can be characterized by a multitude of techniques most of them are qualitative or semiquantitative. Our opportunities are limited to characterize large scale clusters (larger than dimer) or describe the composition of

heteroclusters. Understanding the size and composition of homo- and heteroassociations of ErbB receptors may be essential to inhibit their signal transduction, thus arrest cell division.

Therefore we aimed to

- elaborate a statistically reliable, flow cytometric homo-FRET method for quantitative characterization of homoassociations.
- study the large scale homoclusters of ErbB1 and ErbB2 in quiescent and stimulated human cancer cells using the method mentioned above.
- quantitatively study heteroassociations using the FSAB technique based on photobleaching of a photolabile acceptor molecule by hetero-FRET.
- determine the heteroclustered fraction of ErbB1 and ErbB2 in quiescent and EGF stimulated human breast tumor cells using FSAB method.
- quantitatively examine the homoclusters of ErbB1 and ErbB2 in quiescent and EGF stimulated human cancer cells using also the FSAB technique.

3. Materials and methods

3.1. Cell lines

The quantitative analysis of ErbB2 homoclusters and ErbB1-ErbB2 heteroclusters was performed on SKBR-3 human breast tumor cells which express $\sim 2\times10^5$ ErbB1 and $\sim 10^6$ ErbB2 proteins. The A431 human epithelial carcinoma cell line, expressing $\sim 2\times10^6$ ErbB1 and $\sim 2\times10^4$ ErbB2, was used to study ErbB1 homoclusters in a quantitative way.

3.2. Labeling cells to measure fluorescence anisotropy

Cells were starved in 0.1% serum (FCS) containing medium (DMEM) for 24 hours before stimulation with growth factor, then ~1-1 million trypsinized cells were suspended in 100 µl Hanks-buffer/BSA (1 mg/ml BSA). 20 µg/ml pertuzumab pretreatment on starved SKBR-3 cells was performed at 37 degrees for 15 minutes, then control and pretreated cells were stimulated with 600 ng/ml EGF or 50 ng/ml heregulin for 15 minutes at 37 degrees. Starved A431 cells were stimulated with 600 ng/ml EGF. After the treatments cells were labeled for homo-FRET so that the given receptor (ErbB1 or ErbB2) was labeled by a mixture of Alexa488-conjugated and unlabeled antibodies for 30 minutes on ice in the dark. The ErbB1 receptor was labeled with Mab528 or EGFR455 antibody, while ErbB2 was labeled by trastuzumab. The total concentration of labeled and unlabeled antibodies was 50 µg/ml, and the fraction of labeled antibody varied from 0% to 100% with increments of 10%. Labeled cells were washed with PBS and fixed with 1% formaldehyde in PBS. All samples were kept at 4 degrees before performing the measurements.

3.3. Labeling cells to measure FSAB

Cells grown in chambered coverglass were starved in DMEM containing 0.1% FCS for 24 hours before the experiments were conducted. A portion of the

cells was stimulated with 600 ng/ml EGF for 15 minutes at 37 degrees. For analyzing homoclusters quiescent and stimulated receptors (ErbB1 on A431 cells, ErbB2 on SKBR-3 cells) were labeled with antibodies (Mab528 in case of ErbB1, trastuzumab in case of ErbB2) conjugated with a photostable donor (Alexa546) and a photolabile acceptor (Cy5) at a concentration of 20 μg/ml. For studying heteroclusters SKBR-3 cells were labeled with a mixture of 20 μg/ml Alexa546-trastuzumab or Cy5-trastuzumab and 20 μg/ml Cy5-Mab528 or Alexa546-Mab528 antibodies. Cells washed with PBS were fixed with 1% formaldehyde in PBS. Photobleaching was carried out in the presence of 8×10^{-4} M CBr_4 to increase photobleaching rate of Cy5.

3.4. Flow cytometry and data analysis

Cell-by-cell fluorescence anisotropy measurements were performed in the L-format arrangement on a FACSVantage SE instrument with a DiVa option. Alexa488 was excited by a vertically polarized 488-nm beam, which was produced by letting the 488-nm line of an Ar ion laser pass through a half-wave plate retarder. Emitted photons passed through a 530±15 nm band-pass filter, followed by a broadband polarizing beam-splitter cube separating the vertically and horizontally polarized components. Two polarized fluorescence intensities, designated by I_{vv} and I_{vh}, were detected, where the first and second indices refer to the polarization directions of the exciting laser light and of fluorescence, respectively.

