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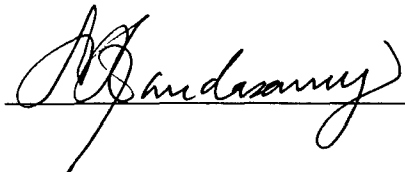
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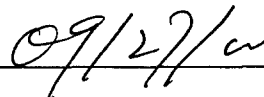
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13. ABSTRACT (Maximum 200 words)

The four erbB receptor tyrosine kinases are activated by dimerization upon binding their cognate ligands, which include epidermal growth factor (EGF) and the neuregulins (NRG's). Each receptor has a large extracellular ligand-binding domain, a single transmembrane domain, and an intracellular tyrosine kinase/regulatory domain. More than 12 different erbB ligands exist, which are thought to activate erbB receptors by inducing receptor hetero- as well as homo-dimerization. A view has emerged in which each ligand induces a specific set of receptor homo- and hetero-dimers, which yield characteristic biological responses. We have set about quantitating the ability of erbB ligands to induce specific erbB receptor dimers using purified isolated receptor extracellular domains. For the erbB1 ectodomain, we established that the extracellular domain is sufficient for EGF-induced receptor homodimerization using X-ray and light scattering, plus analytical ultracentrifugation. NRG1-β1 also induces efficient homodimerization of the erbB4 ectodomain. These are the only ligand-induced homodimerization events detected. The erbB1 ectodomain, despite homodimerizing, does not heterodimerize with any other extracellular domain. However, we detect NRG1-β1 induced ectodomain heterodimers for erbB2/erbB3, erbB2/erbB4, and erbB3/erbB4. Our next plans are to establish how erbB1 transmodulates other erbB receptors without ectodomain heterodimerization, and to test the predictions of our in vitro studies in a cellular context.

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MARK A. LENNON   7/29/99

PI - Signature   Date

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INTRODUCTION

Cellular responses to many extracellular factors that control cell growth and differentiation are mediated by cell-surface receptors with tyrosine kinase activity. The mechanism of transmembrane signaling by receptor tyrosine kinases involves an initial ligand-induced receptor dimerization event that leads to activation of the intracellular tyrosine kinase domain. For a given class of receptor tyrosine kinases, ligand-induced dimerization can involve two receptors that are the same (homodimerization) or different (heterodimerization). It is now appreciated that heterodimerization provides a mechanism for increasing the diversity of signaling through a given family of receptors. In our studies we focus on the four known receptors in the epidermal growth factor (EGF) receptor family - known as erbB1 to erbB4. The erbB receptors have been implicated in a number of human cancers. In particular, erbB2 (also known as Neu, or HER-2) is strongly implicated in breast cancer. Aberrant overexpression of a single member of this family can disrupt normal signaling, in some cases leading to uncontrolled cell proliferation. There are at least 12 different ligands that signal through the erbB family of receptor tyrosine kinases, including EGF, TGFα, and the neuregulins. The ligands differ in their receptor-binding characteristics, and appear to induce formation of distinct combinations of erbB receptor homo- and heterodimers. Their specific biological activities are thought to arise from these differences. We are interested in understanding how the multiple different ligands induce formation of particular receptor dimers. For the EGF receptor (erbB1), we previously showed that the extracellular domain is sufficient for ligand-induced dimerization, and that two EGF molecules are required to form the dimer. Through biophysical analyses of the other erbB receptor extracellular domains, produced in a baculovirus expression system, we have compared ligand-induced receptor homo- and heterodimerization by EGF and neuregulin-1β (NRG1-β), the results of which are described in this report. Our current findings indicate that hetero-oligomerization or transmodulation of erbB receptors differs mechanistically from the accepted ligand-induced homodimerization model established for these and other receptors. Furthermore, while ligand-induced heterodimerization may be relevant for erbB2/erbB3/erbB4, it appears not to be important for the EGF receptor (erbB1). Our next goal is to incorporate the results of these studies of dimerization *in vitro* into an *in vivo* picture of signaling by this class of receptors. By developing this understanding, we hope that approaches will be suggested for specifically modulating erbB receptor signaling when it is disrupted in human cancers.

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BODY OF PROGRESS REPORT

Progress for each task is detailed following description of the task

Task 1.

To determine quantitatively the hierarchy of erbB receptor homo- and heterodimers induced by each ligand in the EGF and NRG family

- *generate s-erbB proteins and erbB ligand in insect cells and yeast/bacteria, and perform preliminary qualitative studies of ligand-induced homo- and heterodimerization (months -18 to 0)*

We succeeded in producing milligram quantities of the four erbB receptor extracellular domains before funding of the award. Each of the four erbB receptor extracellular domains (s-erbB1, s-erbB2, s-erbB3, and s-erbB4) was secreted from Sf9 cells infected with recombinant baculovirus. Each s-erbB protein included the entire extracellular domain of the relevant receptor, followed by a hexahistidine tag to expedite purification from conditioned insect-cell medium. The most C-terminal native amino acid of each protein was K642 (s-erbB1); P647 (s-erbB2); K639 (s-erbB3); and R649 (s-erbB4); where residue numbers include the signal peptide.
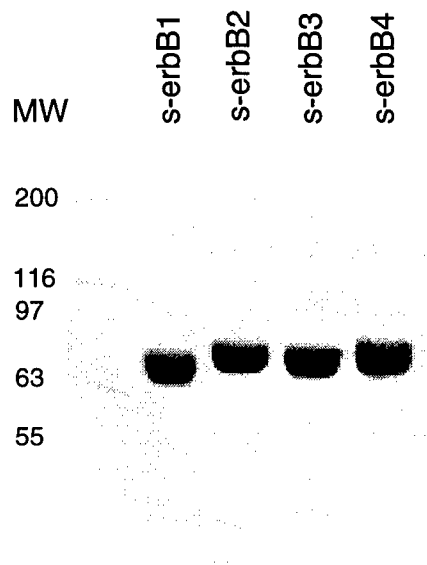

Figure 1

SDS-PAGE of the purified s-erbB proteins used for biophysical analyses of ligand-induced homo- and hetero-dimerization described in this progress report.

Using a Ni-NTA agarose column, followed by gel filtration and a single round of ion exchange (see Experimental Procedures), s-erbB proteins could be prepared from Sf9-cell conditioned, serum-free, medium with yields of 1.5 mg/liter (s-erbB1), 0.3 mg/liter (s-erbB2), 1 mg/liter (s-erbB3), and 0.8 mg/liter (s-erbB4). Significantly higher total yields could be achieved

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using High Five cells from *T. ni*, but the purified protein from these cells was more heterogeneous, and in some cases showed a tendency to aggregate. As a consequence, only protein secreted by Sf9 cells was employed in the studies described here. Figure 1 shows a Coomassie-stained SDS-PAGE gel of the purified s-erbB proteins, which we estimate to be greater than 92-95% pure in all cases.

We have focused most of our studies to date on the central ligands of the erbB ligand family: epidermal growth factor (EGF) and neuregulin-1β1 (NRG1-β1). A number of experiments have also been performed with heparin-binding EGF-like growth factor (HB-EGF) and transforming growth factor-α (TGF-α). In all cases, commercially available ligands (from Intergen or R & D Systems) have been employed. Our own efforts to express large quantities of erbB ligands in *Pichia pastoris* and *E. coli* yielded good quantities of EGF, which we have used for some experiments. However, NRG's, betacellulin, and TGF-α produced in these systems, while bio-active, were insufficiently homogeneous for use in our biophysical studies. Efforts are now underway to produce some of the key ligands not yet studied (notably betacellulin) in an insect cell system.

- *perform binding assays for each ligand to each possible combination of s-erbB proteins using calorimetric and SPR approaches (months 1 to 18)*

We have been able to use surface plasmon resonance (Biacore) to confirm that the s-erbB proteins secreted from Sf9 cells bind to the relevant growth factor ligands. Biacore CM-5 sensor chips were derivatized with the EGF-like domains of EGF or NRG1-β1, or with no ligand, and solutions of the four purified s-erbB proteins were passed over the resulting surfaces. The results of these Biacore studies are summarized in Figure 2 and Table I. As anticipated, s-erbB1 bound significantly to the EGF-derivatized surface, but not to surfaces carrying NRG1-β1 or no ligand. By contrast, s-erbB2 did not bind to any of the surfaces, and significant binding of s-erbB3 and s-erbB4 was seen only with surfaces bearing NRG1-β1. Estimated K_D values for NRG1-β1 binding by s-erbB3 and s-erbB4 were 249 nM and 179 nM respectively, and the estimated K_D for EGF binding by s-erbB1 was 118 nM (Table I). These K_D values all lie well within the range reported (100 to 500 nM) for EGF binding by monomeric s-erbB1 produced in mammalian cells (Greenfield *et al.*, 1989; Günther *et al.*, 1990; Hurwitz *et al.*, 1991; Lax *et al.*, 1991a; Zhou *et al.*, 1993; Brown *et al.*, 1994; Lemmon *et al.*, 1997). A K_D value of 17-35 nM was previously reported for binding of full-length NRG1-β2 to monomeric s-erbB3 from analytical ultracentrifugation studies (Horan *et al.*, 1995). The 10-fold higher affinity seen by Horan *et al.*, may result from their use of full-length NRG1-β2 rather than the EGF-like domain alone. However, the EGF-like domain is known to be sufficient for all known biological activities of

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NRG1 (Holmes *et al.*, 1992). When binding of growth factors to predimerized IgG fusion proteins of erbB receptor extracellular domains is measured, the apparent affinities are approximately 30-fold higher (Jones *et al.*, 1998, 1999; Ballinger *et al.*, 1998; Fitzpatrick *et al.*, 1998). The relative binding affinities listed in Table I agree very well with the relative K_D or IC_{50} values reported from studies of IgG fusion proteins for EGF binding to s-erbB1 compared with binding of NRG1-β to erbB3 and erbB4.

