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Quantifying the effects of co-expressing EGFR-HER2 on HER activation and trafficking

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Abstract

The human epidermal growth factor receptor (HER) system is an intricately regulated system that plays critical roles in development and tumorigenesis. Here, we apply integrated experimentation and modeling to analyze HER receptor activation in a panel of cell lines expressing endogenous levels of EGFR/HER1 and different levels of HER2. A mathematical model that includes the fundamental processes involved in receptor activation and trafficking was used to fit the experimental data, and values of the independent parameters for active receptor dimer formation affinities, trafficking rates and relative phosphorylation levels were estimated. Obtained parameter values quantitatively support the existing ideas on the effect of HER2 on EGFR dynamics, and enable us to predict the abundances of various phosphorylated receptor dimers in the cell lines.

Keywords

EGFR; HER; ErbB; receptor pathways; signal transduction; endocytosis; quantitative network analysis; systems biology; bootstrap confidence intervals

INTRODUCTION

The integration of experimental measurements and computational predictions is being increasingly advocated as a powerful means to dissect the mechanistic details of biological systems [1, 2]. One way to effectively implement this integration is to construct a mathematical model and use it to analyze experimental data and to generate hypotheses. Even though this is conceptually simple, constructing an effective model whose parameters can be reliably estimated from the experimental data is a non-trivial exercise. Inclusion of all the mechanistic details of a biological system often leads to extremely large models even for relatively simple systems and makes parameter estimation unreliable. Thus, an effective model is the one whose size is limited by the scope and limitations of the experimentally measured quantities, while still capturing the fundamental processes that occur in the studied system. Here, we develop such a model to study receptor activation and trafficking patterns

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of the human epidermal growth factor receptors (HER) in human mammary epithelial (HME) cells.

The HER (also known as ErbB) family consists of the epidermal growth factor receptor (EGFR/HER1) and three other members HER2-4, and it is arguably the most important receptor system in the context of development and tumorigenesis [3, 4]. In addition to their normal physiological role in growth, proliferation, and differentiation of epithelial cells, HER receptors play a key role in transformation and over-expression of HER2 is associated with poor prognosis in breast cancers [5].

It is known that, while all members of the HER family display significant homology, each receptor type has distinct properties. EGFR can bind a multitude of ligands, but HER2 has no known activating ligand [4]. While EGFR is rapidly internalized and degraded upon binding its ligand EGF, HER2 does not display significant ligand-induced internalization and recycles rapidly back to the cell surface following endocytosis [6]. Ligand binding induces dimerization of HER family receptors and various homo- and hetero-dimer combinations can be formed [3, 4]. Dimerized receptors undergo rapid phosphorylation and activate downstream signaling pathways such as the MAPK, PI3K/Akt and PKC pathways via the binding of signaling adaptors to phosphotyrosine sites on the receptor cytoplasmic tail [4]. There is considerable evidence to suggest that the numbers and types of dimers formed among the HER family members are the important determinants of the HER-mediated cellular response [3].

Given the diverse properties and interactions among the HER molecules, it is difficult to intuitively identify how overexpressing a particular HER molecule alters receptor activation patterns and affects the overall cell phenotype. Here, we employ model-based analysis to extract active receptor dimer formation affinities, trafficking rates and relative phosphorylation levels from receptor activation data collected in a panel of cell lines expressing endogenous levels of EGFR and varying levels of HER2. We find that: i) the order of the receptor dimer stabilities is $\text{EGFR-EGFR} > \text{EGFR-HER2} > \text{HER2-HER2}$; ii) HER2 preferentially forms EGFR-HER2 heterodimers; iii) HER2 containing dimers are internalized and degraded at a slower rate compared to EGFR homodimers; and iv) the level of HER2 phosphorylation is higher in the EGFR-HER2 heterodimer than in the HER2 homodimer, whereas EGFR phosphorylation levels in the homodimer are comparable to that in the heterodimer. These results validate several existing ideas on how HER2 affects EGFR activation patterns [6–8].