50.000 cells, labeled by a mixture of labeled and unlabeled antibodies, were measured, then the total fluorescence intensity and cell-by-cell anisotropy were calculated using I_{vv}, I_{vh} and the G correction factor. The ReFlex software was used for evaluation. The mean anisotropy values were plotted against the saturation of binding sites with the labeled antibody, and the graph from a single experiment was fitted by the equation, derived by us, describing the dependence of anisotropy of a mixture of monomers and N-mers on the concentration of the antibody. Cluster

size and monomer percentage were determined from the fitting, and the results of three independent experiments were averaged. The standard deviation of measured data was calculated and 250 random data sets were generated using Monte Carlo simulation, which were fitted also by the anisotropy model. Distribution of fitted cluster size and monomer% were calculated. Fitting and Monte Carlo simulation were performed using Mathematica.

3.5. Confocal microscopy and photobleaching

A Zeiss LSM 510 confocal laser scanning microscope was used to image the cells using a 63x oil immersion objective and the pinhole size was adjusted to 2 Airy units. Alexa546, as the photostable donor, was excited by the 543-nm line of a He-Ne laser and its emission was detected in the donor channel at 560-615 nm. Cy5, as the photolabile acceptor, was excited by the 633-nm line of a He-Ne laser and the fluorescence was detected in the acceptor channel above 650 nm. When FRET between Alexa546 and Cy5 was measured, a third fluorescence image (FRET channel), excited at 543 nm and recorded above 650 nm, was also measured in addition to the donor and acceptor channels.

To induce FRET-sensitized bleaching of Cy5, the sample was illuminated at 543 nm and the power of the laser was set to 100%. Bleaching illumination was interrupted every 30 seconds, and donor, FRET and acceptor images were recorded with an attenuated (5%) laser beam. Photobleaching was carried out in the presence of 8×10^{-4} M CBr_4.

3.6. Image analysis

SKBR-3 cells were used for the experiments and ErbB1 receptors were labeled with donor-conjugated antibody (AlexaFluor546-Mab528) and ErbB2 was labeled by acceptor conjugated antibody (Cy5-trastuzumab). Images were recorded in the donor, acceptor and FRET channels before and after every photobleaching step until the fluorescence intensity of the acceptor leveled off.

Image processing was carried out with the DipImage toolbox under Matlab. Segmentation of images into membrane and non-membrane pixels was carried out with the manually-seeded watershed algorithm using a custom-written Matlab program. Image stacks acquired during photobleaching were corrected for shift using the 'correctshift' command of DipImage. FRET efficiency, unquenched donor and direct acceptor intensities corrected for spectral overspill were calculated in the photobleaching stack in the membrane of selected cells double-labeled by donor- and acceptor-tagged antibodies. The fraction of bleached acceptors ($F_{bleached}$) was determined at the time when the FRET efficiency dropped to zero. The fraction of directly bleached acceptors excited at the excitation wavelength of the donor (*BCF* = bleaching correction factor) was determined using a sample labeled with acceptor only.

4. Results and discussion

4.1. Quantitative description of ErbB1 and ErbB2 homoclusters using the flow cytometric homo-FRET method

We have developed a flow-cytometric method for the quantitative analysis of protein homoclustering. The model relies on the correlation between fluorescence anisotropy and the local density of fluorophores. We assumed that a fraction of the investigated protein species is monomeric, while other proteins of the same species form homoclusters with a constant size. This is obviously not true, but given the low number of data points and the measurement error achievable by flow cytometric anisotropy measurements, the simplification above was required to make fitting of the model to measurement data possible.

4.1.1. The size of ErbB2 homoclusters in quiescent and stimulated SKBR-3 cells

When ErbB2 was inactivated by starving cells in 0.1% serum containing medium for 24 hours before the experiment ~60% of ErbB2 molecules were monomeric, but the rest formed huge clusters composed of ~110 ErbB2 proteins.