Table I

Ligand binding by the s-erbB proteins

Ligand	K_D for s-erbB1 (nM)	K_D for s-erbB2 (nM)	K_D for s-erbB3 (nM)	K_D for s-erbB4 (nM)
EGF	118 ± 41	none	> 10^4	> 10^4
NRG-β1	> 10^5	none	249 ± 80	179 ± 10

Summary of K_D measurements made for the four s-erbB proteins to immobilized EGF and NRG1-β1. K_D values listed explicitly represent means of at least four independent determinations, and are quoted alongside their standard deviations.
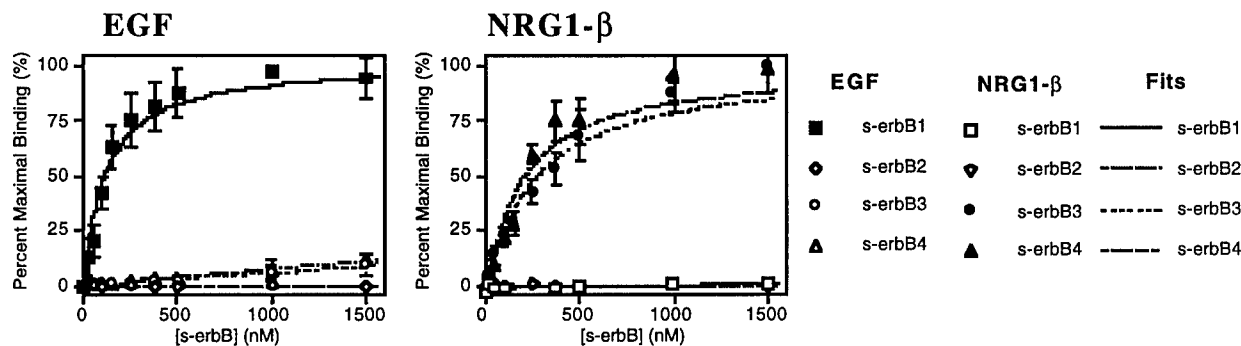
Figure 2

Binding of s-erbB1, s-erbB2, s-erbB3, and s-erbB4 to EGF (left) and NRG1-β1 (right), immobilized on a BIAcore chip. Best-fits to the data, assuming a simple association model, are shown. Errors are standard deviations from the mean of at least 4 independent determinations at each point. K_D values represented by the best fits are list in Table I.

We have not been able to detect any significant difference in binding affinities when passing over mixtures of s-erbB proteins. In particular, a mixture of s-erbB2 and s-erbB4 gave results for NRG1-β binding that were not clearly distinguishable from those determined with s-erbB4 alone (Figure 2), despite the fact that NRG1-β induces s-erbB2/s-erbB4 heterodimer formation. Titration calorimetry was employed to study EGF binding to s-erbB1, and results

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identical to those obtained previously (Lemmon et al., 1997) were obtained. Given the relative difficulty of interpreting calorimetric results for dimerization-coupled binding events, and the large quantities of material required for these studies, we have settled upon BIAcore measurements as a more expeditious approach.

- *using classical multi-angle laser light scattering methods, measure the ability of each erbB ligand to induce homodimerization of each s-erbB protein, by varying both ligand and receptor concentration (months 1 to 4)*

As shown in Figure 3, multi-angle laser light-scattering (MALLS) was measured for a series of samples containing s-erbB protein at 4 μM, to which had been added increasing concentrations of EGF or NRG1-β1. The weight-averaged molecular mass ($M_{\overline{w}}$) for each sample, relative to that measured in the absence of ligand, was determined by extrapolation of a Debye plot to zero angle and was expressed as a fold-increase in $M_{\overline{w}}$ (see Experimental Procedures). As shown in Figure 3, addition of one molar equivalent of EGF to s-erbB1 resulted in a doubling of $M_{\overline{w}}$, as we have observed previously in X-ray scattering studies (Lemmon *et al.*, 1997). No further increase in $M_{\overline{w}}$ was seen when larger excesses of EGF were added, consistent with our previous finding that EGF induces formation of a 2:2 s-erbB1:EGF dimer, but no higher order oligomers (Lemmon *et al.*, 1997).
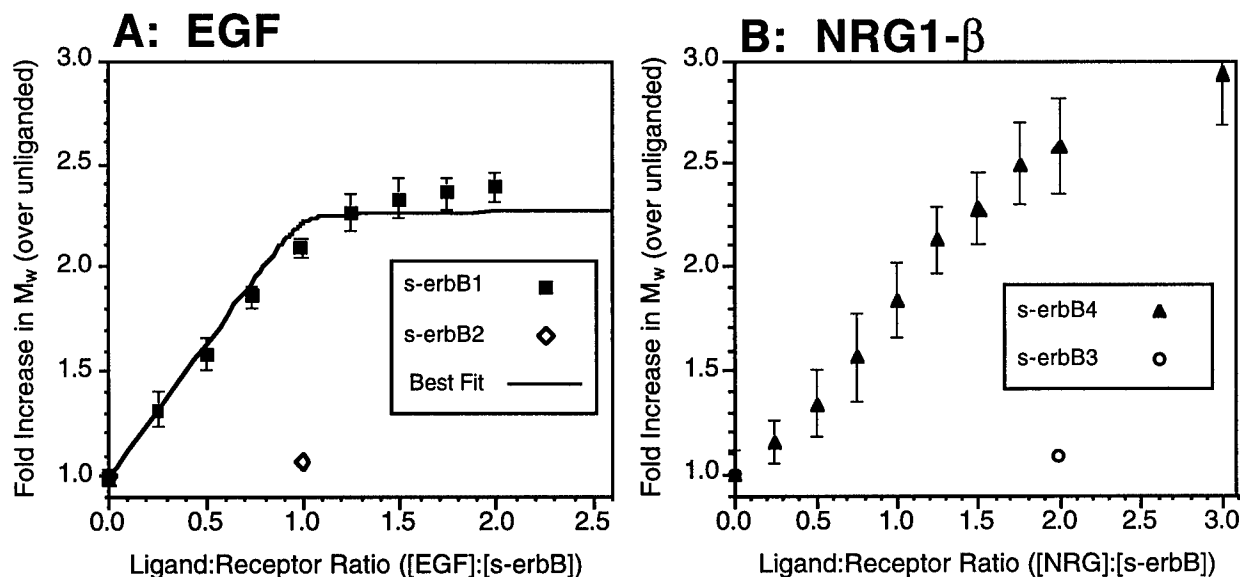

Figure 3

MALLS studies of EGF-induced homo-dimerization of s-erbB1 and s-erbB2 (A) as well as of NRG1-β1 induced homodimerization of s-erbB3 and s-erbB4 (B) (see text).

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Figure 3A shows a best fit to the EGF-induced s-erbB1 dimerization data using a model in which a 1:1 EGF:s-erbB1 complex forms with K_D = 118 nM, and this 1:1 complex dimerizes with K_D less than 100 nM to form the 2:2 EGF:s-erbB1 dimer. This data fitting indicates that our insect cell-derived s-erbB1 dimerizes 20 to 50-fold more strongly than the CHO cell-derived protein studied by Lemmon *et al.*

Analysis of s-erbB4 by MALLS also demonstrated clear dimerization upon addition of NRG1-β1. In this case, the maximum $M_{\overline{w}}$ is not reached until almost two equivalents of NRG1-β1 have been added to the s-erbB4. Furthermore, in some experiments the final $M_{\overline{w}}$ was a little higher than expected for a 2:2 s-erbB4:NRG1-β1 complex. These MALLS experiments, together with our analytical ultracentrifugation studies, argue against the possibility that NRG1-β1 induces formation of s-erbB4 oligomers with order greater than two. However, the data are consistent with a model similar to that described for EGF-induced s-erbB1 dimerization if it is assumed that 20 to 30% of the NRG1-β1 preparation is present as small aggregates and/or is inactive. Given this caveat, we have not attempted to fit the NRG1-β1/s-erbB4 data explicitly, although it is clear that NRG1-β1 induces strong s-erbB4 dimerization.

