HER2, a member of the epidermal growth factor receptor (EGFR) tyrosine kinase family, functions as an accessory EGFR signaling component and alters EGFR trafficking by heterodimerization. HER2 overexpression leads to aberrant cell behavior including enhanced proliferation and motility. Here we applied a combination of computational modeling and quantitative experimental studies of the dynamic interactions between EGFR and HER2 and their downstream activation of ERK to understand this complex signaling system. Using cells expressing different levels of HER2 relative to the EGFR, we could separate relative contributions of EGFR and HER2 to signaling amplitude and duration. Based on our model calculations, we demonstrated that, in contrast with previous suggestions in the literature, the intrinsic capabilities of EGFR and HER2 to activate ERK were quantitatively equivalent. We found that HER2-mediated effects on EGFR dimerization and trafficking were sufficient to explain the observed HER2-mediated amplification of epidermal growth factor-induced ERK signaling. Our model suggests that transient amplification of ERK activity by HER2 arises predominantly from the 2-to-1 stoichiometry of receptor kinase to bound ligand in EGFR/HER2 heterodimers compared with the 1-to-1 stoichiometry of the EGFR homodimer, but alterations in receptor trafficking yielding increased EGFR spatiotemporal patterns cause the sustained HER2-mediated enhancement of ERK signaling.

HER2 (also known as ErbB2) is a key member of the human epidermal growth factor receptor (EGFR) family, which also includes EGFR (HER1/ErbB1), HER3 (ErbB3), and HER4 (ErbB4). HER2 is overexpressed in one-third of all breast cancers and correlates with poor prognosis in that disease (1). Its overexpression is also noted in other carcinomas including those of the lung, pancreas, and colon (2). Lacking a ligand, HER2 generally relies on dimerization with other family members for its activation. Dimerization, in turn, results in phosphorylation of tyrosine residues in the cytoplasmic domain of HER2 and subsequent activation of signaling cascades. The intrinsic affinity of HER2/EGFR heterodimerization is approximately equal to that of EGFR/EGFR homodimerization, although overexpression gives HER2 the overall appearance of being a preferred dimerization partner (3). Moreover HER2 has been reported to mediate enhanced signaling relative to other family members, and HER2-containing heterodimers are often postulated as being exceptionally potent transducers of downstream signaling pathways including the ERK pathway (4–8).

The EGFR family operates within an intricate signaling and regulatory network with complexity at several highly interconnected levels. These include ligand binding, receptor dimerization, receptor trafficking, and recruitment of signaling components into several different cellular compartments. With four distinct receptors that interact in various combinations and more than eight EGFR family ligands, opportunity exists for a large number of possible dimer species feeding into the ERK, phospholipase Cγ, phosphatidylinositol 3-kinase, and other signaling pathways. The amplitude, duration, and quality of signals generated by a given ligand are functions of receptor activation states, quantities, and locations that are determined by regulated intracellular trafficking. Altering the expression level of a single receptor, such as EGFR or HER2, perturbs all of these processes simultaneously. Delineation of the relationship between each signaling determinant and the net output of the system requires a quantitative model that can integrate all of these processes and their key variables.

Endocytic trafficking is responsible for the short and long term regulation of receptor number at the cell surface. It also dictates the distribution of receptors and ligands between surface and internal compartments. It is principally regulated at two steps: (i) internalization wherein ligand binding enhances receptor localization to coated pits, which facilitate endocytosis, and (ii) endosomal sorting wherein receptors and ligands are either targeted for lysosomal degradation or recycled to the surface for successive rounds of trafficking (9, 10). Both steps are subject to interference by overexpression of HER2 (11, 12). Elevated EGFR expression may also alter the endocytic and endosomal sorting dynamics through the saturation of the internalization and sorting machinery involved in these processes (13–15). Elevated HER2 expression, by contrast, inter-
feres with internalization by driving the formation of heterodimers following EGF stimulation that have a reduced internalization rate relative to EGF-EGFR complexes (3). Further elevated HER2 levels can shunt EGF-EGFR complexes to the recycling pathway by saturating EGF domain sorting through competitive interference (5, 11, 16, 17). The net effect of HER2 overexpression is a slower degradation of EGF/EGFR complexes along with a shift in spatial location toward the cell surface (12).

Ligand-induced receptor activation and transphosphorylation stimulate the recruitment of a repertoire of signaling molecules by both EGFR and HER2. EGFR homodimers and EGFR/HER2 heterodimers associate with many of the same signaling molecules including Src, Shc, phospholipase Cγ, Sos, Grb2, Grb7, and Crk (18, 19). However, controlled dimerization of these receptors has indicated the presence of differential signaling between homodimers and heterodimers. For example, c-Cbl is able to associate with homodimers but unable to associate with heterodimers (7, 20, 21). Among the pathways common to both the EGFR and HER2, the ERK mitogen-activated protein kinase pathway is implicated in cell proliferation and tumor progression. The ERK cascade is a central element in the transmission of signals from the cell surface to the nucleus. Overexpression of HER2 has been shown to potentiate and prolong EGF-induced ERK activation (6, 8). This is consistent with the observation that EGFR/HER2 heterodimers result in an increased proliferation and focus forming ability of cells relative to homodimers (7, 20).