The novelty of our work is that we are able to quantify these trends through model-based analysis of a comprehensive set of experimental data. As reaction rates can have strong cell line dependence, use of a closely related library of HER2 expressing cell lines has the unique advantage that the derived rate constants are internally consistent. In addition, our approach enables us to predict the abundances of phosphorylated receptor dimers as a function of the HER2 expression level in HME cells. These dimerization patterns are difficult to measure experimentally, but are necessary to define the contributions of the individual dimer types to downstream signaling events and to the cell phenotype.

MATERIALS AND METHODS

Cell culture and treatment conditions

Four distinct cell lines that expressed different levels of the HER2 receptor were used in our experiments (Table 1). The parental cell line (*Par*) was originally provided by Martha Stampfer (Lawrence Berkeley Laboratory, Berkeley, CA) as HME cell line 184A1-1. It expresses approximately 200,000 molecules of EGFR and low levels of HER2 [7]. This cell line was transduced with the HER2 gene and subcloned to obtain three clones expressing: low (*17L*), medium (*24H*), and high (*A11H*) levels of HER2. Cells were maintained in DFCI-1 medium with 12.5 ng/ml EGF (PeproTech, Rocky Hill, NJ) as described in [9]. At about 70–80% cell confluency, DFCI-1 medium was replaced with bicarbonate-free DFHB minimal medium plus 1% bovine serum albumin. Cells were then quiesced for 12–18 hours before treatment. To activate the HER system, 100 ng/ml EGF was added into the culture medium and cells were incubated at 37°C for fixed amounts of time from 5 to 120 min.

Phosphorylated receptor levels in the internal compartments were determined using an acid-stripping protocol, which selectively dephosphorylates cell surface receptors without altering the phosphorylation of internalized receptors [10]. Following acid stripping, cells were washed 3× with ice cold PBS, incubated at room temperature for one minute to allow surface receptor dephosphorylation, and washed again with cold PBS. Both, the stripped and un-stripped cells were solubilized with ice cold lysis buffer (1% NP-40, 20mM pH 8.0 Tris buffer, 137mM NaCl, 10% glycerol, 2mM EDTA, supplemented with 1mM heat activated sodium orthovanadate and 1% protease inhibitor cocktail III (Calbiochem, La Jolla, CA)) for 20 min.

Receptor mass and phosphorylation measurements

We ran three sets of ELISA assays to quantify the time-dependent receptor mass and phosphorylation levels using R&D DuoSet IC ELISA kits (R&D Systems Inc., Minneapolis, MN):

1. *The EGFR (mR_{1t}) and HER2 (mR_{2t}) receptor masses* were quantified in total cell lysates using capture and probe antibodies specific to EGFR and HER2.
2. *The masses of tyrosine phosphorylated EGFR (mRP_{1t}) and HER2 (mRP_{2t})* were determined by pulling total cell lysates down with a phospho-tyrosine antibody and then probing with EGFR and HER2 specific antibodies. These measurements enable us to quantify the fraction of total EGFR and HER2 that get tyrosine phosphorylated in each cell line.
3. *The extent of EGFR (pRP_{1t}) and HER2 (pRP_{2t}) tyrosine phosphorylation* was assayed by pulling total cell lysates down with antibodies specific to EGFR and HER2, and subsequently probing with a phospho-tyrosine antibody.

To compare across cell lines, ELISA results were normalized based on the total protein present in the cell lysate. Repeating the measurements 2 and 3 above following ligand stripping enabled us to quantify the masses of the phosphorylated receptors ($mRP_{1/2t}$) and the extent of phosphorylation ($pRP_{1/2t}$) in the internal compartments. In summary, 10

different quantities were measured for each cell line, except for the parental cell line where only EGFR activation was assayed since the HER2 expression was low. Thus, we performed 35 independent time-course measurements, with at least two replicates for each time-course.