As also shown in Figure 3, addition of excess EGF to s-erbB2 does not induce its dimerization (Fig 3A), and addition of excess NRG1-β1 to s-erbB3 does not significantly increase $M_{\overline{w}}$ of that protein (Fig 3B). Thus, these data argue that EGF induces homodimerization of only s-erbB1, and NRG1-β1 induces homodimerization of only s-erbB4. Homodimers of neither s-erbB2 nor s-erbB3 can be induced by these ligands or any other tested.
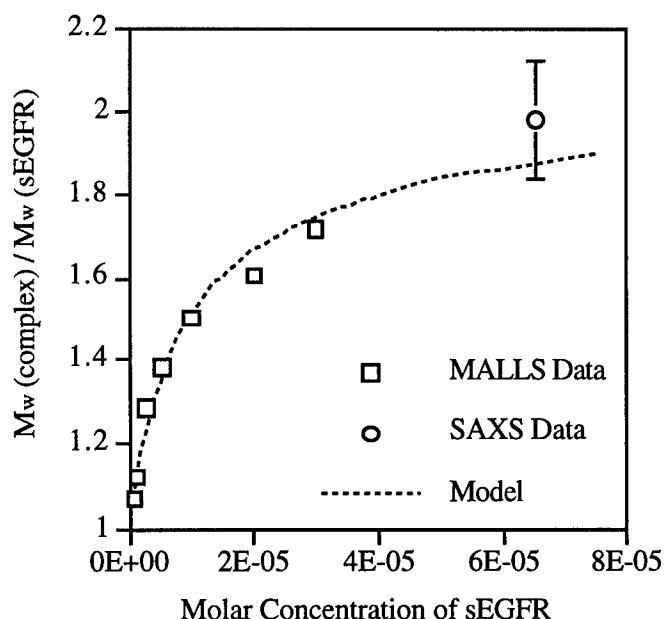

Figure 4
Measurement of $\overline{M}_w$ for a 1:1 EGF/s-erbB1 mixture at a series of different molar concentrations in 50 mM Hepes, pH 7.5, 100 mM NaCl at 25°C. Data up to 30μM were obtained from MALLS measurements, while the data point at 65μM is an average of the relative $\overline{M}_w$ measured for a 1:1 mixture in small-angle X-ray scattering (SAXS) experiments. The solid line represents predicted data from the model presented by Lemmon *et al.*, (1997), with K_γ increased to 8μM and K_β reduced to 25μM as described in the text. The error on the Debye plot fit for each MALLS data point does not extend beyond the symbol size.

To determine K_D values for ligand-induced s-erbB1 and s-erbB4 homodimerization, experiments were performed in which the concentration of a 1:1 receptor:ligand complex was varied, as shown in Figure 4. For the CHO cell-derived s-erbB1 that we employed prior to the beginning of these studies, this approach was useful, and gave a dimerization K_D of approximately 3 μM to 8 μM, depending on the batch of protein employed. The protein that we now produce from insect cells dimerizes much more strongly, such that we have not been able to detect dissociation of the dimer in complex dilution experiments of this sort. Data fitting suggests that K_D for s-erbB1 or s-erbB4 homodimerization, when occupied by EGF or NRG1-β1 respectively, is in the 30 nM range. The precision of this value will be improved through analytical ultracentrifugation experiments to be performed over the coming months.

- *using the results from the homodimerization assay, assess using light-scattering the ability of each erbB ligand to induce each of the 6 possible s-erbB heterodimers. By repeating experiments using different concentrations of receptors and ligands (and different ratios of the two s-erbB proteins), determine binding constants for dimerization and ligand binding (months 1 to 12)*

The most likely relevant erbB receptor heterodimer or oligomer in breast cancer, and the one that was first reported to occur, is the erbB1/erbB2, or EGFR/Neu hetero-oligomer (King *et al.*, 1988; Stern *et al.*, 1988; Wada *et al.*, 1990). Having established (as described above) that EGF induces efficient s-erbB1 homodimerization, and that NRG1-β1 induces efficient s-erbB4 homodimerization, we next tested the ability of these s-erbB proteins to heterodimerize upon growth factor binding. As outlined in the introduction, there is a great deal of evidence for ligand-induced heterodimerization of erbB receptors. One of the first indications for heterodimerization (or transmodulation) came from the finding that erbB2, which does not bind EGF, can nonetheless be activated by EGF in cells that express both erbB1 and erbB2 (King *et al.*, 1988; Stern *et al.*, 1988; Wada *et al.*, 1990; Spivak-Kroizman *et al.*, 1992). ErbB2 is not activated by EGF in cells that do not express erbB1. Transmodulation of erbB2 by erbB1 has been shown to result from EGF-dependent association of erbB1 and erbB2 to form presumed heterodimers that show elevated tyrosine kinase activity, and are extensively autophosphorylated (Wada *et al.*, 1990; Spivak-Kroizman *et al.*, 1992). Supporting the suggestion that erbB1/erbB2 heterodimers might resemble active erbB1 homodimers, erbB2 with a cytoplasmic truncation was reported to act as a dominant-negative inhibitor of erbB1 signaling (Qian *et al.*, 1994) apparently in the same way as similar erbB1 truncation mutants inhibit EGF signaling (Kashles et al., 1991).

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Since EGF efficiently induces s-erbB1 homodimerization (see above), we reasoned from findings in the literature and current models for heterodimerization that EGF should also induce efficient heterodimerization of s-erbB1 and s-erbB2.
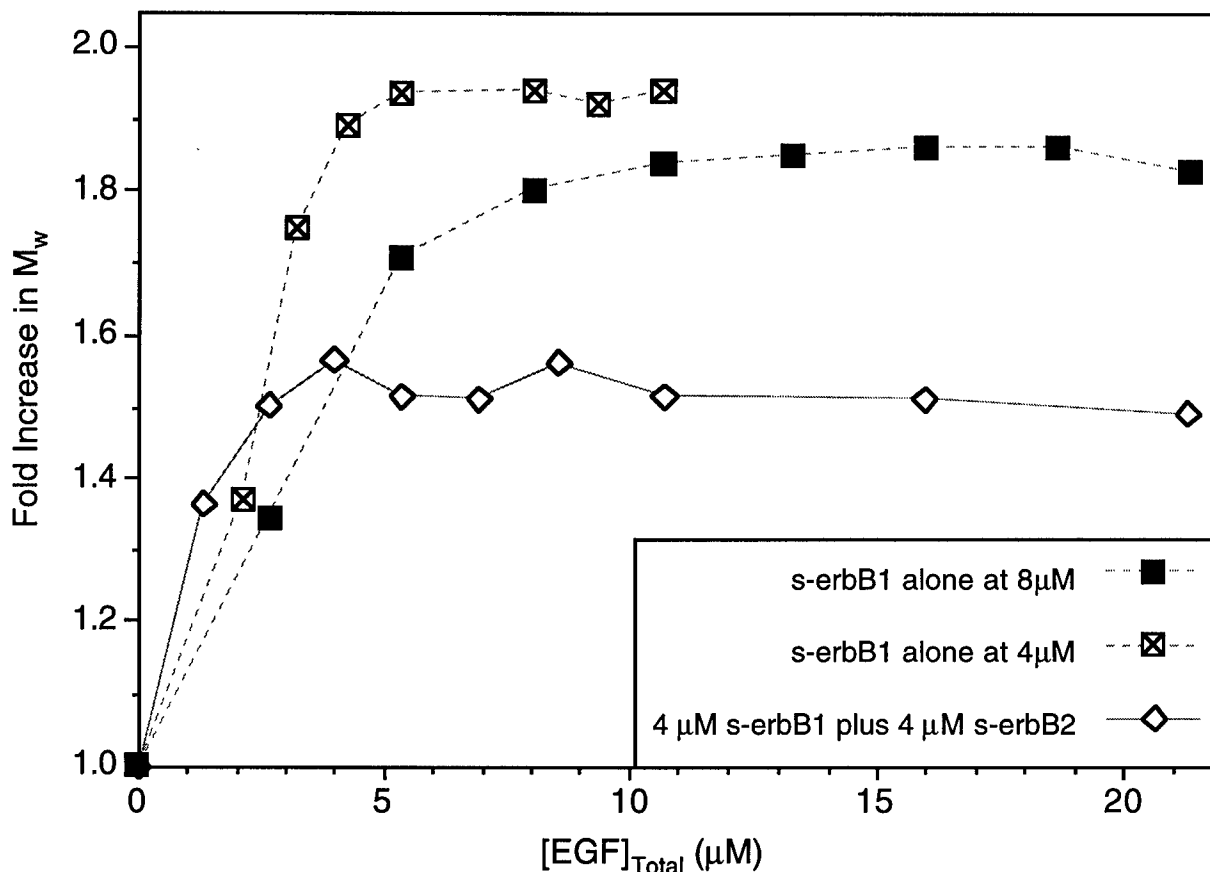

Figure 5

MALLS studies demonstrate that EGF induces complete homodimerization of s-erbB1, but does not induce formation of heterodimers between s-erbB2 and s-erbB1. The weight-averaged molecular mass (Mw) of the s-erbB1/s-erbB2 mixture increases such that after addition of one EGF molecule for each s-erbB1 molecule in the mixture, Mw reaches a maximum coincident with all s-erbB1 forming homodimers, and s-erbB2 remaining monomeric.

