Membrane Binding of ErbB1(645–660) is Due Mainly to Nonspecific Electrostatic Interactions

Fig. 3 shows the ErbB1(645–660) peptide, which corresponds to the reversible membrane anchor region defined in Fig. 1, binds strongly to 5:1 and 2:1 PC/PS vesicles. We also used the centrifugation assay to measure binding of ErbB1(645–660) to 5:1, 10:1, and 1:0 PC/PS vesicles (unpublished data) and deduced the molar partition coefficient $K$ from data similar to those shown in Fig. 3. Fig. S1 shows the value of $K$ increases exponentially with the mole fraction of PS in the membrane, i.e., the binding energy increases linearly with mole fraction of acidic lipid. This behavior, which we have observed with numerous other unstructured basic and basic/hydrophobic peptides that bind electrostatically to membranes, is expected theoretically (Arbuzova et al., 2000). Binding due to nonspecific electrostatic interactions should be independent of the chemical nature of the monovalent acidic lipid in the membrane; ErbB1(645–660) binds with essentially the same affinity to 5:1 PC/PS and 5:1 PC/phosphatidylglycerol vesicles (unpublished data), as expected.

The weak, but measurable, binding of ErbB1(645–660) to PC vesicles ($K \approx 10^2$ M$^{-1}$; Fig. S1) is due to the hydrophobic residues (3 Leu, 1 Ile, 1 Val) in the peptide. Surface pressure measurements on lipid monolayers indicate these hydrophobic residues insert into the lipid head-group region. Adding 1 $\mu$M ErbB1(645–660) to a 2:1 PC/PS monolayer increases the surface pressure significantly, from 30 to 34 mN/m (unpublished data); in contrast, simple basic peptides (e.g., Lys 5, Lys10, Lys13), which bind outside the envelope of the lipid polar head groups, do not increase the surface pressure of a PC/PS monolayer.

We also measured the binding of ErbB1(645–660) to PC/PIP$_2$ vesicles ($K = 6 \times 10^4$ M$^{-1}$ for 99:1 PC/PIP$_2$ vesicles; unpublished data) because several reports suggest ErbB1 may be located in noncaveolar, cholesterol-enriched rafts that also contain an enhanced mole fraction of PIP$_2$. Many different basic or basic/hydrophobic peptides have similar binding affinities for vesicles containing either 20% PS or 1% PIP$_2$ plus PC (see Table III in Wang et al., 2002). In both cases, the binding is due to nonspecific electrostatic interactions.

FRET Shows Membrane-bound ErbB1(645–660) Laterally Sequesters PIP$_2$

We used FRET to show that membrane-bound ErbB1(645–660) laterally sequesters PIP$_2$, even when the monovalent acidic lipid PS is present at 100-fold excess. Note that Fig. S4 shows the membrane-adsorbed basic peptide produces a local positive potential, which will act as a basis for attraction for the multivalent acidic lipid PIP$_2$, as we discuss in detail elsewhere (Gambhir et al., 2004). Fig. S2A shows FRET measurements on 66.7:33:0.3 PC/PS/Bodipy-TMR-PIP$_2$ membranes upon addition of ErbB1(645–660); addition of the labeled peptide
tide produces strong energy transfer from Bodipy- 
TMR-PIP_2 to membrane-bound ErbB1(645–660) as the peptide concentration increases from 0 
(upper curve) to 1000 nM (lower curve). This demonstra-
tes the basic peptide laterally sequesters the PIP_2.
Figs. S2 (B and C) shows the deconvoluted emission 
spectra of the two fluorophores; the donor fluores-
cence decreases (Fig. S2 B) and the acceptor fluores-
cence increases (Fig. S2 C) with increasing [peptide].
We calculated the percent energy transfer as a function 
of peptide concentration for three independent sets of 
measurements and plotted the results in Fig. S2 D; 
~500 nM peptide produces 50% quenching or transfer. We note 
that 100% quenching and FRET can occur because basic or basic/aromatic peptides with a hydrophobic Texas red 
probe can rapidly permeate a phospholipid vesicle (Gambhir et al., 2004). The total PIP_2 concentration in the vesi-
cles is only 600 nM, which suggests membrane-bound ErbB1(645–660) can sequester PIP_2 strongly, even the presence of 180-fold (physiological) excess of monovalent acidic lipid. As expected for a lateral sequestration process 
driven mainly by electrostatics, increasing the salt concentration decreases the FRET (unpublished data).