The overall effect of elevated HER2 expression on EGF signaling can be conceptually divided into two actions. The first is a direct signaling effect wherein heterodimerization allows the activation of two receptor kinases per one ligand molecule; the second is an indirect trafficking effect wherein elevated HER2 expression alters the normal trafficking and down-regulation of the EGFR and thus its consequent signaling. The signaling effect of HER2 overexpression must manifest itself as an increase in signal amplitude, whereas the trafficking effect would operate at longer time scales resulting in an increase in signal duration. These qualitative characteristics have been seen in ERK, p70/S6K, and c-Jun NH2-terminal kinase activation data in response to EGF stimulation (6, 8). However, the trafficking and signaling of EGFR and HER2 are intimately linked through their dependence on dimerization and receptor expression levels. Moreover, the recursive nature of receptor recycling can obscure any simple relationship between expression levels and receptor activity. Consequently these relationships cannot easily be separated experimentally, making computational methodologies necessary.

In this work we determined the quantitative abilities of EGFR and HER2 to activate the ERK pathway. We developed and validated a computational model of EGFR/HER2 trafficking that can predict the levels of various homo- and heterodimers in response to EGF stimulation. By correlating these predicted quantities of activated receptors with experimental ERK signaling data, we were able to computationally separate the direct (signaling) and indirect (trafficking) effects of elevated HER2 expression. In determining the intrinsic signaling abilities of EGFR and HER2 for the ERK pathway, we found that they are quantitatively equivalent. The framework of our model suggests that the short term enhancement of ERK activity by HER2 is predominantly due to the 2-to-1 stoichiometry of receptor kinase to ligand in EGFR/HER2 heterodimers compared with the 1-to-1 stoichiometry in EGFR/EGFR homodimers, whereas the long term maintenance of ERK activity arises almost exclusively from increased EGFR levels as a result of the HER2-mediated reduction in EGFR down-regulation.
then imaged in physiological buffer containing 162 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl$_2$, 10 mM glucose, 10 mM Hepes, pH 7.4. Alexa-546 (LC) was the donor, and Alexa-647 (13AB) was the acceptor of energy. The dyes were excited by a 532 nm laser (Nd:YAG Verdi V-10, Coherent) and 632 nm laser (dye laser CR-599, Coherent), respectively, using a dual dichroic mirror (Chroma Technology). Donor and acceptor emissions were sent simultaneously onto two defined areas on the CCD camera while toggling between the two lasers. The green and red emissions were separated using a dichroic wedge mirror. FRET was detected by Alexa-647 indirect excitation with 532 nm as the energy that was transferred from the excited Alexa-546.

The intensities in the acceptor and FRET channels were used to define a cut-off value, and signals that overlapped in the two channels were further pursued for analysis. The bleed-through of the donor (10%) and the acceptor (7%) into the FRET (red) channel was determined empirically and was subtracted pixel by pixel to obtain the true FRET and the acceptor (7%) into the FRET (red) channel was determined empirically and was subtracted pixel by pixel to obtain the true FRET signal (Fc).

$$F_c = (F_{	ext{FRET}} - 0.10 \times (\text{DONOR}) - 0.07 \times (\text{ACCEPTOR})) \quad (\text{Eq. 1})$$

FRET, DONOR, and ACCEPTOR are the fluorescence intensities in the corresponding channels. FRET efficiency ($E$) was then calculated pixel-by-pixel according to Equation 2 where $\gamma$ is the acceptor to donor ratio of the quantum yields of the fluorophores, the objective, and the CCD camera and equals 0.82.

$$E = \frac{100 \times F_c}{F_c + \gamma \times (\text{DONOR})} \quad (\text{Eq. 2})$$

Model Development and Implementation—The complete receptor trafficking model is a composite of the global trafficking model and receptor interaction module presented previously (3, 12). The global trafficking models (equations shown under “Appendix I”) for EGFR and HER2 were solved at steady state in the absence of EGF stimulation to determine the initial distribution of receptors. Receptor synthesis rates were fit to match reported values of surface EGFR and surface HER2 per cell (12). Once the initial receptor distributions were determined, the global trafficking model equations were solved transiently over a 2-h time period in response to appropriate EGF stimulation. At each time point, the total EGFR, EGFR, and HER2 in each compartment were calculated. These values served as inputs into a quasi-steady dimerization submodel (equations shown under “Appendix II”), which was used to estimate the quantity of each possible receptor species, including EGFR-EGFR homodimers and EGFR-EGFR/HER2 heterodimers, in the surface and internal compartments. The dimerization submodel consists of kinetic equations describing every possible binary EGFR/HER2 interaction with and without EGFR. Assuming a conservation of mass over the time scale of receptor-receptor interaction, that is there is no significant gain or loss of receptors or ligand to trafficking, these reactions can be assumed to be at pseudosteady state, and their solution is readily obtained. All necessary parameter values governing EGFR and HER2 interactions are found in the literature (3, 12).

We hypothesized that the total ERK signal generated is the linear combination of the total number of signaling receptors multiplied by their respective ERK signaling abilities (see Equations 3–6 under “Results”). The total (surface + inside) number of signaling receptors was determined at each time point from the trafficking model for the desired level of HER2 expression. We assumed that three species in our model were capable of recruiting signals: EGFR/EGF-EGFR, EGF-EGFR/EGF-EGFR, and EGF-EGFR/HER2. Thus, we assumed that one active kinase (i.e. EGF-EGFR complex) is sufficient to phosphorylate a dimer and enable signal recruitment. It should be noted that the EGFR/EGF-EGFR species only become significant at ligand concentrations below $K_a$ (1 ng/ml EGF) and could be neglected at saturating ligand concentrations (100 ng/ml EGF).

