Quantitative Analysis of HER2-mediated Effects on HER2 and Epidermal Growth Factor Receptor Endocytosis

DISTRIBUTION OF HOMO- AND HETERODIMERS DEPENDS ON RELATIVE HER2 LEVELS

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Endocytic trafficking plays an important role in the regulation of the epidermal growth factor receptor (EGFR) family. Many cell types express multiple EGFR family members (including EGFR, HER2, HER3, and/or HER4) that interact to form an array of homo- and heterodimers. Differential trafficking of these receptors should strongly affect signaling through this system by changing substrate access and heterodimerization efficiency. Because of the complexity of these dynamic processes, we used a quantitative and computational model to understand their integrated operation. Parameters characterizing EGFR and HER2 interactions were determined using experimental data obtained from mammary epithelial cells constructed to express different levels of HER2, enabling us to estimate receptor-specific internalization rate constants and dimer uncoupling rate constants. Significant novel results obtained from this work are as follows: first, that EGFR homodimerization and EGFR/HER2 heterodimerization occur with comparable affinities; second, that EGFR/HER2 heterodimers traffic as single entities. Furthermore, model predictions of the relationship of HER2 expression levels to consequent distribution of EGFR homodimers and EGFR/HER2 heterodimers suggest that the levels of HER2 found on normal cells are barely at the threshold necessary to drive efficient heterodimerization. Thus, altering HER2 concentrations, either overall or local, could provide an effective mechanism for regulating EGFR/HER2 heterodimerization and may explain why HER2 overexpression found in some cancers has such a profound effect on cell physiology.

In the EGFR family, endocytic trafficking processes can strongly influence cell responses to EGF family ligands. Many cell types express multiple EGFR family members that can interact to form an array of homo- and heterodimers (1). Regulation of the distribution of these receptors among cell compartments can significantly modulate the overall signaling through this system by changing access to heterodimerization partners. Because of the potential complexity of EGFR family interactions associated with concomitant receptor trafficking and signaling, application of quantitative experimental and computational modeling techniques to its analysis should be very useful.

The EGFR family (EGFR/HER1/ErbB-1, HER2/ErbB-2/neu, HER3/ErbB-3, and HER4/ErbB-4) of receptor tyrosine kinases consists of four highly related receptors each with a unique set of functional properties. Following ligand binding, EGFR family receptors interact to form an array of homo- and heterodimers each with a characteristic repertoire of downstream signaling molecules (1). EGFR and HER2 are by far the most studied and have gained particular attention in the process of tumorigenesis. HER2 is commonly postulated to be the "preferred dimerization partner" of all EGFR family receptors (2–4). HER2 behaves much like a receptor subunit, as it binds none of the eight reported EGF family ligands (EGF, transforming growth factor-α, betacellulin, amphiregulin, heparin-binding EGF, epiregulin, and the neuregulins NRG and NRG-2) with high affinity (5).

Overexpression of both the EGFR and HER2 has been associated with cell transformation and tumorigenesis. HER2 is overexpressed in 25–30% of all breast and ovarian cancers and is emerging as an important player in many other cancers as well (6). HER2 overexpression correlates with poor prognoses in breast cancer (7), and its effects in the process of tumorigenesis have been fairly well documented. Increased HER2 expression contributes to cell transformation, anchorage-independent cell growth, increased proliferation and mitogenic sensitivity, as well as increased tumor cell migration and invasiveness (8–11).

Receptor overexpression could potentially influence cell behavior in multiple ways. The presence of excess receptors could recruit additional signaling molecules, resulting in an increase in signal amplitude. Alternatively, excess receptors could saturate and interfere with processes involved in receptor downregulation and signal attenuation (12, 13). This second phenomenon is a receptor trafficking effect that may affect the duration of the signaling by interfering with receptor degradation and ligand dissociation. Studies have shown that trafficking defects in receptors can facilitate cell transformation (14), suggesting that signal duration may be more of a determining factor in mitogenic sensitivity than signal amplitude, particularly at physiological ligand concentrations. It appears likely that receptor expression levels are directly connected to their trafficking behavior which, in turn, affects signaling.
The trafficking behavior of the EGFR, in the absence of other family members, has been well characterized (15, 16). Binding of EGFR ligands to the EGFR promotes receptor homodimerization and phosphorylation of cytoplasmic tyrosine residues that initiate signaling cascades (5, 17). Phosphorylated receptors are rapidly internalized by clathrin-coated pit endocytosis resulting in both short and long term loss of receptor activity. Once internalized, receptors and ligands are sorted in endosomes and either targeted toward lysosomal degradation or follow the recycling pathway back to the surface (16–18). Ligand-induced degradation of receptors can be impaired both at the level of endocytosis and/or endosomal sorting. Disruption of endocytosis has been shown to increase EGF-dependent proliferation (14). Overexpression of EGFR has also been shown to impair their degradation. Endocytic and endosomal sorting machinery have both been shown to exhibit saturation at high receptor levels, apparently due to limiting levels of the regulatory molecules involved in these processes (12, 13, 18, 19).