PLC Hydrolysis Measurements Confirm that ErbB1(645–660) Laterally Sequesters PIP_2

FRET measures lateral interactions between labeled PIP_2 
and labeled membrane-bound ErbB1(645–660), directly, 
but we wanted to confirm the results using an indepen-
dent technique that utilizes unlabeled PIP_2. We examined 
the effect of ErbB1(645–660) on the initial rate of hydro-
lysis of PIP_2 catalyzed by PLC_δ_1, which is a measure of the 
free fraction of PIP_2 in the membrane. Fig. S3 shows that 
2.5 μM ErbB1(645–660) decreases the initial rate of 
hydrolysis approximately twofold, suggesting 50% of the 
PPIP_2 is bound. Texas red-labeled ErbB1(645–660) has a 
significantly larger effect: 1.5 μM reduces the initial rate 
of hydrolysis approximately fourfold. This was expected 
because the hydrophobic probe should insert into the bi-
layer and pull the adjacent Arg residues into the polar 
head group region and enhance electrostatic attrac-
tion of PIP_2 to the basic peptide by a Born charging mech-
anism (Gambhir et al., 2004). The results using two inde-
pendent experimental techniques provide strong evi-
dence that the ErbB basic JM region should laterally 
sequester PIP_2 under physiological conditions (excess of 
monovalent acidic lipid PIP_3). Furthermore, FRET experi-
ments with a reconstituted peptide corresponding to the 
transmembrane + juxtamembrane basic region of ErbB1, 
ErbB1(622–660), also indicate the basic cluster strongly 
sequesters PIP_2 (Sato, T., and P Pallavi, personal commu-
nication; unpublished data).

Electrostatic Potential Profiles

Fig. S4 shows the electrostatic potential profiles adjacent to a bilayer with a bound ErbB1(645–660) peptide and 
unbound Ca/CaM; the electrostatic potential adjacent to 
Ca/CaM (net charge ~16) is negative over most of its sur-
face. The net charge of the Ca/CaM–ErbB1(645–660) complex is also negative (~8), which will repel it from the 
negatively charged membrane.
Fig. S5 depicts the electrostatic profile of the complex between Ca/CaM and a peptide corresponding to a portion of the MARCKS effector region, MARCKS(148–166). Like ErbB1(645–660), this peptide has eight basic residues and binds Ca/CaM with $K_0 \sim 10$ nM; it exists in a mainly extended conformation when it binds Ca/CaM (Yamauchi et al., 2003). The scheme below compares the sequence of these peptides.

![Scheme S1](Image 273x867 to 513x885)

Data from Model Systems Related to the Ligand-independent Activation of ErbB1
As noted in the main text, our model predicts that electrostatic effects can explain ligand-independent activation of ErbB1 by exposure to (a) hyperosmotic conditions, (b) high concentrations of Mg$^{2+}$ or Mg$^{2+}$, and (c) the amphiphilic weak base sphingosine. We performed binding measurements similar to those shown in Fig. 3 at higher salt concentrations; increasing the [KCl] to 200 or 300 mM reduces the molar partition coefficient $\sim$100-fold or $\sim$500-fold, respectively. Specifically, for ErbB1(645–660) and 2:1 PC/PS vesicles, $K = 10^6 \text{ M}^{-1}$, $1.5 \times 10^5 \text{ M}^{-1}$, and $2 \times 10^2 \text{ M}^{-1}$ in 100, 200, and 300 mM salt, respectively. The large change in binding occurs because the counterions screen the negative charges on the membrane; increasing the [KCl] reduces the magnitude of the negative surface potential, and thus decreases the Boltzmann accumulation of the positively charged peptide/protein in the double layer adjacent to the membrane. Theoretical calculations using the Poisson-Boltzmann equation predict that increasing the [KCl] from 100 to 500 mM should decrease the membrane association of ErbB1(645–660) to a 2:1 PC/PS negatively charged membrane to a very small value (from 3 to 0.5 kcal/mol; unpublished data).

