Ph.D. THESIS

Optimization of flow cytometric FRET measurements and their application in the molecular modeling of the nearly full ErbB2 receptor

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1. INTRODUCTION

Investigation of protein-protein interactions is important in understanding the higher organization level of molecular complexes, their structure-function relationships, and the regulation of signal transduction processes in living cells. The fluorescence resonance energy transfer (FRET) based methods are excellent tools for determining conformations and association patterns of biomolecules at the cell surface or inside the cells. With the help of FRET, molecular dimensions can be measured and determined in functioning, live cells.

One of the most effective applications of FRET method is flow cytometric energy transfer (FCET) measurement which provides investigation of molecular proximities of large number of live cell with robust statistics. In recent years, numerous cheaper diode and solid-state lasers with higher power output and matching fluorescent dye pairs have been developed. The high number of combinations raises the question of selecting the optimal dye pair for the flow cytometer with given laser excitations. In our study, we investigated seven fluorescent dyes on three flow cytometers, and we determined crucial parameters for the characterization of FCET measurements.

ErbB2 is the member of the epidermal growth factor receptor (EGFR) family and is responsible for the proliferation and differentiation of cells. Augmented functioning of the receptor can be observed in numerous malignancies for example in breast, ovarian and gastric tumors, which is mostly based on gene amplification and over-expression of the receptor. So far two immunotherapies targeting ErbB2 with humanized monoclonal antibodies have been developed, but the exact mechanism of action of these antibodies – which is probably the function of the spatial arrangement and molecular interaction of the epitopes - is still unknown. In our study, we performed the epitope mapping of ErbB2 extracellular domain in optimized FCET measurements, and we determined the orientation of the ectodomain relative to the cell membrane. We also described the nearly full model of ErbB2 receptor with molecular modeling techniques.

Theory of fluorescence resonance energy transfer and its implementation in flow cytometry

The FRET process is a long-range dipole-dipole interaction in which an excited donor fluorescent molecule transfers its energy to a neighboring acceptor molecule in a non-radiative way. The efficiency of energy transfer is highly sensitive to the distance of the donor and acceptor molecules, and steeply decreases in 1 – 10 nm; accordingly it can be used as a "spectroscopic ruler" and it allows for the prediction of molecular associations.

In the case of an ideal FRET dye pair, the emission spectrum of the donor significantly overlaps with the excitation spectrum of the acceptor. As a result of energy transfer, the fluorescence intensity (and quantum efficiency) of the donor molecule decreases in the presence of an acceptor molecule, which is called donor quenching. In the case of a fluorescent acceptor, the parallel process is the sensitized emission of the acceptor, when augmented fluorescence intensity can be observed at the acceptor emission wavelengths upon the excitation of the donor molecule.

The flow cytometric fluorescence resonance energy transfer (FCET) method provides information of the distribution of transmembrane proteins on the cell surface and conformational changes of bioactive molecules in relatively short time. The FCET method was successfully applied for numerous biological systems, for example for the investigation of association patterns of membrane proteins on immune-competent and various tumorous cells.

The role of ErbB2 receptor tyrosine kinase in tumorous malignancies

The type I family of transmembrane receptor tyrosine kinases comprises four members: epidermal growth factor receptor (EGFR or ErbB1), ErbB2 (HER2 or Neu), ErbB3 (HER3) and ErbB4 (HER4). The ErbB receptors play an important role in the regulation of proliferation, differentiation, apoptosis and migration. Their activation is assisted by more than dozen growth factor-like ligands, for example EGF, TGFα, Neuregulin, HB-EGF, Epiregulin, Amphiregulin and β-Cellulin; except for ErbB2, which doesn't have a known ligand yet. In the EGFR family, the receptors can form both homo- and heterodimers, thus receptors can be trans-activated by certain growth factors that do not even directly bind to them. The composition of dimers (or higher order oligomers) is determined by the relative expression level and affinity of various receptors and the concentration of their respective ligands. ErbB2 is the preferred heteroassociation partner of all other ErbB proteins, which is most prominent in the case of ErbB2-ErbB3 heterodimer, because ErbB3 doesn't possess intrinsic tyrosine kinase activity, thus it can be activated in heterodimers only.

ErbB2 molecule was first recognized as a proto-oncogene mutant. Although similar ErbB2 mutations are quite rare in human tumors, the wild-type ErbB2 is often amplified in genomic level, and thus, but sometimes without amplification, over-expressed in protein level too. It originally functions in embryogenesis, and is rarely expressed in adult tissues. On the other hand, it is highly over-expressed in 25 – 30 % of breast cancers, which is associated with aggressive phenotype, resistance against chemotherapy and poor prognosis. ErbB2

seemed to be an excellent immunotherapic target for which several strategies have been developed in recent years, and one of these is already widely used in clinical practice. These therapies include: antibody therapy, targeted drug delivery by immuno-liposomes, the decrease of gene amplification by antisense oligonucleotides, application of specific tyrosine kinase inhibitors, and specific blocking of heat-shock proteins by half-synthetic drugs.