For clone 24H trafficking model predictions following 2C4 administration, minor adjustment was required since blocking heterodimerization with mAb 2C4 did not quite return the trafficking behavior of clone 24H to that of the parental cell line, most likely due to clonal differences between the two cell lines; experimental measurements of EGFR downregulation in clone 24H following mAb 2C4 pretreatment differ by a factor of 1.34 from that in the parental cell line (12). This correction factor was incorporated into model predictions of ERK signal following mAb 2C4 pretreatment by multiplying trafficking model predictions of the number of actively signaling EGFRs for clone 24H by 1.34.

To use the trafficking model to make predictions for the 1 ng/ml EGF case, adjustments were needed to the internalization rates used in the global trafficking model (12). These internalization rates for EGFR and HER2 were predicted from the internalization model for the parental and clone 24H cell lines (3). HER2 internalization rates were predicted to be 0.04 and 0.03 min$^{-1}$, and EGF-EGFR complex internalization rates were predicted to be 0.17 and 0.12 min$^{-1}$ for the parental and clone 24H cell lines, respectively. Model solutions and manipulation were performed using MATLAB Version 6.5.

RESULTS

To separate the signaling and trafficking effects of HER2 on EGFR signaling we first needed to build a quantitative model of receptor location and dimerization as a function of time. Using this model, we could then associate a signal output from each receptor type at each corresponding time. Calculated estimates of the number of actively signaling receptors were
compared with experimental signaling measurements to backcalculate the individual signal contributions of EGFR and HER2. This deconvolution of signaling can be considered as three processes: (i) signaling data collection, (ii) trafficking model development, and (iii) model/data correlation analysis. For our analysis we examined a downstream signal activated by both EGFR and HER2, namely ERK activity. ERK activity is an important determinant of cell proliferation and thereby a reasonable metric by which to gauge the increased signaling brought about by elevated HER2 expression in the context of tumor progression. For our signaling experiments we used the same set of human mammary epithelial cells exhibiting different levels of HER2 expression as we have used previously to develop the trafficking model (3, 12, 16). Each cell line has roughly $2 \times 10^5$ surface EGFR/cell and surface HER2 expression of $3 \times 10^4$, $2 \times 10^5$, and $6 \times 10^5$/cell for the parental, clone 12, and clone 24H cell lines, respectively (3, 12).

We measured ERK activity in response to 100 ng/ml (16 nM) EGF stimulation in each cell line with an in vitro kinase assay over a 2-h time course (Fig. 1A). At early time points there was an increase in signal amplitude with increasing HER2 expression, corresponding to an increased number of actively signaling receptors. At longer time scales, signal duration increased with increasing HER2 expression, corresponding to the inhibition of receptor down-regulation as a consequence of elevated HER2 expression. Using a lower EGF concentration of 1 ng/ml, we found little difference in ERK activity with clone 24H being marginally higher at longer time points (Fig. 1B).

To probe the role of heterodimerization in ERK activity we repeated the 100 ng/ml EGF experiment following preincubation with mAb 2C4, an antibody that has been shown to bind to HER2 and block heterodimerization (24–27) (Fig. 1C).

### Table 1

| Parameter | Description | Value |
|-----------|-------------|-------|
| $S_H$ | EGFR synthesis rate | 2400/cell min $^{-1}$ $^a$ |
| $k_f$ | EGFR association rate constant | $9.7 \times 10^4$ M$^{-1}$ min$^{-1}$ $^b$ |
| $k_c$ | EGFR dissociation rate constant | 0.24 min$^{-1}$ $^b$ |
| $k_{er}$ | Unoccupied EGFR internalization rate constant | 0.07 min$^{-1}$ $^b$ |
| $k_{c,100}$ | Occupied EGFR internalization rate constant, 100 ng/ml EGF | 0.25 min$^{-1}$ $^b$ |
| Parental | Occupied EGFR internalization rate constant, 1 ng/ml EGF | 0.17 min$^{-1}$ $^c$ |
| Clone 12 | 0.18 min$^{-1}$ $^b$ |
| Clone 24H | 0.10 min$^{-1}$ $^b$ |
| $k_{c,4}$ | Occupied EGFR internalization rate constant following 2C4 treatment, 100 ng/ml EGF | 0.28 min$^{-1}$ $^b$ |
| $k_{er}$ | Unoccupied EGFR endosomal exit rate constant | 0.08 min$^{-1}$ $^b$ |
| $k_c$ | Occupied EGFR endosomal exit rate constant | 0.80 min$^{-1}$ $^b$ |
| $k_{c,100}$ | Occupied EGFR endosomal exit rate constant following 2C4 treatment | 0.04 min$^{-1}$ $^b$ |
| $f_{r,100}$ | Occupied EGFR recycling fraction, 100 ng/ml EGF | 0.03 min$^{-1}$ $^b$ |
| Parental | 0.50$^a$ |
| Clone 12 | 0.52$^a$ |
| Clone 24H | 0.70$^a$ |
| $f_{r,100;2C4}$ | Occupied EGFR recycling fraction following 2C4 treatment, 100 ng/ml EGF | 0.55$^a$ |
| Parental | 0.68$^a$ |
| Clone 24H | 0.47$^b$ |
| $S_H$ | HER2 synthesis rate | 0.54$^a$ |
| Parental | 12/cell min$^{-1}$ $^a$ |
| Clone 12 | 35/cell min$^{-1}$ $^a$ |
| Clone 24H | 140/cell min$^{-1}$ $^a$ |
| $k_{h}$ | HER2 internalization rate constant, no EGF | 0.02 min$^{-1}$ $^b$ |
| Parental | 0.01 min$^{-1}$ $^b$ |
| Clone 12 | 0.01 min$^{-1}$ $^b$ |
| Clone 24H | 0.01 min$^{-1}$ $^b$ |
| $k_{h,100}$ | HER2 internalization rate constant, 100 ng/ml EGF | 0.06 min$^{-1}$ $^b$ |
| Parental | 0.05 min$^{-1}$ $^b$ |
| Clone 12 | 0.03 min$^{-1}$ $^b$ |
| Clone 24H | 0.03 min$^{-1}$ $^b$ |
| $k_{h,4}$ | HER2 internalization rate constant, 1 ng/ml EGF | 0.04 min$^{-1}$ $^c$ |
| Parental | 0.03 min$^{-1}$ $^c$ |
| Clone 24H | 0.07 min$^{-1}$ $^b$ |
| $f_h$ | HER2 recycling fraction | 0.94$^a$ |