Reduced electrostatic interactions between the membrane and the JM and PTK domains also can account for the observation that $\sim$1 mM Mn$^{2+}$ or $\sim$10 mM Mg$^{2+}$ activates ErbB1 in a broken cell preparation. Specifically, addition of 1 mM Mn$^{2+}$ or 10 mM Mg$^{2+}$ to 2:1 PC/PS multilamellar vesicles in 100 mM KCl, pH 7.0, reduces the surface/zeta potential by 25–30 mV, respectively; the same concentrations of divalent cations reduce binding of ErbB1(645–660) to 2:1 PC/PS vesicles by $\sim$100-fold or $\sim$1,000-fold, respectively (unpublished data). Our theoretical calculations indicate that reducing the surface potential of the bilayer by 25–30 mV should also markedly decrease the affinity of the ErbB PTK core for PC/PS membranes, but we have not tested this prediction experimentally.

Earlier work showed adding 2–5 $\mu$M sphingosine to fibroblasts activates ErbB1; we found that adding 2 $\mu$M sphingosine to a solution (100 mM KCl, 1 mM MOPS, pH 7) both reverses the charge of a 2:1 PC/PS multilamellar vesicle (zeta potential changes from $-44$ to $+11$ mV) and markedly decreases the binding of ErbB1(645–660) to PC/PS vesicles (to 25% of its initial value); 5 $\mu$M sphingosine decreases the binding to <5% of its initial value. Furthermore, 5 $\mu$M sphingosine can reverse the association of the JM portion of a reconstituted ErbB1(622–660) peptide in 5:1 PC/PS vesicles; this peptide comprises both the transmembrane and basic JM region. Specifically, it eliminates the FRET observed between Bodipy-TMR-PIP, incorporated into the vesicles and a Texas red probe attached covalently to the COOH terminus of the peptide (Sato, T., and P. Pallavi, personal communication; unpublished data). Thus electrostatic effects could account for ErbB activation by sphingosine in the absence of EGF.

![Figure S5](Image 417x357 to 628x553)
Results of Kinetic Stop Flow Measurements

Our acrylodan-labeled ErbB1(645–660) stop flow data are qualitatively similar to those for an acrylodan-labeled MARCKS effector domain peptide (see Fig. 7 of Arbuzova et al., 1997), which show Ca/CaM removes this peptide from membranes rapidly. As with the MARCKS effector domain peptide (Arbuzova et al., 1997), increasing the fraction of acidic lipid, and consequently the affinity of acrylodan-labeled ErbB1(645–660) for the membrane, decreased the rate at which CaM removes the peptide. Specifically, increasing the mole fraction of PS in the vesicles from 15 to 19% decreased the transfer rate constant from $5 \times 10^7$ M$^{-1}$ s$^{-1}$ to $2 \times 10^6$ M$^{-1}$ s$^{-1}$.

Conversely, decreasing the fraction of acidic lipid, and thus the partition coefficient of the peptide onto the vesicles, increases the transfer rate constant. For 88:12 PC/PS vesicles, the transfer rate constant is $10^7$ M$^{-1}$ s$^{-1}$; for 90:10 PC/PS vesicles it is $6 \times 10^6$ M$^{-1}$ s$^{-1}$, which approaches the diffusion limited value of $10^8$ M$^{-1}$ s$^{-1}$ (Arbuzova et al., 1997). This result agrees with work from other laboratories showing Ca/CaM has forward rate constants of $10^8$ M$^{-1}$ s$^{-1}$ (in solution) for binding to myosin light chain kinase (Bowman et al., 1992), CaM kinase II (Meyer et al., 1992), and the MARCKS-related protein MacMARCKS (Schleiff et al., 1996).

Membrane-permeable CaM Inhibitors Bind to Membranes

Fig. 5 shows that 50 μM W-7 stimulates a small amount of ErbB1 autophosphorylation in Cos1 cells in the absence of EGF; Li et al. (2004) also reported 40 μM W-7 has a biphasic effect on EGF-mediated ErbB1 phosphorylation in N7xHERc fibroblasts (see their Fig. 3). These observations are consistent with our model because W-7 is a weak base that binds to membranes, reduces the magnitude of the negative surface potential of PC/PS vesicles, and inhibits ERB1(645–660) binding to the vesicles at concentrations $\geq 50$ μM (unpublished data). Our model predicts this W-7 activation effect should be stronger in cells that have a significantly higher concentration of ErbB1 than Cos1 cells, and may overwhelm the CaM inhibition effect.