Figure 5 presents our MALLS studies, which show that EGF is not able to induce the expected extracellular domain heterodimerization. MALLS monitors the weight-averaged molecular mass ($M_{\overline{w}}$) of the particles in solution. For a sample containing only 4 μM s-erbB1, $M_{\overline{w}}$ is doubled upon addition of greater than 4 μM EGF (Figure 5 crossed squares). Similarly, for a sample containing 8 μM s-erbB1, $M_{\overline{w}}$ is almost doubled when more than 8 μM EGF is added (Figure 5 filled squares). By contrast, for a sample containing 4 μM s-erbB1 plus 4 μM s-erbB2, $M_{\overline{w}}$ reaches a maximum value when EGF is added to a final concentration of 4 μM (Figure 5, open diamonds). The fold-increase in $M_{\overline{w}}$ in this case is only 1.5. This is the expected result if

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EGF induces s-erbB1 homodimerization while s-erbB2 remains monomeric in the mixture. $M_{\overline{w}}$ is defined:

$$\overline{M}_w = \frac{\sum_i n_i M_i^2}{\sum_i n_i M_i} \qquad \text{for } n \text{ moles of } i \text{ different species with molecular mass } M_i$$

In a sample containing 4 μM monomeric s-erbB2 (80 kDa) plus 2 μM s-erbB1 dimers (160 kDa), $M_{\overline{w}}$ would be estimated as 120 kDa, or a 1.5-fold increase over the monomeric $M_{\overline{w}}$ of 80 kDa. Thus, the MALLS data provide no evidence for EGF-induced s-erbB1/s-erbB2 heterodimerization, and argue that s-erbB1 in the mixture homodimerizes with s-erbB2 as a monomeric "bystander".

These results suggest that the ligand-induced erbB1/erbB2 hetero-oligomers observed by several groups must form through a mechanism distinct from that used by erbB1 for homodimerization upon EGF binding. In the homomeric case, the isolated extracellular domain can recapitulate the interaction. In the heteromeric case it cannot. However, one caveat to this finding is that we have no independent validation of the functional quality of our s-erbB2 preparations. Since erbB2 has no known ligand, we cannot validate the protein by virtue of its ability to bind ligand, as was done for s-erbB1, s-erbB3, and s-erbB4 (Figure 2). However, we do not believe that the s-erbB2 is non-functional, since it is able to form NRG1-induced heterodimers with both s-erbB3 and s-erbB4 (see below). Furthermore, MALLS studies of potential heterodimers formed between s-erbB1 and s-erbB3 or s-erbB4 (with EGF or NRG1-β1) also gave clear negative results (not shown). Thus, in spite of its ability to form EGF-induced homodimers, s-erbB1 does not form heterodimers with other isolated erbB receptor extracellular domains.

MALLS studies have given indications of NRG1-β1-induced heterodimer formation for s-erbB2/s-erbB4 and s-erbB2/s-erbB3. Datasets for these MALLS studies are currently being completed. Moreover, as detailed below, analytical ultracentrifugation studies illustrate the formation of these heterodimers.

- *confirm findings regarding ligand-induced s-erbB homo- and heterodimerization in vitro using analytical ultracentrifugation (month 1-18)*

Using sedimentation analytical ultracentrifugation and multi-angle laser-light scattering (MALLS), we have studied ligand-induced dimerization of each s-erbB protein. Figure 6 shows typical results from sedimentation equilibrium experiments (6,000 rpm) in which samples of each s-erbB protein were centrifuged both with and without the most relevant growth factor ligand.

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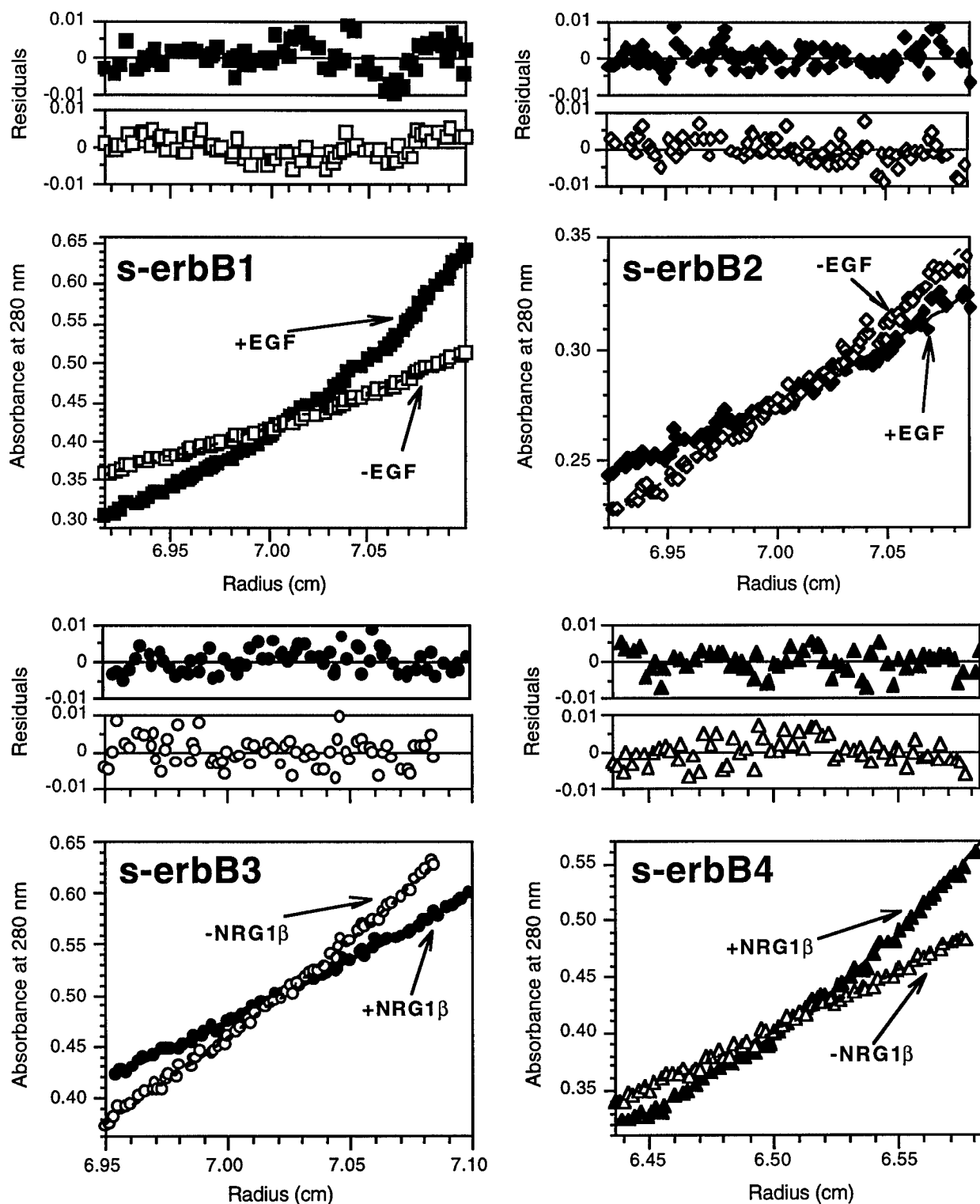
Figure 6

Representative sedimentation equilibrium analytical ultracentrifugation data for analysis of s-erbB homodimerization induced by EGF or NRG1-β1. In each case, open symbols represent the unliganded receptor, which is fit as an ideal single species (molecular mass range from 80 kDa for s-erbB1 to 97 kDa for s-erbB4). Filled symbols represent samples to which has been added a two-fold molar excess of the noted ligand. Fits to these data are with two ideal

species - fixing the Mw of the ligand and floating the Mw of the complex. Fits return Mw of 198 kDa for s-erbB1 (dimer), 77.5 kDa for s-erb2 (monomer), 85 kDa for s-erbB3 (monomer), and 197 kDa for s-erbB4 (dimer). As described in the text, this result is clear from inspection of the curves. All experiments shown were performed at 6,000 rpm (other speeds giving the same result). Residuals for the fits described are shown, and are both small and random, indicative of a good fit.