Kinetic network model and parameter estimation

Our current model is a simplified version of our earlier mechanistic model [11], and it retains only the features of HER activation and trafficking that could be reliably quantified from the collected experimental data. For the network addressed in this study, there are two groups of processes: i) biochemical reactions such as ligand binding, receptor dimer formation and receptor phosphorylation, and ii) receptor trafficking related events such as internalization and degradation. We note that the timescale of the former reaction group (sub-minute) is much shorter than the time resolution of the experiments pursued in this study. Therefore, to be consistent with the experimental design, the current model lumps the receptor-ligand binding and subsequent receptor dimerization and phosphorylation reactions into a single *active receptor dimer formation* step (Fig. 1). We note that there are rigorous models for the EGFR system that include these biochemical reactions explicitly [7, 10–13]. These reactions can also be included in the current model – in fact the introduced additional parameters would improve the fits by increasing the number of degrees of freedom. However, due to the lack of the correct type of data, the estimated rate parameters for these reactions would be highly unreliable. One alternative would be to use fixed parameter values from earlier studies but, as reaction rates can be cell line dependent, this may incorrectly bias the data analysis. As retaining individual reactions is not advantageous for our purpose, only a lumped representation was pursued.

The model (Fig. 1) contains 10 species: Receptor monomers (R_1 : EGFR and R_2 : HER2) and active (i.e., phosphorylated) receptor dimers (R_{11}^* : EGFR homodimers, R_{12}^* : EGFR-HER2 heterodimer, and R_{22}^* : HER2 homodimer) at the cell surface (subscript s) and in the internal (i) compartments. Monomers at the cell surface interact to form active receptor dimers with a forward rate k_{fs} and a dimer-dependent reverse rate k_{rs} . Internalized dimers dissociate with a dimer-dependent rate k_{ri} . Monomers are internalized with rate k_i . Dimers are internalized with a dimer-dependent rate k_e . In the absence of ligand, EGFR and HER2 monomers are assumed to have surface-to-internal receptor ratios of α_1 and α_2 , respectively. Internalized receptors are degraded at a species dependent rate k_d . V_{R1} and V_{R2} are the zero-order synthesis rates for EGFR and HER2.

The phosphorylation levels of receptor molecules in homo- and hetero-dimers are most likely different. We accommodate this important aspect in our analysis by introducing dimer-specific phosphorylation factors pf into our model and use the formula $pRP = 2 \times pf_{homo} \times R_{homo}^* + pf_{hetero} \times R_{hetero}^*$ to convert the abundances of activated dimers to the extents of phosphorylation measured in the ELISA experiments (Supp Mat 1). The pf parameters represent a combination of the average number of phosphorylated sites on a receptor molecule in a given dimer, and the relationship between the number of phosphorylated sites and the experimentally measured ELISA value. The latter quantity is related to the assay protocol. If the measurement efficiency for determining tyrosine phosphorylation was the same across dimer types, then the comparison of pf values would

establish the relative phosphorylation levels of the receptors as a function of their dimer partner. i.e., the *pf* factors are a measure of the relative kinase efficiencies of the partnering receptors.

We determined the optimal values for the 20 unknown model parameters (16 independent kinetic rates and 4 *pf* values) using non-linear least-squares regression to simultaneously fit the EGFR and HER2 receptor mass and phosphorylation data collected in the four cell lines. Experimental replicates were not averaged, and were treated as separate time-series. We employed bootstrap simulations to determine the uncertainties in the estimated parameter values. Detailed discussions of our mathematical model, its governing equations, the procedure to fit the model parameters, and the bootstrap confidence interval calculations are provided in Supplementary Material 1.