The Electrostatic Properties of the JM and PTK Domains are Conserved in the ErbB Family

Fig. S6 illustrates the highly conserved nature of the basic JM region that we postulate binds to membranes and Ca/CaM. Fig. S7 shows the electrostatic profiles of the PTK cores of the ErbB family members; each has a positively charged face that is capable of binding electrostatically to a negatively charged membrane. Thus, if our hypothesis that the PTK domain of ErbB1 adsorbs electrostatically to membranes is correct, it could apply to the PTK domains of other ErbB family members as well.

Predictions of the Model

The curves in Fig. S8 show the predicted percent trans autophosphorylation of ErbB1 as a function of time after adding EGF for three different cases.

**Case 1: Permeabilized Cells Lacking both CaM and Phosphatases (Dashed Line).** The percent phosphorylation increases linearly with time: $dP^*/dt = k_1$, where $k_1$ is a constant and $P^*$ is the fraction of phosphorylated ErbB. We assume $P^* \ll 1$ for simplicity. The results of Ichinoso et al. (2004) support this prediction: phosphorylation increases linearly with time for 1 h after exposing permeabilized cells to EGF.

**Case 2: Permeabilized Cells Lacking only Phosphatases (Solid Line).** The percent phosphorylation increases exponentially with time: $dP^*/dt = k_2 P^*$, where $k_2$ is a constant.

**Case 3: Permeabilized Cells Containing EGF and Phosphatases (Dotted Line).** The percent phosphorylation decreases exponentially with time: $dP^*/dt = -k_3 P^*$, where $k_3$ is a constant.
Rationalization of HeLa cells to EGF. This observation is consistent with the postulate that EGF induces dimerization and thus increases trans autophosphorylation by a local concentration mechanism. The rate of autophosphorylation is low, however, suggesting an autoinhibition mechanism such as the one we postulate in Fig. 1.

Case 2: Cell Exposed to CaM Inhibitors (Dotted Curve). These cells have phosphares, but a greatly diminished concentration of functional CaM. We assume the initial rate of phosphorylation is the same as for case 1 and that phosphares remove phosphates at a rate proportional to the fraction of phosphorylated ErbB: $\frac{dP^*}{dt} = k_1 - k_2P^*$, where $k_1$ is a positive constant and $P^*$ has the form shown by the dotted line, $P^*(t) = P^*(0)(1 - \exp(-t/\tau))$. A steady-state occurs when the fraction of phosphorylated ErbB increases to a level where the rate of hydrolysis by phosphatases ($k_2P^*$) equals the constant rate of trans autophosphorylation ($k_1$). This prediction corresponds well to the observations of Li et al. (2004; see their Fig. 4).

Case 3: Normal Cell (Solid Curve). The fraction of phosphorylated ErbB attains a maximal value (after 1–10 min depending on cell type [EGF]), and then decays to the same steady-state level as in case 2. This behavior has been observed in many cell types (e.g., see Kholodenko et al., 1999; Ichino et al., 2004; Li et al., 2004). Li et al. (2004) report the level of steady-state phosphorylation is identical in cases 2 and 3, as predicted by our model. The initial transient peak could be due to the positive feedback mechanism shown in Fig. 1; when intracellular Ca increases, a low value (e.g., Kholodenko et al., 1999). The initial transient peak could be due to the positive feedback mechanism shown in Fig. 1; when intracellular Ca increases, a low value (e.g., Kholodenko et al., 1999).

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Figure 5B. Predicted patterns of ErbB1 phosphorylation. The percent ErbB1 phosphorylated in a cell is plotted as a function of time after addition of EGF. The dashed line illustrates the prediction for permeabilized cells, which lack both phosphatases and Ca/CaM. The dotted curve shows the prediction for cells exposed to CaM inhibitors. The solid line illustrates the prediction of the model shown in Fig. 1 for an intact cell, which contains both CaM and phosphatases. See text for details.