The association of HER2 with a number of different pathologies could be due to a host of molecular level effects. Following dimerization with another (ligand-bound) EGFR family member, HER2 becomes phosphorylated and is then able to recruit a distinct repertoire of signaling molecules including effectors both overlapping and distinct from EGFR-associated ones (1). In addition to the signal amplification role played by HER2, a second effect is elicited at the level of trafficking. Overexpression of HER2 affects the normal trafficking behavior of the EGFR, disrupting the processes that control receptor degradation (20).

The precise trafficking behavior of HER2 remains unclear. The internalization rates of HER2, HER3, and HER4 have been studied through the construction of chimeras consisting of the EGFR extracellular domain and different HER cytoplasmic domains. All EGFR/ErbB chimeras are internalized severalfold more slowly than the EGFR (21–23). EGF-induced HER2 down-regulation has also been reported (20). However, other investigators have failed to observe any EGF-induced HER2 internalization or EGFR/HER2 internalization (24). In recent work, we have demonstrated the internalization of HER2 in the absence of EGF and its accelerated internalization following EGF stimulation (25). Additionally, we found that overexpression of HER2 decreases the internalization rate of EGF. A quantitative evaluation of EGFR and HER2 internalization in the context of previous literature data, however, has not been done.

In this work, we examine the endocytic portion of the trafficking pathway in detail with the aim of quantitatively understanding how EGFR and HER2 interact in the process of internalization. We found the following: (i) EGFR/HER2 heterodimers are internalized as single entities, with other models of internalization being inconsistent with literature data (25); (ii) EGFR/HER2 heterodimers have a comparable dimerization affinity to EGFR/EGFR homodimers, thus the notion of HER2 as a preferred dimerization partner should be re-assessed; and (iii) there appears to be a threshold level of HER2 above which heterodimerization is maximal but increased HER2 expression is still able to alter signaling through longer term effects.

**Experimental Procedures**

**Reagents and Cell Culture—**Antibody 13A9 against the EGFR (26), mAbs 7C2 and 2C4 against HER2 (27, 28), and the Fab fragment of monoclonal 7C2 were gifts from Genentech. The human mammary epithelial cell lines MTSV1–7 and ce2 have been described previously (29) and were provided as a generous gift from Dr. Joyce Taylor-Papadimitriou. These cells were grown in Dulbecco’s modified Eagle’s medium (Flow Laboratories) containing 10% calf serum (Hyclone) supplemented with 1 μM insulin and 5 μM dexamethasone. ErbB-2 expression in ce2 cells was maintained by the addition of 500 μg/ml G418 (Sigma). Antibodies and EGF were iodinated with IODO-BEADS (Pierce) according to the manufacturer’s directions to specific activities of 2.7 × 10^6 cpm/pmol (mAb), 8 × 10^6 cpm/pmol for the Fab fragment of 7C2, and 1.6 × 10^6 cpm/pmol for EGF.

**Binding Analysis—**Numbers of EGFR and HER2 molecules on the cell surface were determined by steady state analysis (30). Cells were incubated with concentrations from 6.7 × 10^10 to 2 × 10^11 m for 3.5 h at 37 °C. The relative amount of antibody associated with the cell surface was determined by acid stripping, and the data were analyzed as described previously (20). Specific internalization rates (b_i) for the EGFR were determined as described (31) using 17 nM ligand and a 5-min incubation period. Specific internalization rates for the labeled mAbs were determined using a concentration of 1.5 nM antibody and 2-min intervals for a total of 10 min. Values were calculated as regression slope of the integral surface-associated ligand against the amount internalized (31).

**Model Development**

**Constitutive Case—**Our model was designed to output results that can be compared with the internalization experiments used to generate the data (25, 31, 32). The constitutive internalization of EGFR and HER2 is modeled with a set of coupled mass action kinetic equations. Consistent with published reports, we assume that a constitutive level of EGFR and HER2 homo- and heterodimerization takes place (33–35). As diagrammed in Fig. 1 (in the absence of any stimulation), EGFRs are allowed three states: free EGFR (R1), homodimerized with another EGFR (R1R1), or heterodimerized with HER2 (R1R2). EGFR is allowed similar freedom: free HER2 (R2), homodimerized with another HER2 (R2R2), or heterodimerized with EGFR (R1R2). The behavior of each species with regard to internalization is characterized by an internalization rate constant. Each dimerization and uncoupling act is characterized by its own, and not necessarily unique, kinetic rate constant.