RESULTS AND DISCUSSION

EGFR and HER2 receptor activation patterns

Figure 2 presents receptor activation data from two cell lines each for EGFR and HER2. The analysis results and experimental data sets for the entire cell line library (Table 1) are presented in Supplementary Material 1 and 2, respectively. The mean receptor masses prior to ligand addition were used to determine the EGFR and HER2 expression levels in each cell line (Table 1): *Par*, *17L* and *24H* cell lines express comparable levels of EGFR, while *A11H* expresses ~1.5 times more. There is a significant difference in the HER2 expression levels between the cell lines with *A11H* expressing ~13 times the amount of HER2 seen in *17L*. Comparison of the slopes of the receptor mass curves indicates that in general HER2 is degraded at a slower rate than EGFR (Fig. 2; top plots). The middle plots present the masses of phosphorylated receptors, which increase with the total receptor expression level. This is clearly evident in the HER2 data where there is a significant difference in the receptor expression levels between the cell lines. The bottom plots present the extent of EGFR and HER2 phosphorylation. The peak EGFR phosphorylation levels are similar for *Par*, *17L* and *24H*, while the peak value is ~4 times higher for *A11H* (Fig. 2, and Supp Mat 1). The HER2 phosphorylation levels also increase with receptor expression level. Although there are apparent cell-line dependent variations, identifying the molecular processes that are responsible for these variations requires more rigorous data analysis. Towards this end, we pursued a model based analysis of the experimental data.

Estimated parameter values

Our mathematical model was fit to the experimental data to estimate the 20 unknown parameters (Table 2). Since multiple data sets are analyzed simultaneously, as expected, some of the experimental time-courses are captured better than the others, and the fit is by no means perfect for each of the cell lines and measurement types (Supp Mat 1). However, given the fact that we are using a single set of parameters to model the entire data set, the fits are remarkably good. The model captures the variations in activation patterns in spite of the noise that is inherent to these experimental measurements. Bootstrap analysis (Supp Mat 1) indicates that the 95% confidence intervals for most of the parameters in Table 2 are narrow and are within an order of magnitude of the parameter estimate. Although there is

larger uncertainty in the estimates for k_{r11s} , k_{r22s} , k_{r22i} , k_{t2} , k_{d2} , k_{e22} , k_{d22} and pf_{22} . clustering analysis of the bootstrap parameter distributions (Supp Mat 1) reveals that the values presented in Table 2 are in fact good estimates for k_{t2} , k_{d2} , k_{e22} , k_{d22} and pf_{22} . Our analysis also suggests that the value for k_{r11s} is likely to be smaller than ~ 0.2 /min and that k_{r22s} and k_{r22i} are likely to be greater than ~ 1 /min (Supp Mat 1). As discussed below, the estimated parameter values are consistent with previous reports.

Receptor activation—Even though it is only one of the lumped steps in our model, ligand binding measurements can be used to assess the receptor association in our model. Kholodenko et al. report K_D values of 0.6 nM and 10 nM respectively for EGFR ligand binding and receptor dimerization reactions in hepatocytes [12]. French et al. measured the ligand binding affinity to surface EGFR in B82 mouse fibroblast cells to be 4.2 nM for human derived EGF [14]. The lumped active dimer formation reaction in our approach combines the ligand binding, receptor dimerization and phosphorylation reactions, and its dissociation constant K_{D11s} ($=k_{r11s}/k_{fs}$) is 23 nM for EGFR homodimers at the cell surface. French et al. also found the ratio of the ligand dissociation rates in the internal compartments to that at the surface to be in the range 7–31 [14]. The corresponding ratio in our model, k_{r11i}/k_{r11s} , is 6.4. Although these are indirect validations, values of predicted parameters are in agreement with the existing data on EGFR activation.

Since a constant forward rate was used, the ordering of the obtained surface dissociation rates reveals that the lifetime of phosphorylated EGFR homodimers is expected to be longer than that of EGFR–HER2 heterodimers, which in turn have much longer lifetimes than HER2 homodimers. The dissociation rates of internalized complexes also highlight the lack of stability of the HER2 homodimer compared to the other dimer types. Hence, when co-expressed with the EGFR, HER2 molecules are expected to reside in and signal from heterodimers with the EGFR.