The structures of ErbB receptors are very similar to each other, and several studies were published about the crystal structures of the extracellular domain of EGFR, ErbB2 and ErbB3, and also about the tyrosine kinase domain of EGFR. The structure of the receptors consists of the following parts: a ligand-binding extracellular part composed of four domains (L1, CR1, L2 and CR2), a transmembrane helix, a tyrosine kinase domain, and a C-terminal tail containing tyrosine phosphorylation sites. The comparison of the structures of EGFR and ErbB3 monomers and of ligand-bound EGFR dimer revealed that the ligand binds in between domains L1 and L2, and induces a significant conformational change in the extracellular domain of the receptor. In the structure of ErbB2 extracellular domain, however, the ligand-binding domains are directly attached to each other. The structural mechanism for the dimerization of ErbB proteins can be explained with a ligand-induced conformational change. Without ligand, EGFR and ErbB3 is in closed (ligand-free) conformation, that is the cystein-rich, CR1, domain is interacting with a small part of the CR2 domain. In ligand-bound, open state, the dimerization hairpin is protruding from the center of the CR1 domain, and this part is responsible for the formation of EGFR dimer, which was only observed in ligand-bound molecules so far. On the other hand, the extracellular domain of ErbB2 is always in open state, even in monomeric form giving explanation for its preference as a dimerization partner for the other three member of the family.

2. SPECIFIC AIMS

The major goals were to investigate the applicability of newly developed fluorescent molecules and flow cytometers, and to build a model of ErbB2 receptor. We had the following aims during our investigations:

- Characterization of new fluorescent dyes and flow cytometers for flow cytometric energy transfer (FCET) measurements and their influence on the energy transfer efficiency.
- Establishment of objective physical parameters for the selection of optimal FRET dye pair for a cytometer with given optical characteristics.
- Determination of molecular distances of various ErbB2 epitopes from each other and the cell membrane as assessed by epitope specific antibody fragments in FCET measurements.
- Prediction for the full structural model of ErbB2 receptor on the basis of published structural data and FCET measurements.

3. MATERIALS AND METHIODS

Cells

In our studies, SK-BR-3 breast and N87 gastric carcinoma cell lines were used expressing high amount of ErbB2 and MHC Class I molecules on the cell surface.

Antibodies

Monoclonal antibodies, W6/32 (against heavy chain of MHC Class I) and L368 (against β2-microglobulin) were used for the investigation of fluorescent dyes. For the epitope mapping of ErbB2, 4D5 (Trastuzumab, Herceptin™) and 2C4 (Pertuzumab, Omnitarg™) Fab', 7C2 Fab, and F5 scFv antibodies were used. These genetically engineered antibody fragments were tagged with a free SH-side chain cystein at position opposite to the epitope-recognition site (except for 7C2), so the 1:1 dye-to-protein labeling ratio could be established by maleimide-reactive dyes enabling for exact FRET distance calculations.

7C2 Fab fragments were prepared as it was described earlier. Briefly, IgG mAbs were digested with activated papain. The enzyme activity was terminated by addition of iodoacetamide. The reaction mixture was passed through a Sephadex G-100 fine column and the collected Fab fractions were further separated from intact immunoglobulin by using a protein A sepharose column.

For the testing of fluorescent dyes, approx. 1 mg/ml concentration of W6/32 antibodies were labeled with donor (Cy3, Alexa546, Alexa555 és Alexa568) and L368 antibodies were labeled with acceptor (Cy5, Alexa633 és Alexa647) dyes (succinimide-derivatives) as described in product manual. Unreacted dye molecules were removed by gel filtration through a Sephadex G-25 column. The dye-to-protein labeling ratio was determined by spectrophotometry and its values were in the range of 1 to 5, where concentration quenching does not yet have a great effect on the fluorescence quantum yield of the donor and acceptor dyes.

Aliquots of anti-ErbB2 antibodies (4D5, 2C4 and F5; approx. 1 mg/ml concentration) were conjugated with maleimide-derivatives of Alexa488, and Cy3 and Cy5 fluorescent dyes, while for 7C2 the succinimide-derivatives of the same dyes were used as described in product manual. Unreacted dye molecules were removed by gel filtration through a Sephadex G-25 column. The dye-to-protein labeling ratio was determined by spectrophotometry and its values were approximately 1.