Since ErbB2 is a ligandless receptor, its ligand-induced activation can only be achieved by heterodimerization with ErbB1 or ErbB3. When starved cells were stimulated with epidermal growth factor (ligand of ErbB1) or heregulin (ligand of ErbB3) the percentage of monomers did not change significantly, but a substantial decrease in the size of ErbB2 homoclusters was observed. The effect of heregulin was more pronounced than that of EGF, because only 30 ErbB2 receptors remained in the homocluster upon heregulin stimulation, and ~70 proteins after EGF stimulation. Since EGF can induce both ErbB1-ErbB2 heterodimerization and ErbB1 homodimerization, so only a part of lower expressed ErbB1 proteins can recruit ErbB2 from its homoclusters to form ErbB1-ErbB2 heteroclusters. To the contrary, heregulin induces mostly ErbB2-ErbB3 heterodimerization, because

ErbB3 is the preferred heterodimerization partner of ErbB2. According to the literature unstimulated ErbB3 forms homoclusters from which ErbB3 is recruited by heregulin, then heregulin activated ErbB3 recruits ErbB2 from its homoclusters to form ErbB2-ErbB3 heterodimers.

If starved cells were pretreated with pertuzumab, a monoclonal antibody sterically blocking the heterodimerization of ErbB2, the size of ErbB2 homoclusters decreased significantly compared to non-pretreated, starved SKBR-3 cells, and the percentage of ErbB2 monomers slightly increased from ~60% to ~70%. Our previous unpublished results from hetero-FRET experiments showed that pertuzumab slightly blocks ErbB2 homoassociation, therefore it decreases the size of homoclusters and increases the monomer%.

Pertuzumab pretreatment practically abolished the effect of heregulin on ErbB2 cluster size, since roughly 70 receptor formed a homocluster in contrast to homoclusters formed of ~30 molecules upon heregulin stimulation in the absence of pertuzumab. Pertuzumab, an antibody binding to domain II of ErbB2, sterically blocks ErbB2 heterodimerization, therefore smaller decrease in the size of ErbB2 homoclusters was observed upon heregulin stimulation in the presence of pertuzumab, since ErbB2 was able to heterodimerize with heregulin activated ErbB3 to a smaller extent.

The size of ErbB2 homoclusters was the smallest on starved cells, only ~10 ErbB2 formed a homocluster, because receptors were activated by growth factors present in the serum. Ligand bound ErbB1 or ErbB3 recruits ErbB2 from the large scale homoclusters to form heterodimers, therefore fewer ErbB2 molecules remain in homoclusters.

Treatment of non starved cells with pertuzumab significantly increased the size of ErbB2 homoclusters to ~30 proteins. Receptors were activated by growth factors in the serum, therefore they formed heterodimers and little ErbB2 was present in homoclusters. However pertuzumab inhibited ErbB2 heterodimerization, thus more ErbB2 remained in homoclusters.

4.1.2. Correlation between ErbB2 cluster size and activation state

Several growth factors can induce the activation of ErbB2 and during our measurements we have found that the size of ErbB2 homoclusters decreased upon stimulation. Therefore, we investigated the correlation between the level of ErbB2 tyrosine phosphorylation and its homocluster size.

The amounts of total and tyrosine phosphorylated (activated) ErbB2 molecules were determined in parallel with its cluster size on fixed and permeabilized cells. We have found an inverse correlation between the activation state of ErbB2 and its homocluster size: the smaller the size of ErbB2 homoclusters, the higher the activation level of ErbB2 on quiescent and non starved cells was.

4.1.3. The size of ErbB1 homoclusters on quiescent and stimulated A431 cells

If the cells were starved in 0.1% serum containing medium for 24 hours before the experiment, most of the ErbB1 receptors, ~90%, were monomeric, and the remaining ~10% formed homoclusters containing ~4 ErbB1 molecules.

Stimulation of cells with EGF reduced the monomer percentage of ErbB1 to ~70% and increased its homocluster size to ~10 receptors/homocluster, because EGF is the ligand of ErbB1 and can increase the homoassociation of ErbB1.

When the cells were cultured under normal serum conditions (10% FCS), the receptors were activated by growth factors in the serum. The homocluster size of ErbB1 was larger, ~7 proteins/homocluster, and the monomer percentage was lower, 75%, than in starved cells. These changes were caused by the serum activating ErbB1 homoclusterization.

4.2. Quantitative description of ErbB1 and ErbB2 homo- and heteroclusters using the FSAB method using confocal microscopy

During our work so far ErbB1 and ErbB2 homoclusters were analyzed on quiescent and stimulated cells, but we raised the question whether the assumptions about the presence of heteroclusters are true. Since only homoassociations can be measured using homo-FRET, another method had to be applied.