Receptor endocytosis and degradation—Our results reveal that EGFR monomers are internalized (compare k_{t1} and k_{t2}) and degraded (k_{d1} vs k_{d2}) slightly faster than HER2 monomers. The surface-to-internal ratio of EGFR, α_1 ($=k_{d1}/k_{t1}$) and HER2, α_2 ($=k_{d2}/k_{t2}$) in the absence of ligand are 1.1 and 1.2 respectively indicating that 52% of EGFR and 55% of HER2 are at the cell surface in the absence of ligand.

The dimer internalization rates (k_e) quantify the extent of ligand-induced endocytosis, and reveal that phosphorylated EGFR homodimers are internalized much faster than heterodimers and HER2 homodimers. These numbers are in agreement with an internalization mechanism wherein phosphorylated EGFR molecules associate with clathrin-coated pits more efficiently than HER2, and hence, are internalized faster [15].

The dimer degradation rates (k_d) indicate that the EGFR homodimers are degraded much faster than the other dimer types. In cells co-expressing EGFR and HER2, the measured EGFR degradation rate would be within the range defined by k_{d1} ($=0.019$ /min), k_{d11} ($=0.33$ /min) and k_{d12} ($=0.0082$ /min). Thus, depending upon the dimer distribution in the cell, the apparent EGFR degradation would be in the range 0.008–0.33/min. This prediction range

agrees well with the measurements of French et al. who report an EGFR degradation rate of 0.03/min in B82 cells [14].

Phosphorylation efficiencies—The ratios of the pf values enable us to quantify the phosphorylation levels of the receptors as a function of their dimer partner, and establish the relative kinase efficiencies of the partnering receptors. The ratio pf_{11}/pf_{12} is 1.05, indicating that the EGFR molecules have comparable phosphorylation levels in homodimers and heterodimers. The pf_{21}/pf_{22} ratio for HER2 phosphorylation levels is 4.46, which indicates that the phosphorylation levels of HER2 molecules are significantly higher on average in the heterodimers than in the homodimers. We note that our approach cannot distinguish if the difference is due to the differences in the number of tyrosine residues that are phosphorylated, or due to better and more stable phosphorylation of the same residues. We also note that site specific phosphorylation measurements using mass spectroscopy can help us understand the observed differences in the pf values, and there have been significant advances in the application of such techniques to HER family signaling [16].

Receptor dimerization predictions

Once parameterized based on the experimental data, our mathematical model allows us to make quantitative predictions for HER dimerization patterns, which are difficult to measure experimentally (Supp Mat 1). In Fig. 3, we examine the source of the variation in EGFR and HER2 phosphorylation levels across the cell lines. As seen in Fig. 3A, EGFR homodimers account for ~90% of the total EGFR phosphorylation in the parental cell line. The homodimer contribution decreases with HER2 expression due to the formation of increasing numbers of heterodimers. In *A11H* the homo- and hetero-dimer contributions are ~20% and ~80%, respectively. Contribution of the HER2 homodimers to the total HER2 phosphorylation is much less, increasing from nearly 0 in *Par* to ~20% in *A11H*.

These predictions are important because they can be used to directly test the hypothesis that dimer identity is a critical determinant of the HER-mediated cellular response [3]. For example, these predictions can be used to quantitatively relate the activation levels of signaling molecules such as Erk and Akt to the activation levels of particular receptor dimers. Such analyses enable us to decipher the logic of EGFR and HER2 mediated signal transduction in cell lines co-expressing these receptor types, and can reveal the conditions under which crosstalk among the receptors is an important driver of the response (Zhang et al., submitted).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic description of the mathematical model

Utilized model consists of active receptor dimer formation from monomer receptors, receptor endocytosis, trafficking, and degradation. Details of the model are discussed in the main text and in the Supplementary Material 1.