Labeling of cell surface antigens for FCET measurements

Freshly harvested cells were washed twice in ice-cold phosphate buffered saline (PBS; pH 7.4), then 1 million cells were suspended in 50 µl PBS. Cells were incubated for 30 minutes on ice in dark with various conjugates of antibodies or Fab fragments in saturating concentrations. For the double labeling of FRET samples, two fluorescently conjugated antibodies were added simultaneously to the cells. Then cells were washed twice in ice-cold PBS and fixed with 1 % formaldehyde and PBS.

For the FRET based determination of membrane proximities, the cells were doped with 2-(4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diazas-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphatidylcholine (BODIPY-PC (581/591)) as a FRET acceptor for the Alexa488-conjugated anti-ErbB2 antibody fragments. For labeling with BODIPY-PC (581/591), a final concentration of up to 5 µg/ml of dye was added to the cell suspension kept in glass tubes, at a cell density of 10^6 cells/sample (in 50 µl final volume). Labeling was performed for 30 min on ice, and then cells were washed twice with ice-cold PBS, labeled with Fabs for 30 min on ice, and analyzed immediately by flow cytometry. Cells were kept in ice-cold buffer (without fixation) during the flow cytometric analysis.

Flow cytometric energy transfer measurements

For the investigation of new fluorescent dyes, FCET measurements were performed on three commercial flow cytometers: FACSCalibur, FACSVantage SE with DiVa option and FACSArray. The optical setups for the flow cytometers were as follows:

		I_1 donor	I_2 sens. acceptor	I_3 acceptor
FACSCalibur	laser	488	488	635
	detector	FL2	FL3	FL4
	filter	585/42	670LP	661/16
FACSDiVa	laser	532	532	633
	detector	FL4	FL5	FL6
	filter	585/42	650LP	650LP
FACSArray	laser	532	532	635
	detector	Yellow	Far Red	Red
	filter	585/42	685LP	661/16

The energy transfer efficiencies representative of the mean distance between same and different types of anti-ErbB2 antibodies were determined on FACSCalibur with Cy3- and Cy5-conjugated antibody fragments and were calculated on a cell-by-cell basis.

For the FRET based determination of membrane proximities, the fluorescently labeled molecule is surrounded by a large number of acceptor dye labeled lipid, so energy transfer efficiency does not solely depend on the separation of the molecule, but it is also a function of the donor-acceptor ratio. In such cases, only the vertical distance between the plane of the cell membrane and the labeled epitope counts that can be approximated by the theoretical model of Yguerabide. According to the model, in steady state the intensity of the donor changes linearly with the surface density of the acceptor lipid probe, and the degree of this change is the inverse fourth power function of the membrane distance. These experiments were carried out on a FACSStar^{PLUS} flow cytometer with 488 and 514 nm laser excitation. The change in donor fluorescence intensity was plotted versus the surface density of BODIPY-PC (581/591) acceptor lipid probe, and the points were fitted to a straight line. The surface density of BODIPY-PC was calculated from the amount lipid probe and the concentration of cells in suspension, assuming total dye uptake.

Construction of models for ErbB2 domains

Crystal structures of ErbB2 and ErbB1 ectodomains were downloaded from the Protein Data Bank. Invisible loops were built by Modeller and Sybyl. NMR structure of the transmembrane helix of ErbB2 was used together with templates from the transmembrane regions of Glycophorin A, photosynthetic reaction center, and bacteriorhodopsin for construction of transmembrane helix dimers. The following secondary structure prediction methods were applied for the inner juxtamembrane region, located between the transmembrane and the protein kinase domains: GOR, Maxfield-Scheraga, and Qian-Sejnowski methods as implemented in Sybyl; and GOR4, SOPMA, JNET, and, which were available on the ExPASy molecular biology server (http://www.expasy.org). The ExPASy server was also used for BLAST sequence similarity search. The homologous model for the protein kinase domain of ErbB2 was built by Modeller based on the crystal structure of the protein kinase domain of ErbB1 and the sequence alignment made by ClustalW. To eliminate some large unfavorable van der Waals interactions generated by modeling, short minimizations were applied at several stages of the procedure.

4. RESULTS AND DISCUSSIONS

Establishing factors that influence energy transfer efficiency in FCET measurements

We found four parameters that are relevant for determining the practicality of FRET dye pairs, namely normalized fluorescence intensity, spectral overlap integral, normalized energy transfer efficiency, and the *efr* coefficient.