**Ligand-stimulated Case—**By using the constitutive internalization model as a basis, ligand-induced interactions were added (diagrammed in Fig. 1). We have included the complete set of binary interactions between the EGFR and HER2 with and without EGF. Higher order
oligomerization of receptors is neglected, as this is a first approximation of the EGFR/EGFR/HER2 interactions. Additionally, HER2 and HER4 were left out of our analysis because we were examining the effects of EGF stimulation and they are typically expressed at lower levels.

In addition to the constitutive receptor species (R1, R1R1, R1R2, R2, and R2R2) and their interactions, EGFRs now bind ligand (L) to form complexes (R1L). Complexes may homodimerize with another complex to form doubly bound EGFR homodimers (L1R1L1) or heterodimerize with HER2 to form EGFR/EGFR/HER2 heterodimers (R1L2). Additionally, all the existence of singly bound EGFR homodimers (R1L1) is permitted, and each species may be formed in any order. For example, singly bound EGFR homodimers can be formed by ligand dissociation from doubly bound EGFR homodimers or by dimerization of an empty EGFR with an EGFR/EGFR complex. As before, each receptor species may have a unique internalization rate constant, and every interaction is reversible with its own set of kinetic rate constants.

Superimposed on the surface level receptor interactions are two additional processes: (i) the binding of radiolabeled antibodies to HER2, corresponding to the actual experiment; and (ii) the internalization of each receptor species. Under resting conditions, each receptor species is assumed to be at steady state, wherein rates of receptor internalization are perfectly balanced by rates of receptor synthesis and degradation. Thus, internalization and recycling of unlabelled receptor species can be left out of the model. Receptor species that have been bound by EGF, an antibody or antibody Fab fragment, however, are internalized at a rate specific to each species to an inside compartment. Recycling of each species is assumed to be negligible over the 7.5-min time scale of the model/experiment (30).

Inside and surface data are generated with the model and manipulated in the same manner as the experimental data to calculate the observed internalization rate constants for specific EGFR and HER2 expression levels. The model parameters are list in Table I. The overall observed internalization rate for EGF or Fab-bound species is essentially a weighted sum of the individual internalization rate of each EGF- or Fab-bound receptor species. Model equations corresponding to the interactions are drawn in Fig. 1 with parameters fixed, the uncoupling rate constants for EGFR complex homodimers and bound heterodimers (\(k_u\)) are fit to either the EGFR internalization data or the HER2 + EGF internalization data. These values are intended as order of magnitude estimates of the various uncoupling rate constants.

**Co-internalizations**—Mathematical equations corresponding to the model described above were coded into MATLAB version 6.5 (Mathworks, Natick, MA) and solved using ODE solver odes23s. Parameters were fit to experimental data using the lscurvefit routine from the Optimization Toolbox.

**RESULTS**

Recent work has highlighted the importance of receptor internalization in determining the distribution and induced degradation of EGFR in response to EGF stimulation (25). HER2 has emerged as a modulator of EGFR internalization presumably through heterodimerization. Here we explored the importance of heterodimerization in the internalization of both EGFR and HER2 as a function of HER2 expression level using previously published experimental data and a computational model of EGFR and HER2 internalization.

**Parameter Determination from Experimental Data**—Experimental data demonstrating the effect of increasing HER2 expression on the internalization rate constants of HER2 and EGF is derived from Ref. 25 and shown in Fig. 2A. In the absence of EGF, HER2 was internalized slowly at a rate comparable with membrane turnover. The addition of EGF accelerated the internalization of HER2. The internalization rate of EGF showed a marked decrease with increasing HER2 expression, whereas HER2 internalization under similar conditions showed a mild decline in internalization with increasing HER2 expression. In Fig. 2B is experimental data (25) showing the effect of preincubation with heterodimerization-blocking antibodies (2C4) on the internalization rate constant of EGF. Blocking heterodimerization abrogated any HER2-dependent effect on EGF internalization.

The significance of these results has been explored previously from a global, whole cell perspective. Here we seek to explore the mechanistic basis for these findings in order to test different models of internalization, determine which receptor species are dominant under different conditions, and elucidate which interactions dictate internalization behavior. The distribution of receptors between different signaling states (homodimers versus heterodimers, for example) should provide insight into the circumstances that contribute to aberrant cell behavior.

**Models of Receptor Internalization**—The general framework for our internalization models is shown in Fig. 1. Every binary interaction between EGFR and HER2 with and without EGF addition is included. Individual models differ with regard to certain parameter values or assumptions regarding species-specific behavior. The first internalization model that we consider is one in which each dimer species is sufficiently stable to be internalized as a single entity, hereafter referred to as the “coupled internalization” model. This model is fit to the data by first fitting uncoupling rate constants \((k_u R1R1, k_u R1R2, k_u R2R2)\) to the data in the absence of EGF. Holding those parameters fixed, the remaining uncoupling rate constants \((k_u L1R1L1 and k_u R1R2L2)\) are fit to the EGFR data and used to predict the HER2 + EGF internalization data (shown in Fig. 2A). Nearly identical results are obtained if one fits to the
and then predict EGFR data (shown in Fig. 3C).