$^a$ Parameter value fit to receptor expression levels reported in Hendriks et al. (12).

$^b$ Parameter value experimentally measured in Hendriks et al. (12).

$^c$ Parameter value calculated using internalization model in Hendriks et al. (3).

$^d$ Parameter value estimated in Hendriks et al. (12).

$^e$ Parameter value estimated in Hendriks et al. (3).
pharmacological decoupling of HER2 from the EGFR was able to reverse most of the effects of elevated HER2 expression on ERK activity both in terms of peak levels and prolongation of signaling.

The signaling data were analyzed by correlation with the number of actively signaling receptors as estimated from the dynamic receptor trafficking model. The full model was derived from the hierarchical combination of previously published models of EGFR and HER2 trafficking (3, 12). The global trafficking models published previously for EGFR and HER2 contain a defined parameter set that have been experimentally validated (12) (see Table I). These models make a priori predictions of empty EGFR, EGF-EGFR complexes, and total HER2 for surface and internal compartments. Their corresponding equations can be solved transiently to give dynamic predictions of these quantities. However, this model does not give any explicit information about the dimerization state of EGFR or HER2, a required component for the resolution of signaling data.

Dimerization is the initial event in receptor activation that

Fig. 2. Model integration. Computational results for the global trafficking models developed in Hendriks et al. (3) yielded predictions for transient surface and intracellular levels of total EGFR, unoccupied EGFR, and total HER2 in response to EGF stimulation (upper panel). This model gives no explicit information about dimerization state. At each time point, these quantities were input into a quasisteady dimerization submodel developed in Hendriks et al. (12) (middle panel) to determine the entire distribution of each binary receptor species, including the number of homo- and heterodimers (bottom panels).

The time scale for receptor-receptor interactions (<0.1 min) is much faster than the time scales for receptor trafficking and ligand binding (both on the order of 10 min). As such, it can be safely assumed that EGFR-HER2 dimerization reaches a quasisteady state in each compartment before a significant change in receptor number attributable to receptor trafficking. Thus, with the knowledge of the total EGF, EGFR, and HER2 in each compartment at each time point, one can assume a quasisteady state and determine the distribution of each binary receptor species (Fig. 2). The kinetic rate constants for these interactions have been estimated in previous work and are shown in Table I (3, 16). Making quasisteady state predictions at each time point within an experimentally validated model prevents the propagation of error from any erroneous prediction of dimerization (12).

Dimerization is the initial event in receptor activation that
drives phosphorylation of EGFR complexes as well as HER2. Once phosphorylated, these receptors are able to recruit intracellular signaling molecules, including components of the ERK signaling cascade (18). At the molecular level, the proximal signaling species are EGF-EGFR homodimers (including both EGF-EGFR/EGF-EGFR homodimers and EGFR/EGF-EGFR homodimers) and EGF/EGFR/HER2 heterodimers. Using our trafficking model, we could predict the time-dependent profile of the formation of these species as a function of different levels of EGF stimulation. Shown in Fig. 3 are predictions of the number of intracellular and surface-localized EGF-EGFR homodimers and EGF-EGFR/HER2 heterodimers for HER2 expression levels corresponding to the parental (A and C) and clone 24H (B and D) cell lines, respectively. Predictions are shown for 100 ng/ml EGF stimulation (A and B) and 1 ng/ml EGF stimulation (C and D). Note the different y axis scale on A and B versus C and D. Surface homodimers, surface heterodimers, intracellular homodimers, intracellular heterodimers, and the total number of receptor dimers are marked with solid green, solid orange, dashed green, dashed orange, and solid black lines, respectively.

There are several approaches one can take to determine the relative contributions of EGFR and HER2 to the overall ERK signal. First we hypothesized that the total ERK signal generated was equal to the sum of the contribution of EGFR signaling and of HER2 signaling. The EGFR portion of the signal was equal to the total number of active EGFR receptors multiplied by the EGFR activation function, \( \alpha(t) \). Likewise the HER2 portion of the signal was equal to the total number of active HER2 receptors multiplied by the HER2 ERK activation function, \( \beta(t) \).

\[
\text{ERK}(t) = \text{EGFR}(t) \times \alpha(t) + \text{HER2}(t) \times \beta(t)
\]  
(Eq. 3)

\( \alpha(t) \) and \( \beta(t) \) reflect the signal generated by EGFR and HER2, respectively, on a per receptor basis as a function of time. \( \text{EGFR}(t) \) and \( \text{HER2}(t) \) are the number of actively signaling EGFRs (those in EGF-EGFR:EGF-EGFR and EGF-EGFR/EGFR homodimers) and HER2 receptors (those heterodimerized with an EGF-EGFR complex) as determined by the trafficking model.