The fluorescence intensity is very important in FCET measurements, since one measure of FRET efficiency is the decrease of donor signal in the presence of an acceptor. So, it is preferable that the donor signals are at least twice as high as the background intensities to detect donor quenching. The background subtracted intensities were corrected for labeling ratios to obtain the fluorescence intensities for the same number of fluorescent dyes. The normalized fluorescence intensity values were in the range of 5-200 and 60-1100 for FACSCalibur and FACSDiVa, respectively.

Since the rate of energy transfer is influenced by the Förster distance of the dye pair, which contains physical constants distinctive for the dyes, this parameter should be a good tool for characterizing the FRET dye pairs. Unfortunately, the quantum yield highly depends on the environment, so it is difficult to tell the value of Q_D for a given donor dye at the cell surface (or inside the cell) according to the parameters of a non-conjugated dye. In the present study, we used the spectral overlap integral rather than the Förster distance. The J_{DA} overlap integrals were calculated from the spectra provided by the manufacturers of the dyes. The J_{DA} values were in the range of 5-15 x 10^{-13} $M^{-1}cm^3$, and were the highest for dye pairs containing Alexa568 as donor, and lowest for pairs containing Cy3 as donor.

The actual measured FRET efficiency also has to be taken into account in order to find a good dynamic range for the *E* value. Low *E* values compromise the statistical accuracy of the measurements and hinder monitoring changes in the association pattern. High *E* values are also error prone especially above 90 %. To compare the efficiency values between dye pairs, they have to be normalized for their excitation spectrum, quantum yield and the donor-acceptor ratios of the dyes. For the later it can be surmised that almost all donor labeled antibodies are in close proximity of one, and only one acceptor labeled antibody. In such cases, only the ratio of the labeling ratios has to be considered, since the ratio of the number of antibodies is approximately one. Since the FRET efficiency is not linearly proportional to the labeling ratios, the $A = E/(1–E)$ values have to be used for normalization. Dye pairs containing Alexa568 as donor showed the highest FRET efficiency values, in one occasion, for the Alexa568 and Alexa633 pair the normalized FRET efficiency value was even close to

0.80 on the FACSDiVa instrument. The lowest normalized FRET efficiency values (about 0.22) were obtained for dye pairs containing Alexa546 dyes as donor on the FACSCalibur instrument.

Another important factor is the *efr* coefficient. The *efr* coefficient is given by the ratio of molar extinction coefficients of the fluorophores at the donor excitation wavelength, and the larger its value the more reliable the determination of FRET efficiency. As this physical parameter cannot be reliably obtained from observing the absorption spectrum of the dyes, another possibility is to determine the FRET efficiency and vary the *efr* coefficient until the efficiency value equals the one obtained by donor quenching calculation (corrected for antibody competition) and/or the unquenched donor fluorescence intensity equals the fluorescence intensity of the donor only labeled sample. The *efr* coefficients determined this way were in the range of 0.2-17 and were the highest for dye pairs containing Cy3 as donor, and lowest for pairs containing Alexa568 as donor.

The J_{DA} and *efr* coefficient values presented here may be useful in other experiments as well. Since the overlap integrals were calculated from known excitation and emission spectra of the dyes, these values can be used to calculate the Förster distance in the knowledge of the donor quantum yield. The most important feature of the *efr* coefficient is that these values are transitive between the same types of cytometers and can be used for FCET measurements on any cytometer with the same optical setup. The *efr* coefficients obtained here and those derived from the spectra are different, and the ones presented here are more valid for measurements on biological systems, since they are related to fluorescently labeled antibodies attached to the cell surface of live cells.

Selecting the optimal fluorescent dye pair for FCET measurements

Because of the high number of combinations between the four donor and three acceptor dyes, we wanted to narrow the scope of our search to obtain the most suitable dye pair. The dye Cy3 has the lowest overlap integral and FRET efficiency values, and it has the lowest quantum efficiency. Another striking feature is the very high J_{DA} and E values for the Alexa568 dye, however, these values should be contrasted with the extremely low intensity and *efr* values, which result in less reliable FRET efficiency histograms and render FCET measurements practically useless with this dye. Thus, two donor dyes (Cy3 and Alexa568) should be left out from further investigations. Looking at the data of acceptor dyes, we didn't find any significant differences. So for a more thorough investigation, Alexa546 and

Alexa555, as donors, and Cy5, Alexa633 and Alexa647, as acceptors, giving six dye pairs, were used

First we analyzed the intensities, and found that Alexa546 had about twice as high fluorescence intensity as Alexa555. Among the acceptors, we didn't find great differences. Nonetheless, Alexa647 has the highest and Alexa633 the lowest intensity. So the Alexa546-Alexa647 pair is the best choice considering fluorescence intensity.