Finally, we present a model in which the receptor internalization is indirectly related to HER2 expression level of the form $k_{i,j} = a_i + b_i \times \text{(HER2 level)}$, where $i$ refers to either EGFR or HER2, $a_i$ are the receptor internalization rates in the absence of HER2, and $b_i$ are the constants to be fit. Shown in Fig. 3D are the results from fitting parameters to the HER2 data and predicting EGFR data. Here the model is able to capture some of the trends in the data but misses the curvature in the EGFR data. The results are similar for the reciprocal fit.

Experimental Test of the Effect of HER2 Expression on Heterodimerization—We can also use our model to predict quasi steady state distributions of homo- and heterodimers. The time scale for receptor-receptor interactions in the membrane is significantly faster than the time scale for internalization. As such, it can be assumed that the distribution of the various receptor species reaches a quasi steady state prior to internalization. By solving the model at steady state, neglecting internalization, and using the dimerization parameters determined from fitting experimental data, we have calculated the degree of homo- and heterodimerization as a function of HER2 and EGFR expression levels. Shown in Fig. 4A is the percent of HER2 that is homodimerized in the absence of EGF. This is strongly dependent on HER2 level and reaches a maximum value of about 90%, with the most pronounced effects occurring between 0 and about 100,000/cell. In the presence of 1.7 nM EGF, the percent of HER2 that is heterodimerized is shown in Fig. 4B, and the percent of EGFR that is heterodimerized is shown in Fig. 4C. In both cases there was a strong dependence on HER2 level, and modest dependence on EGFR level. The largest effects are observed over the range of $0.2 \times 10^5$ HER2 per cell.

In Fig. 5 we compare the absolute numbers of homo- and heterodimers as predicted by our model for EGF concentration from 1 to 100 ng/ml. These predictions are made with equal affinities for homo- and heterodimerization, as measured from our parameter determination. As expected, for a given EGFR level, increasing HER2 expression increases the relative amount of heterodimers. Surprisingly, for a given HER2 level, increasing EGFR expression also increases the amount of heterodimers under most conditions. Only when the number of occupied EGFR is equal to or greater than the amount of HER2 does the relative amount of heterodimers decrease with increasing EGFR expression (best seen in the 100 ng/ml case). If one were to compare the absolute number of homo- versus heterodimers as an indirect measure of dimerization affinity, the spurious conclusion that heterodimerization is highly favored could easily be made. This apparent preference of EGFR for heterodimerization happens for two reasons. (i) Not all EGFR are occupied, especially at lower EGF concentrations so that the number of EGFR interacting with HER2 may actually be significantly less than expected. This is further exacerbated by the fact that EGF-EGFR complexes and homodimers are cleaved from the surface more quickly than heterodimers. (ii) The equilibrium distribution between EGF-EGFR complexes and homodimers does not completely favor homodimers. Thus, even if EGFR and HER2 levels are comparable, the dynamics and kinetics of the system will favor the appearance of heterodimers in an absolute sense.

To test the predictions made by our model, we obtained a set of mammary epithelial cells that were not used to estimate the original model parameters. These cells were SV40 immortalized MTSV-1 cells and the derivative ce2 line, which was engineered to overexpress HER2 (29). Binding of radiolabeled EGF demonstrated that these cells expressed between 3.5 and $4.5 \times 10^5$ EGFR each (data not shown). Steady state binding of ra-
diolabeled anti-HER2 mAb 7C2 indicated that MTSV cells express surface HER2, whereas ce2 cells express per cell (data not shown; also see Ref. 20). From these relative numbers of EGFR and HER2, we can predict quite distinct degrees of heterodimerization between EGFR and HER2 for the two cell lines (see Fig. 4, B and C). In the presence of saturating EGF, we expect that few (25%) EGFR will be found in heterodimers in MTSV cells, whereas most should be in heterodimers in ce2 cells. Conversely, a large fraction of HER2 will be found in heterodimers in MTSV cells, but only a small fraction will be heterodimerized in ce2 cells. Furthermore, the effect of heterodimerization should be readily observable by its influence on EGFR and HER2 internalization.