We developed the FRET-sensitized acceptor bleaching technique, which was originally proposed by Mekler et al., to characterize the composition of heteroclusters of ErbB1 and ErbB2 in a quantitative way. FRAP measurements proved that fixation with 1% formaldehyde inhibited lateral diffusion of cell surface proteins, therefore only acceptors in the immediate vicinity of a donor are expected to get photobleached by FSAB.

4.2.1. Determination of the ratio of ErbB1 and ErbB2 in homoclusters and comparison with the results of homo-FRET measurements

A431 cells were used to analyze ErbB1 homoclusters, and ErbB2 homoclusters were analyzed on SKBR-3 cells. The cells were starved in 0.1% serum containing medium 24 hours before the experiment, so that the receptors were inactivated. We have found that 83% of ErbB2 molecules form homoclusters on starved SKBR-3 cells, and only 13% of ErbB1 receptors form homoassociations on quiescent A431 cells. These data were consistent with our previous homo-FRET results.

If the cells were stimulated with EGF, the measured fraction of homoclustered ErbB1 was three fold higher than in starved A431 cells, however the fraction of homoclustered ErbB2 decreased to 61% in SKBR-3 cells. These findings are in agreement with our previous results obtained by homo-FRET.

4.2.2. Quantitative analysis of heteroclusters of ErbB1 and ErbB2 in quiescent and stimulated SKBR-3 cells

The SKBR-3 human breast tumor cell line, expressing both receptors at high level, was used to define the absolute number of free and bound ErbB1 and ErbB2 molecules in quiescent and EGF stimulated cells after determining the expression level of both receptors using flow cytometer.

The cells were starved in the presence of 0.1% serum containing medium for 24 hours, and the fraction of ErbB1 heteroclustering with ErbB2 in quiescent and EGF stimulated SKBR-3 cells was determined. ErbB2 was labeled by Alexa546-trastuzumab (donor) and ErbB1 was labeled with Cy5-Mab528 (acceptor). The fraction of heteroclustered ErbB1 in quiescent cells was ~40% (~80.000 proteins) which decreased to 33% (~66.000 ErbB1 molecules) after EGF stimulation.

ErbB1 molecules were labeled by Alexa546-Mab528 (donor) and ErbB2 proteins were labeled with Cy5-trastuzumab (acceptor) in quiescent and EGF stimulated SKBR-3 cells to determine the fraction of heteroclustered ErbB2. Only 10%, ~120.000 ErbB2 receptors form heterocluster with ErbB1 in quiescent cells and this fraction doubled upon EGF stimulation. The fact that the majority of ErbB2 is not heteroclustered with ErbB1 is the consequence of the much higher number of ErbB2 expressed by SKBR-3 cells and the strong tendency of ErbB2 to form large scale homoclusters whose size decreases upon EGF stimulation. We assumed in our homo-FRET measurements that phenomenon is caused by EGF induced ErbB1-ErbB2 heterodimerization. Since our FSAB experiments proved that the fraction of ErbB2 heteroclustered with ErbB1 increased upon EGF stimulation, our previous assumption proved to be true.

The fraction of heterocluster-forming ErbB2 was also analyzed on a cell-by-cell basis, and was found to be independent of the expression levels of ErbB1 and ErbB2. To the contrary, the FRET efficiency was proportional to the acceptor/donor ratio. If free and bound ErbB2 were in equilibrium according to the law of mass action the fraction of heteroassociating ErbB2 would depend on the

expression levels of ErbB1 and ErbB2. The fact that the fraction of bound ErbB2 was independent of the expression levels of ErbB1 and ErbB2 implies that the formation of these heteroclusters does not follow the law of mass action. We believe that the composition of these clusters is established during their export by the vesicular transport system to the cell membrane, and that the density of proteins in the membrane of these vesicles is more or less constant and independent of the number of proteins expressed in the cell membrane.

5. Summary

We presented a statistically reliable, flow cytometric homo-FRET method for quantitative characterization of the homoassociation of ErbB1 and ErbB2. ErbB1 and ErbB2 receptors were labeled by a mixture of unlabeled and fluorescent antibodies and homo-FRET was measured between two spectroscopically identical fluorophores. The excitation energy is distributed in the ensemble of molecules by homo-FRET. The only manifestation of homo-FRET is decreased fluorescence anisotropy which depends on the concentration of fluorophores. The decrease in the concentration dependent anisotropy shows whether the molecule is monomeric, dimeric, trimeric or forms higher order oligomers.