Next, we looked at the spectral overlap integrals (J_{DA}), since they determine the range of sensitivity of energy transfer via the Förster distance. In all of the cases, Alexa633 as acceptor gave the smallest and Alexa555 acting as donor had the highest overlap integral. The other two acceptors (Cy5 and Alexa647) produced similar values, though for Cy5 it was a bit higher. Thus, in this respect, the Alexa555-Cy5 pair was the best dye pair, but the other dyes shouldn't be excluded because of the small differences between the dye pairs (J_{DA} values were in the range of 7.2 - 8.7 x 10^{-13} $M^{-1}cm^3$).

Then we investigated the normalized FRET efficiency values. For the two donor dyes, we obtained fairly similar values on both cytometers, so we don't think that this is a decisive factor in either case. However, there were larger differences between the acceptor dyes, and Alexa633 had the highest, while Alexa647 gave the smallest FRET values. Thus the Alexa555-Alexa633 pair is the most effective when viewing the normalized FRET efficiency values.

The last factor we investigated was the *efr* coefficient, and the biggest differences occurred in this data set. The *efr* coefficients of Alexa555 containing dye pairs were higher than the Alexa546 containing pairs. However, the values with Alexa633 were almost the same on both cytometers, and on FACSDiVa the Alexa546-Alexa647 pair had a higher *efr* value than the Alexa555-Alexa647 pair. The *efr* values for the acceptors tested increased in the order Alexa633, Alexa647 and Cy5 independent of the donor, on both cytometers. So considering the *efr* values, the Alexa555-Cy5 pair seems to be the best choice for FCET measurements.

The three instrument-dependent parameters were also determined for the Alexa546-Alexa647 dye pair on the FACSArray. The normalized fluorescence intensities were even larger than the ones obtained with FACSDiVa, probably due to the cuvette flow system. The normalized FRET efficiency value was larger than for FACSCalibur and FACSDiVa. The *efr* coefficient, determined as described above, was the same as for the FACSDiVa, which can be explained by the similar laser lines and detection optics

To summarize our finding, the two donor fluorophores (Alexa546 and Alexa555) were quite similar in every aspect of our investigation, but Alexa555 was superior in most cases. However the most important parameter in the case of a donor dye is its fluorescence intensity and consequently the signal-to-noise ratio in the donor fluorescence channel, which makes Alexa546 a better partner for FCET experiments. In the case of an acceptor dye, the lack of spectral crosstalk to the donor fluorescence channel and the overlap integral is more important. In this respect, Cy5 seemed to be the best and Alexa633 the worst choice as a FRET acceptor and Alexa647 is very similar to Cy5.

After analyzing the relevant four parameters (normalized fluorescence intensity, overlap integral, normalized energy transfer efficiency and *efr* coefficient), we found that the Alexa555-Cy5 and Alexa546-Alexa647 pairs have the best characteristics. The former, Alexa555-Cy5 pair exhibits the highest overlap integral and FRET efficiency, while the latter dye pair has the highest fluorescence intensity. As the most important parameter in fluorescence measurements is the excitability and detectibility of the fluorescent dye, the Alexa546-Alexa647 pair is the most suitable fluorophore pair for flow cytometric energy transfer experiments. On the other hand, in some microscopic techniques the photo-destructibility of the fluorophores is also important, so the Alexa555-Cy5 pair is the best choice for acceptor photobleaching FRET measurements.

If we compare all data sets, the most significant feature is the pronounced fluorescence intensity (and signal-to-noise ratio) with the 532 nm donor excitation, which is not surprising in view of their excitation spectrum. So we can state that in order to increase the sensitivity of FCET measurements, it is not enough to use fluorophores emitting in the red region of the visible spectrum (where the amount of autofluorescence is smaller) as was described previously, but the flow cytometer has to be also equipped with lasers providing optimal excitation of these fluorophores.

The difference in signal-to-noise ratio also had a great impact on the FRET efficiency values. Both signal-to-noise ratio and FRET efficiency were higher on the FACSDiVa than on the FACSCalibur for all tested dye pairs, although in principle the FRET efficiency values should be independent of the instrument. This discrepancy questions the comparability of FRET results measured at the different research centers and/or on diverse equipment. Generally, FRET efficiencies should be quite similar if measured on the same or same type of instrument, and the differences that arise can be attributed to the biological variance of the samples. However, the change in the excitation wavelength of the donor can seriously influence its quantum yield, which in turn has a large effect, via the Förster distance, on the

value of FRET efficiency. A more ideal excitation wavelength also increases the donor signals and highly narrows the instrument-dependent variance of the FRET efficiency distribution histograms, which can manifest in altered mean FRET efficiencies. Thus a feasible strategy to match data from different laboratories should at least include the comparison of both fluorescence intensities and normalized FRET efficiencies and, if possible, quantum yield data of the fluorophores in use.