Inspection of the raw centrifugation data in Figure 6 shows that addition of a 2-fold molar excess of EGF to s-erbB1, or of a 2-fold excess of NRG1-β1 to s-erbB4 (filled symbols) results in a radial distribution indicative of a species larger than the s-erbB protein monomer. Since the s-erbB proteins have molecular masses of around 85 kDa, and the added ligands have molecular masses of only 6.3 kDa (EGF) and 8.3 kDa (NRG1-β1) , this can only be explained if the s-erbB protein is induced to oligomerize upon addition of the growth factor. Data fitting assuming a single ideal species for the ligand-free receptor gives molecular masses of 80 kDa and 97 kDa for s-erbB1 and s-erbB4 respectively. The best fit to the ligand/receptor mixture is obtained with two ideal species representing excess ligand and the s-erbB dimer. The residuals for these fits (experimental value minus fit value), plotted above the data in Figure 6, are both small and random, indicative of good fits to the data.

By contrast with the case for s-erbB1 and s-erbB4, addition of excess ligand to s-erbB2 or s-erbB3 results in a radial distribution consistent with a single species that is smaller than monomeric s-erbB protein. This is the expected result if the s-erbB protein does not bind ligand, or does not oligomerize upon ligand binding, since the distribution is now contributed to by free ligand that is 10-fold smaller (6 to 8 kDa) than the s-erbB protein (approx. 85 kDa). While best fits to a single ideal species gave molecular masses of 80.8 kDa and 84 kDa for s-erbB2 and s-erbB3 (without ligand) respectively, best fits to the ligand/receptor mixtures were obtained with two ideal species representing excess ligand and the s-erbB monomer (77.5 and 85 kDa for s-erbB2 and s-erbB3 respectively). Again, residuals for these fits are plotted above the data in Figure 3, and are both small and reasonably random, suggesting reasonable fits.

These data confirm the MALLS finding that, while s-erbB1 and s-erbB4 dimerize upon binding to EGF and NRG1-β1 respectively, s-erbB3 does not dimerize when it binds NRG1-β1. The inability of EGF to induce s-erbB2 dimerization is consistent with the lack of significant binding (Figure 2). NRG1-β1 also failed to bind s-erbB2 or to induce its dimerization (Figure 2 and 9). In other sedimentation equilibrium experiments (not shown) we found that s-erbB1 dimerization is also induced by TGF-α and HB-EGF (to which it binds), but not by NRG1-β1 (to which it does not bind). Dimerization of s-erbB4 was seen with NRG1-β1 (to which it binds), but not with HB-EGF, or EGF (to which it does not bind in our hands). Thus, all ligands tested that are capable of binding to s-erbB1 or s-erbB4 also induce their homodimerization. The failure of neuregulins to induce homodimerization of s-erbB3 represents the only example we have seen in

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which binding is observed in the absence of associated homodimerization. Previous studies with full-length NRG1-β1 (Horan *et al.*, 1995) also showed strong binding to s-erbB3 in the absence of induced dimerization.

Also using analytical ultracentrifugation, we have confirmed the inability of EGF to induce s-erbB1/s-erbB2 heterodimerization (Figure 7), and the failure of NRG1-β1 to induce s-erbB1/s-erbB4 heterodimerization (Figure 8). Figure 7 shows plots of the natural logarithm of absorbance against $(r^2-r_0^2)/2$, where r is the radial position in the sample, and r_0 the radial position of the meniscus for datasets collected at 6,000 rpm. For an ideal single species this plot is linear, with a gradient ($M\omega^2(1 - \overline{V}_2\rho)/RT$) that is proportional to the molecular mass (M) of the ideal species. The lines obtained for s-erbB1 alone and s-erbB2 alone are approximately the same, and yield molecular masses of 80.5 and 78.9 kDa respectively. Addition of one equivalent of EGF to s-erbB1 results in near doubling of the gradient of this line, consistent with the ability of EGF to induce complete dimerization of s-erbB1. By contrast, addition of EGF to a 1:1 mixture of s-erbB1 and s-erbB2 increases the gradient of the line to only an intermediate extent (1.3-fold) and causes a greater deviation from linearity. This result is consistent with the interpretation of MALLS studies (Figure 5) that EGF induces homodimerization of s-erbB1 in the s-erbB1/s-erbB2 sample while the s-erbB2 remains monomeric.
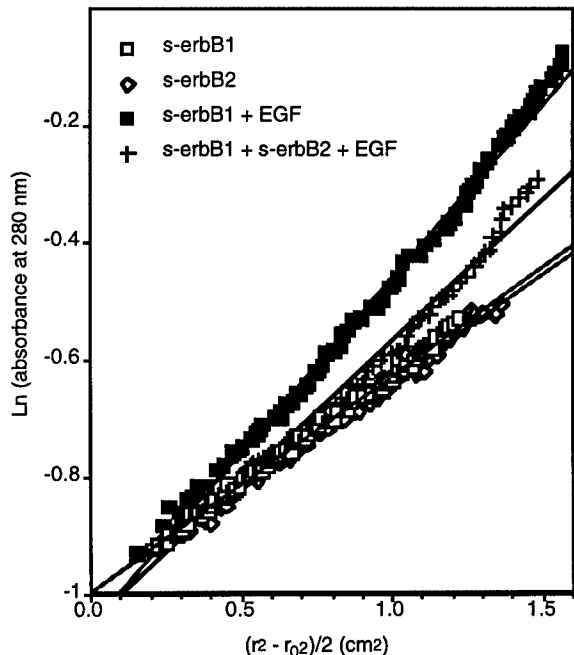

Figure 7

Plots of the natural logarithm of absorbance against the radius squared for analytical ultracentrifugation data. S-erbB1 and s-erbB2 alone yield straight lines in this plot, with gradients proportional to their molecular mass. Addition of EGF to s-erbB1 doubles the gradient showing that dimerization results. For an s-erbB1/s-erbB2 mixture, addition of a 2-fold excess of EGF yields an increase in the gradients of only about 1.3-fold. As in Fig 5, this is expected is the s-erbB1 homodimerizes while s-erbB2 remains monomeric.

These experiments, together with chemical crosslinking studies and co-immuno-precipitation studies (not shown), confirm the MALLS finding that EGF is not able to induce heterodimerization of the isolated extracellular domains of erbB1 and erbB2.

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Contains unpublished data

Heterodimerization of erbB1 and erbB4 upon treatment with EGF or NRG has been reported by several groups (*e.g.* Cohen *et al.*, 1996; Zhang *et al.*, 1996). Since we have been able to show that s-erbB1 and s-erbB4 both bind to their relevant ligands and homodimerize efficiently (Figures 3 and 6), we can be confident that both of these proteins are functionally active. We next used analytical ultracentrifugation to determine whether s-erbB1 and s-erbB4 can form heterodimers upon treatment with EGF or NRG1-β1.
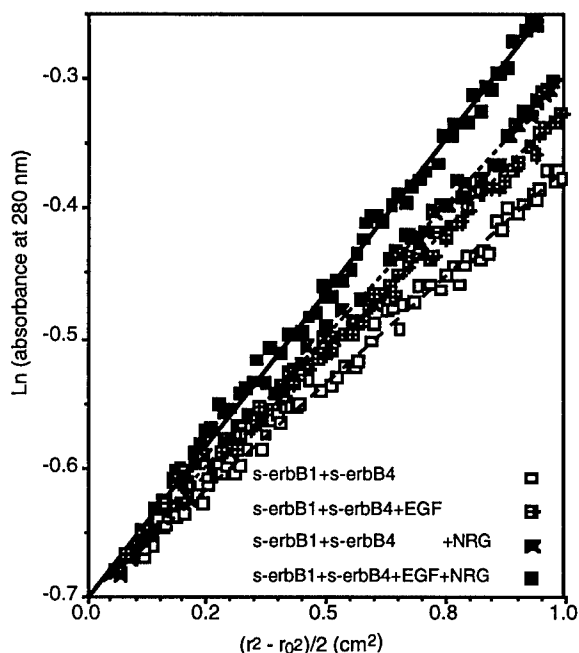

Legend (within figure):
s-erbB1+s-erbB4 □
s-erbB1+s-erbB4+EGF ▣
s-erbB1+s-erbB4   +NRG ▨
s-erbB1+s-erbB4+EGF+NRG ■

Axis labels: Ln (absorbance at 280 nm) vs $(r^2 - r_0^2)/2$ (cm^2)

Figure 8
Analytical ultracentrifugation data, presented as ln(Abs) against r^2 plots, for s-erbB1/s-erbB4 heterodimerization. The s-erbB1/s-erbB4 mixture without ligand gives a straight line with gradient that yields monomer molecular mass. Addition of one molar equivalent (to total receptor) of EGF alone, or of NRG alone results in an increase in molecular mass consistent with homodimerization of one species only. Addition of both EGF and NRG at the same level results in a substantial increase in the gradient, indicating that both species are homodimerizing independently (see text for explanation).