Figure 2. Receptor phosphorylation patterns

Model fits (lines) to the experimental data (points) for EGFR (A & B) and HER2 (C & D) receptor mass and phosphorylation levels. Figure reports representative results from two cell lines each for EGFR and HER2. Results for the other cell lines can be found in Supplementary Material 1. EGFR results are presented for: (A) the parental cell line (*Par*), which has very low HER2 expression, and (B) the *A11H* cell line that expresses high levels of the HER2 receptor. HER2 results are for: (C) *17L* with low HER2 levels, and (D) the *A11H* cell line. In each panel, the top third presents the total receptor mass, the middle third presents the mass of phosphorylated receptors in the cell (circles, solid line) and in the internal compartments (squares, broken lines), and the bottom third presents the receptor phosphorylation level for the cell (circles, solid line) and for the internal compartments (squares, broken lines). The figures present results from multiple biological replicates each of which is denoted using a distinct color.

Figure 3. Dimer contributions to receptor phosphorylation

The fractional contributions of EGFR homodimers to the total EGFR phosphorylation signal (A) and that of HER2 homodimers to the total HER2 phosphorylation signal (B) were computed using the mathematical model with the parameter values in Table 2, and are plotted for the four cell lines indicated. The arrows indicate the direction of increasing HER2 expression level. The respective heterodimer contributions can be obtained by subtracting the values shown in the figure from 100.

TABLE 1

Cell lines used in the study

List of cell lines		Abundance of EGFR and HER2 (molecules/cell) *	
Notation	Description	EGFR	HER2
<i>Par</i>	Parental cell line	2.0×10^5	3.0×10^4 #
<i>17L</i>	Low HER2 expresser	1.8×10^5	1.1×10^5
<i>24H</i>	Medium HER2 expresser	1.6×10^5	6.0×10^5
<i>A11H</i>	High HER2 expresser	3.0×10^5	1.5×10^6

* We assumed an EGFR expression level of 200,000 for the parental cell line and a HER2 expression level of 600,000 for *24H* [7]. Receptor mass data obtained prior to ligand addition ($t=0$) for EGFR and HER2 (see top-third panels in Figs. S1 and S2 of Supp Mat 1) were then used to calculate the total molecules of EGFR and HER2 in the other cell lines.

The HER2 expression level of the parental cell line is an estimate based on [7].

TABLE 2

Parameter values estimated by fitting the model to EGFR and HER2 data*

Receptor Activation		Receptor Trafficking		Phosphorylation factors	
Parameter	Value	Parameter	Value	Parameter	Value
k_{β} (nM/min)	1.8×10^{-3}	k_{t1} (/min)	1.8×10^{-2}	pf_{11}	4.3×10^{-4}
		k_{t2} (/min)	7.0×10^{-3}	pf_{12}	4.1×10^{-4}
k_{r118} (/min)	4.2×10^{-2}			pf_{21}	2.3×10^{-4}
k_{r128} (/min)	6.3×10^{-1}	k_{d1} (/min)	1.9×10^{-2}	pf_{22}	5.1×10^{-5}
k_{r228} (/min)	6.7×10^0	k_{d2} (/min)	8.2×10^{-3}		
k_{r111} (/min)	2.7×10^{-1}	k_{e11} (/min)	1.6×10^{-1}		
k_{r121} (/min)	1.6×10^{-1}	k_{e12} (/min)	5.6×10^{-2}		
k_{r221} (/min)	7.5×10^1	k_{e22} (/min)	8.4×10^{-2}		
		k_{d11} (/min)	3.3×10^{-1}		
		k_{d12} (/min)	8.2×10^{-3}		
		k_{d22} (/min)	8.2×10^{-3}		

* To convert from the number of molecules to nanomolar quantities, we assume a conversion factor of 1,800 molecules=1 nM as in our earlier studies [10, 11]. Note that k_{β} was kept fixed during parameter optimization. Bootstrap parameter confidence intervals along with parameter estimates obtained based on the maximum and median of the bootstrap histograms are provided in Table S1 of the Supplementary Material 1.