Molecular distances between epitopes of ErbB2

For the construction of the model for the extracellular domain of ErbB2 receptor and for its dimer, we used four antibodies: 4D5, 2C4, 7C2, and F5. The humanized version of 4D5 monoclonal antibody (Trastuzumab; Herceptin™) is already used in clinical practice for treatment of breast cancer with high expression levels of ErbB2, while the humanized version of 2C4 antibody (Pertuzumab; Omnitarg™) is in II. phase clinical trial, and is hoped to be useful in lung and prostate cancer phenotypes where ErbB2 expression level is low. The F5 scFv antibody in liposome-attached form is a promising agent for targeted drug therapy.

In our study, we investigated the molecular distances between the four fluorescently labeled antibodies, and the intermolecular distances between these antibodies attached to the same epitope of two different ErbB2 molecules. The FRET efficiency values were in every case above or around the detection limit (5 %), and were significantly different from the donor only labeled sample (negative control). Similar values were obtained for both SK-BR-3 and N87 cell line (henceforward we present data obtained on N87 cell line only).

According to the FRET efficiency histograms corresponding to intra- and intermolecular labeling schemes, the highest transfer efficiency value was obtained between 4D5 and 2C4, but the distribution histogram is quite broad, which cannot be explained by biological variance alone in contrast with the distribution histogram of 4D5-7C2 with the lower mean transfer efficiency value. The most probable explanation is that it contains both intra- and intermolecular interactions that cannot be resolved in flow cytometric methods. On the basis of the molecular distance measurements between the different types of epitopes, the endpoints of 4D5 and 2C4 antibodies are the closest to each other (6.2 nm), while the other distances were close to the detection limit, and quite far from each other.

The intermolecular transfer efficiency (7 – 14 %) and distance (6.8 – 7.7 nm) values were very similar to each other. By theoretical considerations there can be two explanations: first, it is possible that the dye molecules residing at the end of the antibodies are situated at one “side” of the receptor, thus they follow the surface of the receptor at a certain distance;

second, it is also possible that the antibodies are protruding from many sides of the receptor, thus these distances, for a pair of antibodies, represent interactions between two, but actually three or more receptors. This simple scheme is further detailed by the distances of dye molecules from the membrane, which can be used for the determination of inclination angle against the membrane of the antibodies measured at their epitopes.

Molecular distances of ErbB2 epitopes from the membrane

To determine the distances of antibodies from the cell membrane we used the method described by Yguerabide. The essence of the method is that the vertical distance of a point-like fluorophore from a plane of other fluorophores with fluorescence resonance possibility can be measured if we examine the change of transfer efficiency in the function of the surface density of the fluorophore in the plane.

In our experiments, we investigated the vertical distance of four fluorescently labeled antibodies from the plane of cell membrane. The transfer efficiency values, or rather the $E/(1-E)$ values measured for various antibodies were plotted in the function of the surface density of our lipid probe, and the point-series were fitted to a straight line. The vertical molecular distances were calculated from the mean of slopes of fitted lines measured in three independent experiments.

These vertical distance values suggest that the F5 scFv is closest to the cell membrane, while the 2C4 and 7C2 antibodies are moderately, and the 4D5 antibody is quite far from the cell membrane (4.7, 6.1, and 7.4 nm, respectively). This is in contrast with phage display data, which suggested that the epitope of 4D5 is close to the membrane, however this can be reasoned if we surmise that 4D5 Fab' points away from the membrane. These distance values are also supported by the X-ray crystal structure data of ErbB2 ectodomain complexed with 4D5 and 2C4 Fabs published after our measurements, and in further calculations we used the coordinates derived from crystal structures.

Modeling the nearly full length ErbB2 dimer

Although in our experiments, we determined the distances measured between each other and from the cell membrane for all four Fab antibodies, for the molecular modeling calculations we used distance information determined for 4D5 Fab' only. The reason for this is that 2C4 antibody inhibits the formation of homo- and heterodimers according to structural data, the F5 scFv antibody had low affinity and strongly competes with the other antibodies,

and for the 7C2 antibody we couldn't yet determine its epitope because of the lack of available Fab that is labeled with SH-reactive dye in 1:1 ratio.