Figure 8 presents ln(Abs) against $(r^2-r_0^2)/2$ plots for a series of 1:1 s-erbB1/s-erbB4 mixtures. The total receptor concentration is the same in each case. With no added ligand, the gradient of the straight line (proportional to molecular mass when an ideal single species is considered) gives an average monomeric molecular mass of approximately 80 kDa when divided by the appropriate constants. Addition of EGF to a concentration twice that of total receptor (*i.e.* 2 EGF molecules per s-erbB1 plus 2 EGF molecules per s-erbB4) increases the gradient of the straight line by a factor of approximately 1.3, suggesting that some dimerization is induced. Addition of NRG1-β1 to the same final concentration gives a similar result. Note that ligand is not limiting in either of these cases, suggesting that the limited increase in gradient results from homodimerization of only s-erbB1 when EGF is added, and only s-erbB4 when NRG1-β1 is added. If this is true, then an identical sample containing the same total ligand concentration, but as a mixture of EGF and NRG1-β1 should show substantially more dimerization, as both s-erbB1 and s-erbB4 will be capable of homodimerizing independently. The steepest line in Figure 8 shows this to be the case, providing evidence that, as with s-erbB1 and s-erbB2, hetero-

Contains unpublished data

dimerization of s-erbB1 and s-erbB4 does not occur under these conditions - with either EGF or NRG1-β1. In similar experiments we have shown that s-erbB1 also fails to form heterodimers with s-erbB3, regardless of whether EGF or NRG1-β1 is added. Thus, we have failed to detect formation of any heterodimer that includes s-erbB1.

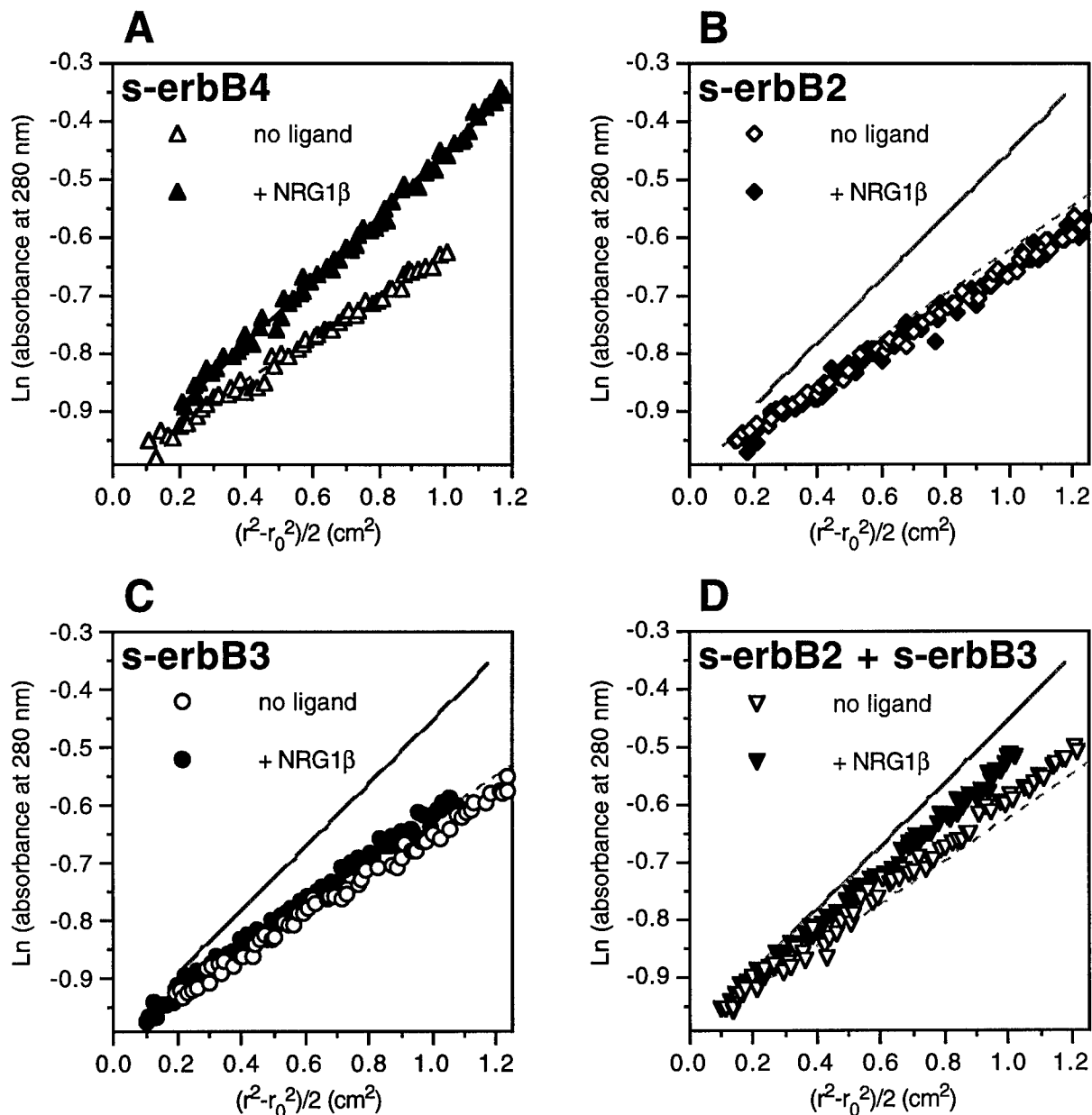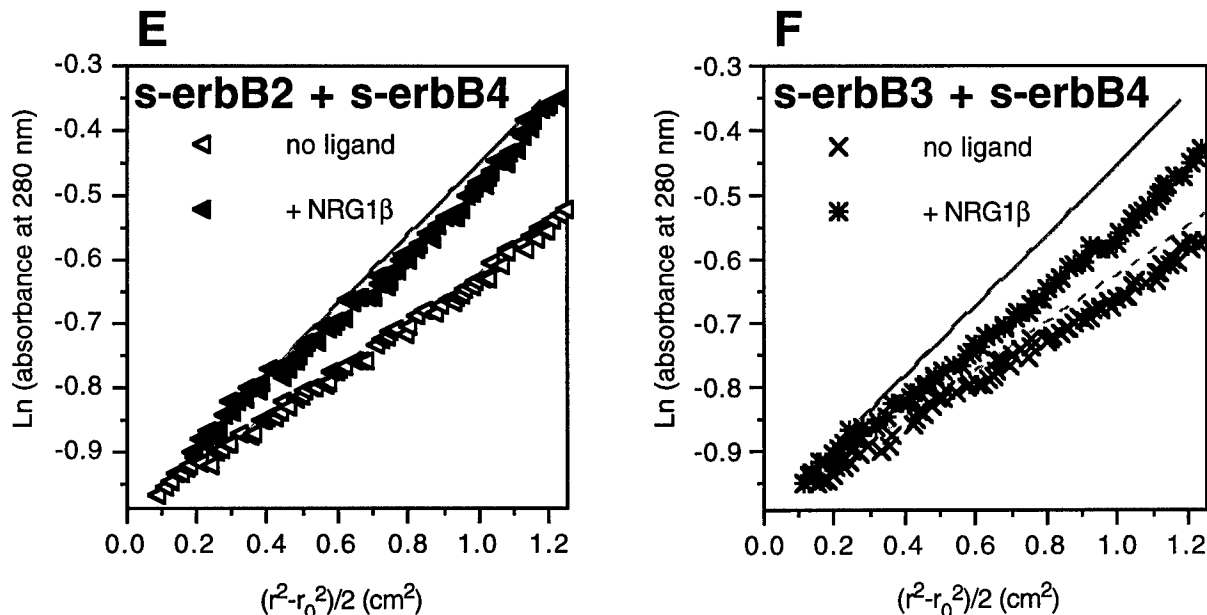## Heterodimerization between NRG-binding receptors and s-erbB2

Figure 9

Analytical ultracentrifugation evidence for formation of s-erbB2/s-erbB3 and s-erbB2/s-erbB4 heterodimers. Panel A shows the increase in gradient of the ln(Abs) against r^2 plot that results from NRG1-β1-induced s-erbB4 homodimerization. These lines are superimposed in grey on all other panels. Panels B and C show that NRG1-β1

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fails to induce homodimerization of s-erbB2 or s-erbB3, while panels D and E demonstrate that NRG1-β1 can induce formation of s-erbB2/s-erbB3 and s-erbB2/s-erbB4 heterodimers. Panel F indicates that s-erbB3 and s-erbB4 do not heterodimerize efficiently upon NRG1-β1 binding.