To verify the predicted effect of EGFR/HER2 heterodimerization on HER2 internalization, ce2 cells were incubated in 200 ng/ml 125I-7C2 or 125I-2C4 antibody for 3 h and then the amount of internalized antibody was then determined as a function of time following the addition of 100 ng/ml EGF. As shown in Fig. 6A, the addition of EGF increases the amount of internalized 7C2 antibody. In contrast, there was no change in the amount of internalized 2C4, verifying that heterodimerization

### Table I

| Model parameter | Description | Value |
|-----------------|-------------|-------|
| Binding parameters | | |
| \( k_{on} \) | EGF association | \( 9.7 \times 10^7 \) (M min\(^{-1}\)) |
| \( k_{off} \) | EGF dissociation | \( 0.24 \) min\(^{-1}\) |
| \( k_{onFab} \) | Fab association | \( 1.4 \times 10^7 \) (M min\(^{-1}\)) |
| \( k_{offFab} \) | Fab dissociation | \( 0.30 \) min\(^{-1}\) |
| Internalization rate constants (estimated from experimental data) | | |
| \( k_{R1} \) | Unoccupied EGFR internalization rate constant | \( 0.08 \) min\(^{-1}\) |
| \( k_{R2} \) | HER2 internalization rate constant, No EGF | \( 0.01 \) min\(^{-1}\) |
| \( k_{R1R2} \) | EGFR/HER2 unbound heterodimer internalization rate constant | \( 0.03 \) min\(^{-1}\) |
| \( k_{R1L} \) | EGFR-EGFR complex internalization rate constant | \( 0.28 \) min\(^{-1}\) |
| \( k_{R1R2L} \) | EGFR-HER2 bound heterodimer internalization rate constant | \( 0.10 \) min\(^{-1}\) |
| Dimerization/uncoupling parameters (fit to experimental data) | | |
| \( k_c \) | Receptor dimerization rate constant | \( 1 \times 10^{-3} \) (/cell min\(^{-1}\)) |
| \( k_{R1R1} \) | EGFR/EGFR unbound homodimer uncoupling rate constant | \( 10 \) min\(^{-1}\) |
| \( k_{R1R2} \) | EGFR/HER2 unbound heterodimer uncoupling rate constant | \( 10 \) min\(^{-1}\) |
| \( k_{R2R2} \) | HER2/HER2 homodimer uncoupling rate constant | \( 1 \) min\(^{-1}\) |
| \( k_{R1R2L} \) | EGFR-HER2 bound heterodimer uncoupling rate constant | \( 0.1 \) min\(^{-1}\) |
| \( k_{LR1R1L} \) | EGFR homodimer uncoupling rate constant | \( 0.1 \) min\(^{-1}\) |

* Set to diffusion-limited value.
tion between EGFR and HER2 is necessary to stimulate HER2 internalization.

To measure the internalization of both HER2 and the EGFR, we used radiolabeled mAbs against these two receptors. The internalization rate constant ($k_e$) for HER2 was measured over a 10-min period either radiolabeled 7C2 or 2C4 anti-HER2 antibodies in both the presence and absence of EGF. Because mAb 7C2 has no effect on HER2 heterodimerization, it should indicate the internalization rate of the entire population of HER2 (27). In contrast, mAb 2C4 blocks HER2 heterodimerization and thus should indicate the internalization rate of non-dimerized HER2.

Shown in Table II are the internalization rate constants of mAbs against HER2 and EGFR in the presence and absence of EGF. We found that in the absence of EGF, the values of $k_e$ were very similar between the different anti-HER2 mAbs in either cell type. Essentially identical values were obtained when using Fab fragments of 7C2 (data not shown). The anti-EGFR mAb 13A9 was also internalized at a similar rate. The observed values (generally between 0.02 and 0.04 min$^{-1}$) are similar to values reported previously (20, 22) and are consistent with internalization by constitutive turnover of the plasma membrane (39).

The addition of EGF increased the values of $k_e$ of mAb 7C2 2-fold in both cell types but had little if any effect on the heterodimerization-blocking mAb 2C4, again confirming the importance of heterodimerization for EGF-induced internalization of HER2. EGF stimulated the internalization of the anti-EGFR mAb 13A9 3–4-fold. The internalization rate of the bound 13A9 was very similar to that observed for EGF itself (Table II), indicating that the behavior of the mAbs accurately reflects that of the receptor population.

The above data demonstrate that EGF stimulates HER2 internalization through heterodimerization but does not indicate the stoichiometries of the internalized EGFR/HER2 heterodimers as a function of HER2 expression. To address this important point, we brought parallel plates of cells to steady state with either radiolabeled 7C2 or 13A9. The fraction of the total HER2 and EGFR population tagged with the antibodies was determined by conducting saturation binding experiments with a parallel set of cells. After the cells reached a steady state distribution of labeled mAbs between the cell surface and intracellular compartment, a saturating amount of EGF was added. The change in intracellular anti-HER2 and anti-EGFR mAbs was then quantified and corrected for their relative fractional occupancies.