In this analysis, we related events at the level of receptor interactions and trafficking to the downstream signaling event of ERK activation, leaving out the precise mechanisms of signal transduction. The validity of this approach relies on a tight and fast coupling between receptor dynamics and ERK activation as well as the requirement that our defined relationships do not change as a function of secondary parameters, such as cell type or receptor expression levels. In the end, the ability to make experimentally verified predictions attests to its applicability.
HER2 can be pharmacologically decoupled from EGFR via pretreatment with monoclonal antibody 2C4. The crystal structure of the 2C4-HER2 complex has revealed that 2C4 binds to the dimerization arm of HER2 and sterically interferes with its dimerization (27). Our ERK activity experiments were performed with saturating concentrations of 2C4 and sufficiently long preincubation to ensure that HER2 dimerization was maximally disrupted (data not shown). Decoupling HER2 from the system allowed further simplification of Equation 3 to Equation 4.

\[ \text{EGFR}(t) \times a(t) = \text{ERK}(t) \quad (\text{Eq. 4}) \]

The functions \( a(t) \) and \( \beta(t) \) are properties of a given cell type since they reflect the make up of intracellular signaling components downstream of receptor activation. Since the cell lines used were all derived from a common parent and vary only in their HER2 expression level, it was assumed that the functions \( a(t) \) and \( \beta(t) \) were conserved for all cell lines used: parental, clone 12, and clone 24H. Because the ERK pathway can be recruited by receptors both on the surface and within endosomal compartments (28, 29), the total number (inside + surface) of EGF-EGFR complexes and total number of actively signaling HER2 molecules in complexes were used for this analysis. A complete list of model assumptions and hypotheses is shown in Table II.

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Using the ERK data from the parental cell line following 2C4 treatment in response to 100 ng/ml EGF (Fig. 1B) and predictions of the number of active EGFRs from the trafficking model (Fig. 4A), we could explicitly calculate \( a(t) \) (Fig. 5A, dotted line). If we begin with the assumption that EGFR and HER2 have comparable ERK signaling abilities (i.e. \( a(t) = \beta(t) \)) we could use \( a(t) \), \( \beta(t) \), and the trafficking model to make a priori predictions of the ERK signal for the parental, clone 12, and clone 24H cell lines in response to 100 ng/ml EGF including the effect of 2C4 on clone 24H. As shown in Fig. 5, C–F (see dotted lines), these predictions show excellent agreement with the experimentally observed data. Here we used one set of ERK data and with our trafficking model successfully predicted four additional ERK profiles.

As an alternate approach, \( a(t) \) could be calculated from a different data set using ERK data from clone 24H following 2C4 treatment in response to 100 ng/ml EGF (Fig. 5A, solid line) along with the same predictions from the trafficking model. Assuming that \( a(t) = \beta(t) \), we were able to successfully generate a priori predictions of the ERK signal for the parental, clone 12, and clone 24H cell lines, including the effect of 2C4 on the parental cell line (Fig. 5, B, D, E, and F).
Global trafficking model
The transient solution of the trafficking model is a good approximation of EGFR and HER2 levels and compartmental location as a function of time. The trafficking model is also applicable at low EGF concentrations provided that the internalization rate constants are adjusted. This model is developed and validated in Hendriks et al. (12).

Dimerization submodel
Dimerization reactions are fast relative to trafficking steps such that dimerization is at quasisteady state relative to internalization, recycling, and degradation. Dimerization of EGF-bound species results in instantaneous receptor phosphorylation of both receptors and enables signaling to ERK. The explicit deactivation of signaling species is not included; it is implicitly represented in the decrease of $a(t)$ and $b(t)$ at long times.

The endosomal compartment is sufficiently well mixed such that the dimerization submodel can also be applied to this compartment.

Rate constants characterizing dimerization are the same for the surface and endosomal compartments.

This submodel is a portion of the internalization model developed and validated in Hendriks et al. (3).

Signaling analysis
EGFR homodimers (EGFR/EGFR and EGFR-EGFR/EGFR) and EGFR/HER2 heterodimers (EGF-EGFR/HER2) are the only species in our model that are capable of signaling to ERK.

EGFR homodimers contain two active EGFRs that are capable of signaling to ERK, and EGFR/HER2 heterodimers contain one active EGFR and one active HER2 that are able to signal to ERK.

Both surface and endosomal species are able to signal to ERK and do so with equal ability (i.e. surface EGFR homodimers and endosomal EGFR homodimers signal to ERK equally well).

EGFR vs. HER2 signaling comparison
Active EGFR and HER2 have independent abilities to signal to ERK. For each ligand concentration, active EGFR has an intrinsic ERK signaling ability, $a(t)$.

For each ligand concentration, active HER2 has an intrinsic ERK signaling ability, $b(t)$.

The ERK signal generated is the sum of the signal through EGFR (equal to the number of active EGFR molecules multiplied by its intrinsic ERK signaling ability) and the signal through HER2 (equal to the number of active HER2 molecules multiplied by its intrinsic ERK signaling ability; see Equation 3).