In quiescent A431 cells most ErbB1 receptors are monomeric and stimulation with EGF or serum leads to an increase in the cluster size of ErbB1 due to ligand-induced homodimerization of ErbB1. On the contrary, most of the ErbB2 proteins are inactivated and present in large homoclusters in unstimulated SKBR-3 cells whose size decreases upon EGF, heregulin or serum stimulation. We attribute this phenomenon to the recruitment of ErbB2 to heterodimers with ligand-activated ErbB1 and ErbB3 resulting in the removal of ErbB2 from homoclusters.

We developed the FRET-sensitized acceptor bleaching (FSAB) technique to quantitate the ratio of ErbB1 and ErbB2 in their heteroclusters by confocal microscopy. Briefly, a photostable donor excites a photolabile acceptor by FRET, and the acceptors within FRET distance to the donor will get photobleached and the fraction of acceptor molecules in the vicinity of donors can be determined. AlexaFluor546 and Cy5 fluorophores were used as a photostable donor and a photolabile acceptor, respectively.

In unstimulated SKBR-3 cells almost half of ErbB1 receptors form heteroclusters with ErbB2 and after EGF treatment the fraction of heteroclustered ErbB1 did not change significantly, because EGF induces the formation of both ErbB1 homodimers and ErbB1-ErbB2 heterodimers. On the contrary, only 10% of

ErbB2 is in heteroclusters with ErbB1 in quiescent SKBR-3 cells and this fraction doubles upon EGF stimulation, because ligand-activated ErbB1 recruits ErbB2 proteins from the large ErbB2 homoclusters.

The large-scale clusters described by us are the place of signal transduction mediated by ErbB receptors where rearrangements take place upon ligand binding. The large ErbB2 homoassociations present in quiescent cells ensure that ErbB2 proteins are easily accessible for ligand-activated ErbB1 and ErbB3 to form heteroclusters. The receptors in large clusters are present in high local concentration which is favorable for signal transduction activated by growth factors. In this way the clusters are the places of physiological and pathological signal transductions. A better understanding of these clusters may shed light on the mechanisms of malignant cell proliferation and its possible inhibition.

6. Publications

6.1. Publications used in the dissertation:

1. **Szabó Á.**, Horváth G., Szöllősi J., Nagy P.: Quantitative characterization of the large-scale association of ErbB1 and ErbB2 by flow cytometric homo-FRET measurements. Biophysical Journal (2008) 95:2086-2096
   IF: 4.683

2. **Szabó Á.**, Szöllősi J., Nagy P.: Coclustering of ErbB1 and ErbB2 revealed by FRET-sensitized acceptor bleaching. Biophysical Journal (2010) 99:105-114
   IF: 4.39 (2009)
   Cumulative impact factor: 9.073

6.2. Posters:

1. **Ágnes Szabó**, Gábor Horváth, János Szöllősi, Péter Nagy: Quantitative characterization of the large-scale association of ErbB1 and ErbB2 by flow cytometric homo-FRET measurements. Regional Biophysics Conference (RBC), Balatonfüred, Hungary, 2007. (Award for best poster)

2. **Ágnes Szabó**, Gábor Horváth, János Szöllősi, Péter Nagy: Quantitative characterization of the large-scale association of ErbB1 and ErbB2 by flow cytometric homo-FRET measurements. XXIV Congress of the International Society for Advancement of Cytometry (ISAC), Budapest, Hungary, 2008.

3. **Ágnes Szabó**, János Szöllősi, Péter Nagy: FRET-sensitized acceptor bleaching reveals the large-scale co-clustering of ErbB1 and ErbB2. European Biophysics Congress (EBSA), Genoa, Italy, 2009.

4. **Ágnes Szabó**, János Szöllősi, Péter Nagy: FRET-sensitized acceptor bleaching reveals the large-scale co-clustering of ErbB1 and ErbB2. Methods and Applications of Fluorescence (MAF-11), Budapest, Hungary, 2009.

5. **Ágnes Szabó**, János Szöllősi, Péter Nagy: FRET-sensitized acceptor bleaching reveals the large-scale co-clustering of ErbB1 and ErbB2. XXV Congress of the International Society for Advancement of Cytometry (ISAC), Seattle, USA, 2010. (Outstanding poster award)

6. **Ágnes Szabó**, János Szöllősi, Péter Nagy: FRET-sensitized acceptor bleaching reveals the large-scale co-clustering of ErbB1 and ErbB2. Membrane Transport Conference, Sümeg, Hungary, 2010.