As shown in Fig. 6B, the steady state distribution between the cell surface and the intracellular compartment of antibodies bound to HER2 and EGFR was very similar, with 20–30% being intracellular. Following addition of EGF, however, there was a rapid shift of the bulk of EGFR to an intracellular compartment, accompanied by a relatively minor shift of HER2 to an intracellular compartment (Fig. 6B, inset). Internalization of both EGFR and HER2 was significantly less in the case of the HER2-overexpressing ce2 cells. 15 min following EGF addition...
addition, 70% of the EGFR but only 30% of the HER2 had been internalized in MTSV cells. In the case of ce2 cells, 55% of the EGFR had been internalized but only 12% of the HER2. By considering the initial numbers of EGFR and HER2 in the two cells, we calculate that EGF addition stimulated the internalization of 3.2 and $3.7 \times 10^5$ EGFR in MTSV and ce2 cells, respectively, and stimulated the internalization of 0.2 and $3.1 \times 10^5$ HER2 in the same cells. Thus we calculate that in MTSV cells, only about 6% (±2% S.D.) of the internalized EGFR are internalized as heterodimers, but in ce2 cells 83% (±33% S.D.) are internalized as heterodimers. Our model predicts 20 and 80% of the EGFR to be in heterodimers for MTSV and ce2 cells, respectively. These predictions are slightly different from the experimental calculations, but if we assume that some heterodimers dissociate prior to internalization and consider the experimental error, then the match between predictions and our experimental tests is quite good.

**Model Insights**—Receptors comprise the primary layer of input to the intracellular signaling machinery of the cell. The generated signals are a function of the distribution of receptor species (complexes versus heterodimers) as well as receptor location (surface versus internal). By using our coupled internalization model of internalization and our derived parameter values, we can gain insight into how varying levels of receptor expression could affect cell signaling. For this analysis, we will only examine short term signaling in response to 10 ng/ml EGF stimulation. We restrict ourselves to short time scales because our model neglects the effects of receptor recycling. Recycling is very important in determining the overall system behavior, and it acts on the time scale of minutes to hours. The early events of ligand binding, receptor dimerization, and activation develop very quickly, over a time scale of seconds with internalization acting on the time scale of minutes (40, 41). As such, the short term responses, on the order of 10 min, are sufficient to determine the nature of the initial input to the signaling machinery.

One manner in which HER2 heterodimerization could amplify extracellular signals is by allowing one molecule of EGF to recruit and activate two receptors (one EGFR and one HER2). Consequently, the total number of receptors actively signaling in response to 10 ng/ml EGF should increase with increasing HER2 expression level, as is seen in Fig. 7A. The distribution of these receptors between homo- and heterodimers is shown in Fig. 7B. Increasing HER2 expression causes a dramatic increase in the number of heterodimers formed but elicits a relatively small decrease in the amount of homodimers formed.

### Table II

| Labeled probe | EGF | Cell type | MTSV | Ce2 |
|---------------|-----|-----------|------|-----|
| 7C2 (HER2)    |     |           | 0.035 | 0.023 |
| +             |     |           | 0.088 | 0.049 |
| 2C4 (HER2)    |     |           | 0.043 | 0.031 |
| +             |     |           | 0.049 | 0.030 |
| 13A9 (EGFR)   |     |           | 0.028 | 0.054 |
| +             |     |           | 0.119 | 0.132 |
| EGF           |     |           | 0.148 | 0.112 |

### FIG. 7. Signaling receptor dimers. Model predictions in response to 10 ng/ml EGF stimulation for varying levels of HER2 expression (30,000, 100,000, 200,000, and 600,000 per cell) are shown. A, total number of receptor dimers recruited (EGFR homodimers and EGFR/HER2 heterodimers); B, time course of homodimers (solid lines) and heterodimers (dotted lines); C, time course of the fraction of total receptor dimers that are heterodimers; D, time course of the internalization flux of equivalent homodimers (solid lines) and heterodimers (dotted lines).

The fraction of total signaling species that is heterodimerized is shown in Fig. 7C. Because homodimers and heterodimers are believed to have different signaling abilities and different signaling strengths, this plot demonstrates how the nature of EGF-induced signaling may change with increasing HER2 expression. At short time points the EGF binding has not yet reached a steady state so that there is a large excess of HER2 relative to occupied EGFR, even for the parental cell line. At longer time scales the distribution of signal plateaus with a roughly even distribution for the low HER2 expression (30,000/cell) and a shift in distribution for HER2 expression levels over 100,000 per cell.