The functions $a(t)$ and $b(t)$ change with time, but each function is assumed to be the same for all cell lines: parental, clone 12, and clone 24H. These functions are also specific to the ligand concentration used, i.e. there is an $a_{100 \text{ ng/ml EGF}}(t)$ and an $a_{1 \text{ ng/ml EGF}}(t)$.

Our assumption of equal affinities of homo- and heterodimerization were based on previous work in which rate constants for homo- and heterodimerization were determined indirectly (3). To verify this crucial aspect of our model we adopted a more direct, FRET-based approach. The heterodimerization of EGFR with HER2 was identified and quantified by FRET between the Fab fragments of 13A9 and 7C2 antibodies tagged with Alexa dye-647 and Alexa dye-546, respectively. Fig. 7A shows the donor and acceptor emissions for clone 12, also shown as contour maps in Fig. 7B. FRET occurrence is detected by the emission in the acceptor channel following donor excitation. After background and bleed-through subtraction, FRET efficiency was calculated for each overlapping pixel (Fig. 7C). The weighted average of FRET efficiency was then calculated and designated as the FRET efficiency of the cell.

EGFR homodimerization was identified and quantified using a mixture of the Fab fragments of 13A9 antibody tagged with Alexa dye-647 or Alexa dye-546 following the procedure described above. The average FRET efficiency for homo- and heterodimerization with and without EGF stimulation was analyzed pixel by pixel for each cell (Fig. 5A).

Because FRET efficiency values depend on the distance between the epitopes of the antibodies within a pair as well as on the number of the FRET pairs per pixel, it was necessary to correct for the distance differences between the epitopes of the two, 13A9-13A9 and 13A9-7C2, pairs. It was found that, across all cells, the maximal FRET efficiency at maximal intensity was 25% higher for the 13A9-7C2 pair when compared with the 13A9-13A9 pair, a difference that we attributed to the difference in distance within the two pairs. Therefore, the heterodimerization-over-homodimeriza-
The FRET efficiency ratio was multiplied by 0.75 to correct for the difference in the distance between the fluorophores in the two pairs. Using this approach, it was found that the FRET efficiency ratios before and after adding EGF were close to 1.0 (Fig. 8B), indicating that the same number of homodimers and heterodimers were formed in these cells. Since clone 12 has roughly equal quantities of EGFR and HER2, this is indicative of having comparable affinities for homodimerization and heterodimerization and validates a critical assumption in our model.

As a final verification of our finding that EGFR and HER2 have quantitatively equivalent ERK signaling abilities, we also applied an alternate methodology. For the parental and clone 24H cell lines we now had two equations with two unknowns (α(t) and β(t)), which are assumed to be conserved across these cell lines) that could be explicitly solved for both α(t) and β(t). The values calculated for the 100 ng/ml EGF case are shown in Supplemental Fig. S3A. This analysis was also applied to the 1 ng/ml EGF data shown in Supplemental Fig. S3B. In both cases, there was no significant difference in the EGFR versus HER2 ERK signaling abilities. To test the calculated values of α(t) and β(t), we applied them along with our trafficking model to make an a priori prediction of the ERK signal generated per EGFR calculated from either the parental + 2C4 data (dotted line) or the clone 24H + 2C4 data (solid line) in response to 100 ng/ml EGF treatment. B–F, dotted lines are predictions made using the trafficking model and the signal per EGFR calculated in A from the parental + 2C4 data. Solid lines are predictions made using the trafficking model and the signal per EGFR calculated in A from the clone 24H + 2C4 data. Model predictions are shown for the assumption that the signal generated on a per HER2 basis is equal to that of EGFR. E and F, predictions are also shown for the case where the signal per HER2 is 2 times (dashed line) and 3 times (dash-dotted line) that of EGFR.
signal in response to 100 ng/ml EGF for a cell type expressing an intermediate level of HER2 (clone 12: $2 \times 10^5$ HER2/cell) as shown in Fig. 9A.

As a further validation of our model, we sought to predict the effect of blocking EGFR/HER2 heterodimerization on ERK activity. Following pretreatment with mAb 2C4, the ERK signal reflects signaling through only the EGFR since 2C4 blocks HER2 activation (24). As shown in Fig. 9B we compared the EGFR portion of the original ERK signal to the 2C4 data (from Fig. 1B) having adjusted the model for minor alterations in receptor trafficking (discussed under “Model Development and Implementation”) observed in the presence of 2C4 (12). The predicted time profile and magnitude of ERK activity agreed quite well with the experimental data.

The literature suggests that HER2 has a heightened ERK signaling ability relative to EGFR (4–8). From our analyses and given the assumptions inherent in our model, we conclude that EGFR and HER2 are essentially identical in their intrinsic quantitative abilities to generate signals through the ERK pathway. Furthermore HER2-mediated effects on EGFR trafficking can account for the observed differences in ERK signal as a function of elevated HER2 expression.

**DISCUSSION**

The complex set of EGF, EGFR, and HER2 interactions offers great potential for the generation and regulation of cellular signals. Complexity resides at many levels and time scales including, but not limited to, a combinatorial set of inputs through various dimerization possibilities, receptor or dimer-specific trafficking behavior, and possible differences in signaling activities generated by each receptor. In the face of such complexity it is extremely difficult to intuitively estimate the impact of changes in molecular parameters, such as HER2 expression, on cell signaling and cell behavior. The combined application of quantitative experimental and computational techniques allowed us to unravel some of this complexity and gain insight into how elevated HER2 expression affects the dynamics of the system as a whole.