Our model-building procedure for ErbB2 dimer started with the extracellular domains of ErbB2. It was assumed that the ErbB2 dimer could adopt a conformation similar to that of the EGFR dimer in the crystal form. Two copies of this ErbB2 model complexed with Herceptin Fab' were aligned to the crystal structure of the EGFR dimer A- and B-chains, respectively, and the conformations of the dimerization loops were changed to those found in the EGFR dimer by Modeller. The alignment regions consisted of residues only from CR1 domains (dimerization loops were also excluded), since different relative position of L- and CR-domains in EGFR and ErbB2 may result in bad fitting of the two monomers. The dimer was translated and rotated to the center of the coordinate system in such a way that the C_2 symmetry axis of the dimer was in line with the z axis and the x,y plane served as a model for the outer surface of the cell membrane.

Although this dimer configuration appears to be a good candidate based on the analogy to the EGFR homodimer, it should be noted that other configurations for the ErbB2 homodimer should also exist so that the experimentally measured 7.1 nm intermolecular distance between bound Herceptin Fab'. This 7.1 nm distance can be conceived as a weighted average from dimers predicted by the model and other dimers (or higher oligomers) that, themselves, would yield smaller intermolecular distances. Therefore it is predicted that the modeled EGFR-like dimer configuration exists as a subclass among many others, and/or as a dynamic entity that could be interconverted to other dimeric forms.

Besides the possible dimerization of ErbB2 ectodomains, the transmembrane domain can also dimerize. Transmembrane α-helix pairs from photosynthetic reaction center protein with resolved structures served as a template for constructing the dimer of ErbB2 transmembrane domain. Two copies of the NMR structure of the transmembrane helix of ErbB2 were aligned to each helix-pair template and a short minimization was applied. The transmembrane helix dimer was positioned below the ectodomain dimer in the same rotational axis, therefore each dimer interface could enhance the effect of the other dimerization region. More than one dimerization interface, acting synergistically, may help the formation of tightly bound ErbB2 dimers or heterodimers.

The distance between the ectodomain and the membrane was set based on FRET measurements: the ectodomain dimer complexed with Herceptin Fab' was moved up along the z axis until the vertical distance of the free end of the Herceptin Fab' from the cell membrane (x,y plane) reached the experimental value determined by FRET. The

transmembrane helix dimer was moved down along the z axis until the N-terminal hydrophobic segment of the helices reached the x,y plane. The Protein Loop Search algorithm of Sybyl was applied to fill the gap between the C-terminal end of the ectodomain and the N-terminal end of the transmembrane domain. To satisfy the symmetry requirements of the structure, one monomer containing the full extracellular and transmembrane domains was duplicated and aligned to the other monomer, which was deleted after the transformation.

The C-terminal part of the CR2 domain is not ordered, or does not exist in crystal structures of ErbB molecules, and may form a flexible arm together with the outer juxtamembrane region. The flexibility of this region may be required in dimer formation for the correct positioning of the first monomer (ErbB2) relative to the variable second monomer (ErbB1–4). Our flexible model does not rule out the possibility of interaction between CR2 domains of homo- or heterodimers of ErbB molecules, as suggested by immunoprecipitation and mutational studies.

Secondary structure of the juxtamembrane region located between the transmembrane and the protein kinase domains was predicted by several methods of Sybyl or of the ExPASy molecular biology server (http://www.expasy.org). All prediction methods showed high probability of a-helix for the N-terminal half of the sequence. The proximity of the α-helical transmembrane segment together with the consensus result of several secondary structure prediction methods and the three-dimensional structure of the 3HLA fragment suggested that this 3HLA fragment may serve as a good template for homologous modeling of the inner juxtamembrane region of ErbB2.

Two copies of the model of the juxtamembrane segment were superimposed with the modeled transmembrane helix dimer using the four N-terminal residues, which were common in the models of the juxtamembrane region and the transmembrane helix. Model building was continued with the superposition of two copies of the protein kinase domain using the common four terminal residues. Interestingly, using this approach, the two kinase domains formed a good, symmetric back-to-back dimer without any unfavorable interaction. In our model the localization of the kinase domain was designated solely based on the position of the transmembrane domain dimer and the shape of the model of the inner juxtamembrane region. Based on the model, kinase domains may interact and may serve as a third dimerization interface. The interaction of kinase domains is also supported by experimental data

The models of the extracellular dimer and intracellular dimer were superimposed based on the common transmembrane regions. This nearly full-length ErbB2 dimer consisted of the whole ectodomain, the outer juxtamembrane region, the transmembrane domain, the

inner juxtamembrane region, the protein kinase domain, and a part of the regulatory C-terminal tail of the ErbB2 molecule. We have built an ErbB2 homodimer structure with three potentially interacting regions. This ErbB2 homodimer, taking into account the similar structures of the ligand-bound forms of the other ErbB molecules, may serve as a starting model for heterodimers of ErbB2 with other members of the ErbB family. The back-to-back kinase dimer arrangement could be responsible for the *trans*-phosphorylation of the cytoplasmic regulatory tails but not of the kinase domains themselves, which requires the involvement of other kinase molecules.