Both the number of surface complexes and number of heterodimers reaches a quasi steady state over the short time scale. The fluxes of receptor species, defined as the rate at which a given receptor species is being internalized (flux = number of species present × (species internalization rate constant)), parallels this result. Shown in Fig. 7D is the internalizing flux of heterodimers and the flux of equivalent EGF-EGFR homodimers, defined as (R1L + R1R1L + 2 × LR1R1L) / 2 to enable the direct comparison with the flux of heterodimers, so chosen because all of these species are postulated to have equivalent internalization behavior. More EGFR homodimers are being internalized than heterodimers at low HER2 levels, but as HER2 levels increase, the flux of EGFR homodimers decreases, matched by a concomitant increase in the flux of heterodimers (Fig. 7D). This is reflected in our observation that at low levels of HER2 expression, only a small fraction of EGFR is internalized as heterodimers, whereas high levels of HER2 expression result in almost all EGFR being internalized as heterodimers. Interestingly, het-
Cell signaling is a complex dynamic process initiated by ligand-receptor binding followed by the recruitment of a cascade of signaling molecules. Signals are transmitted to the nucleus by way of protein modifications and translocations, causing altered gene and protein expression and ultimately resulting in a cell response. This process is regulated at many levels, including negative feedback in signaling pathways as well as at the level of receptor trafficking. One of the most direct ways to regulate the system output is to regulate the system input. For the EGFR family of receptor tyrosine kinases, the system input is modulated by the formation of receptor homo- and heterodimers, each with potentially different signaling capabilities. This allows signaling to be regulated by controlling the degree of homo- and heterodimerization as well as by controlling receptor distribution between cellular compartments.

To understand how endocytosis and endosomal sorting modulates input into receptor signaling networks, we have compared various models of EGFR and HER2 internalization. With these models we are able to assess critically some of the current hypotheses regarding HER2 internalization and heterodimerization as well as to predict the impact of elevated HER2 expression on intracellular signal generation.

**HER2 Internalization**—For the most part, studies of HER2 internalization have confined themselves to examining the behavior of HER2 (or EGFR/HER2 chimeras) in isolation. Although there is some disagreement regarding the exact rate of internalization (42–44), the generally accepted conclusion is that HER2 is “internalization impaired” relative to the EGFR (21, 22). Our previous work supports this notion (25). Because HER2 appears to require dimerization for activation, the internalization behavior of heterodimers is likely to be of greater interest. Wang et al. (24) have studied the internalization behavior of EGFR/HER2 heterodimers and concluded that they were not endocytosed in response to EGF stimulation. Our modeling demonstrates that this contention is inconsistent with quantitative internalization data. Instead, heterodimers appear to be internalized at a rate that is lower than EGFR homodimers but still rapid enough to induce a significant redistribution of receptors.

Our model and experimental data are consistent with the hypothesis that heterodimers are sufficiently stable to traffic as single entities. It appears that heterodimers are internalized at a reduced rate, at about half to one-third of the rate of EGFR complexes. This is consistent with the observation that EGF can induce down-regulation of HER2 in the absence of rapid HER2 internalization. Although heterodimers are internalized relatively slowly, they are still internalized faster than the rate of HER2 loss (45). Our model was able to recapitulate the complex interplay between the EGFR and HER2 as demonstrated by its ability predict an independent set of data using a constant parameter set, as well as predict the effect of blocking heterodimerization. Finally, we were able to validate model predictions with experimental measurements of the stoichiometry of EGFR and HER2 internalization. None of the other models that we explored had this ability. Thus, our model of internalization is a reasonable basis for studying the regulatory influence of the trafficking pathway.

**HER2 Dimerization**—It is commonly understood that HER2 is the preferred dimerization partner of all EGFR family receptors, and dimerization takes place with a strict hierarchy (2–4). This implies that occupied EGFR, in the presence of all EGFR family members, prefers to heterodimerize with HER2 rather than EGFR homo- and heterodimerize with another occupied EGFR or other EGFR family member. We interpret this to mean that following EGF stimulation, the affinity for EGFR/HER2 dimerization should be greater than that of EGFR-EGFR dimerization. Our mathematical modeling of receptor dimerization and the effects of elevated expression levels allow us to obtain estimates of these dimerization affinities and thereby critically evaluate this notion.

Our experimentally derived receptor dimerization and uncoupling parameters are the first reported estimates of these values in whole cells with native receptors. EGFR dimerization has been monitored previously in live cells but in the absence of HER2 (46). Other studies have examined receptor dimerization with solubilized fragments of various ErbB receptors (47, 48). Although we determined the parameters values indirectly, they are consistent with reports that in the absence of EGF, EGFR and HER2 homodimerize with comparable affinities (34). We also found that the addition of EGF increased both EGFR homo- and heterodimer affinities by 10–100-fold relative to empty receptors and found them to be of equal magnitude. Due to the uncertainty in the parameter fitting heterodimerization could still be slightly preferred, as suggested in the literature although it appears unlikely (2–4). If EGFR heterodimerization affinity is postulated to be 10-fold greater than that for homodimerization (k_{R1R2L}/k_{L1R1L} = 0.1), the model fits the experimental data 24% worse (data not shown). By comparison, if EGFR homodimerization affinity is 10-fold greater than that for heterodimerization (k_{R1R2L}/k_{L1R1L} = 10), the model fit differs by less than 2% from the fitted parameters (data not shown). This suggests that the local relative concentration of EGFR and HER2 is the primary determinant of the fraction of homo- versus heterodimers rather than the relative affinities of their interaction. Finally, these studies do not provide estimates of the dimerization affinity of HER2 with HER3 or HER4, although it is reasonable to expect that they would be of the same order of magnitude as EGFR/HER2 dimerization.