In this study we adopted an integrative modeling approach enabling us to relate upstream events at the level of ligand binding and receptor interaction to downstream signal activation. This is different from previously published models of ERK signaling that incorporate each molecular interaction involved in ERK activation (30–34). This is also the first modeling attempt to incorporate the effects of both trafficking and HER2 expression level effects into the analysis of ERK signaling. By considering different time scales, we were able to separate the effect of dimerization and trafficking events. This yielded a comprehensive model that provided detailed predictions of receptor dimerization state while remaining constrained by a defined and experimentally validated trafficking model.

From the trafficking model prediction, it was clear that EGF concentration plays an important role in determining the relative distribution between homo- and heterodimers (see Fig. 3, C and D). When EGF concentration was low, the number of EGF-EGFR complexes was small. Thus, even cells that express relatively high levels of HER2 cannot form a significant number of heterodimers. Consequently at low EGF concentrations the parental and clone 24H cell lines had little functional difference in their receptor trafficking (see Figs. 3 and 4), and this was borne out in their similar ERK responses (see Fig. 1).

Thus, when ligand concentrations are quite low, elevating HER2 expression above a basal level would have little effect on ligand-dependent ERK signal amplitude. However, autocrine production of ligand in both normal and pathological conditions could amplify the effect of HER2 overexpression by increasing local ligand concentrations.

**FIG. 6.** Determination of HER2/EGFR ratio. Model predictions of the 100 ng/ml EGF-stimulated ERK signal for clone 12 (solid lines) and clone 24H (dashed lines) were made using varying ratios of HER2/EGFR signal per receptor. For each ratio, the model fit of the data was calculated, normalized to the best fit value, and plotted as a function of the HER2/EGFR ratio (clone 12, solid lines; clone 24H, dotted lines). A, the goodness of fit for the assumption that the affinity for EGFR homodimerization is equal to that of heterodimerization. B, the goodness of fit for the assumption that the affinity for EGFR heterodimerization is 10-fold higher than that of homodimerization. C, the goodness of fit for the assumption that the affinity for EGFR heterodimerization is 10-fold lower than that of homodimerization.
Clearly directly relating receptor dimerization state with ERK activity is a simplification as there are many steps in between. The advantage to this approach is that it allowed us to construct a model in which individual molecular steps did not have to be described explicitly, which is especially useful in cases where the reaction networks are poorly understood. This approach assumed that receptor kinase activation is the rate-limiting step in ERK activation (35). Our success in predicting the ERK signal for several different conditions speaks to its validity in our specific case. This led us to conclude that, in 184A1 human mammary epithelial cells, there is a tight correlation between receptor dynamics and downstream ERK signal. For this to occur the transfer of information from ligand binding to ERK activation must be quite rapid, on the order of 1 min, which has also been suggested by other published works (33, 36). Finally negative feedback pathways must be sufficiently weak to prevent the receptor dynamics and ERK activity from becoming effectively uncoupled.

FIG. 7. FRET-based detection of dimerization. The heterodimerization of EGFR with HER2 was identified and quantified by FRET between the Fab fragments of 13A9 and 7C2 antibodies tagged with Alexa dyes-647 and -546, respectively. A, donor and acceptor emissions are sent simultaneously to two areas on the CCD camera. The green laser directly excites the donor (I), and the red laser directly excites the acceptor (II). FRET occurrence is detected by the emission of the acceptor with donor excitation (III). B, contour maps were generated from each of the three channels (I–III) and are superimposed. Signals that overlap in the acceptor (II) and FRET (III) channels were further analyzed. C, after background and bleed-through subtraction, FRET efficiency was calculated for each overlapping pixel using the equations described under “Experimental Procedures.” The weighted average of FRET efficiency was calculated and designated as the FRET efficiency of the cell. AF, Alexa Fluor.

FIG. 8. FRET-based comparison of homo- and heterodimerization. A, the average FRET efficiency in clone 12 was analyzed pixel by pixel for each cell (n = 20 cells for each condition). The FRET efficiency for EGFR homodimerization was determined using the Fab fragments of 13A9 antibody tagged with either Alexa dye-646 or -647. The FRET efficiency for EGFR-HER2 heterodimerization was determined using the Fab fragments of antibodies 13A9 tagged with Alexa dye-647 and 7C2 tagged with Alexa dye-546. B, FRET efficiency was corrected for the distance between epitopes of the antibodies within a pair as described in the text. The heterodimerization-over-homodimerization FRET efficiency ratio was multiplied by 0.75 to correct for the difference in the distance between the fluorophores in the two pairs.
plasmatic domains (38) and the experimental findings that, once initiated by different receptors, the ERK pathway appears to proceed independently of the specific receptor kinase. If HER2 is quantitatively equivalent to EGFR in terms of ERK activation this begs the question, what then is the evolutionary role of HER2? HER2 may be playing more a qualitative role in EGFR signaling, providing linkage to other pathways as well as by shifting receptor localization via trafficking effects. However, the EGFR-sparing effects of HER2 co-expression would be magnified over time into a major quantitative advantage particularly in cases of autocrine stimulation in which receptor internalization and degradation play a major role in limiting tumorigenic transformation (39, 40).