5. SUMMARY

Our investigations were aimed to construct the molecular model of ErbB2 receptor and its dimer by fluorescence resonance energy transfer (FRET) based epitope mapping and molecular modeling methods, and to establish the applicability of newly developed fluorescent dyes and flow cytometers in flow cytometric FRET (FCET) measurements. Our results are the follows:

- We established crucial parameters for the flow cytometric investigation of membrane protein associations, which are the normalized fluorescence intensity, the spectral overlap integral (or critical Förster distance) determining the magnitude of transfer transition, the normalized FRET efficiency, and the *efr* coefficient accounting for the excitability of fluorescent dyes.
- On the three flow cytometers we tested, we found that the Alexa546-Alexa647 and Alexa555-Cy5 donor-acceptor dye pairs are optimal for FCET measurements.
- In FCET measurements, we determined the molecular distances of various, fluorescently labeled epitope-specific anti-ErbB2 antibodies from each other and the cell membrane.
- On the basis of FCET molecular distances and published structural data, we constructed the nearly full-length model of ErbB2 transmembrane receptor by molecular modeling methods, and this model consists of the extracellular domain, the transmembrane region, the tyrosine kinase domain, and the linker regions in-between these domains. We also constructed a possible dimer of ErbB2 receptor according to the FRET efficiency values measured between antibody-bound ErbB2 molecules, and we assessed many possible dimerization surfaces, namely at the outer juxtamembrane sequence, the transmembrane, and the tyrosine kinase regions.

6. UTILIZATION OF RESULTS

By comparing new fluorescent dyes and flow cytometers, we developed a process for their characterization in FRET measurements, which can be implemented for any dyes and flow cytometers developed in the future. Among the tested new flow cytometers, there is one which enables for even automatic measurements and analysis of large number of samples.

Our results contribute to the understanding of the role and mechanism of epidermal growth factor receptors in tumorous malignancies, since they point out the variegation of receptor interactions. We established the first model in the receptor family that contains the nearly full-length model of ErbB2 receptor.

7. PUBLICATIONS

The thesis is based on the following publications:

1. Bagossi P., Horváth G., Vereb G., Szöllősi J., Tőzsér J.: Molecular modeling of nearly full length ErbB2 receptor. *Biophysical Journal* (2005) 88:1354-1362.
IF: 4.463 (JCR 2003)

2. Horváth G., Petrás M., Szentesi G., Fábián Á., Park J.W., Vereb G., Szöllősi J.: Selecting the right fluorophores and flow cytometer for fluorescence resonance energy transfer measurements. *Cytometry* (2005) (*accepted, Epub ahead of print*)
IF: 2.095 (JCR 2003)

Other publications:

1. Sebestyén Z., Nagy P., Horváth G., Vámosi G., Debets R., Gratama J.W., Alexander D.R., Szöllősi J.: Long wavelength fluorophores and cell-by-cell correction for autofluorescence significantly improves the accuracy of flow cytometric energy transfer measurements on a dual-laser benchtop flow cytometer. *Cytometry* (2002) 48:124-135.
IF: 2.095 (JCR 2003)

2. Nagy P., Vereb G., Sebestyén Z., Horváth G., Lockett S.J., Damjanovich S., Park J.W., Jovin T.M., Szöllősi J: Lipid rafts and the local density of ErbB proteins influence the biological role of homo- and heteroassociations of ErbB2. *J. Cell Science* (2002) 115(Pt 22):4251-4262.
IF: 7.250 (JCR 2003)

3. Szentesi G., Horváth G., Bori I., Vámosi G., Szöllősi J., Gáspár R., Damjanovich S., Jenei A., Mátyus L.: Computer program for determining fluorescence resonance energy transfer efficiency from flow cytometric data on a cell-by-cell basis. *Computer Methods and Programs in Biomedicine* (2004) 75:201-211.
IF: 0.724 (JCR 2003)

4. Diermeier S., Horváth G., Knuechel-Clarke R., Hofstaedter F., Szöllősi J., Brockhoff G.: Epidermal growth factor receptor coexpression modulates susceptibility to Herceptin in HER2/neu overexpressing breast cancer cells via specific erbB-receptor interaction and activation. *Experimental Cell Research* (2005) 304(2):604-619.

IF: 3.949 (JCR 2003)