Our model calculations regarding the extent of whole-cell heterodimerization assumes that all receptors are uniformly distributed on the cell surface. It is clear, however, that locally high concentrations of HER2, such at membrane subdomains (49, 50), could facilitate the formation of heterodimers. In some tumors, levels of both EGFR and HER2 can vary over several orders of magnitude. Thus, relative expression levels could be the principal factor governing the pattern of homo- and heterodimerization in transformed cells. Unfortunately, most studies investigating the ability of different HER family members to form homo- and heterodimers do not quantify the relative level of receptor expression. This omission obscures the intrinsic propensity of different receptors to form different pairs of homo- versus heterodimers (51, 52). Our work strongly suggests that relative expression levels are critical determinants in this process.

**Signaling Implications**—Our predictive model for the distribution of EGFR and HER2 homo- and heterodimers provides a tool for generating testable hypotheses regarding the contribution of this aspect of receptor behavior to intracellular signaling. The model makes obvious predictions that one would expect from increasing HER2 expression, for example, an increase in heterodimerization. However, we can also gain some non-intuitive insight into system dynamics. For example, we found that the dynamics of EGFR/HER2 receptor distribution between the cell surface and endosomal compartment are principally controlled by the level of HER2 expression that, in turn, controls the degree of heterodimerization. Fig. 4, B and C, shows that there is a threshold level of HER2 expression...
appears to be a threshold level of HER2 (roughly 10^5 for these expression levels and simple mass action kinetics. (iii) There preferred dimerization partner as concluded previously, rather targets for therapeutic intervention.

these two effects may be difficult to separate from an experimental point of view, but a computational modeling approach should aid in determining which are the true factors controlling cell behavior and which are the best targets for therapeutic intervention.

Conclusions—The principle findings of this study can be summarized as follows. (i) EGFR/HER2 heterodimers are internalized as a single entity, with other models of internalization being inconsistent with the data (25). (ii) HER2 is not a preferred dimerization partner as concluded previously, rather it has a comparable dimerization affinity as an EGFR with the appearance of preferential dimerization arising from relative expression levels and simple mass action kinetics. (iii) There appears to be a threshold level of HER2 (roughly 10^5 for these cells with 2 x 10^5 EGFR) above which there is little effect on the degree of heterodimerization (see Fig. 4), but increased HER2 expression may still affect the quantity and nature of signals generated (see Fig. 7). All of these conclusions underscore the importance of relative EGFR and HER2 expression levels. Consequently, it is not enough to merely examine HER2 levels when interpreting downstream signaling data (or looking at a patient population), but the entire complement of interacting receptors should also be determined and taken into consideration.

Acknowledgments—We thank Genentech for the generous donation of antibodies and Mark Sliwkowski for helpful interactions.

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(−100,000/cell) beyond which there is little alteration in receptor behavior and that EGFR expression has only a minor effect on the degree of heterodimerization. This conclusion is supported by the data presented in Fig. 5 and in Table II that show similar levels of enhancement of EGFR-stimulated HER2 internalization over a 16-fold range of HER2 expression levels. Of course, increasing receptor expression (EGFR and/or HER2) will increase the magnitude of the signals that can be generated. However, this prompts an important question, What is more likely to cause aberrant cell behavior, increased signal strength, or a change in the nature of the input signal (i.e. a shift from homodimers to heterodimers or a shift from the cell surface to endosomes)? We might anticipate that cells would be more likely affected by qualitative rather than quantitative changes in signal input. These two effects may be difficult to determine from an experimental point of view, but a computational modeling approach may aid in determining which are the true factors controlling cell behavior and which are the best targets for therapeutic intervention.

Conclusions—The principle findings of this study can be summarized as follows. (i) EGFR/HER2 heterodimers are internalized as a single entity, with other models of internalization being inconsistent with the data (25). (ii) HER2 is not a preferred dimerization partner as concluded previously, rather it has a comparable dimerization affinity as an EGFR with the appearance of preferential dimerization arising from relative expression levels and simple mass action kinetics. (iii) There appears to be a threshold level of HER2 (roughly 10^5 for these cells with 2 x 10^5 EGFR) above which there is little effect on the degree of heterodimerization (see Fig. 4), but increased HER2 expression may still affect the quantity and nature of signals generated (see Fig. 7). All of these conclusions underscore the importance of relative EGFR and HER2 expression levels. Consequently, it is not enough to merely examine HER2 levels when interpreting downstream signaling data (or looking at a patient population), but the entire complement of interacting receptors should also be determined and taken into consideration.

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