The excellent agreement between the predicted and actual dynamics of ERK signaling in cells expressing varying numbers of HER2 molecules shows the power inherent in our systematic and quantitative approach to dissecting receptor signaling pathways. The apparent complexity of the EGFR-HER2 system obscures the relatively simple principles that govern it: namely EGFR and HER2 are equivalent in stimulating the ERK pathway. The EGFR is internalized faster than HER2, but heterodimers are internalized at an intermediate rate. HER2 interferes with lysosomal targeting of the EGFR. Homodimerization between occupied EGFR occurs with affinity that is at least equal to that for heterodimerization with HER2 (3, 16). Each of these properties is quantifiable and contributes to the overall system behavior. We note that several of these observations are in direct opposition to previous reports in the literature, highlighting the difficulties in understanding a complex system in the absence of an adequate model. Finally the nature of the signaling output appears to be determined primarily by the dynamics of the molecular interactions and less by the precise magnitudes of individual interaction parameters. This principle has been especially apparent in the study of EGFR and HER2 trafficking (3, 16).

The relative ability of EGFR and HER2 to recruit other signaling pathways remains to be determined. However, we built the framework required and demonstrated one possible approach for such analyses in mammary epithelial cells. This approach could also be extended to other cell types, but it would require the recharacterization of the trafficking behavior for optimal results as trafficking rate constants do vary from cell type to cell type. Finally extending this approach to studying two dissimilar receptors is another possibility, but it would have to be done with care and proper experimental validation as some of the underlying assumptions would have to be reassessed. Nonetheless, provided that receptor activation is the limiting step in signal activation for both receptors, this approach would provide a reasonable first step toward parsing such cross-talk and should be particularly valuable in efforts to quantitatively interpret the effects of receptor interactions in signal transduction.

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APPENDIX I

The global trafficking model is taken directly from Hendriks et al. (3). It is solved transiently and then forms the input into the dimerization submodel (see “Appendix II”). The global trafficking model equations are presented below as Equations 7–12,

\[
\frac{dR_t}{dt} = S_t - k_{RsL} + h_{a}C_t - k_{Rs}R_t + k_{af}R_t
\]  

(Eq. 7)
\[
\frac{dCs}{dt} = k_{RsL} - k_{c}Cs - k_{c}Cs + k_{s}f_{w}Ci 
\]
(Eq. 8)
\[
\frac{dRs}{dt} = S_H - k_{a}HS + k_{a}f_{w}Hi 
\]
(Eq. 9)
\[
\frac{dHi}{dt} = k_{a}HS - k_{s}f_{w}Hi - k_{a}(1-f_{w})Hi 
\]
(Eq. 12)

where \( R_s \) is surface unoccupied EGFR, \( Cs \) is surface EGFR-EGFR complexes, \( Hs \) is surface HER2, \( R_i \) is intracellular unoccupied EGFR, \( Ci \) is intracellular EGF-EGFR complexes, and \( Hi \) is intracellular HER2. EGFR-EGFR complexes include those present in all forms: undimerized, homodimerized, and heterodimerized with HER2. HER2 includes all forms: free, homodimerized, and heterodimerized with EGFR or EGF-EGFR complexes. All parameters and their values are listed in Table I.

**APPENDIX II**

The dimerization submodel is solved at quasi-steady state with total numbers of free EGFR, occupied EGFR, and total HER2, for either the surface or intracellular compartment, coming from the results of the global trafficking model. Dimerization submodel equations are adapted from Hendriks et al. (12). Dimerization submodel equations are presented below as Equations 13–21.

\[
\frac{d(R1)}{dt} = -k_{i}(R1)(R2) + k_{a}null(R1R2) - 2k_{i}(R1)(R1) + 2k_{s}null(R1R1) 
\]
(Eq. 13)
\[
\frac{d(R2)}{dt} = -2k_{i}(R2)(R2) + 2k_{a}null(R2R2) - k_{i}(R1)(R2) + k_{a}null(R1R2) 
\]
(Eq. 14)
\[
\frac{d(R1R1)}{dt} = k_{i}(R1)(R1) - k_{a}null(R1R1) 
\]
(Eq. 15)
\[
\frac{d(R1R2)}{dt} = k_{i}(R1)(R2) - k_{a}null(R1R2) 
\]
(Eq. 16)
\[
\frac{d(R2R2)}{dt} = k_{i}(R2)(R2) - k_{a}null(R2R2) 
\]
(Eq. 17)
\[
\frac{d(R1L)}{dt} = -2k_{i}(R1L)(R1L) + 2k_{a}null(LR1R1L) - k_{i}(R1L)(R2) 
\]
+ \( k_{a}null(R1L)(R1L) - k_{i}(R1L)(R1L) \) (Eq. 18)
\[
\frac{d(R1L1)}{dt} = + k_{i}(R1L)(R2) - k_{a}null(LR1R2L) \]
(Eq. 19)
\[
\frac{d(R1L1L)}{dt} = + k_{i}(R1L)(R1L) - k_{a}null(LR1R1L) \]
(Eq. 20)
\[
\frac{d(LR1L1L)}{dt} = k_{i}(R1L)(R1L) - k_{a}null(LR1R1L) \]
(Eq. 21)
Parsing ERK Activation Reveals Quantitatively Equivalent Contributions from Epidermal Growth Factor Receptor and HER2 in Human Mammary Epithelial Cells

Bart S. Hendriks, Gayla Orr, Alan Wells, H. Steven Wiley and Douglas A. Lauffenburger

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