E

s-erbB2 + s-erbB4

◁   no ligand

◀   + NRG1β

Ln (absorbance at 280 nm)

$(r^2-r_0^2)/2$ (cm^2)

F

s-erbB3 + s-erbB4

✕   no ligand

✻   + NRG1β

Ln (absorbance at 280 nm)

$(r^2-r_0^2)/2$ (cm^2)

Using a similar approach to that used for investigating s-erbB1/s-erbB2 and s-erbB1/s-erbB4 heterodimerization, we next tested the ability of s-erbB3 and s-erbB4 to heterodimerize with one another and with s-erbB2. Recent studies by Sliwkowski's laboratory (Fitzpatrick *et al.*, 1998; Jones *et al.*, 1999) have shown that dimeric IgG fusion proteins containing s-erbB2 together with either s-erbB3 or s-erbB4 have an enhanced affinity for NRG compared with those containing just s-erbB3 or just s-erbB4. This finding has been interpreted to suggest that s-erbB2/s-erbB3 and s-erbB2/s-erbB4 heterodimers represent high-affinity NRG receptors. In Figure 9, ln(Abs) versus radius^2 plots are shown for each pairwise combination of s-erbB2, s-erbB3, and s-erbB4 with and without NRG1-β1. In panel A, NRG-induced homodimerization of s-erbB4 is clearly seen by the approximately 1.6-fold increase in gradient upon addition of a 2-fold excess of NRG1-β1. No such increase is seen upon addition of NRG1-β1 to s-erbB2 (panel B) or s-erbB3 (panel C). In panel D it can be seen that NRG1-β1 induces a small increase in the gradient of the ln(Abs) against r^2 line for an s-erbB2/s-erbB3 mixture. The increase is less dramatic than that seen for s-erbB4, and there may be some tendency for these two s-erbB proteins to interact with one another in the absence of ligand (the gradient is slightly greater than that for the ligand-free mixture than for any unliganded s-erbB protein). However, the NRG-induced shift in gradient shown here is reproducible, and suggests some weak ligand-induced heterodimerization of s-erbB2 and s-erbB3.

Contains unpublished data

Comparing panel F with Figures 7 and 8, it can be seen that the s-erbB3/s-erbB4 mixture behaves, when NRG1-β1 is added, in the same way as an s-erbB1/s-erbB4 mixture with NRG1-β1 or either a s-erbB1/s-erbB2 or s-erbB1/s-erbB4 mixture when EGF is added. In other words, addition of NRG1-β1 appears to induces s-erbB4 homodimerization while s-erbB3 remains monomeric. There is therefore no evidence for s-erbB3/s-erb4 heterodimerization. However, panel E of Figure 9 appears almost identical to panel A, despite the fact that the mixture contains s-erbB2 that neither binds NRG1-β1 nor is induced to dimerize by this ligand. The only explanation for this result is that NRG1-β1 induces efficient heterodimerization of s-erbB2 and s-erbB4. This finding allays our fears that negative results with s-erbB1/s-erbB2 heterodimerization simply reflect a non-functional s-erbB2 preparation.

EXPERIMENTAL PROCEDURES

Generation of s-erbB Constructs

A fragment of human erbB1 cDNA directing expression of residues 1-642 (1-618 of the mature sequence) followed by a hexahistidine tag and stop codon was subcloned into pFastBac1 (Life Technologies Inc). The 1955-base pair fragment was generated by PCR, introducing a unique *Bgl II* site immediately before the initiation codon and a unique *Xba I* site that follows the introduced stop codon. The 1955-base pair *Bgl II/Xba I* digested PCR product was ligated into *Bam HI/Xba I* digested pFastBac I. To minimize the risk of PCR artifacts, a 1260-base pair *Eco RI/Apa I* fragment of this PCR-derived clone was swapped for the equivalent region from the original erbB1 cDNA.

A fragment of human erbB2 cDNA directing expression of residues 1-647 (1-628 of the mature sequence) was generated similarly. In this case a unique *Xba I* site was introduced before the initiation codon, and a unique *Hind III* site was introduced to follow the histidine tag and stop codon. The 1980-base pair *Xba I/Hind III* digested PCR product was ligated into *Xba I/Hind III*-digested pFastBac I. An 1880-base pair internal fragment of this PCR product, extending from an *Nco I* site at the initiation codon to a unique *Sph I* site, was then swapped for the equivalent fragment from the original erbB2 cDNA.

Fragments encoding human erbB3 residues 1-639 (1-620 of the mature protein) and human erbB4 residues 1-649 (1-624 of the mature protein), with a unique *Bam HI* site at one end and *Xba I* site at the other, were generated by PCR, and ligated into *Bam HI/Xba I* digested pFastBac I. The sequence of all PCR-derived fragments and their cloning boundaries were confirmed by standard manual or automated dideoxynucleotide sequencing methods.

Protein Production

Typically 5 - 10 liters of Sf9 cells were grown as a suspension culture in Sf900-II medium (Gibco/BRL) using a number of 1 liter spinner flasks. Each 1 liter flask contained less than 500 ml of medium to ensure adequate aeration. When a cell density of 2.5×10^6 cells/ml (viability >98%) was reached, freshly amplified high-titer virus stock was added to a multiplicity of infection (MOI) of approximately 5. Cultures were incubated for a further 96 hours. Clarified conditioned medium was then diafiltered against 3.5 volumes of 25 mM Tris-HCl, 150 mM NaCl, pH 8.0 (buffer A), using a Millipore Prep/Scale-TFF 30 kDa cartridge, and was concentrated to approximately 300 ml prior to loading onto a 5 ml Ni-NTA Superflow column (Qiagen). After extensive washing with buffer A, the column was washed sequentially with 2 column volumes of buffer A containing 30, 50, 75, 100 and 300 mM imidazole, pH 8.0. Typically the majority of the protein eluted in the 75 and 100 mM fractions. Fractions were concentrated in a Centriprep 30 (Amicon), and loaded onto a Pharmacia Superose 6 gel filtration column. The s-erbB proteins eluted as approximately 85 kDa species, and were greater than 95% pure at this stage of purification. For s-erbB1 and s-erbB4, appropriate gel-filtration fractions were pooled, diluted 1.5 fold with 50 mM MES pH 6.0, and were loaded on to an BioScale-S2 cation exchange column (BioRad), pre-equilibrated with 25 mM MES pH 6.0. Protein was eluted with a gradient in NaCl, s-erbB1 eluting at approximately 200 mM NaCl, and s-erbB4 at approximately 300 mM NaCl. Attempts to purify s-erbB2 and s-erbB3 by ion exchange led only to precipitation of the proteins at the low salt concentration required for binding to the column. Purified s-erbB proteins are buffer exchanged into 25 mM Hepes, 100 mM NaCl, pH 8.0, concentrated to between 20 and 100 μM, and stored at 4° C. Purity was checked by SDS-PAGE, and concentrations were determined by absorbance at 280 nM using calculated extinction coefficients. Molar extinction coefficients used were s-erbB1, 58900; s-erbB2, 63310; s-erbB3, 68430; s-erbb4, 73550.

Multi-angle laser light-scattering (MALLS) studies

MALLS is our primary approach to study s-erbB homo- and heterodimerization. This technique is both more sensitive and more rapid than small-angle X-ray scattering that we have used before, allowing more experiments to be done, and over a wider range of protein concentrations. A DAWN DSP Laser Photometer from Wyatt Technologies (Santa Barbara, CA) is used, which is ideally suited to these experiments (Wyatt, 1993). The DAWN contains a glass flow-cell (volume 70 μl), around which are 17 usable photodiode detectors at different angles from 15° to 160°. Scattering of light (633 nm) from a 5mW He-Ne laser is measured simultaneously at each of these angles, and normalized for variations in laser intensity as well as geometric effects (using an isotropic scatterer). The DAWN is used in micro-batch mode, samples being

Contains unpublished data

introduced into the flow-cell via a 0.1μm filter with a syringe pump. To avoid introduction of air-bubbles, samples are degassed under vacuum, and introduced via a low dead-volume multi-port valve, which is loaded with several samples and purged of air prior to a series of measurements. A sample of 300 μl is more than sufficient to flush and equilibrate the flow-cell for stable scattering measurements, which themselves are observed in real time. With adjustments to the gain of the detector amplifiers, scattering from sEGFR samples of less than 0.01 mg/ml (0.1 μM) to greater than 10 mg/ml (100 μM) can be measured accurately. Data are collected and analyzed using the ASTRA software supplied with the instrument. For micro-batch experiments, we inject a series of samples with fixed sEGFR concentration and increasing concentrations of EGF. Scattering data at all 17 angles are collected until the response is stable. For a region of the normalized data after equilibration for each injected sample, the software is directed to calculate a Debye plot for each time point. In the Debye plot, $R(\theta)/K^{*}c$ is plotted against $\sin^2(\theta/2)$, where:

θ is the scattering angle

$R(\theta)$ is the excess intensity (I) of scattered light at that angle $(= I(\theta)_{sample}/I(\theta)_{buffer})$

c   is the <u>mass</u> concentration of the sample

K^{*} is a constant equal to $4\pi^2 n^2(dn/dc)^2/\lambda_0^4 N_A$, where n = solvent refractive index, dn/dc = refractive index increment of scattering sample, λ_0 = wavelength of scattered light, N_A = Avogadro's number

Since:

$$\frac{K^{*}c}{R(\theta)} = \frac{1}{\overline{M}_w P(\theta)} + 2A_2 c \qquad\qquad 1$$

where A_2 is the second virial coefficient, $\overline{M}_w$ is the weight-averaged molecular mass, and:

$$P(\theta) = 1 - \frac{16\pi^2 R_G^2 \sin^2(\theta/2)}{3\lambda^2} + \ldots \qquad\qquad 2$$

then, extrapolating to zero angle $\theta = 0$ $(P(\theta) = 1)$:

$$\frac{K^{*}c}{R(\theta)} = \frac{1}{\overline{M}_w} + 2A_2 c \qquad\qquad 3$$

By extrapolating the Debye plot to zero angle (when $R(\theta)/K^{*}c = \overline{M}_w$), the weight-averaged molecular mass $(\overline{M}_w)$ of the molecule in the scattering sample can be measured directly if the value of the virial coefficient (A_2) is known for the protein. We have measured A_2 for s-erbB1 (and the 1:1 EGF:s-erbB1 complex) from Zimm plots, obtaining a value of 6.5×10^{-5} mol.ml.g^{-2}. Even at the highest s-erbB1 concentrations studied (3mg/ml), A_2 contributes less than 3% to the apparent weight-averaged molecular mass. For monitoring s-erbB protein dimerization, we are interested

only in relative values of $\overline{M}_w$. If the concentration of receptor is fixed, errors in determination of the degree of glycosylation will not be important.

Analytical ultracentrifugation studies

Sedimentation equilibrium experiments employed the XL-A analytical ultracentrifuge (Beckman). Samples were loaded into six-channel epon charcoal-filled centerpieces, using quartz windows. Experiments were performed at 25°C using three different speeds (6,000 and 9,000, and 12,000 r.p.m.), detecting at 280 nm, with identical results. Solvent density was taken as 1.003 g/ml, and the partial specific volumes of the s-erbB proteins were approximated from their amino acid compositions and the assumption of approximately 20% carbohydrate, as 0.71 ml g^{-1} for the purposes described in this report. Experiments were performed at 5 μM or 10 μM protein. Data were fit using the Optima XL-A data analysis software (Beckman/MicroCal) to models assuming a single ideal or non-ideal species for unliganded s-erbB proteins. When ligand was added, a two-species fit was used, in which one of the species was the excess ligand, which sediments as a 6 kDa (EGF) or 8 kDa (NRG) species. Knowing the K_D values from our BIAcore studies, the amount of free ligand is also known. The molecular mass of the s-erbB species is allowed to float in these fits. Fits were judged by the occurrence of randomly distributed residuals, examples of which are shown in Fig 6. Where possible, simple interpretation of analytical ultracentrifugation experiments was made by inspection.

BIAcore studies

Experiments employed a BIAcore 2000 instrument, and were all performed in 10 mM Hepes buffer, pH 7.4, containing 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20 at 25°C. Ligands were crosslinked to the hydrogel matrix of BIAcore CM5 Biosensor chips activated with *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*'-[3-(diethylamino)propyl]carbodiimide (EDC). EGF at 200 μg/ml in 10 mM sodium acetate, pH 4.0 was then injected at 5 μl/min for 10 minutes. Non cross-linked EGF was removed, and unreacted sites were blocked with 1 M ethanolamine, pH 8.5. The signal contributed by immobilized EGF ranged from 150 RU to 400 RU, depending on the specific chip. For immobilization of NRG1-β1, the procedure was essentially the same, except that immobilization was performed in 10 mM sodium acetate at pH 4.8.

The purified s-erbB proteins at a series of concentrations were each flowed simultaneously over the EGF and NRG (and mock/control) surfaces at 5 μl/min for 7 minutes, by which time binding had reached a plateau in each case. The RU value corresponding to this plateau was taken as a measure of s-erbB protein binding, and was corrected for background non-specific binding and bulk refractive index effects by subtraction of data obtained in parallel using the mock-coupled

hydrogel surface. RU values were then converted into percentage maximal binding. This conversion was performed separately for each surface (since levels of immobilization varied). 100% binding was defined for an NRG surface as the highest corrected signal seen with s-erbB3 and s-erbB4 (which were always the same to within 10%), and for an EGF surface the highest corrected signal seen with s-erbB1. Buffer washes between runs were sufficient to bring th eRU value back down to baseline. Data were plotted as s-erbB concentration against percent maximal binding, and fit to a simple binding equation, in ORIGIN (MicroCal) to estimate K_D.

KEY RESEARCH ACCOMPLISHMENTS

- Produced and purified milligram quantities of the four erbB receptor extracellular domains for biophysical and structural studies

- Demonstrated that EGF induces homodimerization of the erbB1 (EGF receptor) extracellular domain, but of no other s-erbB protein

- Demonstrated that NRG induces homodimerization of the erbB4 extracellular domain, but of no other s-erbB protein

- Demonstrated that the erbB3 extracellular domain binds NRG1-β1 without being induced to dimerize, and measured K_D values for ligand binding by other s-erbB proteins.

- Showed, for the first time, ligand-induced heterodimerization of isolated erbB receptor extracellular domains, with NRG1-β1 inducing heterodimerization of s-erbB2 with either s-erbB3 or s-erbB4.

- Just as importantly, we have shown that s-erbB1 does not participate in any heterodimerization reactions, contrary to the expectations in the field.

REPORTABLE OUTCOMES

1. Results were presented at an invited lecture at the 1999 Gordon Conference on "Ligand Recognition and Molecular Gating" in Ventura, CA, March 7-12, 1999

2. Manuscript entitled: "Extracellular domains of erbB receptors are sufficient for ligand-induced homodimerization, but only neuregulin-induced heterodimerization", to be submitted to *EMBO Journal*, September 1999.

CONCLUSIONS

Our studies to date alter the view of erbB receptor activation by EGF and NRG family members. ErbB1 and erbB4 are the central receptors in the system, binding to and being homodimerized by EGF and NRG respectively. Transmodulation of erbB2 by NRG, to which it does not bind directly, appears to involve NRG-induced formation of heterodimers with erbB3 or erbB4. These heterodimers can be recapitulated using isolated extracellular domains. So far, these findings - observed *in vitro* for the first time - are consistent with expectations from previous cellular studies. A major contribution here is to show that the s-erbB2/s-erbB4 heterodimer, for example, is indeed a hetero**DI**mer, and not a larger aggregate. This question has not been addressed before. A major surprise in our studies was the inability of s-erbB1 to form heterodimers, in spite of the fact that interactions (of unknown stoichiometry) between erbB1 and erbB2 sparked much of the debate about erbB receptor heterodimerization.

Our findings argue:

1. That erbB1 (EGFR) activation is mechanistically distinct from EGF-induced transmodulation of erbB2, erbB3, and erbB4. We hypothesize that oligomers involving erbB1 homodimers must be formed, and we are performing experiments to test this.

2. That EGF and NRG activate erbB2 through distinct mechanisms: one (NRG) through ligand-induced heterodimer formation, and the other (EGF) through an as-yet-unclear mechanism. Experiments, described in Tasks 2 and 3 of the approved Statement of Work will now be performed to distinguish between these possibilities.

3. While our supposition going into this project was that quantitative differences would explain the diversity of signaling in this system, the studies to date have identified qualitative differences that are more likely to be of use in design of therapeutic strategies.

So What ?

A major aim in breast cancer is to inactivate or otherwise remove erbB2/Neu/Her2 in the 30% or so of cases where its over-expression is seen. Herceptin has this as the basis of its efficacy. Our studies are bringing new insights into how erbB2 is regulated in cells. In particular we find that there are TWO mechanims for erbB2 transmodulation by other receptors in the erbB family. Since breast cancer cells differ in their complement of other erbB receptors, these mechanisms are likely to be of different degrees of importance in different cases (T47D cells and SKBR-3 cells will differ, for example). Understanding the mechanisms, which we are beginning to do, will allow us to begin our approaches to designing new strategies for intervention when erbB2 is inappropriately active. Knowing when the different mechanisms are most important will allow consideration of approaches that are much more selective and specific than can possibly be true with antibody-based therapies.

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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)   28 May 02

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has
reexamined the need for the limitation assigned to technical
reports written for Grant DAMD17-98-1-8232. Request the limited
distribution statements for Accession Documents Number ADB258643
and ADB266202 be changed to "Approved for public release;
distribution unlimited." These reports should be released to the
National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at
DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
    Information Management